STEADY STATE ELECTROLYTE FLUXES ACROSS IN VITRO RABBIT ILEUM

by

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Errata

Pg 31 In 10 for Cl- independent entry read Cl-independent Na entry.

Pg 131 In 8 for ration read ratio

Pg 135 In 21 for $P_{Cl} = 0.018$ cmhr $^{-1}$
read $P_{Cl} = 0.015$ cmhr $^{-1}$

Pg 138 In 10 for Na deprivation (zero choline)
read Na deprivation (zero Na, choline media)

Pg 143 In 28 for both unidirectional fluxes
read both unidirectional cl fluxes

Table 24 In 1 prefix μ is omitted from μmoles
In 4 theophylline is mis-spelt

Fig 11 for x axis exp $\frac{(-2F\Psi_{1-3}/RT)}{}$
should read exp $(ZF\Psi_{1-3}/RT)$

Fig 27 for y axis units are μmoles $cm^{-2}hr^{-1}$

ABBREVIATIONS

c-AMP: cyclic 3'5' adenosinemonophosphate

Na-K ATPase: Na-K adenosinetriphosphatase

Ionic signs are omitted from Na^+, K^+, Ca^{2+}, Cl^-, and SO_4^{2-}.

CONTENTS

CHAPTER ONE

INTRODUCTION

A. General Considerations

Whilst all cells possess the ability to utilize metabolic energy to create and maintain non-equilibrium gradients between the cytoplasm and the extracellular fluid, the cells of epithelia possess the additional specialization of net transport of salt, water and various chemicals across the epithelium (Schultz and Curran (1968)).

This function of epithelia is exemplified by small intestine which, in man, absorbs seven litres of fluid and one mole of NaCl per twenty four hours (Turnberg (1973)).

Ussing (1972) considers that epithelia may be treated as membranes of the second order with respect to the transported species, individual membrane-covered cells being arranged in membrane-like sheets. Many studies of epithelial function consider the epithelium as a black-box membrane (Ussing (1972); Schultz and Curran (1974)).

The second order membrane concept is embodied in the short-circuit technique introduced by Ussing and Zerahn in 1951 for the study of ion-fluxes across the isolated frog skin. This general method has now been successfully applied to a wide variety of epithelia including such diverse tissues as frog cornea (Zadunaisky (1966)) and frog choroid plexus (Prather and Wright (1970)).

The Ussing technique places the isolated epithelium between two solutions whose thermodynamic properties are defined and controlled within narrow limits. The electro-chemical gradient may thus be set at any desired value in a way that is virtually impossible for the membranes of single cells. The net movement of an ionic species may be measured and compared with that predicted by simple theory in

order to establish the existence or otherwise of active transport processess occurring within the epithelium.*

The experiments described in this thesis concern the measurement of both Na and Cl fluxes across isolated in vitro rabbit ileum by the use of techniques derived from those described by Ussing and Zerahn (1951).

The usefulness of the black-box membrane or second order membrane concept is limited in a study of transintestinal ion flux, since in reality net ion fluxes are determined by flux across at least two cellular membranes arranged in series. Leaky epithelia such as intestine are also characterized by leakage or shunt pathways for ion flows which circumvent the cellular membranes, (Frömter (1972); Frömter and Diamond (1972); Frizzell and Schultz (1972)).

The interpretation of ion flows in terms of unidirectional fluxes across the two individual epithelial cell membranes is the primary objective of this work.

B. Ion permeation in rabbit terminal ileum**

1. A parallel pathway model for rabbit ileum

Structurally terminal ileum is a complex tissue consisting of the absorptive layer, the lamina propria and the muscularis mucosae,

*Rosenburg (1948, 1954) has suggested that active transport may be defined as one that brings about net transfer of a substance against an electro-chemical potential difference of that substance. Although this definition is limited (Schultz and Curran (1968)) it is adequate for the purpose of this thesis.

**Recent reviews (Schultz and Curran (1974); Schultz, Frizzell and Nellans (1974)) provide comprehensive treatments of ion permeation in intestine. The following discussion emphasizes new findings.

together with the submucosal layers of muscle. The absorptive layer consists of a single sheet of columnar epithelium cells, though the cell population is heterogeneous. Villous crypts contain undifferentiated cells in the process of division and maturation. Cell maturation is concurrent with migration to the villous tip, from where senescent cells are extruded into the intestinal lumen (Trier (1968)).

Analysis of ion fluxes through such a complex tissue may be at best speculative. Two simplifying assumptions can be made:

1. The absorptive layer can be functionally represented as being composed of a single cell layer of homogeneous composition.

2. The rate-limiting step for transepithelial ion flux is transfer across the absorptive layer. The statement implies that flux is not rate-limited by the mucosal or serosal unstirred layers. In this respect the serosal unstirred layer is quantitatively more important.*

Net transepithelial ion flux in the absence of external electrochemical gradients can be regarded as due to asymmetries generated in the absorptive layer. Two limiting cell membranes control ion flux across the absorptive layer, namely the mucosal and the basal-lateral membranes. There is every reason to believe that the transport properties of these two membranes are different, (Schultz and Curran (1974)).

A dual membrane model for the intestine cannot, however, be reconciled with a large body of evidence that suggests a significant proportion of transepithelial ion flux circumvents the limiting membranes of the absorptive cell layer.

*Stripping of the submucosal muscle layers is performed in all experiments described in this thesis. This reduces the serosal unstirred layer.

Determinations of the transepithelial resistance* have consistently yielded values that are less than 200 Ωcm^{-2} (Clarkson and Tools (1964); Clarkson (1967)) and in most instances which are less than 75 Ωcm^{-2} (Schultz and Zalusky (1964); Field and McColl (1973); Field (1971); Schultz and Curran (1974); Barry, Smyth and Wright (1965)).

These values of resistance contrast with resistance determinations made on cell membranes in a variety of tissues (Cole and Hodgkin (1936) (squid axon); Frömter (1972) (gall-bladder epithelial cell membrane)). Values for cell membrane resistance range from 1 to 10k Ωcm^{-2}.

This difference between cell membrane resistance and the transepithelial resistance of intestine suggests that the rabbit small intestine is effectively short-circuited by high conductance (low resistance) pathways for ion movement. These are in parallel to the high resistance pathways afforded by the limiting cell membranes of the epithelial cell layer. Similar conclusions have been made for necturus gall-bladder (Frömter (1972)) and for proximal kidney tubules (Windhager et al (1967)).

Support for the existence of a parallel shunt pathway for ion movements in ileum come from the observations of Clarkson (1967); Frizzell and Schultz (1972) and Desjeux, Tai and Curran (1974). These authors have shown that both unidirectional (mucosal to serosal and serosal to mucosal) fluxes and net fluxes of K, Na and Cl across in vitro rat and rabbit small intestine are comprised of at least two components. The first component varies as the electrical potential across the intestine is changed in the range $\pm$ 40mv. This component

*All resistance values are expressed relative to the chamber surface area. No correction for tissue morphology is made.

adheres strictly to the laws of electrodiffusion. The second component is insensitive to the transepithelial electrical potential. These results are entirely consistent with the existence of parallel pathways for ion movements across intestine.

Additional evidence suggesting the existence of a low resistance shunt pathway is derived from studies of the transepithelial and transmembrane electrical potential differences (Rose and Schultz (1971)). The existence of an electrical potential across the mucosal membrane of -10 to 36mv. (see below) may only be reconciled to a recorded transepithelial potential difference of +2 to+5mv if there exists a high conductance pathway between the mucosal and basal-lateral membranes. The transmural potential difference of the small intestine is known to increase immediately after the addition of an actively transported sugar into the solution bathing the mucosal surface (Barry et al (1964); Smith (1966)). Although the exact nature of this potential is not fully understood, it is clear that the magnitude of the sugar-dependent change in transepithelial electrical potential is only reconcilable with the presence of a high-conductance shunt pathway that attenuates the potential difference across either or both membranes of the epithelial cell (Schultz and Curran (1974); Schultz (1974)).

Similar conclusions have been made in other epithelia in which low-resistance extracellular pathways occur (Maruyama and Hoshi (1972)).

The anatomical location of the shunt pathway is most probably the 'tight' or limiting junctions between the cells of the absorptive layer. This has been shown using voltage scanning experiments in other low-resistance epithelia, such as necturus gall-bladder (Frömter and Diamond (1972); Frömter (1972)). The use of the electron-dense ion lanthanum to evaluate tight-junction permeability

has proved to be particulary useful in this respect. Lanthunum has been observed by electron microscopy to easily penatrate the tight-junctions of rat proximal tubules (Tischer and Yarger (1973, 1975); Martinez-Palomo and Erlij (1973)), amphibian kidney tubules (Whittembury and Rawlins (1971)), rabbit gall-bladder and intestine (Machen, Erlij and Wooding (1972)). Since lanthanum has a larger ion radius than Na or K, the transepithelial penetration of these ions via the tight-junction can be assumed to occur, so providing the anatomical basis for the inferred high-conductance pathway.

2. The paracellular (shunt) pathway in rabbit ileum

(i) The relative conductance of the shunt pathway compared with the transepithelial conductance

Rose and Schultz (1971) demonstrated that the application of a 200 μamp pulse of direct current to *in vitro* terminal rabbit ileum changed both the transepithelial and the transmucosal membrane potential differences. The observed change in transepithelial P.D. was fourteen-fold greater than the change in transmucosal membrane P.D. This result demonstrates that the greater part of total tissue conductance can be ascribed to ion passage through the high-conductance extracellular pathway.

By assuming that ion flux through the shunt pathway obeys the laws of electrodiffusion (i.e. the Ussing flux-ratio criterion (Ussing 1949)), Frizzell and Schultz (1972) were able to measure the total conductance due to Na, K and Cl through the shunt pathway in relation to the total tissue conductance. They concluded that 85% of tissue conductance was due to ionic diffusion through the shunt pathway. Estimates of shunt and transcellular resistance were made; these were 110 and 1000 Ωcm^{-2} respectively. These conclusions have recently been confirmed (Desjeux et al (1974)).

The significance of these findings to the measurement of transepithelial fluxes is of profound importance, particularly with regard to cation fluxes (see below). The relatively small P.D.'s across intestine will markedly effect paracellular ion flows. Voltage-dependent ion drift can be eliminated by applying a short-circuiting current across the tissue (see Chapter 2). This does not, however, effect diffusional movement through the shunt pathway. No statement may be made concerning transcellular fluxes unless measurements have been made to determine the contribution of the paracellular shunt pathway to the measured transepithelial fluxes.

(ii) Ion-selectivity properties of the paracellular (shunt) pathway

The permeabilities of Na, K and Cl determined by Frizzell and Schultz (1972) for the shunt pathway are 0.035, 0.04 and 0.019cm hr^{-1} respectively. (This is a ratio of 1.0:1.14:0.55). The free-solution mobility ratio for these ions is markedly different:- 1.00:1.47:1.52 (Robinson and Stokes (1965)). Rabbit ileum is, therefore, cation-selective. The ratio of Na:K permeability also indicates that ion permeation follows an intermediate ion-selectivity sequence (Eisenman (1962); Diamond and Wright (1969)). This suggests that the shunt pathway affords a watery environment for ionic diffusion and that Na and K traverse this pathway in their hydrated forms (Frizzell and Schultz (1972)). Similar conclusions have been made for rat jejenum (Munck and Schultz (1974)).

Since the transepithelial conductance is primarily determined by the properties of the shunt pathway, it follows that both dilution and biionic potentials elicited across the epithelium will reflect the properties of the shunt pathway with regard to ion-selectivity (Schultz and Curran (1974)). Thus, Frizzell and Schultz (1972) have shown that

dilution of the NaCl in the mucosal bathing solution by isosmotic replacement with mannitol will elicit a large serosa negative electrical P.D. (-18mv.). This indicates that the shunt pathway is cation selective. Similar observations were made by earlier workers (Smyth and Wright (1966)) who studied streaming potentials.

In addition to mannitol replacement the use of KCl in isosmotic replacement of NaCl in the mucosal solution elicits a small serosa positive P.D. (Frizzell and Schultz (1972)). This demonstrates that the ratio of ionic permeabilities for the shunt pathway is $P_K > P_{Na} > P_{Cl}$. Furthermore, by using the Goldman-Hodgkin-Katz equation (Goldman (1943); Hodgkin and Katz (1949)) and incorporating the values of P_K, P_{Na} and P_{Cl} that were obtained from the potential-sensitive flux determinations, it was possible to obtain a good fit between the predicted and experimental values for the dilution and biionic potentials.

Eisenman (1968) considers possible mechanisms by which cation permeation and selectivity could be conferred on biological membranes:

1. Cation permeation could be mediated by mobile negatively charged carrier molecules within the membrane phase. Examples of electrically neutral molecules which act as moble carriers for cations are known - e.g. valinomycin (Kinsky (1970)).

2. Cation permeation could be mediated by trans-membrane pores which were lined with negatively charged groups. The requirements for charged pores are known to be formed by the antibiotic nystatin (Cass, Finkelstein and Krepsi (1970); Holz and Finkelstein (1970); Marty and Finkelstein (1975)).

What evidence exists to differentiate between these mechanisms for the shunt pathway of rabbit ileum? In their formulation of a theory of ion permeation through neutral membranes Barry and Diamond

(1971) have outlined certain experimental requirements that allow differentiation between the various alternatives.

Rabbit ileum behaves as a simple ohmic resistor (Frizzell and Schultz (1972); Schultz and Zalusky (1964)); that is to say there is an instantaneous current-voltage relationship. This result excludes the possibility that ionic permeation through the shunt-pathway is controlled by fixed negative-charges of the ion-exchange type (Barry and Diamond (1971)). Similarly, neutral mobile carriers would only show a linear current-voltage plot for thin membranes and this should deviate from linearity at high voltages (Szabo et al (1969)).

Simply, we may conclude that ion permeation through the shunt-pathway of the rabbit small intestine is controlled by fixed neutral pores. (This point is more extensively discussed by Schultz and Curran (1974)).

Of interest in this respect, is the observation of Smyth and Wright (1966) that a reduction in the pH of the bathing medium to pH 3.0 changes the selectivity of rat small intestine so that the epithelium becomes anion-selective. Similar observations have been recorded in gall-bladder epithelium (Wright and Diamond (1968)). This evidence suggests that ion permeation is not controlled by fixed dipoles (e.g. carbonyl groups). An alternative model is to consider that the pore is lined with dissociated oppositely charged groups in equal numbers (zwitter-ions). The negative members could be aligned towards the centre of the channels, whereas the positive groups could extend outwards. Macroscopic neutrality could thus be conserved.

The conclusion that ions permeate through the shunt-pathway in their hydrated form suggests that the tight-junctions have a finite permeability for water movement. The observations that rabbit ileum is impermeable to lysine (a spherical molecule of ionic radius

of ~4nm (Munck and Schultz (1969)), and that rat jejenum is impermeable to triethylammonium, lysine and diaminobutyric acid (Nellans, Frizzell and Schultz (1974); Munck and Schultz (1974)) suggest an equivalent pore diameter for the tight-junction of 10 - 15 nm. This value for pore size cannot exclude the possibility that a significant proportion of transmural water movements occurs by way of the tight-junctions. In necturus kidney tubules the water permeability coefficient for the whole epithelium is one order of magnitude greater than that of the cellular membranes (Whittembury et al (1973)).

(iii) Factors affecting ion permeation through the paracellular pathway

Increasing interest has been directed towards ion flux through the paracellular pathway, particularly with regard to changes in transepithelial transport of ions and water (Boulpaep (1972); Humphreys and Early (1971); Powell (1974)).

The paracellular pathway is a series array of the tight or limiting junctions and the underlying lateral intercellular space. Any discussion of changes in the properties of this pathway must, therefore, take adequate account of the contribution of the lateral space.

The overall permeability of the paracellular pathway P_s is given by the formula:

$$1/P_s = 1/P_{tj} + 1/P_{ls}$$

where P_{tj} is the permeability of the tight-junction and P_{ls} is the permeability of the lateral space.

(a) Lateral Space Permeability An estimate of P_{ls} may be made in a similar fashion to that outlined by Smulders et al (1972) and by Desjeux et al (1974)

$$P_{ls} = DA/L$$

where D is the free-diffusion coefficient of the solute of interest, A is the area of the lateral spaces and L is the lateral space length. Using the data of Marsh and Swift (1969) for human intestine, and of Trier (1968), Desjeux et al estimated that P_{ls} ranged from 0.033 cm hr^{-1} to 0.077 cm hr^{-1} for distended spaces. They concluded, therefore, that 85% to 90% of all resistance to flux through the paracellular route resided in the tight-junction. That is to say the lateral spaces do not normally rate-limit ion flux through the paracellular pathway. However, collapse or reduction in the width of the lateral space will decrease P_{ls} and so provide a mechanism for rate-controlling ion movements through the shunt pathway.

Smulders, Tormey and Wright (1972), using combined studies of tissue morphology and of flux estimations, have shown that variation in the widths of the lateral spaces changes both ion and non-electrolyte permeation in rabbit gall-bladder. Mucosal hypertonicity was shown to collapse the lateral spaces and reduce ion and non-electrolyte permeability to a third of control values. Conversely, serosal hypertonicity increased the measured permeabilities. The increase was quantitatively smaller than the decrease observed in the presence of mucosal hypertonicity. Such non-linearity may easily be reconciled with a model that postulates that lateral space permeability is rate-limiting when the spaces are collapsed, but to have a smaller effect when either cellular permeability or tight-junction permeability rate-limit permeation. Similar effects were also reported for the effect of osmotic gradients upon the tissue hydraulic permeability (Wright, Smulders and Tormey (1972)).

Comparable observations with those of Smulders et al and Wright et al have been in frog intestine (Loeschke, Bentzel and Csaky (1970) and Loeschke, Hare and Csaky (1971)).

Application of electrical current is also known to change the dimensions of the lateral spaces in frog gall-bladder in a similar fashion to osmotic gradients (Bindslev, Tormey and Wright (1974)). Such changes in lateral space dimensions result in changes in the electrical resistance of the tissue. The time course of these changes is of long duration (~twenty minutes). Current dependent changes in tissue resistance have been observed in other low-resistance epithelia (Spring (1973); Kidder and Rehm (1970)).

Current induced changes in tissue morphology and resistance are of particular interest with regard to the present study, in that a method of measuring paracellular ion permeability requires the passage of high current densities (Frizzell and Schultz (1972); Mandel and Curran (1972, 1973)). Separation of active (transcellular) from passive (paracellular) fluxes using this method may, therefore, be unsatisfactory for low resistance epithelia such as intestine, since paracellular ion fluxes will not necessarily reflect control conditions in the absence of the passage of electric current. A new method of measuring paracellular Na permeability in which these difficulties do not arise is described in Chapter 4.

A question of significance is the physiological relevance of changes in the dimensions of the lateral cell spaces. It may be noted that lateral-space collapse is a consistent morphological feature of cholera and theophylline treated intestine (Dibona et al (1974)). The relationship of this effect to the secretory process is discussed in Chapter 6. Boyd, Cheeseman and Parsons (1975a,b) have examined the effect of Ringer Na replacement on the inulin space in vascular perfused bullfrog intestine. Ringer Na replacement reduces the inulin space for this tissue, indicating lateral space collapse. The effect will modify the passive permeability properties of the intestine (see below and Chapter 4).

(b) Tight-Junctional Permeability Modification of the permeability properties of the paracellular pathway may also occur as a consequence of change in the tight or limiting junction. In high resistance epithelia such as frog skin and toad urinary bladder, hypertonic urea solutions bathing the mucosal (lumen or skin side) increase both epithlial conductance and permeability (Ussing and Windhager (1964); Dibona and Civan (1973)). These changes are thought to result from an increased leakiness of the tight-junctions. This has been visualized under the electron microscope as an opening of the tight junctions (Erlij and Martinez-Palomo (1972)) in frog skin. Aortic hypertonic mannitol solutions result in a graded increase in inulin permeability in the doubly-perfused toad kidney (Perez-Gonzalez and Whittembury (1974)). Also current passage in frog gall-bladder results in a decrease in resistance that cannot be accounted for on the basis of change in the lateral spaces alone (Bindslev et al (1974)).

Modification of junctional permeability may occur in the presence of transintestinal pressure gradients. Mucosal (distension pressure) has been shown to have no effect on transintestinal water flux in rat when either glucose was absent from the bathing media or when phlorrhizin was present (Smyth and Taylor (1957)). In in vitro canine intestine elevation of mucosal pressure does not increase transintestinal water flux (Hakim, Lester and Lifson (1963); Hakim and Lifson (1969)). Small increments in serosal pressure, however, produce marked reductions in transintestinal water movement. Similar findings have been reported for hamster intestine (Wilson (1956)) and rabbit gall-bladder (Tormey and Diamond (1967); Dietschy (1964)). It is likely that a change in junctional permeability gives rise to the effect of serosal pressure upon water flux since solutes such as Na, Cl, urea glucose and inulin experience large solvent drag effects on the application of serosal pressure (Hakim and Lifson (1969)).

Examination of the role of hydrogen-bonding in alkali-metal discrimination in the tight-junctions of rabbit gall-bladder (Moreno and Diamond (1974)), together with a study of nitrogenous cations as probes of the cation-selective channel (Moreno and Diamond (1975)) led to the discovery of a compound, 2,4,6, triaminopyrimidine, which blocks gall-bladder cation permeability (Moreno (1974); Moreno (1975a,b)). Triaminopyrimidine is thought to block cation permeability by forming H-bonds with the proton-acceptor acidic channel ligands conferring cation-selectivity to the tight-junction. The use of triaminopyrimidine allows an experimental modification of paracellular permeability. The action of triaminopyrimidine and its use in assessing paracellular Na permeability in rabbit ileum is discussed in Chapter 4.

(iv) <u>The role of the paracellular pathway in the net transepithelial flows of NaCl and water: effect of bulk flow on ion permeability in the paracellular pathway.</u>

Curran and McIntosh (1962) were the first to propose that active transport of salt into a central compartment delimited by two membranes of differing salt and water permeability could explain the coupling between salt and water flows in many epithelia. The theoretical aspects of this model have been analysed by Patlak, Goldstein and Hoffman (1963).

No attempt was made to fit the model to epithelial geometry. Kaye et al (1966) postulated that the central compartment composed the lateral spaces of rabbit gall-bladder epithelium since distension of these spaces during transport suggested their intimate involvement in the transport process. The double membrane model of Curran and McIntosh requires that the central compartment be well-stirred, a condition unlikely to be realized in the lateral spaces.

The involvement of the lateral spaces in the transport process has been confirmed in rabbit gall-bladder (Tormey and Diamond (1967)). Together with detailed studies on the pathways for ion and water movement across gall-bladder epithelium (Diamond (1962a,b)), Diamond and Bossert (1967) proposed that the lateral spaces acted as local osmotic compartments. Solute transport into the lateral spaces establishes a standing gradient of salt along the channel length. Progressive dilution of the salt concentration by osmosis ensures that the absorbate tonicity approaches physiological values at the end of the lateral space.

Diamond and Bossert (1967) developed this model mathematically, demonstrating that the osmolarity of the fluid emerging from the channel's open end depended upon the channel length, radius and water permeability, solute transport rate and diffusion coefficient within the lateral space. Isotonic absorbates could be achieved with decreased channel radius or solute diffusion coefficient; or increased channel length or water permeability. The model assumed that the tight-junctions were impermeable to water and ion movements and that active-pumping was localized to the proximal portion of the channel. The requirement for isotonic absorbates was stressed since experiments using the 'unilateral' gall-bladder preparation had shown that absorbate osmolarity was equivalent to the bathing solution osmolarity within the gall-bladder for a wide range of bathing solution osmolarities (Diamond (1964)).

Hill (1975a,b) has criticized the standing gradient model. Using the Segal isotonic approximation (Segal (1970)) and choosing 'realistic' values for channel length and radius Hill concludes that isotonic absorbates may only be realized if improbably large values for cell membrane osmotic permeability exist.

Several points are relevant with regard to the Hill analysis:

1. Anisotonic (hypertonic) absorbates from epithelia have been noted, e.g. dog ileum (Visscher et al (1944)), human jejunum (Soergel et al (1968)), rat small and large intestine (Powell and Malawer (1968)) and rabbit gall-bladder in the presence of impermeant solute (Whitlock and Wheeler (1964)).

2. The isotonic approximation assumes that osmotic equilibration occurs rapidly in the lateral space, this assumption is probably invalid (i.e. 1 above). Osmotic equilibration may also occur in underlying submucosal tissue (Marro and Germagnoli (1966)).

3. No attempt has been made to include the possible effect of tight-junction permeability in the analysis.

Modification of the Diamond and Bossert model is, however, necessary in that two assumptions regarding most leaky epithelia made by Diamond and Bossert are invalid; namely the tight-junction closing the lateral space is permeable to both salt and water (see above) and the distribution of active transport sites along the serosal border is not discrete (i.e. concentrated at the proximal end of the channel) but rather is continuous, (Stirling (1972)). Also of importance in a reconsideration of the Diamond and Bossert model is the role of the underlying tissue or basement membrane, and the nature of pressure-modulated changes in epithelial transport properties in conditions such as saline loading (see above).

Such modifications to the simpler model of Diamond and Bossert have been attempted (Sackin and Boulpaep (1975); Huss and Marsh (1975)).

Sackin and Boulpaep develop two general models; a continuous model which is a derivative of the standing gradient model but assuming different boundary conditions; and a discontinuous model which is derived from the Curran and McIntosh model but which incorporates additional compartments identified with epithelial structures. The

behaviour of these models is such that changes in net transport during saline diuresis are adequately described without additional assumptions concerning change in junctional permeability (see above). The existance of a 'basement' membrane results in deviation of absorbate tonicity from isosmotic values, the degree of deviation depends on the basement membrane permeability coefficients for salt and water. Concentration profiles in the lateral space generated by the continuous model are flat; this is a consequence of salt and water influx across the tight-junction into the lateral space and of a uniform pump-site distribution.

The model developed by Huss and Marsh is similar in detail and conclusions. Pressure within the osmotic channel (lateral space) is treated as a variable (independent and dependent). The lateral space is treated as a distensible compartment with a deformation under pressure similar to a thin-walled tube. The major conclusions of this model are:

1. that basement membrane parameters (salt and water permeability) control absorbate tonicity.

2. that NaCl diffusion across the tight-junctions controls in part the rate of absorption.

3. that NaCl concentration variation within the lateral spaces controls NaCl diffusion across the tight-junctions; variation in transmural pressure modulates NaCl concentration.

4. that no pressure-induced change in tight-junctional permeability is needed for pressure to modulate absorption.

It is clear from the above considerations that ion (Na) flux through the paracellular pathway in actively transporting tissues may not be simple. In particular, asymmetry in flux across the tight-

junction is to be expected.* This could arise from

1. back diffusion of Na through the tight-junction from regions of high NaCl concentration located within the lateral spaces.

2. solute-solvent coupling within the tight-junction due to osmotic water flow across the junction into the lateral spaces.

Evidence bearing on these effects in rabbit ileum is presented in Chapters 3 and 4. Solute-solvent coupling interactions have been demonstrated in the paracellular pathway of various epithelia with imposed transepithelial gradients (Berry and Boupaep (1975) (necturus proximal tubule); Fordtran, Rector and Carter (1968) (human small intestine); Levitt, Hakim and Lifson (1969) (*in vivo* dog jejunum)).

3. Transcellular Ion Movements

(i) Measurement of ion fluxes across the mucosal and basal-lateral borders of the epithelial cell

In short-circuited rabbit ileum with identical mucosal and serosal bathing solutions it is generally agreed that there is a net absorption (mucosa to serosa movement) of both Na and Cl (Field et al (1971, 1972); Field (1971); Schultz and Curran (1974)). This represents an active movement of Na and Cl. The existence of active Cl movement was not a consistent feature of early *in vitro* work (see Chapter 5).

*Frizzell and Schultz (1972) and Desjeux, Tai and Curran (1974) have examined Na permeability through the paracellular pathway. These authors consider that Na flux conforms with the laws of simple diffusion since agreement with the Ussing flux ratio is observed. In these experiments solute-solvent interactions are probably second-order effects in comparison with the electrical gradients used to measure paracellular ion flux.

Active ion movements result from asymmetries generated in the transcellular pathway, in particular, across the mucosal and basal-lateral boundaries of the epithelial cell-layer. The transport processes involved in active ion movements may be located at either or both of these cellular membranes. It is evident that the mucosal and basal-lateral membranes differ in their physiological function since biochemical studies have shown that these membranes are markedly different in their lipid and protein compositions (Kawai et al (1974); Douglas et al (1972); Fujita et al (1972, 1973); Stirling (1972)).

Separation of diffusional flux through the shunt pathway from total ion flux, allows the assessment of transcellular ion movements (see above and Chapter 4). Measurement of the ion fluxes across both cell membranes requires special methods.

Frizzell et al (1973) and Nellans et al (1973) have measured the unidirectional influx of Na and Cl across the mucosal boundary. These authors established that their measurements provided a true unidirectional flux, since uptake was linear over the period of measurement (thirty seconds). Together with estimates of the transmural bidirectional fluxes (mucosa to serosa and serosa to mucosa) for both Na and Cl, these measurements provide the basis for measuring efflux across the mucosal boundary. Estimates may also be made of flux across the serosal boundary (Schultz and Curran (1974); Frizzell et al (1973); Schultz et al (1967).

Inaccuracies arise as a result of the need for measurements from two or three separate experiments.

Naftalin and Curran (1974) have developed a method for estimating the unidirectional fluxes of galactose across the mucosal and basal-lateral cell membranes from measurements of the steady state transmural bidirectional fluxes together with an estimate of the ratio of isotope

originating from the mucosal and serosal solutions within the tissue fluid. All these measurements may be made from a single piece of tissue in a single experiment. The application of this methodology to ion fluxes is considered in Chapters 2,4,5 and 6.

(ii) The electrochemical gradient across the mucosal and basal-lateral cell membranes

Any study of ion movement across a membrane must examine the driving forces for ion movement that exist across the membrane. Considerable attention has been paid to the measurement of trans-membrane potentials and intracellular ion concentrations (activities) in intestine. A consensus has not yet been reached.

With regard to the measurement of electrical potentials the lack of agreement may be partially due to the difficulty in measuring the electrical potential across both the mucosal and serosal (basal-lateral) membranes simultaneously; penetration of the epithelial cells by micro-electrodes from the basal-lateral side being difficult in mammalian preparations due to the presence of submucosal tissue layers.

Rose and Schlütz (1970, 1971) have measured the transmural and transmucosal potential differences in rabbit ileum in vitro simultaneously. Transmucosal potential was measured by 3M KCl glass micro-electrodes to be -36mv (cell interior negative). The transmural P.D. was +2 to +5mv (serosa positive). The potential difference across the basal-lateral membrane was inferred to be ≃ -39mv (cell interior negative) since the transmural P.D. represents the difference between the transmucosal and transmural potential differences (Rose and Schultz (1971); Schultz (1972)). The sign and magnitude of the transmucosal P.D. may be entirely due to the potential across the serosal membrane and to the existence of the shunt pathway which allows coupling between the basal-lateral membrane and the mucosal membrane (Schultz (1972)).

Replacement of Na by choline in the Ringer's solution bathing the mucosal surface leads to a hyperpolarization of the transmucosal potential, indicating that Na conductance contributes to this potential. Addition of actively transported sugars leads to a mucosal depolarization. Consideration of the change in transmural P.D. in both these cases indicates that there is a concurrent change in the potential across the basal-lateral membrane (i.e. a depolarization in the presence of actively transported sugar, and a hyperpolarization upon Na replacement). In the presence of metabolic inhibitors and ouabain Rose and Schultz reported that a sugar-dependent depolarization of the mucosal membrane could be elicited. No change in transmural potential occurred. This led to the suggestion that a portion of the trans-serosal potential was electrogenic in nature.

Similar findings to those of Rose and Schultz have been reported for bullfrog small intestine (White and Armstrong (1971) and for newt kidney tubule (Maruyama and Hoshi (1972)).

Barry and Eggenton (1972a,b) have measured the transmucosal, trans-serosal and transmural potential differences of rat jejunum using everted sac preparations stripped of their serosal muscle layers. Transmucosal and trans-serosal potentials were taken to be the difference between a central micro-electrode within the epithelial cell and indifferent electrodes in the mucosal and serosal solution respectively. These authors found a low potential across both mucosal and serosal boundaries (-9 and -12mv respectively, cell interior negative). Addition of actively transported sugars to the bathing media resulted in increases in the transmural potential; no change in transmucosal potential was observed. A hyperpolarization of the serosal potential thus accounted for the change in transmural potential. Metabolic inhibitors and ouabain (1.0mM) abolished the sugar-dependent trans-

serosal hyperpolarization. Mucosal Na replacement by mannitol or Tris Cl resulted in Cl transmucosal hyperpolarization. No change in the serosal membrane potential was recorded. These observations led Barry and Eggenton to suggest that rat jejunum is a 'tight' epithelium without a significant shunt-pathway.

Similar results to those of Barry and Eggenton have been reported in a variety of tissues, such as tortoise small intestine (Gilles-Baillien and Schoffeniels (1965); Wright (1966); tortoise and hamster small intestine (Wright (1966) and rat jejunum and small intestine (Lyon and Sheerin (1971)).

A consistent feature of all studies of electrical potential differences across the mucosal and basal-lateral membranes is the electrogenic nature of the Na-K ATPase located on this membrane (see Thomas (1972)). This is evident from the action of ouabain and of metabolic inhibitors upon transmembrane and transmural potentials (see above). The lack of effect of a change in trans-serosal P.D. when transmucosal P.D. is changed is entirely consistent with the presence of a high-conductance shunt-pathway; the shunt will attenuate change in potential across either membrane (Schultz and Curran (1974)). Disagreement thus centres around the magnitude of the potential across the mucosal and serosal membranes and the effects of actively transported sugars on transmucosal potential. Differences may represent species variation.

Recently (Zeuthen and Monge (1975)) a study has been made of the electrical potentials in epithelial cells of rabbit ileum in vivo. These authors examined the potential profile across epithelial cells using micro-electrodes advanced into the epithelial cells at 5 μm gradations. The position of recording within the epithelial cells was determined by iontophoretically injecting dye (Porscine yellow) followed later by histological examination. The main conclusions were

that the electrical potential across the mucosal membranes is low (-6.6mv cell interior negative), whereas that across the serosal membrane is large (-44mv cell interior negative). Furthermore, there was a smooth gradient of potential along the epithelial cell. Since no attempt was made in previous studies to locate, histologically, the position of recording, Zeuthen and Monge conclude that Rose and Schultz (1971) consistently recorded close to the serosal membrane, whereas Barry and Eggenton (1972) recorded close to the mucosal membrane. Gradients of electrical potential have been observed by Lyon and Sheerin (1971) in rat intestine and by Chouldhury and Snell (1965) in toad-bladder epithelium.

Clearly more work is needed to define the electrical potentials existing in intestinal epithelial cells.

The measurement of intracellular ion concentrations within intestinal epithelial cells present certain difficulties. A large proportion of tissue ion content is due to non-epithelial tissue (muscle layers and connective tissue) and to the presence of an extracellular space that comprises 30% of the tissue water (Koopman and Schultz (1969)). Additionally, ion-binding or sequestration within the intracellular or extracellular compartments will effect measurements of ion concentrations based upon measurements of total tissue ion content (Rotunno et al (1973)).

Schultz et al (1966); Koopman and Schultz (1969); Frizzell et al (1973) have approached the measurement of intracellular epithelial Na, K and Cl concentrations from volume-averaged determinations of tissue ion content corrected for extracellular ion content on the basis of the inulin space. The contribution of non 'epithelial' tissue was reduced by the use of strips of rabbit intestine from which the serosa and muscle layers had been removed.

For Na and K determinations it was shown that both isotopic and and flame photometer measurements gave identical results (Schultz et al (1966)). This indicates that the tissue cation content is freely exchangeable. However, for Cl determination it was demonstrated that chemical analyses of tissue Cl gave consistently greater values compared with isotopic determinations. This indicates that a portion of the total tissue Cl is bound (Frizzell et al (1973)). Values for exchangeable intracellular Na, K and Cl concentrations in a medium containing 140mM Na, 10mM K and 145mM Cl were 40-50mM $[Na]$, 100mM $[K]$ and 58mM $[Cl]$.

The effect of various actively-transported sugars and amino acids upon 'intracellular' Na and K concentrations has been examined by Koopman and Schultz (1969). All actively-transported solutes led to a decline in intracellular $[K]$. An increase in intracellular $[Na]$ was observed in the presence of galactose. This is considered in detail in Chapter 3.

Frizzell et al (1973) report that theophylline and cyclic AMP result in a significant decrease in intracellular Cl concentration. The effect of theophylline upon the mucosal potential difference was not examined.

More direct determinations of intracellular ion activities may be made by ion-sensitive micro-electrodes (Hinke (1959); Thomas (1970)).

Lee and Armstrong (1972) have measured Na and K activities within bullfrog small intestine epithelial cells <u>in vitro</u> by glass micro-electrodes. Bathing Ringer's included 140mM Na and 5mM K. They conclude that intracellular K activity is six-fold greater than Na activity (0.084:0.014). Cpmparison of ion activities with ion concentrations determined in a separate experimental series utilizing inulin as the extracellular marker (Armstrong et al (1970)) indicated

that a significant proportion of 'intracellular' Na was osmotically inactive (the activity coefficient was $\simeq 0.5$) whereas the greater proportion of the intracellular K was osmotically active (the activity coefficient was $\simeq 1.0$). A possible explanation for the discrepancy between Na activity and concentration is considered in Chapter 3. The actively transported sugar 3-O-methyl glucose led to a decrease in both Na and K activities.

Zeuthen and Monge (1975) have examined intracellular epithelial ion activity in in vivo rabbit ileum. Bathing mucosal solutions contained 145mM Na, 5mm K and 150mM Cl. They conclude that there exists along the length of each epithelial cell a gradient of potassium and Cl activity. No measurements of Na activity were made. K activity increased from 50mM at the mucosal pole of the epithelial cell to 160mM at the serosal pole. The volume-averaged concentration is similar to that found by Koopman and Schultz (1969) and to Schultz et al (1966). Cl activity decreased from 80mM at the mucosal pole of the cell to 10mM at the serosal pole.

The existence of ion activity and electrical gradients within the epithelial cell is controversial. Clearly, their existence would require a reassessment of the driving forces involved in trans-membrane ion movement. Confirmation is needed.

(iii) Na and Cl fluxes across the mucosal and basal-lateral membranes

(a) Mucosal fluxes of Na and Cl The electrochemical gradient for Na is directed inwards across the mucosal membrane into the cell. The magnitude of the fluxes across the mucosal membrane is dependent on the membrane permeability. The ratio of influx/efflux may be computed from the Ussing (1949) relationship:

$$\frac{\text{influx}}{\text{efflux}} = \frac{Na(out)}{Na(in)} \cdot \exp \frac{F\Delta\Psi}{RT}$$

where Na(out) is the bulk Na concentration in the mucosal solution, Na(in) is the Na concentration (activity) in the cellular compartment and $\Delta\Psi$ is the trans-membrane potential. F, R and T have their usual meanings.

The Ussing ratio ranges from approximately 6 to 20 for a Ringer containing 140mM Na depending on the value of potential and activity difference chosen (see above).

Schultz et al (1967) and Curran et al (1967) have measured Na influx across the mucosal membrane by measuring a unidirectional (thirty second) uptake of ^{22}Na from the mucosal solution. Correction of the measured influx for a component of influx into the shunt-pathway gave influx across the mucosal membrane as 13 μmoles cm^{-2} hr^{-1}. This uptake was a linear function of the mucosal Ringer Na concentration (Curran et al (1967)). However, this probably represents a saturable process with a low affinity for Na, since a significant proportion of influx was subject to inhibition by Li, K or guanidinium (Schultz et al (1967); Frizzell and Schultz (1972)). Influx is not affected by transconcentration effects (Schultz et al (1967)) nor is it affected by 0.1mM ouabain (Chez et al (1967)).

Net Na flux determined in identical experimental conditions to those in which unidirectional influx measurements were made, was found to be 1-3 μmoles cm^{-2} hr^{-1}. Efflux across the mucosal membrane was, therefore, 10-12 μmoles cm^{-2} hr^{-1} (Schultz et al (1967)). The observed ratio of fluxes across the mucosal membrane (influx/efflux) is 1.2 - 1.6. It may be concluded that Na flux across the mucosal membrane does not adhere to the laws for a simple diffusional flux. Efflux from the cell into the mucosal solution may involve an active process.

From measurements of the electrical potential and freely-exchangeable intracellular Cl concentration, Nellans et al (1973) concluded that the electrochemical potential of the exchangeable Cl pool was greater than that of the bathing medium (but see above). The existence of Cl absorption (mucosal to serosa movement) indicates that the observed flux ratio for the mucosal membrane (influx/efflux) is > 1. This indicates that Cl influx may be an active process. Nellans et al (1973) have measured unidirectional Cl influx across the mucosal membrane. Influx is a saturable function of Ringer Cl with a Vmax of 19.8 μmoles cm^{-2} hr^{-1} and a K_M of 28mM. Cl influx is inhibited by other anions in the sequence. $SCN > NO_3 > I > Br$. Cl influx is not subject to trans-concentration effects (Nellans et al (1973)).

Measurement of the effect of replacement of Na by choline and of Cl by SO_4 upon Cl and Na influxes across the mucosal boundary (Nellans et al (1973)) have indicated that there is a reduction of Na influx by Cl-replacement and a reduction of Cl-influx by Na replacement. The magnitude of these reductions is indicative of a coupled process for NaCl entry. Nellans et al developed a kinetic model involving random combination of Na and Cl with a membrane component to form a ternary complex. The ternary complex could then act as a carrier for NaCl across the mucosal membrane. Net Cl movement may be directed against an electrochemical gradient for Cl, since movement of the ternary complex could utilize the inwardly-directed electrochemical gradient for Na. Na may be partially replaced by Li, and Cl may be partially replaced by I in the coupled process. The NaCl influx process is unaffected by metabolic inhibitors such as cyanide.

Na and Cl movements may, therefore, involve three pathways for movement across the mucosal cell membrane.

(b) Serosal fluxes of Na and Cl Na movement from the cell into the serosal solution is directed against a large electrochemical gradient. The existence of a net Na flux from mucosa to serosa in control tissues indicates that efflux > influx, hence it is likely that Na movement is active.

Extrusion of Na from the cells across the basal-lateral membrane appears to be mediated by a Na-K ATPase. This has been located at the serosal membrane by biochemical (Fujita et al (1972)), autoradiographic (Stirling et al (1972)) and physiological methods (Schultz and Zalusky (1964); Csaky and Hara (1965)).

Schultz and Zalusky (1964) and Frizzell and Schultz (1972) consider that serosa to mucosa Na flux is entirely via the extracellular shunt-pathway. These authors consider, therefore, that Na flux is completely rectified across the basal-lateral membrane. Schultz et al (1967) estimate flux from the cell to the serosal solution to be 2-4 μmoles cm^{-2} hr^{-1} whereas influx across the basal-lateral membrane into the cell is only 1 μmole cm^{-2} hr^{-1}. The question of a significant flux from serosal solution to the cell has recently been examined by Desjeux et al (1974). The application of the new methodology described in Chapter 2 provides new evidence concerning the nature of Na fluxes across the basal-lateral cell membrane (Chapter 4).

Little is known about Cl fluxes across the basal-lateral membrane. Frizzell et al (1973) consider that Cl flux across this membrane is entirely passive. Keynes (1969) has suggested a possible mechanism for coupling of net Cl and Na movements in leaky epithelia such as intestine. Electrogenic movement of Na across the basal-lateral membrane may generate a passive net flow of Cl. Transepithelial potentials of $\sim$ 2mv may be reconciled with the attenuation of the electrogenic P.D. within the lateral space by the cable properties of

the basal-lateral membrane. The space-constant for this membrane should be extremely short. Such a passive mechanism of coupling may explain the tight stoichiometry observed for the coupled NaCl influx process (see above).

(iv) The effect of secretory agents such as theophylline, cholera toxin and cyclic adenosine monophosphate (c-AMP) upon trans-cellular ion fluxes

Although the small intestine is normally viewed as an absorptive organ, active secretion of salt and water may occur in physiological and pathological situations (Schultz et al (1974); Hendrix and Bayless (1970)).

Most investigations on the effect of secretagogues, such as theophylline, cholera toxin and cyclic AMP, upon intestinal ion transport have concentrated upon the measurement of the transepithelial bidirectional (mucosa to serosa and serosa to mucosa) fluxes of both Na and Cl (Sheerin and Field (1975); De Jonge (1975); Powell, Binder and Curran (1973); Al-Awquati, Cameron and Greenough (1973); Field (1971)).

Field and his co-workers have shown that theophylline (Sheerin and Field (1975)), cholera toxin (Field, Fromm, Al-Awquati and Greenough (1972)) and 3'5' c-AMP (Field (1971)) abolish the net absorption of Na in rabbit ileum by decreasing mucosa to serosa (m-s) Na flux without concurrently changing serosa to mucosa (s-m) flux. Net Cl absorption is changed to net secretion by theophylline, cholera toxin and c-AMP as a result of a decreased m-s Cl flux, together with an increased s-m flux. Sheerin and Field (1975) have recently reported that the increase in s-m Cl flux due to theophylline is quantitatively more important than the decrease in m-s flux. This view is supported by the findings of De Jonge (1975) who found that both Na and Cl secretion due to cholera in rat small intestine were

primarily mediated by increases in both the s-m fluxes of both Na and Cl. A range of effects of secretagogues upon bidirectional ion fluxes has been reported and is likely to occur as inter-animal variation in a single experimental series (Sheerin and Field (1975)).

As previously emphasized, the interpretation of measurements of transmural ion fluxes are complicated by the presence of both extracellular and transcellular pathways for ion flow. For secretion by intestine there also exists the possibility that there is an anatomical separation between the pathways for absorption and secretion. Recent evidence by De Jonge (1975) supports the notion that absorption and secretion are properties of the same population of epithelial cells.

The secretory state induced by theophyllins and cholera enterotoxin is associated with changes in the paracellular pathway; these result in:

(a) time-dependent changes in transepithelial resistance (Powell (1974); Nellans, Frizzell and Schultz (1974)),

(b) changes in passive ionic selectivity (Powell (1974)).

The role of the paracellular component of transepithelial ion movement, therefore, needs to be carefully assessed in relation to the observed changes in bidirectional fluxes.

Direct measurements of influx across the mucosal boundary have previously been made (Frizzell et al (1973); Nellans et al (1973)) in an attempt to define the nature of action of secretory agents on the basis of unidirectional fluxes across a single membrane. These studies have indicated the presence of a mucosal process providing coupled (electroneutral) flows of Na and Cl ions; separate entry pathways for Na and Cl alone were also postulated (see above). Theophylline and c-AMP were shown to reduce the magnitude of the neutral coupled influx

of NaCl. Nellans et al (1974) have attempted to relate these influx studies to bidirectional flux measurements. They conclude that theophylline mediated Cl secretion (due primarily to a decrease in m-s Cl flux) and the abolition of net Na absorption (due to a decrease in m-s Na flux) can be explained by the inhibition of a neutral transport process for NaCl at the brush-border. Efflux is presumed to be unaffected by theophylline, c-AMP or cholera toxin. Serosal to mucosal Na flux, in this model, is thought to be due entirely to movement through the paracellular pathway (Schultz and Zalusky (1964); Frizzell and Schultz (1972)). Cl-independent entry at the mucosal membrane is recirculated through the NaCl coupled process back into the mucosal solution.

Data in contradiction to this simple model have recently been supplied by Nellans, Frizzell and Schultz (1975). They report that acetazolamide, though inhibiting the NaCl entry process, does not result in Cl secretion. Theophylline in addition to acetazolamide does, however, result in net Cl secretion. They conclude that net secretion may involve de novo stimulation of Cl exit across the mucosal boundary.

The concept of a separate pump for secretion is implicit in the views of Powell, Binder and Curran (1972 and 1973) and Binder, Tai and Curran (1973). These workers have proposed that secretion, in both guinea-pig and rabbit ileum, is mediated by a neutral NaCl pump on the mucosal membrane. Serosa to mucosa Na flux was viewed to be transcellular (rather than extracellular as envisaged by Schultz and co-workers).

In support of a separate pump Al-Awquati, Field and Greenough (1974) have shown that ethacrynic acid inhibits Cl secretion and

reverses the decrease in Na flux induced by theophylline, c-AMP and cholera toxin.

Field (1971) has provided evidence that the secretion induced by theophylline is not neutral, i.e. it is electrogenic, resulting in an increased open-circuit potential and short-circuit current. However, the dependence of the electrical potential and short-circuit current upon Na suggests that these changes are secondary to a neutral system (Sheerin and Field (1975)).

The question concerning the route of serosa to mucosa Na flux (transcellular or paracellular) in the presence of secretory agents and control tissues has recently been examined in detail, (Desjeux, Tai and Curran (1974)). These workers conclude that a significant transcellular pathway for Na exists in the serosa to mucosa direction. Theophylline was shown to stimulate this transcellular Na flux. A dependence on Ringer HCO_3 was demonstrated since in HCO_3-free media s-m transcellular flux was abolished.

If s-m Na flux were mediated entirely by Na diffusion through the paracellular pathway then a net secretion of Na could never be observed under voltage-clamp at zero P.D. Sheerin and Field (1975) have shown a net secretion of Na in the presence of theophylline in high $[HCO_3]$ buffers (50mM). This result thus favours the view that net secretion is mediated by a transcellular route. The route of serosa to mucosa Na flux is examined in Chapter 6.

The application of the new methods of measuring unidirectional ion fluxes across the mucosal and basal-lateral membranes provides new information concerning the mechanism of reversal of the absorptive state of ileum.

(v) Coupled transport of sodium and organic solutes: interaction between Na and galactose transport in ileum

Crane (1962 and 1965) first proposed that the difference in Na concentration across the mucosal membrane could provide the basis for active intestinal sugar transport. Influx of solute is influenced by the extracellular Na concentration whereas efflux of solute is influenced by the intracellular Na concentration. Net solute movement is thus achieved by the difference in local Na concentrations across the mucosal membrane (Schultz and Curran (1970)); this is maintained by the Na-K ATPase at the basal-lateral membranes.

A test of the Na-gradient hypothesis is to consider the energetic adequacy of the Na-gradient in explaining the observed intracellular solute accumulation.

In a simple isolated cell system such as is available with Ehrlich ascites tumour cells, this question has not yet been satisfactorily answered. Schafer and Heinz (1971) have shown that the amino acid α-aminobutyric acid (AIB) is taken up into Ehrlich cells against its own concentration gradient in the presence of adverse Na and K gradients. Gibb and Eddy (1972) suggest that the operation of an electrogenic Na pump in Ehrlich cells may cause hyperpolarization of the cell membrane resting potential, thus allowing the concentration of AIB since the electrochemical gradient for Na is inwardly directed. Heinz (1974) considers that nuclear sequestration of Na within the nucleus of Ehrlich cells may lead to an underestimate of the driving force for Na-dependant solute accumulation. Geck et al (1974) provide evidence that AIB accumulation is not coupled to metabolic energy via hydrolysis of ATP. This suggests that ion gradients have to supply all the energy required for the observed solute accumulation. Heinz and Geck (1974) suggest that the Na gradient is indeed adequate to

explain the observed solute accumulation. A metabolic linkage cannot be ruled out since Geck et al (1972) have found that the efficiency of coupling between Na and AIB flows decreases in the presence of metabolic inhibitors.

Consideration of the energetic adequacy of the Na-gradient hypothesis in ileum cannot, as yet, be made due to the difficulties in defining the distribution and activities of the intracellular solutes (see above).

Schultz and Curran (1970 and 1974) have reviewed the evidence for the existence of the Na-gradient mechanism for solute uptake in ileum. The evidence, though indirect, supports the Na-gradient hypothesis. Unidirectional Na influx across the mucosal membrane of *in vitro* terminal rabbit ileum has been stoichiometrically related to sugar and amino acid influx, (Goldner et al (1969); Curran et al (1967)). Reversal of the normal Na-gradient leads to a transient depletion of tissue solute (alanine) (Hajjar et al (1970)). Alanine gradients from cell to mucosal solution and mucosal solution to cell have also been shown to drive Na influx and Na efflux across the mucosal membrane (Curran et al (1970)). Ouabain and other metabolic inhibitors do not markedly effect the unidirectional sugar and Na influx (Goldner, Hajjar and Curran (1972)).

Recent attempts to examine the validity of the Na-gradient hypothesis have centred around the study of brush-border membrane vesicles prepared from rat small intestine (Hopfer et al (1973)) and rabbit renal cortex (Aronson and Sacktor (1974)). These vesicles possess an intact 'carrier' for D-glucose which shows many characteristics of the native system such as dependence on medium $[Na]$, specificity for sugars, inhibition by phlorizin and inhibition by other actively-transported sugars. Control experiments using various

external solution osmolarities indicate that transport of sugar and Na is due to uptake into an intervesicular space rather than representing binding to the isolated membranes. Apparent 'uphill' movement of sugar was observed. This declined over a period. The extent of the dependence of uphill movement on the Na-gradient cannot, however, be assessed since both these parameters are time-dependent. 'Uphill' transport may represent an artifact of vesicular swelling. Volume studies of these vesicles are needed.

Most data challenging the Na-gradient hypothesis may be considered to be inconclusive. For instance, Kimmich (1970) has studied the uptake of sugars into isolated intestinal epithelial cells of the chick. Reversal of the normal Na-gradient did not prevent net accumulation of D-galactose. Conversely, ouabain inhibited sugar uptake even in the presence of ion gradients favouring accumulation. These studies have been extensively criticized since the chick cell in culture looses its normal polarity with respect to sugar movements.

In vivo studies in human, dog and rat intestines have revealed that total replacement of luminal Na by mannitol does not affect the rate of active sugar absorption (Saltzman et al (1972)). Schultz et al (1974) have stressed the importance of Na flux from the blood (serosal) side of the gut to the lumen through the high conductance shunt-pathway. The existence of an unstirred layer close to the mucosal surface may thus result in local Na concentrations in the region of the brush-border approaching those of the plasma.

Recently, more serious data have been obtained which contradict the Na-gradient hypothesis (Naftalin and Holman (1974)). Using methods to measure the four unidirectional fluxes of galactose across the mucosal and basal-lateral membranes (Naftalin and Curran (1974)) it was shown that raising Ringer $[Na]$ from 0 to 140mM resulted in a

reciprocal rise in the entry permeability and a fall in the exit permeability of the brush-border membrane for galactose. Tissue [Na] increased as extracellular [Na] was raised. The Na-gradient hypothesis predicts that in this situation galactose exit permeability across the brush-border should also increase. The maximal permeability ratio of the brush-border to galactose exceeded the inverse Na distribution ratio between the Ringer's solution and the tissue twenty-fold. Ouabain was found to abolish the Na-dependent increase in entry permeability and the Na-dependent decrease in exit permeability. Similar results have been obtained with 3-O-methyl-D-glucose and β-methyl-D-glucose (Holman and Naftalin (1976)).

A model (the convective-diffusion model) has been described to explain the reciprocal rise and fall in entry and exit permeability upon activation of the Na-pump (Naftalin and Holman (1974)). This involves mass flow of water across narrow channels in the brush-border generated by the activity of the Na-K ATPase. Transcellular water movements probably result from deposition of hypertonic NaCl within the lateral spaces by Na-pump activity.

Uphill accumulation of sugars within the epithelial cell is thought to result from reflection of sugar at the serosal pole of the cell. Net transepithelial flux of galactose is sufficiently high and serosal permeability is sufficiently low to be consistent with this view (Holman and Naftalin (1975a)).

Inhibition of mucosal galactose transport by ouabain or by competition with other sugars results in a reciprocal increase in exit permeability and a decrease in entry permeability across the mucosal boundary (Holman and Naftalin (1975a)). Similar observations on galactose fluxes across the brush-border of hamster jejunum have been made (Baker, Lo and Nunn (1974)). Mucosal anaerobiosis for ten

minutes resulted in a fall in galactose entry permeability and an increase in galactose exit permeability across the brush-border. These changes occurred without significant change in intracellular ion concentration. This is strong evidence against the Na-gradient hypothesis and lends support to the convective-diffusion model.

Naftalin and Holman (1976) have shown that net absorption and accumulation of D-galactose, β methyl D-glucose and 3-O-methyl-D-glucose are observed even when Na in the mucosal solution is replaced by choline. Leakage of Na from the serosal solution was insufficient to account for the observed net absorption and accumulation. This result indicates that active sugar transport can occur in the direction opposite to that of the brush-border Na-gradient.

The validity of the Na-gradient model versus the convective-diffusion model is further investigated by examining the effects of galactose upon Na fluxes (Chapter 4) across both mucosal and basal-lateral membranes.

CHAPTER TWO

MATERIALS AND METHODS

A. Materials

1. Chemicals

All chemicals used were of ANALAR grade except where stated. The base 2,4,6, triaminopyrimidine was obtained from Aldrich Chemicals. The manufacturer's assay gave the purity of this compound as 99%. Ouabain (stropthanthin G), D-galactose and theophylline were obtained from B.D.H. Ltd. 2, 5, diphenyl-oxazole (P.P.O.) was obtained from Fisons (Loughborough).

2. Radioisotopes

^{14}C-labelled D-galactose, ^{3}H-labelled D-galactose, ^{125}I-labelled albumin, ^{14}C-labelled inulin, ^{22}Na, ^{24}Na, ^{36}Cl and ^{82}Br were all obtained from the Radiochemical Centre Amersham. ^{125}I-labelled albumin, ^{22}Na, ^{24}Na, ^{36}Cl and ^{82}Br were obtained as aqueous isotonic (300 m.osmolar) solutions in NaCl (or NH_4Br for ^{82}Br). All dilution of isotopes was made with the standard Ringer's solution (see below). ^{14}C-labelled D-galactose, ^{3}H-labelled D-galactose and ^{14}C-labelled inulin were obtained as solids in vacuo; these were dissolved with the standard Ringer's solution.

3. Experimental Animals

Male, white New Zealand rabbits (2 to 3kg) were obtained from a local supplier. Only healthy rabbits were used for experimental purposes. All rabbits were maintained prior to experimentation on a normal laboratory diet with unrestricted access to water.

B. Methods

1. Unidirectional flux calculations

Fig. 1 shows a three compartment system representing a piece of ileum (2) contained between two bathing solutions, namely the mucosal (1) and serosal (3) solutions. It is assumed that the absorptive layer is composed of a single homogeneous layer of cells, that compartments 1, 2, and 3 are well stirred and that the mucosal and serosal unstirred layers do not rate-limit transepithelial ion flux.

Transepithelial ion movements from mucosa to serosa (J_{13}) and from serosa to mucosa (J_{31}) are the result of the four unidirectional fluxes J_{12}, J_{21}, J_{23} and J_{32} across the mucosal and serosal boundaries (that is the mucosal and basal-lateral (serosal) cellular membranes of the epithelial cell layer).

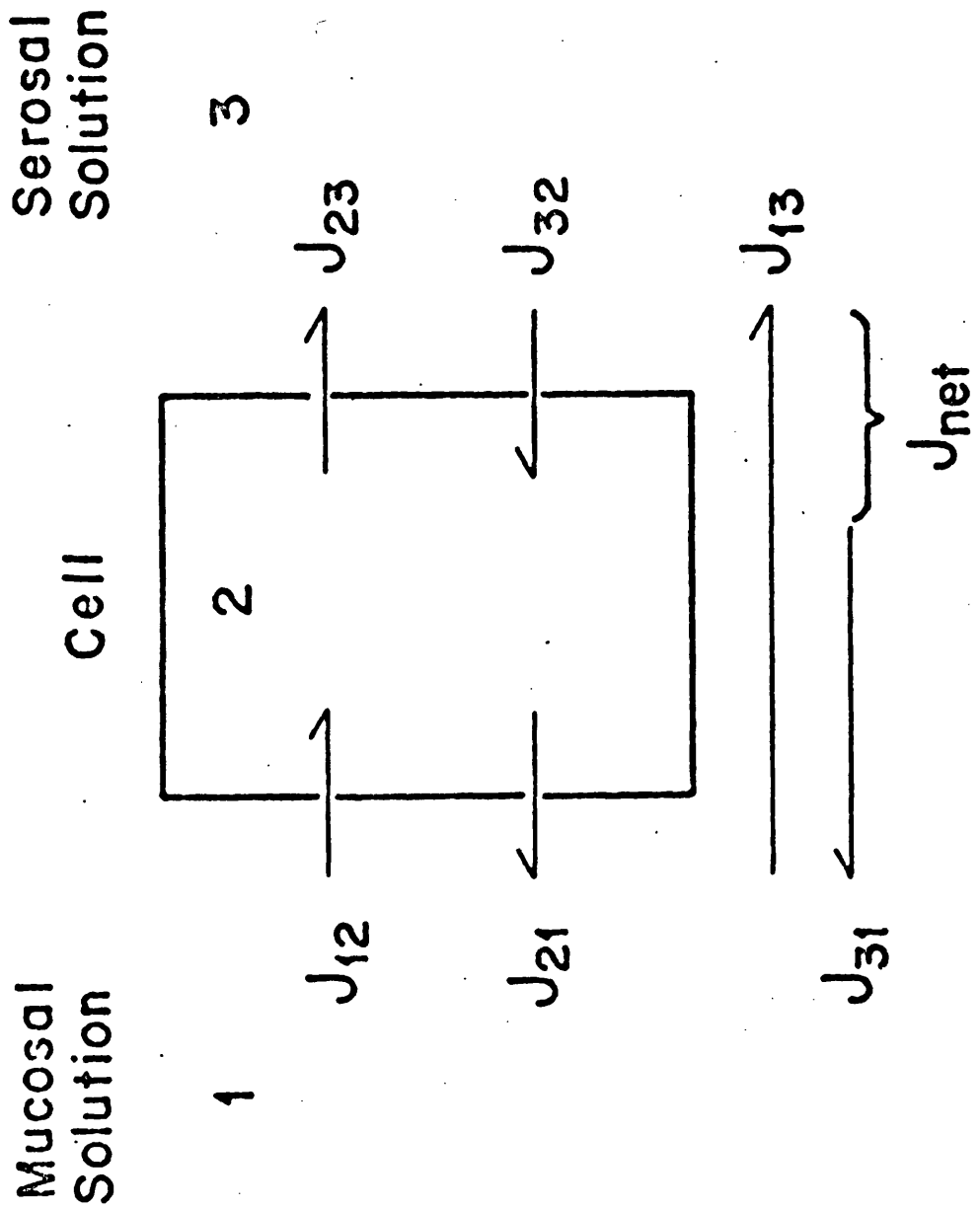
Naftalin and Curran (1974) following the approach of Ussing and Zerahn (1951) (later developed by Schultz et al (1967)) have theoretically demonstrated that the four unidirectional fluxes of D-galactose across the mucosal and serosal cellular membranes may be calculated from three independent measurements in a single experiment. At steady-state both the mucosa to serosa flux J_{13} and the serosa to mucosa flux J_{31} are measured using ^{3}H-labelled and ^{14}C-labelled galactose tracers respectively. Following these measurements an estimate is obtained of the ratio (R) of the specific activities of radioisotopes originating from the mucosal and serosal solutions within the cellular compartment (2).

With equal concentrations of galactose in the mucosal and serosal solutions the ratio R is given by the following relationship:

$$R = \frac{(cpm)^{I}_{2}}{(cpm)^{C}_{2}} \times \frac{(cpm\ /\ cm^{3})^{C}_{3}}{(cpm\ /\ cm^{3})^{T}_{1}}$$

FIGURE 1

3 compartment representation of <u>in vitro</u> rabbit ileum illustrating the measured and calculated unidirectional fluxes.



where subscripts 1, 2 and 3 refer to the mucosal, cell and serosal compartments respectively; superscripts T and C refer to ^{3}H and ^{14}C-labelled D-galactose respectively. The four unidirectional fluxes may be calculated from the following relationships (Naftalin and Curran (1974)).

$$J_{12} = J_{31} R + J_{13}$$

$$J_{21} = J_{31} (1 + R)$$

$$J_{23} = J_{13} (1 + 1 / R)$$

$$J_{32} = J_{31} + J_{13} / R$$

The three assumptions regarding the derivation of these equations are of relevance in the application of the method to the measurement of unidirectional Na and Cl fluxes:

1. There is no significant shunt permeability for ion flux compared with transcellular permeability.

2. The tissue behaves kinetically as a single compartment (such that no inhomogeneity in the tissue isotope R exists within the tissue).

3. The measured variables J_{13}, J_{31} and R are at steady-state (i.e. the net-flux across the tissue is equal to the net-flux across the individual boundaries).

It is known (see Chapter 1) that transepithelial ion flux in low-resistance epithelia may be largely via extracellular channels. In rabbit ileum there is good reason to believe that these channels are cation-selective (Chapter 1). In consideration of transepithelial Na fluxes it is clear that the extracellular pathway has high permeability for Na in comparison with transcellular Na fluxes (Frizzell and Schultz (1972)). Extracellular Cl permeability is lower than Na permeability. The bulk of

transepithelial Cl flux is transcellular (Frizzell and Schultz (1972), Munck and Schultz(1974)).

Estimation of transcellular Na fluxes, therefore, requires a measurement of the component of flux which traverses the tissue by the extracellular pathway. Unidirectional flux calculations made from uncorrected bidirectional flux measurements (J_{13}, J_{31}) would yield incorrectly high values. This problem is considered in detail in Chapter 4; a new approach to the estimation of transcellular Na flux is described utilizing the tight-junction agent 2,4,6, triamino-pyrimidine. Estimation of transcellular Cl fluxes, in contrast, is not dependent on the measurement of paracellular Cl permeability.

The second assumption, namely that the tissue behaves kinetically as a single compartment, requires experimental verification since it is likely that sequestration of tissue ions occurs between intracellular ion pools and extracellular regions that are hypertonic (Diamond and Bossert (1967), Barry, Smyth and Wright (1965)). (See also Chapter 3). Possible inhomogeneity within the tissue of the tissue ration R is tested for both Cl and Na.

The last assumption concerns the existence of a steady-state for both Na and Cl fluxes. This may be verified for both Na and Cl fluxes in control tissues since the net ion flux measured in 2 periods of $\frac{1}{2}$ hour, following a 20 minute preincubation period, is invariant. The absolute magnitude of the bidirectional ion fluxes (J_{13}, J_{31}) however, increases with respect to time. This is due to conductance changes (see Chapter 6) possibly associated with the development of a free-solution shunt pathway. Averaged fluxes over the $2\times\frac{1}{2}$ hour flux periods were always used in the calculation of the unidirectional fluxes.

2. Experimental

(i) Ringer's Solutions

Ringer's solutions were of the following compositions (concentrations in mM.)

(a) Standard NaCl Ringer: 140 NaCl, 10 $KHCO_3$, 0.4 KH_2PO_4, 2.4 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, gassed with 95% O_2 : 5% CO_2 to pH7.4

(b) Zero Na Ringer: (or variable $[Na]$ at constant $[Cl]$ and $[HCO_3]$): as (a) but 140 NaCl replaced isosmotically with choline chloride.

(c) Zero Cl Ringer: (or variable $[Cl]$ at constant $[Na]$ and $[HCO_3]$): as (a) but NaCl replaced by Na_2SO_4, $MgCl_2$ replaced by $MgSO_4$ and $CaCl_2$ replaced by $CaSO_4$ made isosmotic by addition of mannitol.

The base 2,4,6, triaminopyrimidine was neutralized by addition of HCl prior to use. Control solutions contained mannitol in equivalent quantities to maintain isotonicity. No correction for control solution tonicity was made in respect of additions of D-galactose or theophylline. Drugs and sugar-containing solutions were added to both bathing solutions in the Ussing-type chambers.

(ii) Animals

Rabbits were killed by intravenous injection of Nembutal (Na pentobarbital). The terminal ileum was rapidly excised, washed free of intestinal contents by ice-cold Ringer's of standard composition, and stripped of its serosa and muscle layers by the method outlined by Powell, Binder and Curran (1972). The tissue was then opened along its mesenteric border and mounted as a flat sheet in the Ussing chambers or on the perspex formers (see below).

(iii) Ion Flux Measurements

(a) Ussing Chambers. The Ussing chambers were designed to measure isotopic bidirectional transmural fluxes across six adjacent pieces of intestine. The exposed tissue area in each chamber pair was 1.76 cm^2. Fig. 2 shows the plan and cross-section of the chambers. The temperature of the solutions within the chambers is maintained constant by forced circulation of thermostated water through conduits cut into the lucite block. The range of temperature variation from 37°C recorded in any single chamber over the whole period of incubation never exceeded $\pm$0.2°C. Gassing and mixing within each chamber is achieved by continuous gas supply bubbled directly into the chambers. The end of the gas lead is surrounded with a cuff of plastic tubing (0.5cm in diameter) which forms a chimney for the gas bubbles; this achieves the dual purpose of forming a fluid stream due to gas uplift and of protecting the tissue against the effects of vigorous gassing. The use of Indian ink in a separate experiment gave a mixing time of $<$ 2 seconds.

The geometry of the chambers was such that the tips of the agar-KCl bridges (1mm in internal diameter) of the potential-sensing electrodes were held within 2mm of the tissue surface approximately at the centre of the exposed tissue circle, and that the agar-KCl bridges (1mm in internal diameter) of the current passing electrodes were held at the back of each flux chamber immediately opposite the approximate centre of the exposed tissue circle. This arrangement ensures (Kidder (1973):

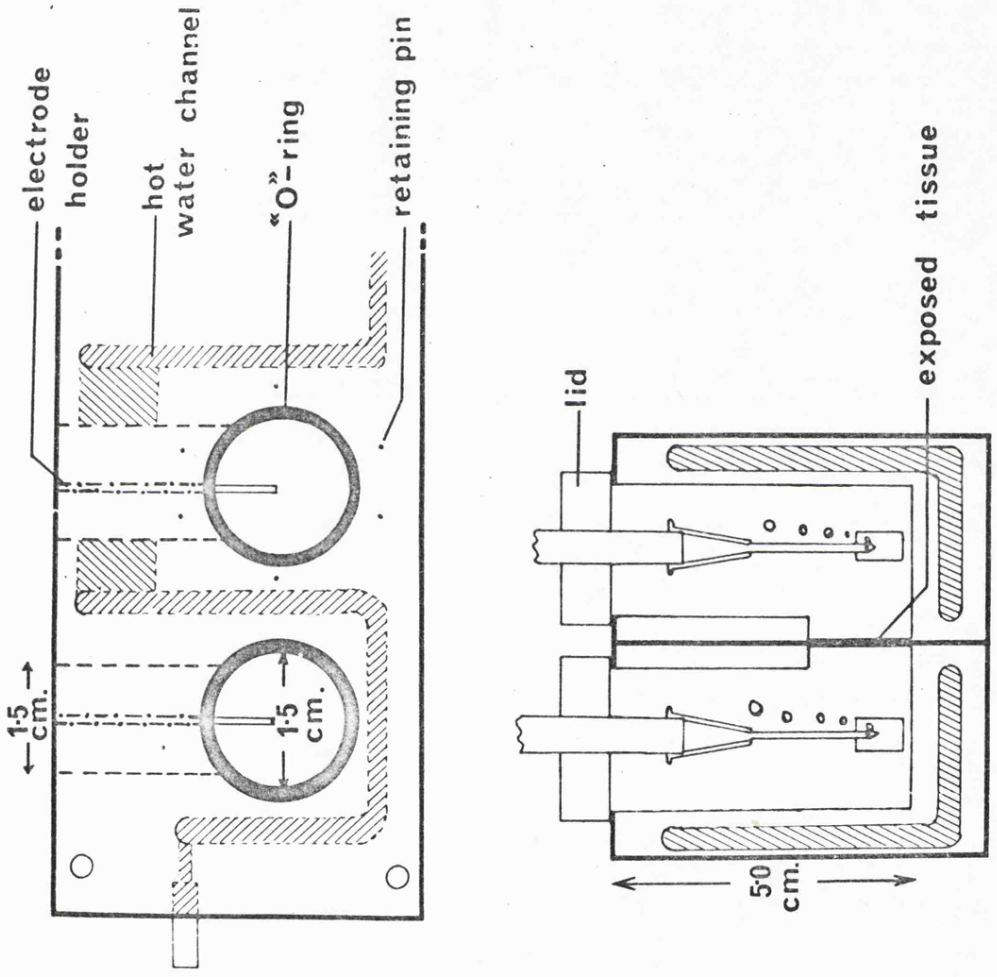
1. Optimum arrangement for transepithelial electrical potential measurement.

2. A homogeneous clamp-current density over the whole exposed tissue surface on voltage clamping.

3. Minimal area for diffusional isotope loss into the KCl-agar bridges.

FIGURE 2

Plan and cross-section of the Ussing-type chambers used in this study.



Electrical isolation of each chamber pair in the block of six was achieved by trimming surplus interconnecting tissues and isolating the remainder by the use of oil-containing conduits running between each chamber pair.

(b) Electrodes and Voltage-Clamp. Since the small trans-epithelial electrical potential differences recorded in high-conductance rabbit ileum are an important determinant of net ion flows (particularly of Na^+) all tissues in flux determinations were continuously voltage-clamped at zero potential difference ($\psi_{13} = 0$).

Fig. 3 shows a schematic drawing of the voltage-clamp apparatus, associated electrodes and Ussing chamber pair.

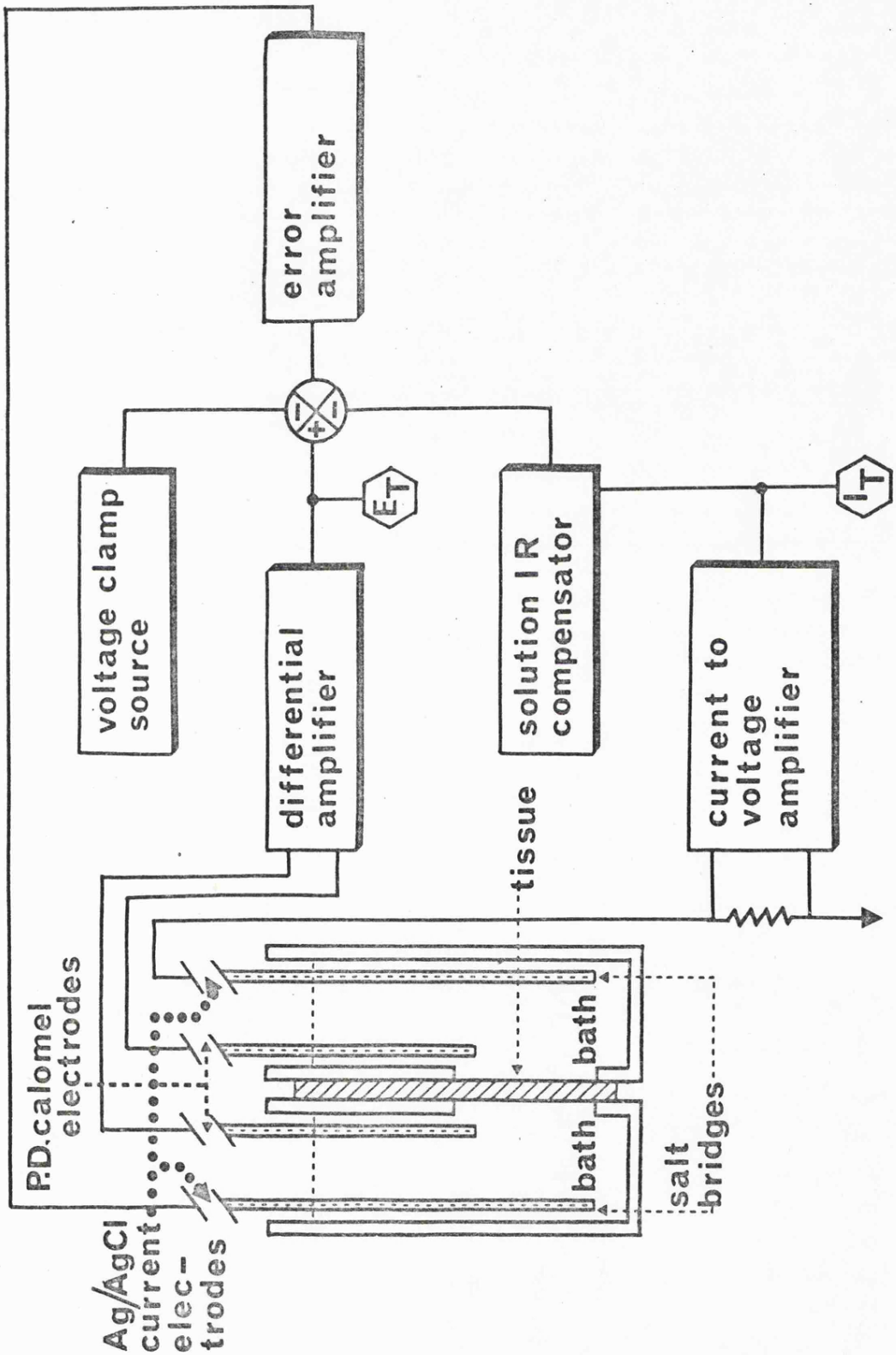
Current passing and potential sensing electrodes were connected to the Ringer's solution by 3M KCl-agar bridges of resistance $50\Omega cm^{-1}$. The use of low resistance 3M KCl bridges was necessitated by the small diameter of bridge used; this in itself was a consequence of the need for high-accuracy isotopic flux determinations. The use of Ringer-agar bridges resulted in high-bridge resistance, this in turn led to increased noise, and decreased accuracy of potential measurement.

The use of 3M KCl bridges reduces the junction-potentials developed between bridge and Ringer solution within the Ussing chamber (Caldwell (1968)). The importance of this is minimal in situations where symmetrical electrode arrangements and solutions are used since junctional potentials subtract. The use of 3M KCl bridges is important however, in situations where asymmetric solutions are used e.g. the measurement of diffusion and biionic potentials (see below).

Potential-sensing electrodes were reversible electrodes of the calomel type, (Hg / $HgCl_2$ /KCl). The resistance of a typical

FIGURE 3

Schematic diagram of the chamber/electrode/voltage clamp apparatus. E_T and I_T represent monitor points for potential and current respectively.



electrode pair was $\sim$ 10KΩ. Electrode pairs were stored connected in series (back to back). All electrode pairs used in experiments had potentials $<$ 1mV. A battery arrangement provides for compensation of residual potentials in the calomel electrode/bridge/chamber circuit in the range $\pm$2mV. This is adjusted prior to tissue mounting.

Current passing electrodes were large area Ag/AgCl electrodes. These were made by direct deposition of AgCl from molten AgCl on to bright silver wire/plates. The resistance of a typical electrode pair was $\sim$ 50KΩ.

The voltage-clamp device illustrated in Figures 3 and 4 provides facility for continual measurement of transepithelial current whilst clamping the tissue at zero potential, (Rothe et al (1969)). A signal proportional to the P.D. across the tissue is provided by the output of 3 type 741 operational amplifiers arranged in cascade to provide a differential amplifier (Fig. 4) of x100 gain and on imput impedance of $1 \times 10^6 \Omega$. This is fed to the sum-junction of the error amplifier, the output of which maintains the sum junction at virtual ground by means of the feed-back resistor. The current driven through the external circuit is proportional to the potential maintained at the output stage. Since intestinal resistance is of the same order as the solution resistance between the potential sensing electrodes and since passage of current through the external circuit will generate a potential across the solution resistor, correction must be made for the potential developed across the solution if a true transepithelial zero potential is to be attained (Clarkson (1967)). The current to voltage amplifier provides a signal that may be set to exactly nullify the effect of the solution resistor through a feed-back circuit. This is achieved by use of the 25KΩ variable potentiometer which is set to give a zero reading at $g\xi E$ on passage of a variable current through

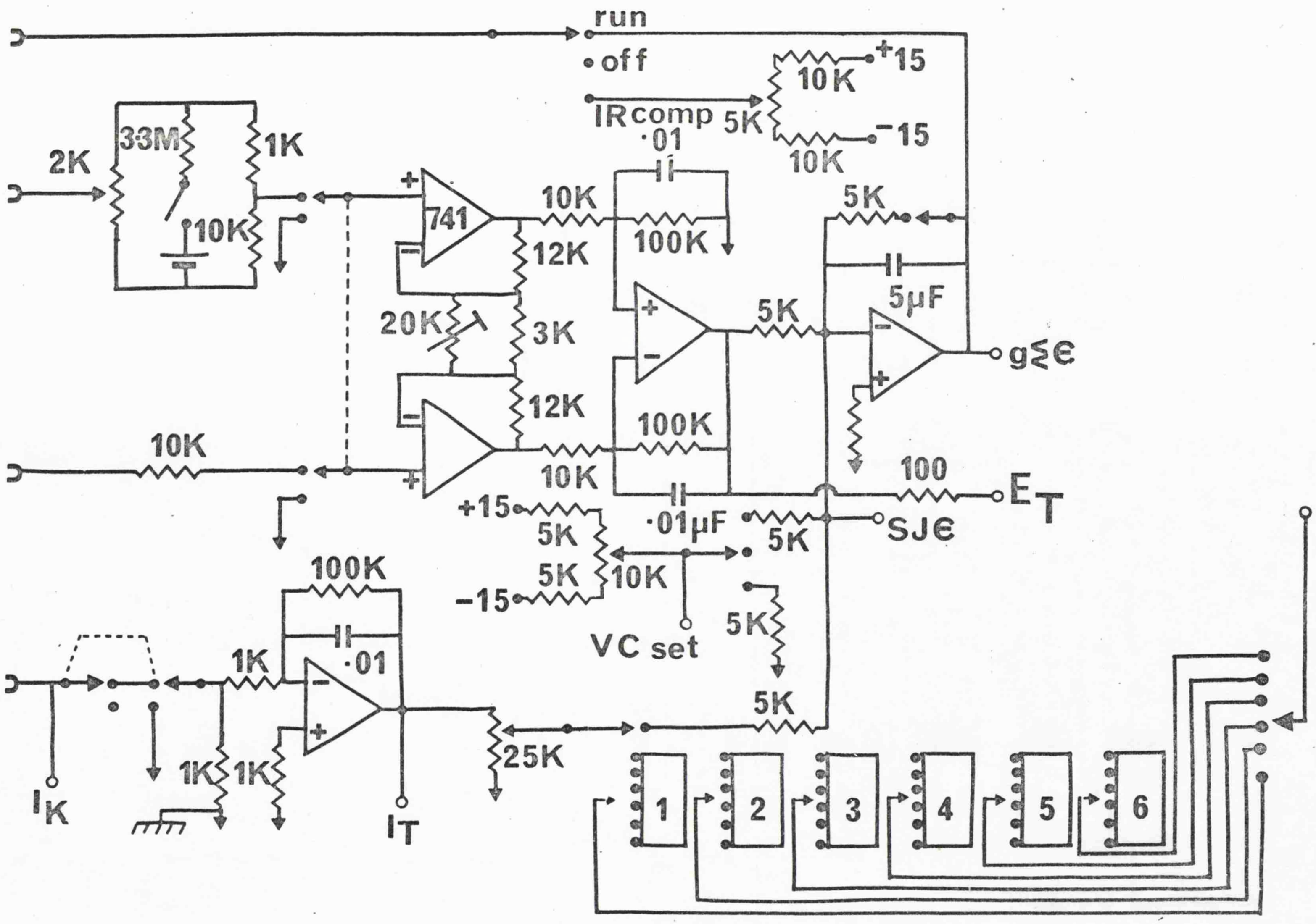
FIGURE 4

Circuit diagram of the voltage-clamp device illustrating potential offset, differential amplifier, error emplifier, current to voltage amplifier, voltage-clamp source and monitor points. All operational amplifiers were 741 type (Motorola MC1 741 L) possessing low current offset and low thermal drift (Rothe et al (1969)). Six voltage-clamp devices were arranged to run off a single power source. The switch circuit (bottom right) enabled monitoring of any single-circuit and any single monitor point within that circuit.

Monitor point

$g \lessgtr E$	output of error amplifier (gain x 1)
I_T	output of current to voltage amplifier (25mV to 1 μamp)
ET	output of differential amplifier (gain x 100)
SJE	output of voltage-clamp source ($\pm$40mV)
Vc_{set}	output of voltage-clamp source in 'off' position
I_K	short-circuit monitor point (between 1K and earth)

Short-circuit current was measured directly at I_K as the potential drop across a precision 1KΩ resistor ($\pm$0.1%) by a Philips Digital Voltmeter (PM 2452) of input impedence 1MΩ. Open-circuit potential was measured at ET by the use of a digital panel meter (Exel type XL 1000) of input impedance 5MΩ.



run
off
IR comp
·01
5K
10K
+15
-15
10K
2K
33M
1K
10K
741
10K
12K
100K
20K
3K
5K
5K
5μF
g≷ε
12K
100K
10K
10K
100
E_T
+15
5K
·01μF
5K
SJε
5K
10K
-15
VC set
5K
100K
1K
·01
5K
25K
1K
1K
I_K
I_T
1
2
3
4
5
6

the bath prior to the experiment (IR comp Fig. 4)*

The voltage-clamp device this enforces the condition $E_T - E_{IR} - E_{VC} = 0$ where E_T is the measured potential difference across the calomel electrodes, E_{IR} is the potential due to the solution resistor and E_{VC} is the desired transepithelial potential. Facility is provided (VC set) to enable E_{VC} to be set to any desired value in the range $\pm$40mV. The current generated from the power supply ($\pm$15V) effectively limited this range to $\pm$15mV.

A dummy membrane circuit (495Ω, 13.3mV open circuit potential, 26.9μamps short circuit current) was used for calibration purposes.

(c) Procedure for flux measurements. ^{22}Na and ^{24}Na (or ^{36}Cl and ^{82}Br) were added to the serosal and mucosal solutions respectively in quantities sufficient to give increments, due to flux across the tissue, of at least 2000cpm in 1cm^3 of solutions within the contralateral chamber during 30 minutes. The volume of isotope containing solution added never exceeded 0.1cm^3.

The initial volume of Ringer in each chamber was 7.5cm^3.

*This method of compensation may over-estimate the value of the solution resistor (Nellans, Frizzell and Schultz (1974)) since the intestine when mounted will occupy a finite space so displacing fluid. As pointed out by Kidder (1973) this concerns the question whether the epithelial cell layer is to be voltage-clamped or whether the whole gut thickness is potential-clamped. Compensation for fluid displacement by the gut thickness may not be warranted if only the epithelial cell layer is required to be clamped. No such correction was made in this study.

$1cm^3$ samples were taken 20 minutes, 50 minutes, and 80 minutes following addition of radioisotopes. Fluxes were averaged over the two ½ hour periods. Flux is calculated from the rate of tracer appearance on the 'cold' contralateral side and the specific activity of the 'hot' side. Flux is expressed as μ moles hr^{-1} cm^{-2} area of exposed ileum. No correction was made for tissue geometry which effectively increases the total membrane area exposed.*

(iv) Tissue extraction: the determination of the tissue isotope ratio (R).

At the end of the final ½ hour period for bidirectional flux measurement the chambers were disconnected from the gas-lifts and the 'hot' solutions were removed. Both sides of the chamber were washed rapidly with ice-cold isotonic choline chloride solution (for Na flux determinations) or with ice-cold isotonic mannitol solutions (for Cl flux determinations). This procedure ensures that the tissue is free of adherent 'hot' solution. (Since only the ratio of tissue isotope specific activities is required, loss of tissue ion is unimportant). The chambers were opened, the exposed tissue was cut out, washed for 30 seconds in cold wash-media (see above) and then blotted carefully on both sides with Whatman No. 1 paper to remove excess moisture. The tissue was weighed in a tared $5cm^3$ conical flask to

*The methods for determining galactose fluxes using ^{14}C and ^{3}H-labelled galactose are identical to those for ion flux measurement. No voltage-clamp was used in these experiments since voltage-clamping is known not to affect sugar fluxes (Schultz and Curran (1970)).

obtain the tissue wet weight and then extracted by shaking for 2 hours in $4cm^3$ of 0.1 M HNO_3. Samples of the extract were obtained for radioisotope counting. The extracted tissue was dried for 16 hours at 80^oC and the dry weight determined. Tissue water was calculated as the difference between the wet and dry weights.

(v) Distribution of the tissue isotope ratio R within the tissue.

Operationally and experimentally rabbit ileal tissue may be divided into 2 parts (Schultz, Fuiz and Curran (1966)).

(a) the epithelial cell layer and underlying tissue to the lamina propia,

(b) the remaining submucosa and muscle.

Each part has an associated extracellular space which may be freed of cation/anion by washing in ice-cold choline or mannitol solutions (see below and Chapter 3). Hence, estimates of the tissue isotope ratio R may be made in each of 4 separate tissue compartments for Na and Cl by scraping the mucosa from the submucosal layers (Schultz et al (1966)) prior to and following a period of washing in ice-cold choline chloride/mannitol.

(vi) Kinetic measurements of net cation loss

(a) Tissue mounting and incubation. Ileum, prepared as above, was mounted on a perspex former covered with $\frac{1}{2}$" spikes approximately 3cm apart to maintain an even tension. The former was then laid in a bath containing the appropriate Ringer at 37^oC for a preincubation period of 1 hour. Access of well stirred solution to both epithelial surfaces was ensured by vigorous gassing with 95/5% O_2/CO_2. Following preincubation the former was transferred to another bath containing the ice-cold stirred wash medium. Measurement of net cation loss was performed discontinuously at time intervals of

up to 80 minutes by estimating [Na] and [K] in extracts of separate pieces of ileum. The ion and water content of the tissue were also monitored during the preincubation period.

(b) Estimation of Na and K. Separate pieces of ileum were blotted on Whatman No. 1 filter paper and placed in tared 10cm^3 conical flasks and weighed. 4cm^3 of extraction fluid (normally 0.1 M HNO_3 but H_2O was used in experiments involving ^{125}I Albumin) were then added to each flask and extraction was carried out overnight. The [Na] and [K] of aliquots of extract were determined by flame photometry. Tissues were then dried over a sandbath at 95^{o}C to determine the tissue dry weight.

(c) Determination of the tissue extracellular space. The extracellular space was determined from the distribution ratio of ^{14}C-inulin between the bathing solution and the tissue extracellular space. ^{125}I Albumin was also used in some experiments for comparative purposes. Inulin and albumin were added to the preincubation solutions only in tracer quantities.

(vii) Methods used to characterize the ionic conductance of the paracellular (shunt) pathway in rabbit ileum.

The ionic conductance of the paracellular route in rabbit small intestine may be measured by three methods (Schultz and Curran (1974)).

(a) Voltage dependent bidirectional transmural fluxes.

The approach outlined by Frizzell and Schultz (1972) was used. Unidirectional transmural mucosa to serosa flux J_{13} determined as a function of the applied electrical potential difference. For the cation Na it has been shown (Frizzell and Schultz (1972)) that:

$$J^{Na}_{T_{1-3}} = J^{Na}_{C_{1-3}} + J^{Na}_{d_{1-3}} \cdot \mathcal{E}^{-\frac{1}{2}}$$

where: $J^{Na}_{T_{1-3}}$ is the total transepithelial unidirectional flux from mucosa to serosa.

$J^{Na}_{C_{1-3}}$ is that portion of mucosa to serosa flux permeating the epithelium by the transcellular route.

$J^{Na}_{d_{1-3}}$ is the diffusional component of mucosa to serosa flux via the high conductance extracellular pathway at zero potential difference.

$\mathcal{E}^{-\frac{1}{2}} = \exp(ZF\Psi_{1-3}/RT)$ where Z,F,R and T have their usual meanings. Ψ_{1-3} is the transepithelial potential difference.

A plot of J_{1-3} versus $\mathcal{E}^{-\frac{1}{2}}$ will yield a straight line with a slope equal to $J_{d_{1-3}}$ and with an intercept on the y-axis of $J_{C_{1-3}}$.

The experimental protocol was designed to measure bidirectional fluxes (as described above) at 2 non-zero clamping potentials, following a preincubation period of 20 minutes. Flux at zero P.D. was determined prior to and subsequent to the non-zero potentials. This provided an internal experimental control. Flux periods were of 20 minutes duration.

(b) Dilution and biionic potentials. Transepithelial P.D.'s were recorded in open circuit during isosmotic replacement of the mucosal bathing solution (initially 140mM NaCl Ringer). Isosmotic replacement of NaCl was made with mannitol,choline chloride, KCl, or Na_2SO_4. All solutions contained 0.1mM ouabain to abolish the spontaneous P.D. across the tissue and so eliminate possible

complications from changes in cell potentials (Frizzell and Schultz (1973)). Saturated (3M) KCl bridges minimized errors due to asymmetric junction potentials. Absolute errors using this approach are approximately 2-3mV (Barry and Diamond (1970)) but are constant in relation to the action of agents such as triaminopyrimidine (Chapter 4) and theophylline (Chapter 6).

(c) Conductance measurements. 2 methods were routinely used. Short-circuit current was measured under voltage clamp ($\Psi_{13} = 0$) during flux measurements; every 10 minutes the clamp was released for sufficient time to allow a steady-state open circuit voltage to be developed (approximately 20 seconds). Conductance is then simply obtained by the quotient of short-circuit current and open circuit potential. Alternatively, conductance measurements were made under voltage-clamp by measuring the current required to clamp the tissue to short (10 second) depolarizing and hyperpolarizing (2mV) steps of command potential applied to the tissue intermittantly. An instantaneous value for conductance may thus be obtained. (The 90% response time for the voltage-clamp was < 0.1 seconds).

(viii) Radioisotope counting.

(a) ^{22}Na and ^{24}Na. ^{24}Na was counted in a Packard Tricarb Liquid Scintillation Counter by its Cerenkov radiation. All samples counted for ^{24}Na activity were diluted with $10cm^3$ of distilled water. Background subtraction of activity due to the presence of ^{22}Na was made after recounting samples following complete decay of all ^{24}Na activity (2 weeks following the first count). ^{24}Na activity was corrected for decay during the time taken for the initial count. ^{22}Na activity was determined following the decay of all ^{24}Na activity, from its β emissions using the Tricarb Counter. All samples for β counting were diluted with $10cm^3$ of scintillation cocktail.

Errors due to quench were evident in samples for counting due to:

(a) differing media between flux samples and tissue extracts, and

(b) due to storage.

All activity was corrected for differences in quench between samples by calibrating the Tricarb external standard against ^{22}Na samples of known activity quenched by varying amounts of chloroform or of triaminopyrimidine.

(b) ^{36}Cl and ^{82}Br. ^{82}Br was estimated from its γ radiation in an Ecko γ-counter. Since ^{36}Cl emits no γ-rays this provides 100% separation of ^{82}Br activity. Appropriate corrections were made for background γ activity and decay of ^{82}Br activity during the counting period. ^{36}Cl was counted by its β activity after one month had elapsed to allow ^{82}Br activity to decay to insignificant levels. β - activity was determined using the Packard Tricarb Counter. ^{36}Cl activity was corrected for quench by the external standard which had been calibrated by known activities of ^{36}Cl quenched with either chloroform or triaminopyrimidine. No dilution of smaples was made for γ-counting. All samples for β-counting were diluted with $10cm^3$ of scintillation cocktail.

(c) ^{3}H and ^{14}C-labelled D-galactose. ^{3}H and ^{14}C-labelled sugars were counted in the Packard Tricarb Spectrometer set to provide maximal discrimination between ^{3}H and ^{14}C; this provided exclusion of ^{3}H activity from the ^{14}C-channel. Cross-over of ^{14}C activity into the ^{3}H-channel was corrected by standard double-label techniques. The external standard was previously calibrated with samples of known activity of single-labelled isotopes quenched with varying quantities of chloroform.

(d) ^{14}C-labelled inulin and ^{125}I-labelled Albumin.

Experiments involving extracellular space markers were singly-labelled only. ^{14}C and ^{125}I were determined from their β activities using the Packard Tricarb Spectrometer. All samples were diluted with $10cm^3$ of scintillation cocktail. All activity was corrected for differences in quench between samples using the external standard.

(e) Scintillation Cocktail. The composition of the scintillation fluid is that given by Fox (1968): $500cm^3$ toluene, $500cm^3$ Triton x-100, and 3.5g of 2,5, diphenyloxazole (P.P.O.). Due to difficulties in the supply of Triton x-100, Tergitol 15-S-9 (Union Carbide) and Fisons emulsifying agent were used as substitutes in identical quantities to Triton x-100.

(ix) Statistics

Statistical variance of the mean of grouped results were routinely expressed as the standard error of the mean (S.E.M.). Statistical significance was tested by the use of the student's 't' test (unpaired means solution) using 2-tailed and 1-tailed tests where appropriate. Calculated values of 't' were compared to the tabulated values (Fisher and Yates (1949)).

Least-square regression analyses were made using the Wang Laboratories Incorporated 700 C Calculator using Wang 700 series software (Package 98).

The kinetic parameters K_m and V_{max} for saturation-type kinetics were obtained using a Wang program (No. 1047A/GS2) which utilizes a matrix method to obtain the best-fit solution to the data. The method gives equal weights to all the data points.

The statistical significance of the effects of triamino-pyrimidine, theophylline and galactose on time-dependent conductance changes (Chapter 6) were tested by analysis of variance using an

unweighted means solution with repeated measures of a single factor e.g. time. The statistical significance of the calculated F-ratios were obtained from Fisher and Yates' (1949) F tables. Calculations were all made using Wang software (Package 9A).

CHAPTER THREE

FACTORS AFFECTING THE COMPARTMENTALIZATION OF Na WITHIN RABBIT ILEUM IN VITRO

A. Introduction

Transcellular flow of water through epithelial cells is presumed to be a response to osmotic pressure acting across the basal-lateral cell borders resulting from deposition of hypertonic NaCl within the lateral spaces by Na-pump activity (Diamond and Bossert (1967)).

Good evidence for the existence of such a mechanism would be the finding of extracellular hypertonicity that was dependent on Na-K ATPase activity. Machen and Diamond (1969) have made an indirect measurement of the concentration of NaCl within the lateral spaces of rabbit gall-bladder; they conclude that the lateral space is some 80 m. osmoles more concentrated than the bathing media. Similarly, Rotunno et al (1973) Zybler et al (1973) and/have inferred that a high concentration of Na is localized within the intercellular space of frog skin, however, they attribute this to ion complexation. Wall et al (1970) have sampled intercellular spaces of cockroach rectum by micro-electrodes and found them to be around 130 m. osmoles hypertonic to the bathing media.

Very recently Zeuthan and Monge (1975) have reported on the basis of ion sensitive micro-electrode studies in rabbit ileum, that intracellular K is present at hypertonic concentrations at the basal pole of the epithelial cells and that the extracellular Cl concentration in the region immediately adjacent to the basement membrane is approximately 300mM.

This work attempts to measure extra and intracellular action concentrations by compartmental analysis. Since sugars are known to stimulate net flux of water and NaCl across intestine (Esposito et al (1969); Barry et al (1965); Fullerton and Parsons (1956)), the effects

of a non-metabolized sugar, D-galactose, (Naftalin and Curran (1974)) on the tissue distribution and concentrations of Na and K were studied. Also of interest are the effects of theophylline, which reverses the normal absorptive state of intestine with respect to NaCl and water (Field (1974)) and also ouabain, which inhibits tissue Na-pump activity (Schatzmann (1953)).

B. Results

1. Compartmental Analysis of Tissue Cation Content

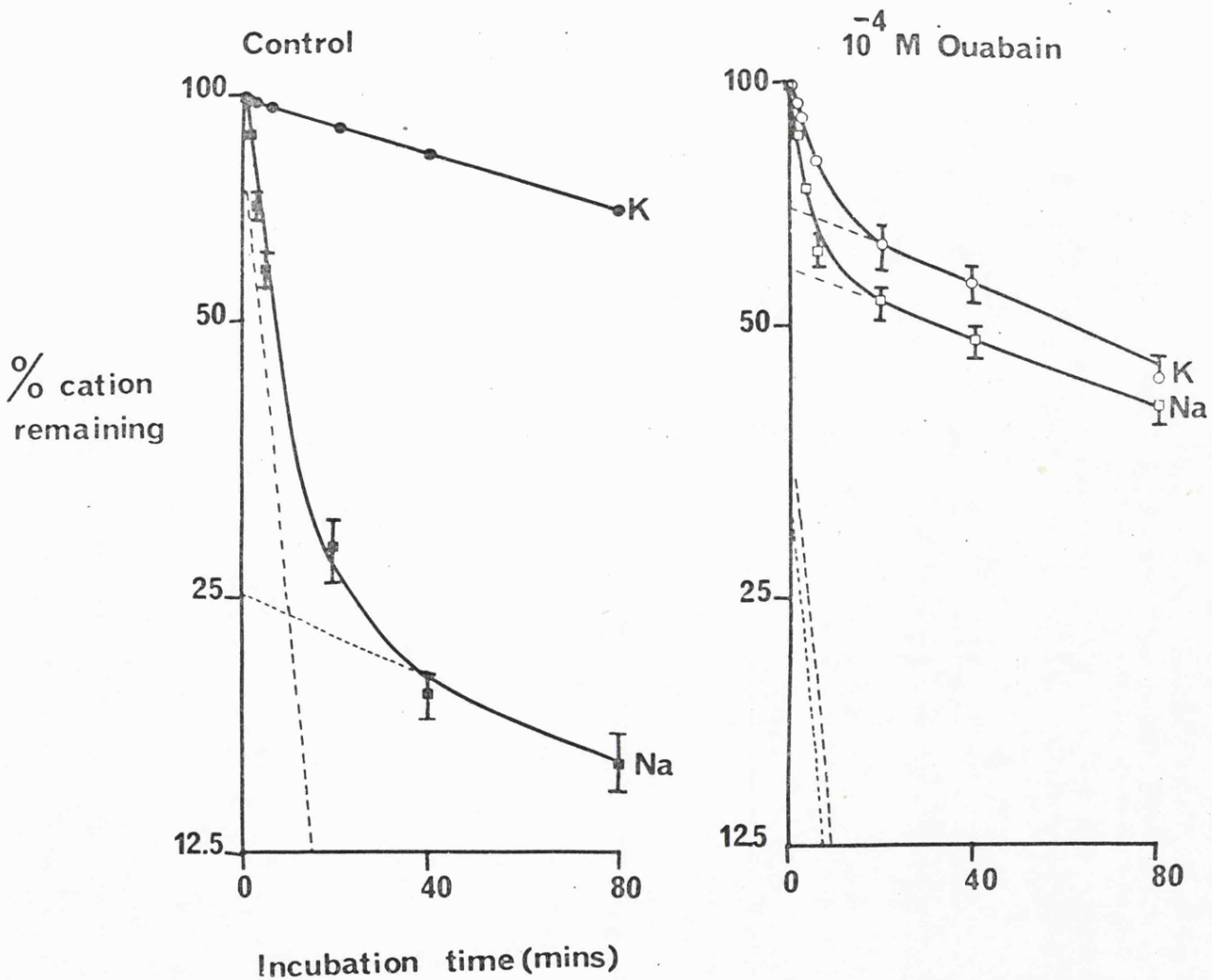
(i) Controls

Figures 5 (a) and (b) show the wash-out curves of tissue Na and K for control tissues and tissues preincubated in the presence of 0.1mM ouabain. Percentage loss is plotted semilogarithmically against time. In control tissues net Na loss is best fitted by a double exponential function of time. Kinetically, therefore, tissue Na exists in two compartments. The $t_{\frac{1}{2}}$ for the fast component is 6.86 $\pm$ 1.69 mins. (S.D.) whilst for the slow component the $t_{\frac{1}{2}}$ is 65.9 $\pm$ 29.3 mins. (S.D.). K loss, in contrast, is best fitted by a single exponential whose $t_{\frac{1}{2}}$ is 96.8 $\pm$ 46.8 mins. (S.D.). This result indicates that K is contained within a single intracellular compartment. Since the rate constant for K loss is similar to the slow component for net Na loss, this component of Na loss can be equated with loss from the intracellular pool. Extrapolation to zero time gives the original amount of intracellular Na. The remaining Na is adequately fitted by a single exponential function of time. Since the $t_{\frac{1}{2}}$ of the component is rapid, this Na must be located within a compartment that is relatively open to the bathing solutions. Identification of this compartment as the tissue extracellular space follows from comparison of the $t_{\frac{1}{2}}$ of inulin loss, (8.1 $\pm$ 3.6 minutes (S.D.)) (see section 2 below) to the $t_{\frac{1}{2}}$ of the fast Na exit component (Table 1).

Figure 5

(a) Wash-out curves for Na (■) and K (●) from stretched rabbit ileum following 1 hour preincubation in standard Ringer. % loss is plotted semi-logarithmically against time of incubation in the wash media. Error bars denote ± S.E.M. for each point (n=27); these lie within the points where not shown. Dashed lines indicate the least-square regression lines for the two components of net Na loss. Solid lines are drawn by eye.

(b) Tissues were preincubated in the presence of 0.1mM ouabain for one hour prior to incubation in the wash media. Curves show the wash outs for Na (□) and K (○). Each point represent the mean of 9 determinations. Error bars denote ± S.E.M. (n=9). Dashed lines denote the least-square double-exponential fit for the data.


Control
10^{-4} M Ouabain
100
50
25
12.5
% cation remaining
K
Na
0
40
80
Incubation time (mins)

(ii) Effect of 0.1mM ouabain

0.1mM ouabain causes a change in the wash-out kinetics for Na and K from the tissue (Fig. 5 (b)). Both net Na and K losses are best fitted by double exponential functions of time. The amount of Na exiting with a long $t_{\frac{1}{2}}$ increases compared with controls ($P < 0.001$) whereas the amount of K exiting with a similar $t_{\frac{1}{2}}$ decreases ($P < 0.001$). This result is consistent with the well-known action of ouabain in inhibiting Na-pump activity (Schatzmann (1953)), so causing cellular K loss and reciprocal Na gain, and supports the intracellular locations for cations inferred from the wash-out kinetics. That net K loss is best fitted by a double exponential function in the presence of ouabain, suggests that the K remaining within the tissue has equilibrated with the extracellular compartment.

Since the intestine is a complex folded tissue (Trier (1968)) the rate constants for both the extracellular and intracellular spaces are lumped parameters representing loss from many different, but operationally similar, compartments. Small changes in the rate constant for the extracellular component may indicate a change in the location of the bulk of extracellular Na within the extracellular space. The $t_{\frac{1}{2}}$ for loss of extracellular Na in the presence of ouabain is greater than control values. (Table 1). The difference between these values is significant ($P < 0.01$). This result may indicate a shift in extracellular Na away from regions of active Na pumping in ouabain-treated tissues.

(iii) The effects of the tight-junction agent 2,4,6, Triaminopyrimidine

The direction of loss of Na from the region of high Na concentration located within the lateral spaces will be determined primarily, by the resistance of the tight-junctions. If this resistance is low, Na loss will be rapid, since net loss will occur through

the tight-junctions rather than by Na traversing the relatively long distance across the submucosal tissue layers. The base 2,4,6, triaminopyrimidine specifically increases the resistance of intestine by reducing the Na conductance of the tight-junctions (Moreno (1975)), (see also Chapter 4). That 2,4,6, triaminopyrimidine has no effect on Na pump-activity can be seen in Table 3 which shows that 2,4,6, triaminopyrimidine has no significant effect upon intracellular cation concentrations. 2,4,6, triaminopyrimidine has no significant effect upon the rate of net Na loss from the extracellular space ($p > 0.4$) of stretched ileum indicating that the serosal resistance to net Na loss is low compared with the resistance of tight-junctions. Incubating ileum without stretching it on the former, (see Methods) causes tissue curling and an increased serosal exit resistance. In this case the fast extracellular component of net Na loss coming from the mucosal surface of control tissues is completely eliminated by 2,4,6, triaminopyrimidine; thus demonstrating that the extracellular component of net Na loss in unstretched tissue is located distal to the tight-junction.

(iv) Effects of galactose and theophylline (10mM)

Both 10mM galactose and theophylline significantly increase the rate of loss of Na from the extracellular space of stretched tissue (Table 1). The galactose dependent increase in rate constant may be associated with an increased tissue permeability due to changes in the widths of the lateral spaces (Naftalin and Curran (1974)) (see below section 2). Theophylline is thought to reduce the width of the paracellular pathway through increased cellular c-AMP levels (Dibona et al (1974)). The observed decrease in the $t_{\frac{1}{2}}$ compared with controls, may be due to partial exclusion of extracellular Na from epithelial layers. Since theophylline treated tissue with

TABLE 1

	$t_{\frac{1}{2}}$ (minutes) ± S.D.
1. CONTROL (9,108)	6.86 ± 1.69
2. + 20 mM 2,4,6, Triaminopyrimide (3,36)	5.57 ± 1.68
3. + 0.1 mM ouabain (3,36)	2.36 ± 0.56^{xxx}
4. + 10 mM Galactose (4,48)	3.51 ± 1.68^{xxx}
5. + 10 mM Theophyline (3,36)	1.21 ± 0.64^{xxxx}
6. + 10 mM Theophyline + 20 mM galactose (3,36)	2.96 ± 0.61^{xxx} (6-4 N.S. $p > 0.8$)

Rate constants ($t_{\frac{1}{2}}$) for loss of Na from the extracellular tissue compartment of rabbit ileum into ice-cold choline chloride buffer.

Numbers in parentheses represent the number of experiments followed by the number of experimental data points involved in the regression analyses.

xxx = $p < 0.01$

xxxx = $p < 0.001$

by students 't' test (unpaired data) of experimental against control tissues.

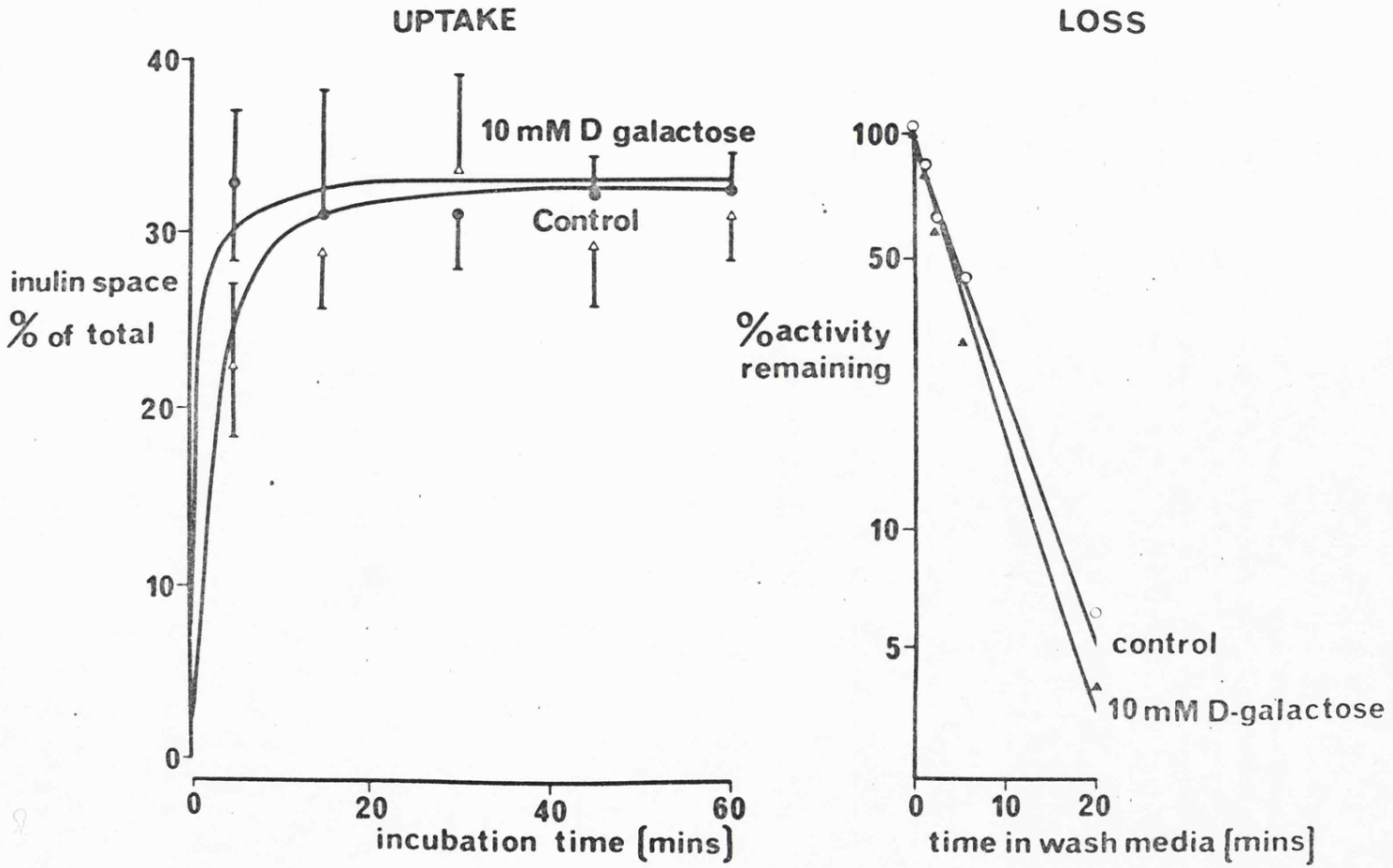
galactose present has a $t_{\frac{1}{2}}$ for extracellular Na loss that is not significantly different from controls with 10mM galactose present in the Ringer ($p > 0.8$) (see also sections 3 (iii) and 4 (iv)), it can be inferred that galactose reverses this effect of theophylline.

2. Extracellular Space Determinations: choice of an extracellular marker

The calculation of intracellular and extracellular cation concentrations from the kinetic data depends upon the accuracy of the estimated extracellular space. Schultz et al (1966) have shown that inulin is an adequate marker for the extracellular space of rabbit ileum. This is confirmed in the present study. Figure 6 (a) shows that equilibration of ^{14}C inulin activity with the extracellular space is rapid and that no slow entry component is discernable. Inulin loss into the ice-cold wash medium is also shown (Fig. 6 (b)). The percentage activity of ^{14}C inulin remaining in the tissue is plotted semi-logarithmically against the incubation time. Loss is rapid and is adequately fitted by a single exponential function of time. Virtually all activity is lost after 20 minutes incubation. The $t_{\frac{1}{2}}$ for inulin loss is 8.17 $\pm$ 3.6 minutes (S.D.). This finding shows that kinetically, the extracellular space behaves as a single compartment with respect to inulin (see section 1 (i)).

Equilibration and loss of ^{14}C labelled inulin activity are facilitated by the presence of 10mM galactose. This finding correlates with the observation that apparent access of galactose to the serosal membranes of the epithelial cells from the serosal fluid is a function of net galactose flux (Naftalin and Curran (1974)) (see also section 3).

The inulin space for controls expressed as a percentage of the total tissue water is 33.21 $\pm$ 2.97% (S.E.M.). This value agrees


UPTAKE
LOSS
40
30
20
10
0
inulin space
% of total
10 mM D galactose
Control
0
20
40
60
incubation time (mins)
100
50
10
5
%activity
remaining
control
10 mM D-galactose
0
10
20
time in wash media (mins)

closely with values determined by other workers (Schultz et al (1966)). The ^{125}I Albumin space is 28.2 ± 2.4% (S.E.M.) of the total tissue water. These two values are not significantly different, ($p > 0.2$) (Table 2). Equilibration of ^{125}I Albumin with the extracellular space is similar to inulin, in having a single rapid time constant. The difference between the inulin and albumin spaces may be interpreted as being due to partial exclusion of albumin (Maizels and Remington (1959)) on the basis of its greater molecular weight.

Examination of Table 2 shows, however, that the ^{125}I Albumin space responds less readily than the inulin space to changes in tissue H_2O observed during incubation with galactose. For this reason inulin was routinely used as the extracellular marker and all calculations were based on these measurements.

3. Factors Affecting Tissue Water

(i) D-galactose

D-galactose causes tissue swelling as judged by increments in the tissue wet:dry weight ratio at the end of the preincubation period (Tables 2 and 3). This increased tissue water is a saturable function of the galactose concentration of the preincubation medium (Fig. 7 (a)) at Ringer $[Na]$'s of 140, 75, and 25mM. The calculated Km values (galactose concentration giving half maximal swelling) at these Na concentrations are 3.06 ± .2 (SE), 3.1 ± .9 SE) and 3.2 ± .2 (SE) mM respectively, which are in agreement with the observed Km's for galactose influx across the brush-border of the epithelial cells (Naftalin and Curran (1974); Goldner et al (1969)). The amount of tissue swelling at constant galactose concentration of 20mM is dependent on the Ringer $[Na]$; on reducing the $[Na]$ below 75mM, the absolute amount of swelling is reduced (Fig. 7 (b)). The Km value for this relationship is 45 ± 9 (SE) mM. This value is similar to

TABLE 2

	wet weight : dry weight ratio	extracellular water expressed as a % of the total.	ratio of the weight of extracellular water : tissue dry weight.
^{14}C inulin CONTROLS	6.62 ± 0.38 (24)	33.21 ± 2.97 (24)	2.37 ± 0.26 (24)
^{125}I Albumin CONTROLS	6.53 ± 0.21 (18)	28.20 ± 2.40 (18)	2.19 ± 0.18 (18)
^{14}C inulin + 10 mM D-galactose	9.62 ± 0.43 (21)	31.34 ± 2.09 (21)	2.83 ± 0.22 (21)
^{125}I Albumin + 10 mM D-galactose	9.47 ± 0.50 (14)	25.50 ± 0.95 (14)	2.36 ± 0.18 (14)

Comparison of the ^{14}C inulin and ^{125}I Albumin spaces for control tissues ± 10mM D-galactose.

All errors denote ± S.E.M., figures in parentheses are the number of experimental data points.

Table 3

Effects of D-galactose, Na replacement, ouabain, 2,4,6, triaminopyrimidine and theophylline on the extracellular space. All measurements were made after 45-60 minutes of incubation. Errors are given as the S.E.M. Asterisks denote values for experimental determination that are significantly different from control values (student 't' test unpaired data).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.005$

**** $p < 0.001$

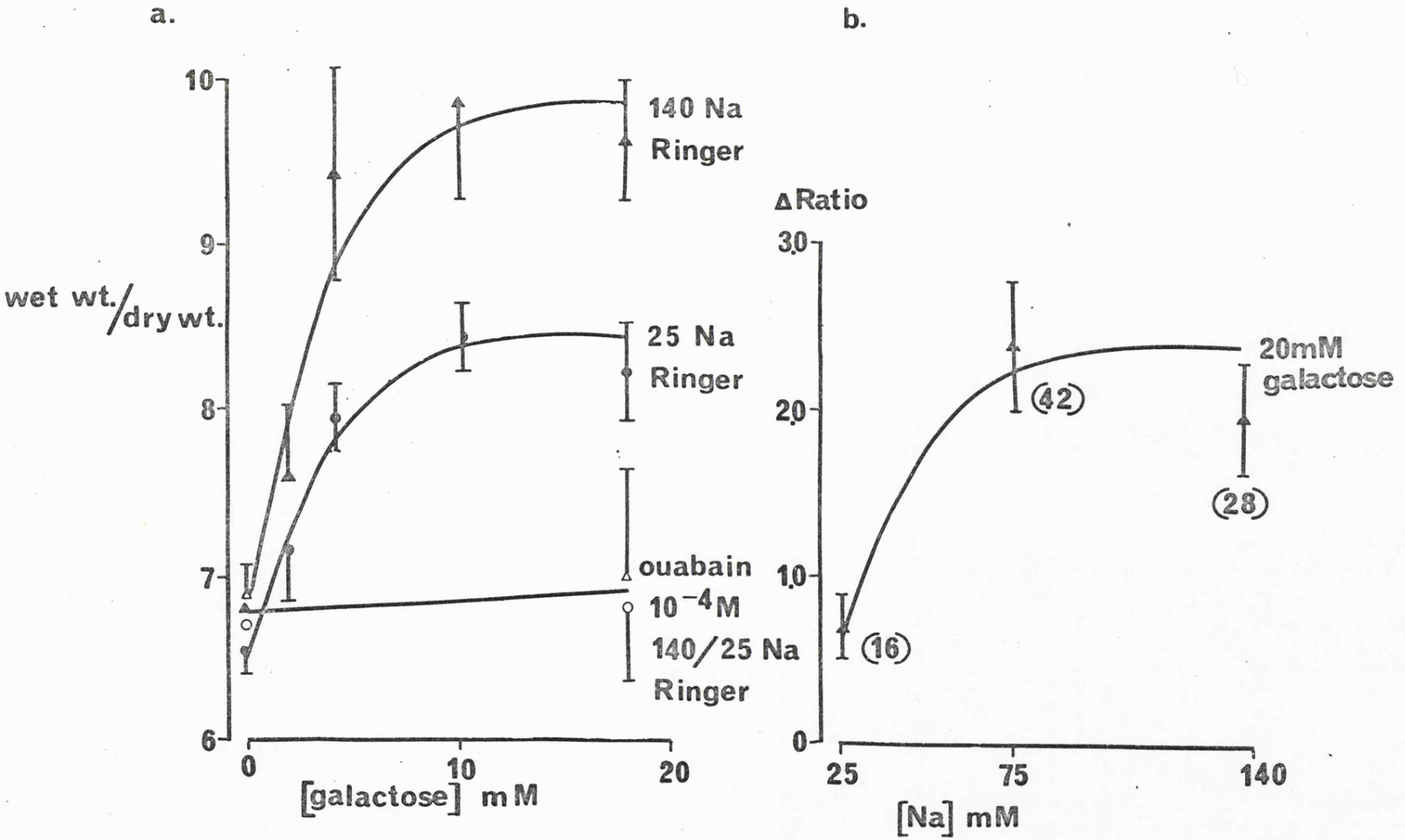
TABLE 3

Experimental Conditions	n	Wet weight to dry weight ratio	Inulin 'space' as a % of total water	Weight of extracellular H_2O : tissue dry weight
Control (Standard Ringer)	24	6.62 ± 0.38	33.21 ± 2.47	2.37 ± 0.25
+ 10mM galactose	21	9.62 ± 0.43***	31.34 ± 2.09	2.83 ± 0.22 ($p<0.1$)
+ 0.1mM ouabain	17	6.59 ± 0.46	46.10 ± 2.56**	2.98 ± 0.18 ($p>0.1$)
+ 0.1mM ouabain + 10mM galactose	19	6.98 ± 0.56	45.60 ± 2.32**	2.89 ± 0.14 ($p>0.1$)
75mM Na Ringer	12	7.01 ± 0.46	36.16 ± 4.56	2.39 ± 0.26
75mM Na Ringer + 20mM galactose	12	9.71 ± 0.46****	35.30 ± 7.40	2.89 ± 0.32 ($p>0.1$)
25mM Na Ringer	8	6.52 ± 0.16	29.31 ± 2.89	2.19 ± 0.19
25mM Na Ringer + 20mM galactose	8	8.25 ± 0.25**	29.80 ± 4.03	2.46 ± 0.26
140mM Na + 10mM Theophylline	18	5.86 ± 0.25 ($p<0.1$)	25.56 ± 1.80*	1.87 ± 0.14 ($p<0.1$)
+ 10mM Theophylline + 20mM galactose	6	9.05 ± 0.56***	34.82 ± 6.70	3.15 ± 0.61 ($p>0.2$)
+ 20mM triaminopyrimidine	14	7.16 ± 0.34	31.11 ± 2.20	2.14 ± 0.16
+ 20mM triaminopyrimidine + 20mM galactose	10	10.80 ± 0.96****	32.50 ± 3.80	2.73 ± 0.19 ($p<0.2$)

Figure 7

(a) The effect of D-galactose (0 to 20mM) on tissue water (the tissue wet weight : dry weight ratio) following preincubation in Ringer solutions containing [Na] 's of 140mM (▲) and 25mM (●). Values for 75mM Na are coincident with those for 140mM Na and are omitted for clarity. The effect of 0.1mM ouabain is also shown at 140mM Na (△) and 25mM Na (○) ± 20mM galactose.

(b) Increments in the wet : dry weight ratio for paired date ± 20mM D-galactose as a function of the [Na] of the bathing media. Figures in parentheses are the number of experimental points. Error bars denote ± S.E.M.


a.
b.
wet wt./dry wt.
10
9
8
7
6
140 Na
Ringer
25 Na
Ringer
ouabain
10⁻⁴M
140/25 Na
Ringer
0
10
20
[galactose] mM
ΔRatio
3.0
2.0
1.0
0
(42)
(16)
(28)
20mM
galactose
25
75
140
[Na] mM

that determined for the [Na] giving maximal stimulation of both convective and diffusive permeabilities for galactose entry across the mucosal brush-border (Naftalin and Holman (1974)).

Table 3 shows that galactose-dependent swelling is paralleled by an increased extracellular space. Since no increase in the ratio of extracellular water/total tissue water is observed, it may be concluded that galactose causes concurrent increases in both the cell water and the extracellular space.

(ii) 0.1mM Ouabain

The data of Table 3 shows that the addition of 0.1mM ouabain to the preincubation medium has no significant effect on tissue water as judged by the wet weight : dry weight ratio ($p > 0.9$). Ouabain also abolished the galactose-dependent increase in wet:dry weight ratio.

With ouabain present the extracellular space increases to 46% of the total wet weight compared with control values of 33.2% ($p < 0.01$). These last two results in conjunction indicate cell shrinkage.

Ouabain is known to cause tissue swelling (e.g. in brain (Towfighi and Ganatas (1973)) and red cells (Poznansky and Soloman (1972)). Also Schultz et al (1966) has shown cell swelling with ouabain in mucosal slices of intestine. The apparent difference of the present results with these findings may result from the necessary stretching of the ileum in the present study.

(iii) 10mM Theophylline

Theophylline causes a reduction in the tissue wet : dry weight ratio compared with control values ($p < 0.1$) (Table 3). This is accompanied by a reduction in the amount of extracellular water as judged by the inulin space ($p < 0.05$). These effects are, therefore, in agreement with previous observations (Holman and Naftalin (1975b)).

Addition of 20mM galactose reverses the theophylline dependent reductions in the wet to dry weight ratio and the inulin space. These results are consistent with the view (Dibona et al (1974)) that theophylline-mediated secretion is associated with the reduction of the extracellular spaces. Galactose may reverse this effect by stimulation of net water and NaCl absorption.

4. Calculated Intracellular and Extracellular Cation Concentrations

(i) Control Values

The calculated intracellular and extracellular Na concentrstions for controls are 30.68 ± 4.13 (S.D.) and 180.0 ± 13.3 m. equiv/litre respectively (Table 4). The extracellular Na concentration is significantly greater than the [Na] of the Ringer's solution ($p < 0.01$); hence the previously held assumption that the Na concentration of the extracellular space is equivalent to that of the bathing medium (Schultz et al (1966)) is invalid. The use of this assumption will lead to an overestimate of the intracellular [Na]. The calculated intracellular [Na] in this study is approximately 50% below previous estimates (Schultz et al (1966); Koopman and Schultz (1969)). The intracellular [K] concentration is 115.69 ± 5.61 (S.D.) m equiv/litre cell H_2O, a value which agrees closely with previous data (Koopman and Schultz (1969)). Estimates of intracellular [K] are not subject to error from elevated extracellular concentrations, hence this agreement is to be expected. The hypertonicity of the extracellular space is at least 80 m osmoles, a value similar to that deduced by Machen and Diamond (1969) for the lateral spaces of rabbit gallbladder.

(ii) 0.1mM Ouabain

Ouabain causes a redistribution of both Na and K within the tissue (Table 4). The intracellular [K] falls to 27.9 ± 5.9 (S.D.)

Table 4

Calculated intracellular and extracellular cation concentrations based upon the extracellular space determinations of Table 3. Errors are given as the standard deviation.

n = number of experiments; figures in parentheses are the number of data points involved in the regression analyses.

Asterisks denote values for experimental determinations that are significantly different from control values.

x = $p < 0.05$

xx = $p < 0.02$

xxx = $p < 0.01$

xxxx = $p < 0.001$

TABLE 4

EXPERIMENTAL CONDITIONS	n	intracellular [Na] m. equiv/l cell H_2O	extracellular [Na] m. equiv/l H_2O	intracellular [K] m. equiv/l cell H_2O
CONTROL 140 mM Na	9	30.68 ± 4.13 (108)	180 ± 13.2 (81)	115.6 ± 5.6 (189)
+ 10 mM D-galactose	4	42.06 ± 8.40 (48) xxx	236.5 ± 22.7 (36) xxxx	102.1 ± 4.69 (84) xxx
0.1 mM ouabain	3	137.10 ± 17.26 (36) xxxx	146.4 ± 16.2 (27) xx	27.9 ± 5.9 (63) xxxx
0.1 mM ouabain 20 mM D-galactose	3	143.2 ± 21.6 (36) xxxx	149.6 ± 18.0 (27) xxx	22.4 ± 4.3 (63) xxxx
75 mM Na	2	30.34 ± 6.4 (24)	115.5 ± 13.6 (18)	109.9 ± 3.2 (42)
75 mM Na + 20 mM D-galactose	2	29.5 ± 3.8 (24)	148.8 ± 15.0 (18)	114.1 ± 2.7 (42)
25 mM Na Ringer	2	12.8 ± 3.6 (24)	36.2 ± 12.4 (18)	115.6 ± 4.6 (42)
25 mM Na + 20 mM D-galactose	2	14.9 ± 4.1 (18)	40.4 ± 9.6 (24)	110.4 ± 4.3(42)
140 mM Na + 10 mM theophylline	3	(N.S. $p < 0.4$) 35.9 ± 4.2 (27)	(N.S. $p > 0.4$) 192.0 ± 21.6 (36)	(N.S. $p < 0.2$) 108.9 ± 7.2 (63)
10 mM theophylline + 20 mM D-galactose	3	42.1 ± 6.4 (27) xx	226.1 ± 20.4 (36) xxx	105.6 ± 4.4 (63) x
140 mM Na + 20 mM triaminopyrimidine	2	(N.S. $p < 0.1$) 37.1 ± 3.3 (24)	(N.S. $p < 0.1$) 158 ± 12.3 (18)	(N.S. $p < 0.5$) 111.6 ± 10.3 (42)
20 mM triaminopyrimidine + 20 mM galactose	2	45.80 ± 4.6 (24) xxx	245.1 ± 11.1 (18) xxx	97.67 ± 7.5 (42) xx

m./equiv whilst there is a concurrent increase in the cell [Na] to 134 ± 17.6 (S.D.) m. equiv. Ouabain also abolishes the observed hypertonicity of the extracellular space. The [Na] of the extracellular space falls to 146 ± 16.0 m. equiv. This is not significantly different from the Na concentration of the bathing fluid ($p > 0.8$) but is significantly below the control values ($p < 0.02$). Intracellular Na and K concentrations were probably not at equilibrium at the end of the preincubation period, since intracellular [K] is significantly larger that the concentration contained within the bathing medium ($p < 0.05$).

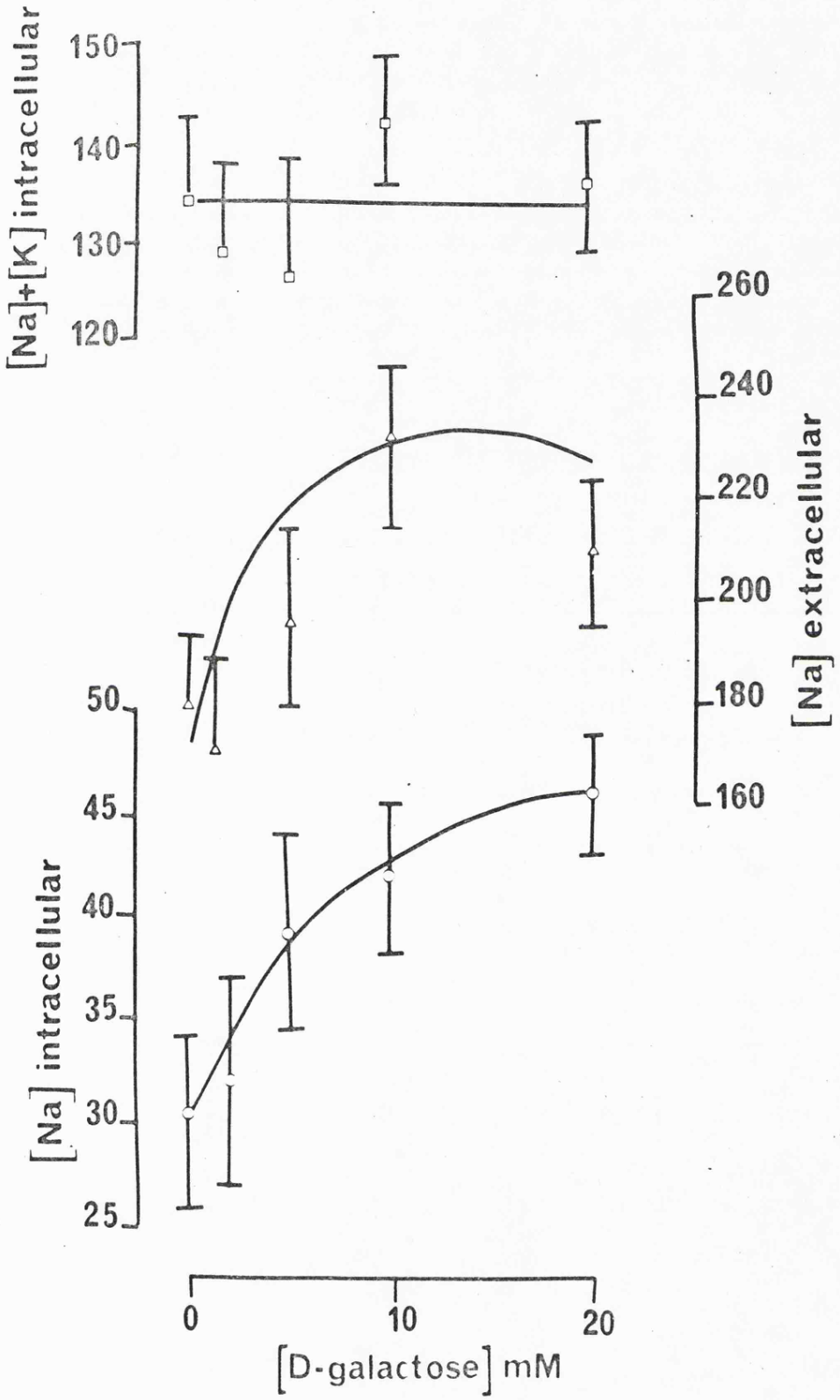
(iii) D-galactose

Both intracellular and extracellular Na concentrations are saturable functions of the D-galactose (Fig. 8). Intracellular [Na] increases from 30.68 ± 4.13 (S.D.) m. equiv. to 42.06 ± 8.4 (S.D.) m. equiv. at 10mM galactose. Extracellular [Na] increases from 180 ± 13.2 (S.D.) m. equiv. to 236.5 ± 22.7 m. equiv. with 10mM galactose present in the Ringer ($p < 0.001$).

It can be seen that the large increases in tissue [Na] observed by Koopman and Schultz (1969) in the presence of galactose, are not solely due to an increased intracellular Na content, but are due, primarily, to an increased extracellular [Na]. There is a reciprocal decrease in the intracellular K concentration as intracellular [Na] increases. This result agrees with the observations of Brown and Parsons (1959) and Koopman and Schultz (1969). The sum of intracellular [Na] and [K] is invariant with galactose concentration (Fig. 8). Galactose-dependent tissue swelling can, therefore, be inferred to be due to partial osmotic equilibration of both the extracellular and cell fluid, with the bathing medium.

Figure 8

The effect of D-galactose on the calculated values of intracellular $[Na]$, intracellular $[Na] + [K]$ and extracellular $[Na]$. Error bars denote $\pm$ S.D. for each point ($N \leq 42$).


[Na]+[K] intracellular
150
140
130
120
260
240
220
200
180
160
[Na] extracellular
50
45
40
35
30
25
[Na] intracellular
0
10
20
[D-galactose] mM

Variation in the Ringer [Na] in the range 0 to 140m. equiv causes saturable increases in the extracellular hypertonicity in the presence and absence of galactose (Fig. 9). The hypertonicity of the extracellular fluid is virtually abolished by reduction of Ringer [Na] to 25 m. equiv. This result confirms those already obtained with 0.1mM ouabain (i.e. that extracellular hypertonicity is dependent on the action of the Na-pump). Galactose probably stimulates the Na-pump by increasing influx of Na into the cell.

Figure 10 shows the variation of intracellular Na and K concentrations as a function of the preincubation Ringer [Na]. 20mM galactose has no significant effects upon intracellular Na or K until Ringer [Na] is raised above 75 m. equiv.

In Ringer containing 140m. equiv. Na and 20mM galactose the intracellular [Na] is raised to 46 $\pm$ 84 mM (S.D.) whilst intracellular [K] falls to 102 $\pm$ 4.6 m. equiv. (S.D.). This result shows that in 140 m. equiv. Na Ringer, the passive galactose-stimulated net Na entry starts to saturate the capacity of Na extrusion mechanism.

(iv) <u>The effects of 10mM theophylline</u>

The calculated intracellular Na and K concentrations of tissues in the presence of 10mM theophylline (Table 4) are not significantly different from control values ($p > 0.2$ for both Na and K). Extracellular [Na] is unaffected by theophylline ($p > 0.4$). Thus, reduction in the volume of the extracellular space does not alter extra-cellular hypertonicity. 20mM galactose in the presence of theophylline increases both intracellular and extracellular Na concentrations by similar amounts to those observed in control tissues (Table 4). Intracellular [K] also falls slightly, following addition of galactose.

Figure 9

Effect of variation in the [Na] of the preincubation medium on the calculated [Na] of the extracellular space expressed as the increment above the preincubation solution [Na] for controls (●) and in the presence of 20mM of galactose (▲). Error bars denote ± S.D. ($N \leqslant 23$).

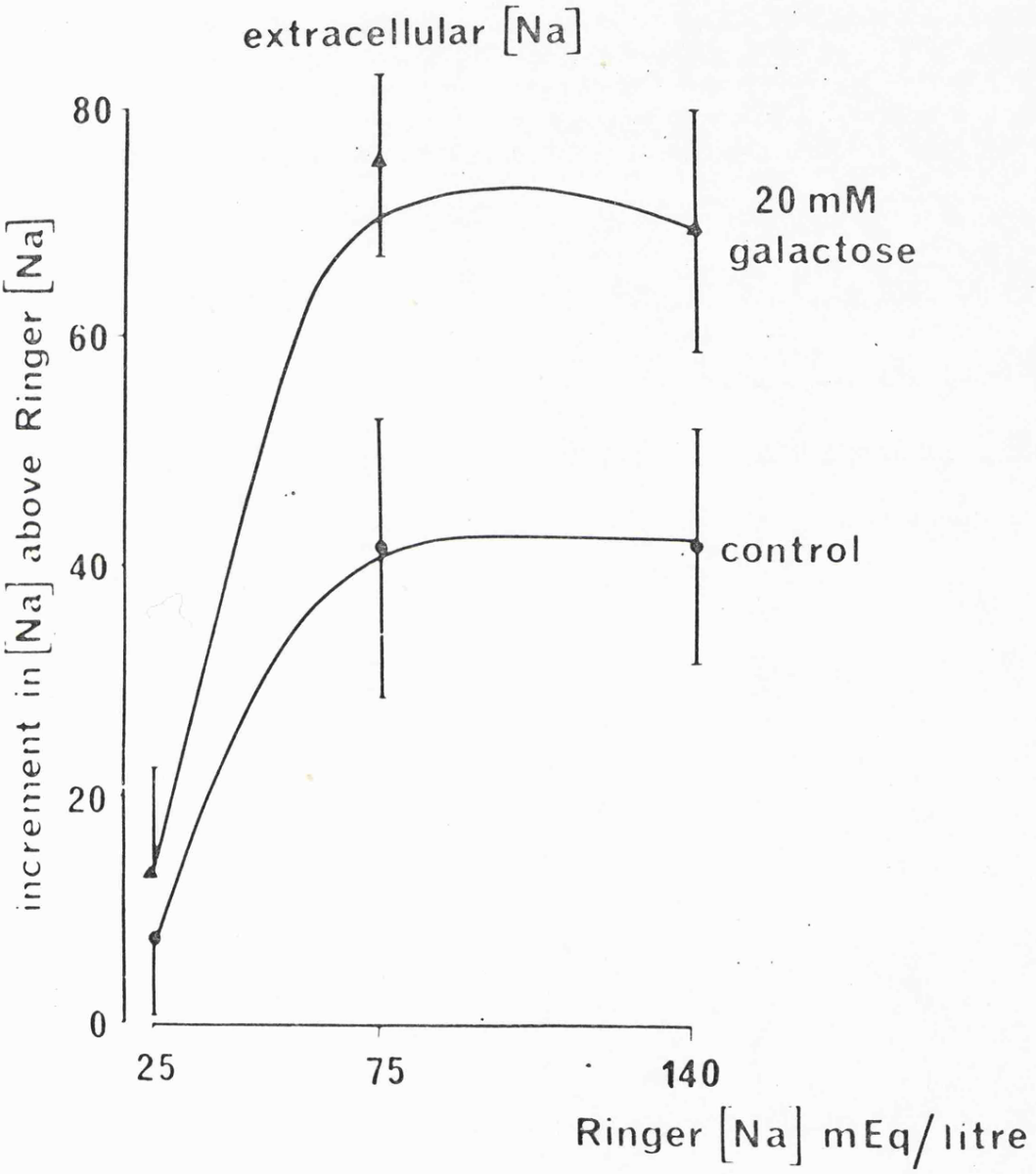
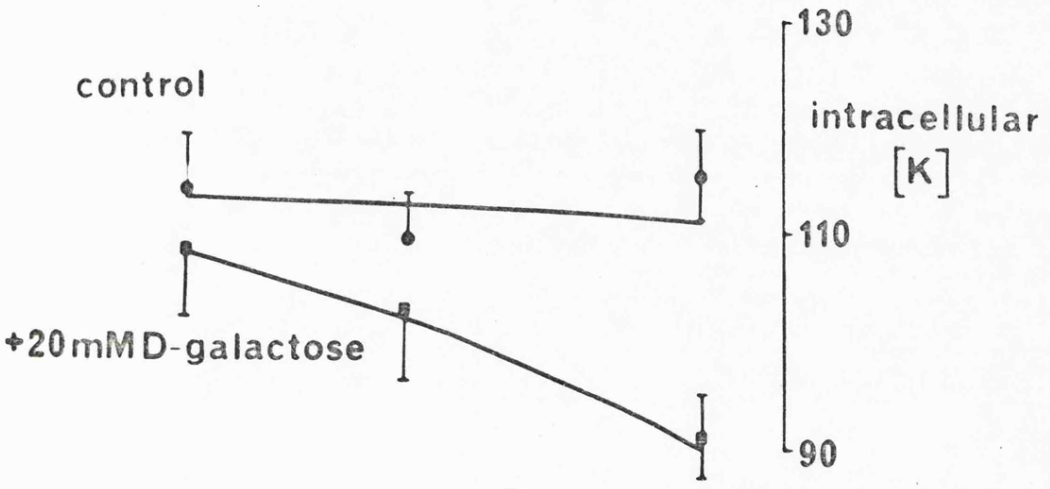
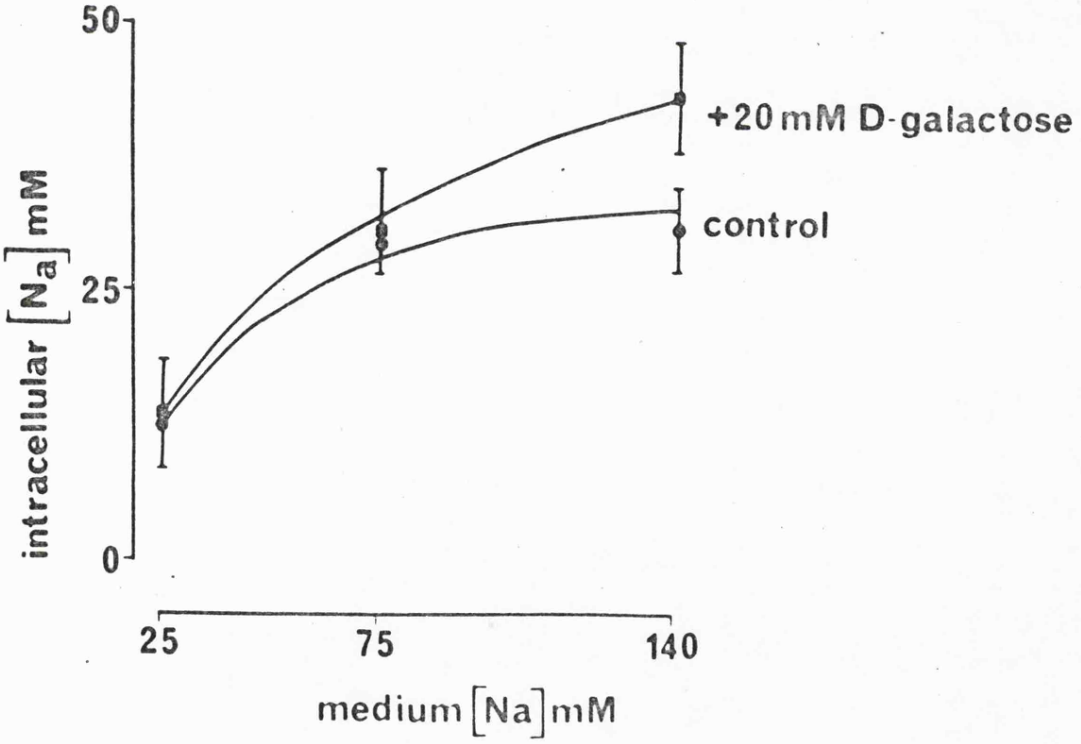

extracellular [Na]
80
60
40
20
0
increment in [Na] above Ringer [Na]
20 mM
galactose
control
25
75
140
Ringer [Na] mEq/litre

Figure 10

Effect of variation in the $[\overline{Na}]$ of the preincubation medium on the calculated intracellular cation concentrations $\pm$ 20mM D-galactose. Error bars denote $\pm$ S.D. ($n \leq 23$).


control
+20mM D-galactose
130
intracellular
[K]
110
90


50
25
0
intracellular [Na] mM
+20 mM D-galactose
control
25
75
140
medium [Na] mM

C. Discussion

It has been shown that the extracellular space of rabbit ileum contains NaCl at a concentration which is at least 80 m. osmoles hypertonic to the external Ringer. This observation directly confirms the standing-gradient model of fluid absorption by Diamond and Bossert (1967). Previously, the only indirect support for this hypothesis has been obtained from experiments with mammalian epithelia (Machen and Diamond (1969)), although direct estimates of the osmolarity of the intercellular space of cockroach rectum has yielded evidence of a solution which is 130 m. osmoles hypertonic (Wall et al (1970)). However, uncertainty as to the composition of this fluid remains.

The estimates of extracellular fluid hypertonicity in this study rely on the accuracy of the extracellular space determination. If the inulin space understimates the true extracellular space by 30% in Ringer, or by 70% in Ringer + 20mM galactose, the $[Na]$ in the space could be isotonic. However, errors of this magnitude would mean that the monovalent cation concentration of the cell fluid would be 170 and 230 m. equivs. respectively, which are improbably large. The calculated tonicities of the intracellular cations on the basis of the measured space are consistent with the mean cell solute concentration being isotonic with the external Ringer. Further confirmation of the reliability of the results is provided by the observation that, either addition of ouabain, or reduction of Ringer $[Na]$ to 25m. equiv. abolishes the hypertonicity of the extracellular fluid compartment. These results indicate that the extracellular hypertonicity is produced by the action of the Na-pump which Diamond and Bossert (1967) predicted would deposit hypertonic NaCl into the lateral intercellular space.

Rather than an overestimate of the extracellular fluid hypertonicity, the data reported here is probably an underestimate of the hypertonicity present in the lateral intercellular spaces of actively transporting epithelia. Small intestinal transport activity is thought to be localized to the apical part of the intestinal villus, consequently fluid coming from inactive regions will tend to dilute the hypertonic reabsorbate towards isotonicity (McElligot et al (1975)). Hence the lumped estimate of the extracellular fluid concentration will underestimate the $[Na]$ in the actively transporting region of the tissue and overestimate the concentration in parts distant to the active pump sites.

It is pertinent to ask whether the hypertonicity of the extracellular fluid is a resultant of the _in vitro_ incubation of ileum. _In vivo_, capillary circulation will reduce the effective unstirred layer formed by the submucosal tissues. The existance of a large serosal unstirred layer could markedly increase the salt concentration in the lateral spaces provided that the rate of active salt transport into the spaces remained constant. Huss and Marsh (1975) have shown that a decrease in osmotic permeability of the basement membrane can theoretically increase the hypertonicity within the lateral spaces. An equivalent effect is observed on decreasing the salt permeability for the basement membrane. In both these situations the theoretical absorbate tonicity is hypertonic. Powell and Malawer (1968) have shown that the ileal absorbate from isotonic solutions placed in the lumen of rat ileum _in vivo_ is hypertonic by $\sim$100 m. osmoles. This demonstrates that anisotonic absorbates, and hence anisotonic tissue extracellular spaces, exist even in a state of capillary perfusion. The present observation of extracellular hypertonicity is unlikely to result from the _in vitro_ incubation of ileum.

Estimates of small intestinal intracellular [Na] based on the assumption that the extracellular fluid is isotonic to the external bathing medium are invalidated by the findings reported here. The high cell [Na] reported by Koopman and Schultz and the large galactose-dependent increase in tissue [Na] , can now be ascribed to an increase in NaCl deposition within the extracellular fluid, rather than to an increase in intracellular [Na] induced by inhibition of the Na-pump resulting from reduced cell [ATP] , as they suggest. It is of interest that the low value of intracellular [Na] found here accords with direct determinations of intracellular Na activity made with Na-sensitive microelectrodes in bullfrog small intestine (Lee and Armstrong (1972)). The intracellular activity coefficient of Na in this study was low ($\approx$0.5); however, this low activity coefficient may be in error, since the overall intracellular [Na] from which the activity coefficient was determined, was obtained by assuming that the extracellular fluid was isotonic with the bathing medium.

The large increment in [Na] in the extracellular space following addition of 20mM galactose to the preincubation Ringer may provide an explanation for the observed stimulation of net water and salt absorption by sugars (Esposito et al (1969)). An increase in the osmotic-gradient across the basal-lateral border will increase net water flow across this border from the cell fluid. The cell water will be replaced in turn by increased flow across the brush-border as a consequence of the increase in intracellular solute concentration.

A likely reason for the galactose-dependent increase in Na accumulation in the extracellular space may be that there is an increase in passive net Na influx across the brush-border as a result of sugar linked coupled transport (Goldner et al (1969) and see Chapter 4). The increase in intracellular [Na] will increase the

Na-pump activity at the lateral-basal border of the cell, provided that the pump is not already saturated (Glynn (1968)).

Naftalin and Holman (1974) and Holman and Naftalin (1975a) have proposed that the asymmetric sugar flux across the brush-border membrane and intracellular sugar accumulation are a consequence of mass water flow across the epithelial cell. The observed extracellular hypertonicity found in this study is consistent with this model of sugar transport. Extracellular hypertonicity provides the driving force for sugar transport.

The hypertonic concentration of Cl in the extracellular fluid found by Zeuthen and Monge (1975) who used microelectrode methods, provides independent support for the present finding of extracellular hypertonicity.

CHAPTER FOUR

THE MEASUREMENT OF THE UNIDIRECTIONAL Na FLUXES ACROSS THE MUCOSAL AND SEROSAL BORDERS OF RABBIT ILEUM: THE USE OF 2,4,6, TRIAMINO-PYRIMIDINE AND THE EFFECTS OF D-GALACTOSE

A. Introduction

Estimates of the unidirectional Na fluxes across the mucosal and basal-lateral membranes have previously relied upon measurements from three separate experiments performed on separate pieces of tissue (Schultz and Curran (1968); Schultz et al (1967)). A method for measuring the entry and exit fluxes of sugars simultaneously across the mucosal and serosal borders of rabbit ileum has recently been described (Naftalin and Curran (1974) and see also Chapter 2). An inherent assumption of this method is that there is no significant route for transepithelial sugar movement other than the transcellular pathway. Application of this method to Na flux measurements therefore requires the determination of the diffusional Na flux through the shunt pathway. An alternative approach is to reduce Na flux through the shunt pathway to insignificant levels.

Frizzell and Schultz (1972) have approached the determination of the shunt permeability to Na by measuring the unidirectional influx of Na as a function of the applied transepithelial potential difference. The shunt permeability to Na at zero P.D. (i.e. the diffusion component via the shunt) may then be determined from the functional relationship between influx and transepithelial P.D. Errors may arise, not only from within and between experimental variance, but also from current induced changes in tissue resistance (Bindslev et al (1974) and see also Section 1), and permselectivity (Weidner and Wright (1975)). The use of low transepithelial potentials ($\pm$ 10mV) (Desjeux, Tai and Curran (1974)) does not eliminate this effect due to high

tissue conductance.

Moreno (1974, 1975a,b) has recently found that 2,4,6, triaminopyrimidine specifically reduces the Na conductance of the shunt pathway in the gall bladder and other low-resistance epithelia e.g. rabbit ileum. It was, therefore, considered that triaminopyrimidine, by eliminating or significantly reducing the shunt component of transepithelial Na flux, would be useful in the measurement of transcellular Na fluxes in rabbit ileum.

Recently, the Na-dependence of all four unidirectional fluxes of D-galactose have been examined (Naftalin and Holman (1974), Holman and Naftalin (1975a). Specifically the exit flux across the mucosal boundary was shown to decrease as intracellular $[Na]$ was raised. This result contradicts that predicted by the Na-gradient hypothesis. No information exists concerning the effects of D-galactose on the Na exit flux across the mucosal boundary of sheets of tissue. Furthermore, the finding that extracellular hypertonicity is increased when galactose is added to the Ringer (see Chapter 3) suggests that there is a galactose-dependent stimulation of Na-pump activity.

This Chapter describes the effects of D-galactose on the four unidirectional fluxes of Na across the mucosal and serosal borders of rabbit ileum, and upon the Na fluxes through the shunt pathway.

B. Results

1. Effects of 2,4,6, triaminopyrimidine on the shunt conductance

(i) Voltage dependent mucosa to serosal Na fluxes J_{13}

Frizzell and Schultz (1972) have shown that the transcellular Na flux J_{C13} may be experimentally separated from paracellular shunt flux (J_{D13}) by varying the applied potential (Ψ_{13}) difference and measuring the total transepithelial flux (J_{T13}). The basis of this approach is that application of an imposed potential difference will change ion flux through the low resistance (high conductance) paracellular route, but will not effect ion flux through the high resistance transcellular route. A plot of ion flux J_{13}(total) against a function of the applied potential difference will yield a straight line, with an intercept equal to flux through the cellular route (J_{C13}) and a slope which is numerically equivalent to paracellular flux at zero P.D. (see Methods).

Figures 11 (a) and (b) show the variation of J_{13} when plotted as a scatter diagram against the applied potential difference, (expressed as the function $\exp(-zF\Psi_{13}/RT)^{\frac{1}{2}}$) for control tissues and tissues incubated in the presence of 20mM triaminopyrimidine. The regression equation for controls gives the intercept (J_{C13}) as 6.14 μmoles cm^{-2} hr^{-1} and the slope (J_{D13}) as 5.44 μmoles cm^{-2} hr^{-1}. These values are similar to those obtained by other workers (Frizzell and Schultz (1972); Desjeux, Tai and Curran (1974)).

20mM triaminopyrimidine causes a significant change in the slope of the regression line (J_{D13}) ($p < 0.05$). Indeed, paracellular Na flux (J_{D13}) in the presence of triaminopyrimidine does not differ significantly from zero ($p > 0.3$). A small increase in transcellular Na flux J_{C13} is observed in the presence of triaminopyrimidine. This increase is not significant ($p > 0.2$) and represents variance from extrapolation

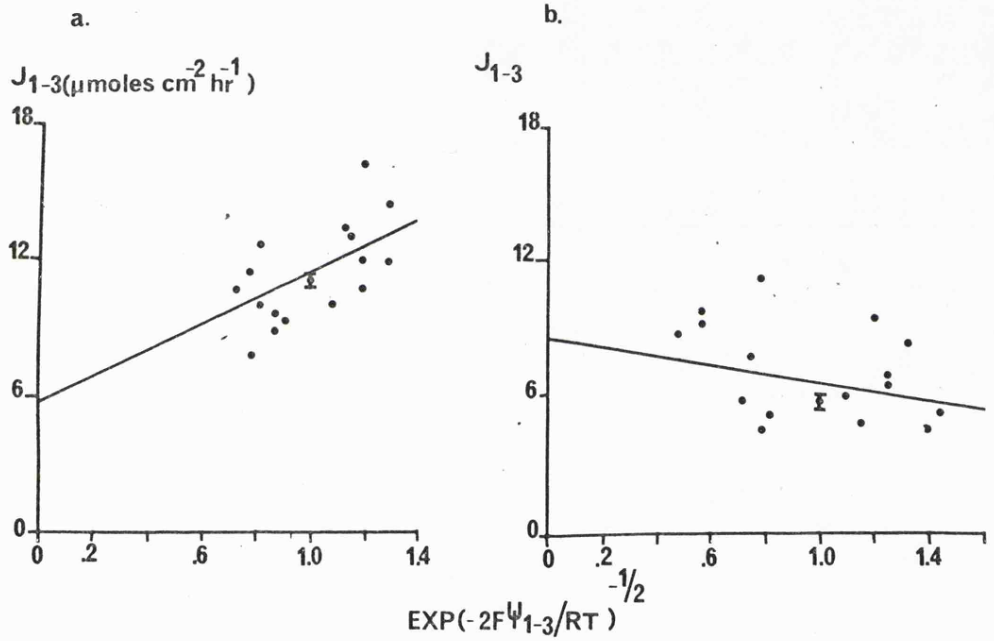
Figure 11 (a)

Relationship between mucosa to serosa flux, J_{13} and the applied potential difference expressed as the relationship $\exp(-ZF\Psi_{13}/RT)^{-\frac{1}{2}}$ for control tissues. Data were pooled from four experiments (eight tissues) and normalized about the flux at zero potential difference. The solid line is the least-square regression line for the data ($J_{13}^{Na} = 6.14 + 5.44^{-\frac{1}{2}}$ $r = 0.46$ $p < 0.05$).

Figure 11 (b)

Relationship between flux J_{13} and the applied potential difference for tissues incubated in the presence of 20mM triaminopyrimidine. Data were normalized from four experiments (eight tissues). The least square line for the data gives

($J_{13} = 9.57 - 2.18^{-\frac{1}{2}}$ $r = 0.27$ $p > 0.05$ N.S.).


a.
b.
J_{1-3}(μmoles cm^{-2} hr^{-1})
J_{1-3}
18
12
6
0
0
.2
.6
1.0
1.4
$EXP(-2F\Psi_{1-3}/RT)^{-1/2}$

at the regression line to the intercept. Transcellular Na flux in the presence of triaminopyrimidine is likely to be no different from control values since J_{13} (total) plus triaminopyrimidine at zero potential (7.02 μmoles cm^{-2} hr^{-1}) is similar to J_{C13} derived for the regression equation for control tissues from Figure 7 (a) (6.4 μmoles cm^{-2} hr^{-1}).

The major effect of triaminopyrimidine is the reduction in Na flux through the paracellular pathway.

(ii) <u>The effect of an applied electrical potential difference on net Na flux</u> (J_{net})

The effect of transepithelial potential upon the net Na flux is shown in Figure 12 for experiments in which the bidirectional (m-s and s-m) Na fluxes were measured simultaneously. The applied potential is expressed relative to the serosal solution. Data for control and triaminopyrimidine-treated tissues (20mM) are shown. Net Na flux is related to the potential difference by the following least-squares linear relationship.

$$J_{net} = -0.29 \Psi_{13} + 3.93$$

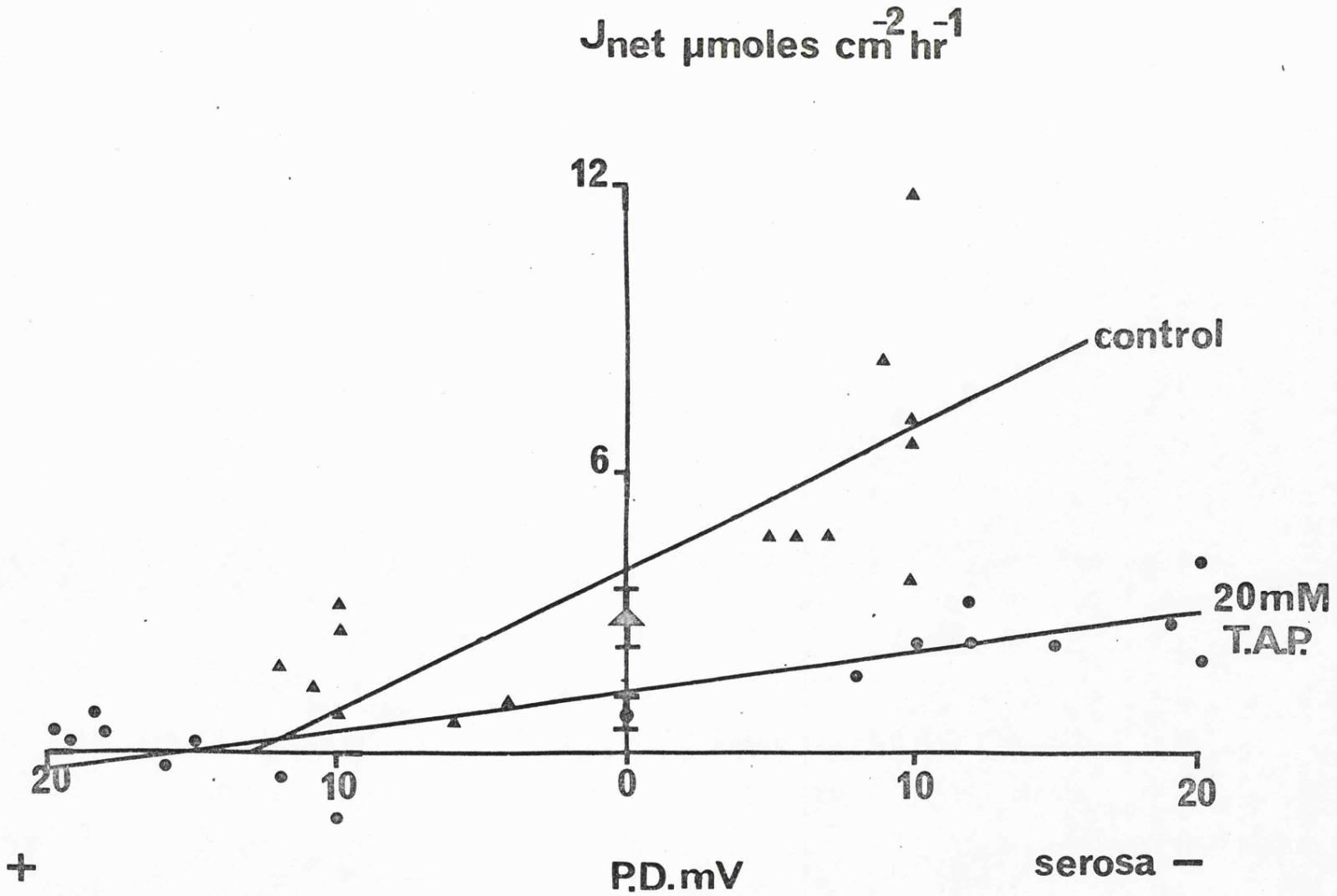
Similar relationships have been obtained by other workers, notably Desjeux, Tai and Curran (1974) (slope = -0.3, intercept = 2.83); Schultz and Zalusky (1964) (slope = -0.32, intercept = 3.56) and Clarkson for rat ileum (1967) (slope = -0.13, intercept = 2.01).

The presence of 20mM triaminopyrimidine reduces the intercept (net flux at zero P.D.) to 1.08 μmoles cm^{-2} hr^{-1}. This result may indicate an effect of triaminopyrimidine on a passive component of net Na movement.

The potential-sensitive net flux (as denoted by the slope of the regression line) is markedly reduced by triaminopyrimidine. Indeed net flux in the presence of triaminopyrimidine is virtually insensitive to the transepithelial electrical gradient. This contrasts with the

Figure 12

The effect of the applied transmural potential difference upon net Na flux (J_{net}) for tissues incubated in the presence and absence of 20mM triaminopyrimidine (TAP). All potentials are expressed relative to the serosal bathing solution. (A negative potential refers to a cathodic electrode). The least-square regression lines for the data are shown. $J_{net} = -0.29 \Psi_{13} + 3.93$ μmoles cm^{-2} hr^{-1} (controls) $r = -0.64$; $n = 24$; $(p < 0.01)$. $J_{net} = -0.076 \Psi_{13} + 1.12$ μmoles cm^{-2} hr^{-1} (for tissues incubated in the presence of 20mM triaminopyrimidine) $r = -0.85$; $n = 22$; $(p < 0.01)$; Ψ_{13} is the transepithelial potential difference.


Jnet µmoles cm-2 hr-1
12
6
control
20mM
T.A.P.
20
10
0
10
20
+
P.D.mV
serosa —

effect of P.D. upon control tissues and lends support to the notion that the primary action of triaminopyrimidine is to reduce Na flux through the paracellular pathway.

(iii) Conductance determinations

The effect of ion substitution of the bathing Ringer's solution upon the transepithelial resistance provides a means of evaluating the relative ionic permeabilities of the shunt pathway since almost all (85%) of tissue conductance is attributable to this pathway in rabbit ileum (Schultz and Curran (1974)). Substitution of Na by an impermeant cation will give a value for that part of transepithelial conductance that is dependent upon Na. It is assumed that conductance is linearly related to ionic concentration (in accord with the data of Schultz and Zalusky (1964)).

Table 5 shows the open-circuit potentials and conductance measurements for tissues incubated in Ringer's solution containing 140, 75 and 25mM Na in the presence and absence of 20mM triaminopyrimidine following a ten minute preincubation period.

The open-circuit potential changes from a positive to negative value as Ringer $[Na]$ is reduced from 140 to 25mM. Concurrently the conductance decreases; this result is consistent with the presence of a cationic-selective shunt across the intestine that is impermeable to choline (Schultz and Curran (1974); Nellans et al (1974)).

20mM triaminopyrimidine decreases tissue conductance at 140mM Na compared with controls ($p < 0.001$). There is no significant effect of triaminopyrimidine at a Ringer $[Na]$ of 25mM ($p > 0.4$). The triaminopyrimidine-dependent reduction in total tissue conductance (3.03 mmhos cm^{-2}) at 140mM Na is equivalent to a reduction in Na permeability of 0.0216 cm hr^{-1}.

20mM triaminopyrimidine has no significant effect upon the open-circuit potential (Table 2). This result shows that the product of

Table 5

Conductance measurements ± 20mM triaminopyrimidine. NaCl replacement was made with choline Cl. (Ringer pH 7.4). Errors are expressed as ± S.E.M. Numbers in parentheses represent the number of separate determinations. Determinations of conductance and P.D. were made 5 to 15 minutes subsequent to the start of each experiment.

Table 5

	[Na] mM	Open Circuit P.D. (mV)	Conductance mmhos cm^{-2}
CONTROL	140	1.48 ± 0.14 (43)	10.39 ± 0.26 (46)
	75	0.97 ± 0.21 (30)	9.52 ± 0.61 (26)
	25	-0.26 ± 0.28 (15)	7.14 ± 0.62 (15)
+20 mM Triminopyrimidine	140	1.34 ± 0.24 (31)	7.29 ± 0.51 (38)
	75	0.707 ± 0.212 (22)	7.26 ± 0.41 (22)
	25	-0.65 ± 0.33 (10)	6.57 ± 0.79 (10)

P.D. and conductance (i.e the short circuit current, which is equivalent to the rate of active ion transport) changes. That is to say, 20mM triaminopyrimidine causes a fall in net Na flux. This result confirms direct measurements of net flux (section 1 (ii)).

(iv) Mannitol dilution and biionic potentials

An alternative approach to those previously described for the measurement of the relative ionic permeabilities of the shunt pathway, is to measure the change in transepithelial electrical potential difference due to unilateral changes in the ionic composition of the bathing media (usually the mucosal solution). The resulting potentials may be analysed in terms of the Goldman-Hodgkin-Katz formulation. All experiments were performed in the presence of 0.1mM ouabain to obviate possible potential changes due to active transcellular processes.

Table 6 shows the biionic potentials recorded after isosmotic replacement of the Ringer in the mucosal solution by the following solutions; choline chloride, KCl, Na_2SO_4, and mannitol. As found by other workers (Frizzell and Schultz (1972)) mannitol replacements gave a larger serosa negative (-18mv) potential. The presence of 20mM triaminopyrimidine reduced this potential to -9.8mv. The difference compared with control values is significant ($p < 0.02$). That this reduction is due specifically to a reduction in paracellular Na permeability without concurrent changes in the Cl permeability is evident from the following observations:

1. the choline/Na biionic potential is reduced by a similar amount compared with the mannitol dilution potential.

2. The K/Na and the SO_4/Cl biionic potentials are unaffected by triaminopyrimidine ($p > 0.7$, $p > 0.2$ respectively).

Table 6

Mannitol dilution potentials and biionic potentials ± 20mM triaminopyrimidine (present in both mucosal and serosal solutions). All measurements refer to mucosal replacement of 140 NaCl Ringer and are expressed relative to the mucosal solution. All solutions contained 0.1mM ouabain. Potentials were recorded after a twenty minute preincubation period during which time the spontaneous P.D. had declined to zero. Errors are expressed as the S.E.M. Numbers in parentheses are the number of experiments. P values are for Student's t test for the effect of triaminopyrimidine.

Table 6

MUCOSAL DILUTION	CONTROL	20mM TRIAMINOPYRIMIDINE	P
MANNITOL	-18.28 ± 2.6 (6)	-9.88 ± 1.26 (6)	< 0.02
CHOLINE Cl	-21.64 ± 1.45 (12)	-11.9 ± 1.57 (12)	< 0.001
KCl	$+4.72 \pm 0.31$ (6)	$+4.52 \pm 0.26$ (6)	> 0.7
Na_2SO_4 *	$+6.04 \pm 0.47$ (12)	$+4.56 \pm 0.98$ (12)	> 0.2

*Isotonicity to control 140mM NaCl Ringer was ensured by addition of Mannitol.

These findings are, therefore, in accordance with the effects of triaminopyrimidine on frog gall-bladder observed by Moreno (1974, 1975 a,b). The Na permeability for the shunt pathway across control tissues is 0.038 cm hr^{-1} (derived from the slope of the voltage-dependent flux measurements and the relationship $P_{Na} = J_{Na}/C_{Na}$).

A triaminopyrimidine-dependent reduction in the mannitol dilution potential of the observed magnitude is consistent with a reduction of paracellular Na permeability of 0.015 cm hr^{-1} (this value was calculated from the Goldman-Hodgkin-Katz equation assuming that P_{Cl} = 0.019 cm hr^{-1} (Frizzell and Schultz (1972)). The observed decrease in P_{Na} due to triaminopyrimidine is slightly smaller than that decrease due to triaminopyrimidine deduced from conductance measurements (section 1 (iii)).

The residual Na permeability of 0.023 cm hr^{-1} in the presence of 20mM triaminopyrimidine for the paracellular pathway is larger than that indicated by voltage dependent flux measurements (section 1 (i)). This difference may be due to systematic errors arising from current induced changes in tissue resistance (section 1 (vii)) and to the large inaccuracies associated with voltage dependent flux measurements. The magnitude of Na and Cl permeabilities, however, may indicate the existance of a parallel pathway other than the tight junction (see Chapter 6).

(v) <u>The effect of triaminopyrimidine (0 to 20mM) upon paracellular Na permeability</u>

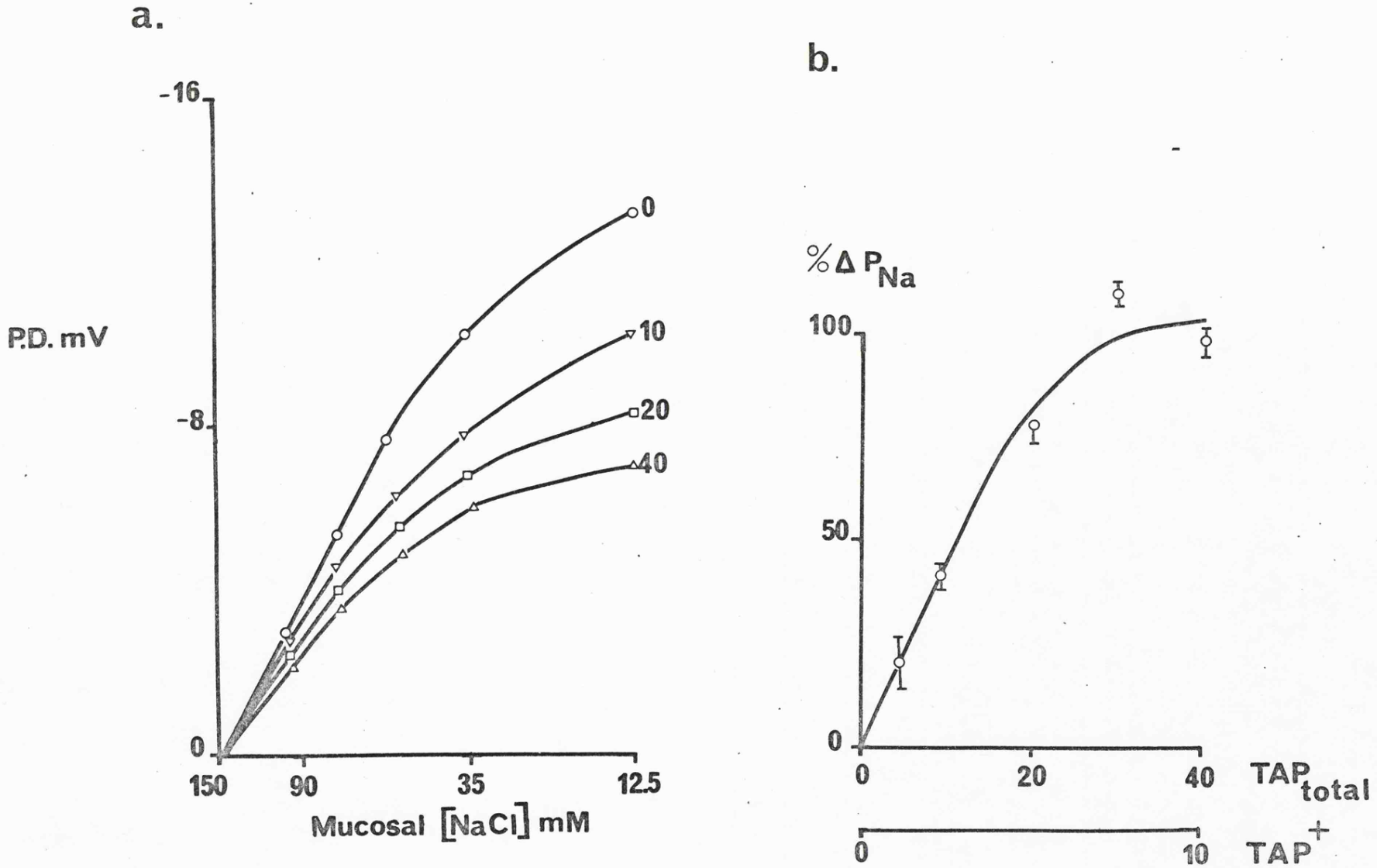
Mannitol dilution potential measurements provide a convenient method for the assessment of paracellular Na permeability. Figure 13 (a) shows the pooled data for six experiments (72 tissues) in which the mannitol diffusion potentials were obtained at varying levels of [triaminopyrimidine]. As triaminopyrimidine concentration is raised the magnitude of the dilution potential at any given level of

Figure 13 (a)

The effect of triaminopyrimidine (TAP) on the open-circuit transepithelial potential differences obtained by mucosal dilution of NaCl with isosmotic replacement with mannitol. Triaminopyrimidine was tested at 0 (○), 5, 10 (▽), 20 (□), 30 and 40 (△) mM. Potentials at 5 and 30mM are omitted for clarity. Each point represents the mean of pooled data from six experiments (two tissues per concentration per experiment).

Figure 13 (b)

The effect of triaminopyrimidine (TAP) upon paracellular Na permeability (expressed as the % change of the maximum change in Na permeability observed). Error bars denote the standard error for each point (dF = 22). Permeabilities were calculated as described in the text (TAP^+ denotes the monoprotonated form of TAP).


a.
-16
-8
0
P.D. mV
0
10
20
40
150
90
35
12.5
Mucosal [NaCl] mM
b.
%Δ P Na
100
50
0
0
20
40
TAP total
0
10
TAP+

mucosal Ringer [NaCl] is reduced.

Calculation of the Na permeabilities at constant [Na] is possible from the Goldman-Hodgkin-Katz equation. Figure 13(b) shows the variation in Na permeability (expressed as a percentage of the maximum decrease obtained) as a function of the triaminopyrimidine concentration. The upper scale concentration represents total base added, whereas the lower scale represents the calculated concentration of the monoprotonated base (see below). It is clear that the reduction in Na permeability due to triaminopyrimidine follows saturation-type kinetics. The K_m for this relationship is 5.54 $\pm$ 2.00 (SE)mM (total base) (1.38 $\pm$ 0.49 (SE) for the monoprotonated form). The latter value is in agreement with the K_m value for the fractional reduction in Na permeability derived by Moreno (1975a) for the effect of triaminopyrimidine on frog-gall bladder. Moreno reports that 100% inhibition of tight junction conductance for Na is possible. However, Moreno divides the passive tissue paracellular conductance of Na into two pathways, i.e. a tight-junction route and a so-called leakage pathway. The residual permeability to Na reported in section 1 (iv) in the presence of triaminopyrimidine may constitue this leakage pathway in ileum (see also Chapter 6).

(vi) Effect of Ringer pH on the triaminopyrimidine-dependent reduction in paracellular Na permeability

The base 2,4,6, triaminopyrimidine (TAP) may exist in solution as a neutral species, a monocation, or a dication depending upon the solution pH. Roth and Strelitz (1969 and 1970) give the acid dissociation constants pK_1 and pK_2 (i.e. base (TAP) $\overset{pK_1}{\rightleftharpoons}$ monocation (TAP^+) $\overset{pK_2}{\rightleftharpoons}$ dication $(TAP^+)^+$) as 6.74 and 1.31 respectively.

Figure 14 shows the effect of variation in Ringer pH * upon the evoked mannitol dilution potentials at 0, 5 and 25mM triaminopyrimidine.

It is clear that pH variation over the range pH 6 to 8 has no effect upon the evoked dilution potential in the absence of triaminopyrimidine. This finding is similar to that found by Smyth and Wright (1966) for streaming potentials in rat small intestine and by Wright and Diamond (1968) for rabbit gall-bladder dilution potentials.

At pH 7 both 5 and 25mM triaminopyrimidine reduce the evoked dilution potential compared with controls (0 triaminopyrimidine). The reduction in permeability is similar to that observed at pH 7.4.

Elevation of Ringer pH to 8 decreases the reduction in the evoked dilution potential observed at both 5 and 25mM triaminopyrimidine compared with the observed effects at pH 7.

A reduction of Ringer pH to 6 causes an increased effect of triaminopyrimidine in reducing the evoked dilution potential at 5mM, but not at 25mM.

Figure 14 shows the relative permeability changes observed due to the presence of triaminopyrimidine (5 or 25mM) expressed as a fraction of the permeability change due to 25mM triaminopyrimidine at pH 7 plotted as a function of Ringer pH.

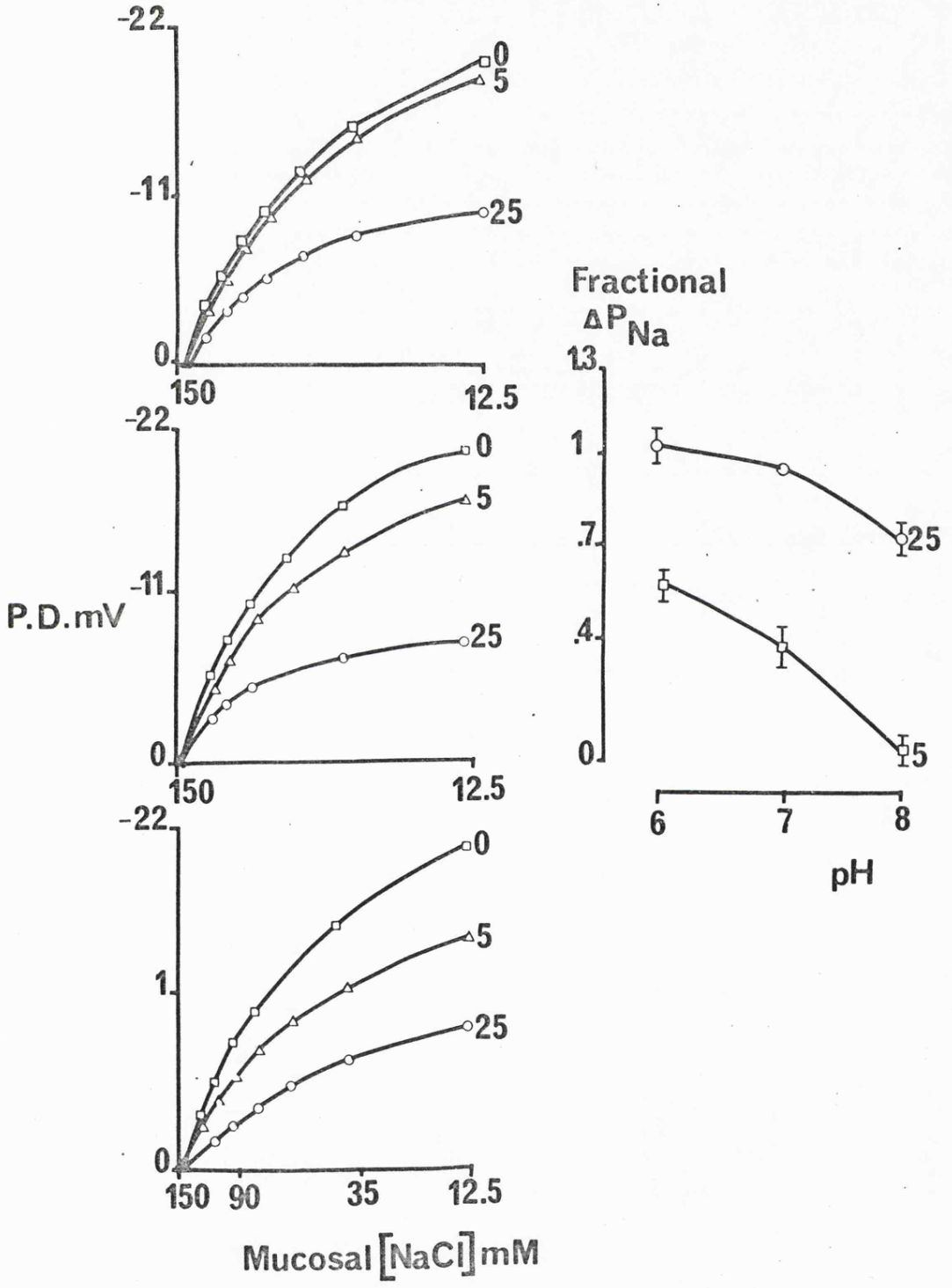
It is clear that the bathing medium pH is an important determinant in the action of triaminopyrimidine. That no change in the fractional permeability at 25mM triaminopyrimidine is seen on reducing the pH of the bathing medium from 7 to 6 indicates that the active form of triaminopyrimidine is present in these conditions at saturating concentrations.

*Ringer pH was varied by adjusting the ratio of Ringer KH_2PO_4 : K_2HPO_4 at constant $[K]$.

Figure 14

The effect of variation in Ringer pH upon the mannitol dilution potentials. Ringer pH was varied by adjusting the $K_2HPO_4/K_2H_2PO_4$ ratio at constant [K] (HCO_3-free buffer). Ringer pH was tested at pH 6.0 (bottom left), pH 7.0 (middle left) and pH 8.0 (top left). Two concentrations of triaminopyrimidine were used, 5 and 25mM. Each point is the mean of three experiments (four tissues).

The insert (middle right) shows the fractional change in Na permeability plotted as a function of the Ringer pH at 5 and 25mM triaminopyrimidine. The fractional change in Na permeability is expressed relative to the change in Na permeability at pH 7, 25mM triaminopyrimidine. Error bars denote the standard error for each point (n = 6).


-22
-11
0
0
5
25
150
12.5
-22
-11
0
0
5
25
150
12.5
P.D.mV
-22
1
0
0
5
25
150
90
35
12.5
Mucosal [NaCl] mM
Fractional
ΔP Na
1.3
1
.7
.4
0
25
5
6
7
8
pH

Since the concentrations of TAP, TAP^+ and TAP^{++} will all change as Ringer pH is varied it is pertinent to ask which is the physiologically active form of the base.

The concentration of TAP, TAP^+ and TAP^{++} are given by the following equations (Moreno (1975a)) where

$$TAP\ (total) = TAP + TAP^+ + TAP^{++}$$

1. $$TAP = TAP\ total\ /1 + 10^{(pH - pK_1)} . (1 + 10^{(pH - pK_2)}$$

2. $$TAP^+ = TAP\ total\ /1 + 10^{(pH - pK_1)} + 10^{-(pH - pK_2)}$$

3. $$TAP^{++} = TAP\ total\ /1 + 10^{(pH - pK_2)} . (1 + 10^{(pH - pK_1)}$$

Since the experiment to determine the concentration dependence of the action of triaminopyrimidine was at pH 7.4, the above equations may be used to determine the appropriate concentrations giving the observed decrease in fractional Na permeability assuming that TAP, TAP^+ or TAP^{++} is the active species.

Such a treatment yields values for K_m of 3.86mM, 1.38mM and 1.09×10^{-6}mM for TAP, TAP^+ and TAP^{++} respectively.

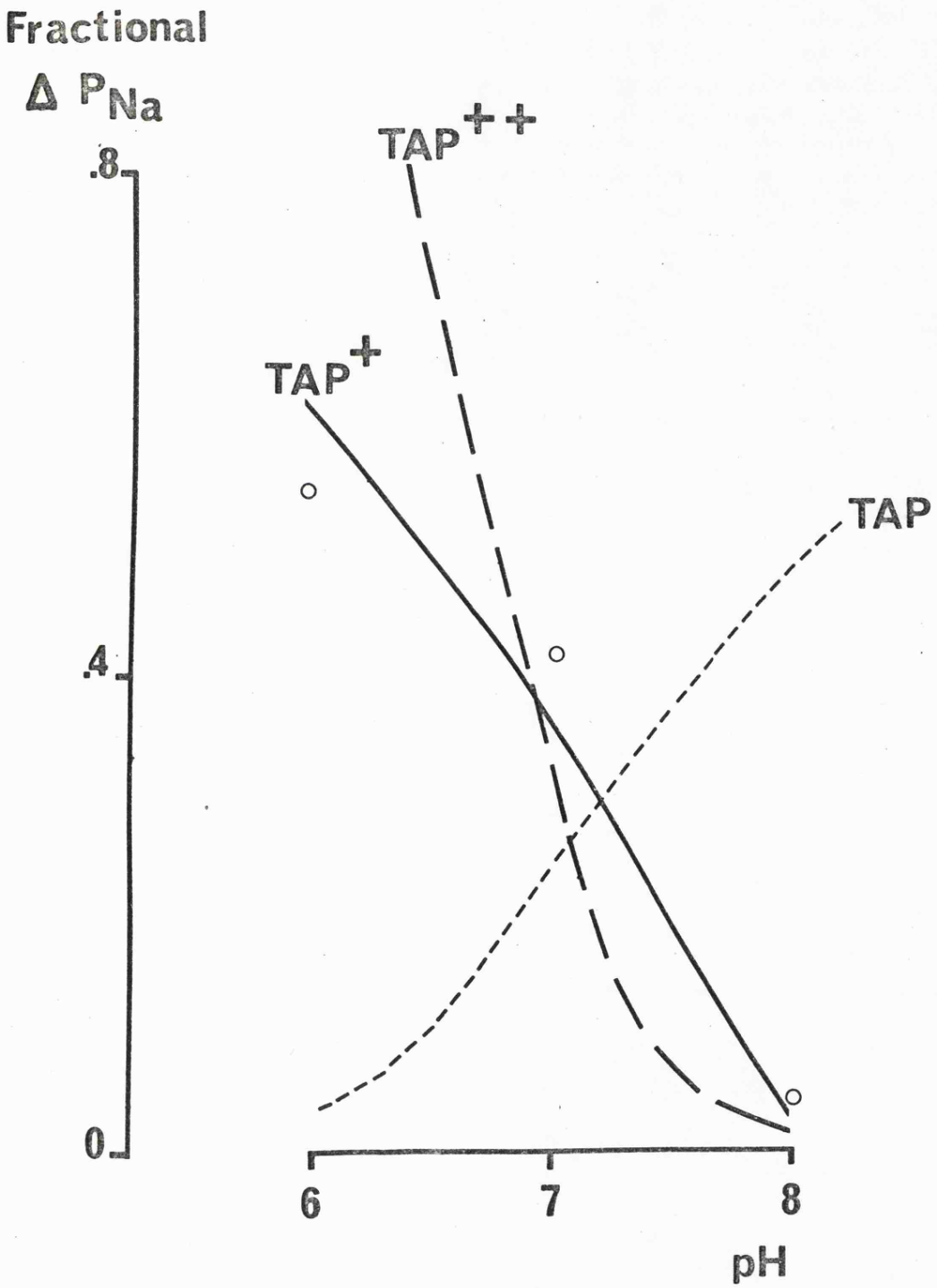
At a constant $\left[TAP\ total\right]$ of 5mM, variation in pH from 6 to 8 will change the respective concentrations of TAP, TAP^+ and TAP^{++} present in solution. These values may be calculated from equations 1 to 3.

By using the respective K_m values a theoretical fractional change in Na permeability may be calculated for each form of triaminopyrimidine over the range of pH of interest (6 to 8).

Figure 15 shows the results of this treatment compared with the experimental change in fractional Na permeability observed for 5mM triaminopyrimidine. The experimental results are consistent with the monoprotonated form of the base being the physiologically active species. This conclusion agrees with that of Moreno for the effect of triaminopyrimidine on gall-bladder Na permeability.

Figure 15

Theoretical relationships between the fractional change in Na permeability (ΔP_{Na}) and the neutral (TAP), monocationic (TAP^{+}) and dicationic (TAP^{++}) forms of triaminopyrimidine (see text for details). Circles are the experimentally observed balues for ΔP_{Na} from Figure 10.


Fractional
Δ P_{Na}
.8
.4
0
TAP^{++}
TAP^{+}
TAP
6
7
8
pH

(vii) Current-induced changes in transepithelial resistance-effect of 20mM triaminopyrimidine

The effective resistance of the paracellular pathway to ion flow in ileum is determined largely by the resistance of the tight-junction (Desjeux, Tai, and Curran (1974)). The locus of action of triaminopyrimidine may, therefore, be supposed to be the tight-junction. Structurally, the paracellular route in ileum and other low resistance epithelia e.g. gall-bladder, consists of a series array of the tight-junction and lateral intercellular space. (See also Chapter 1).

Smulders, Tormey and Wright (1972) have shown that the resistance and permeability of gall-bladder can be modified by the application of osmotic gradients. In gall-bladder these changes are due to variations in the dimensions of the lateral intercellular spaces. Thus, in certain instances, permeation through the paracellular route may be rate-limited not by the tight-junction, but by the lateral intercellular space.

Bindslev, Tormey and Wright (1974) have shown that long duration D.C. current pulses have similar effects on tissue resistance in frog gall-bladder as do osmotic gradients. These effects are also associated with changes in the dimensions of the lateral spaces. Weidner and Wright (1975) report that the ion selectivity ratios of gall-bladder show small changes due to passage of current, an effect, which they suggest, is due to closure of the lateral spaces.

Could the mode of action of triaminopyrimidine be due to structural alterations in the lateral space, rather than to changes in the tight-junction?

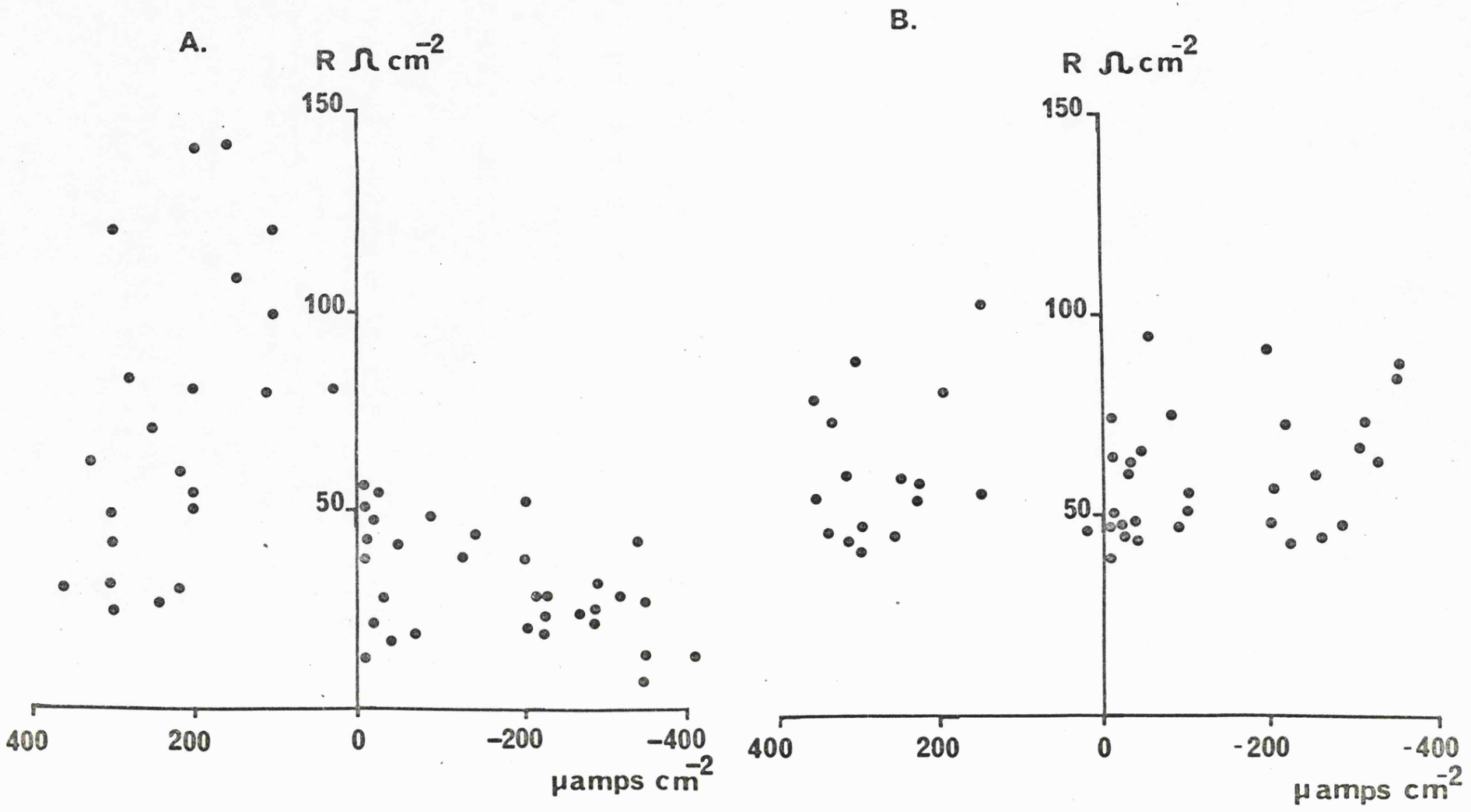
Since intestine is a high-conductance tissue, applied currents in the order of 200-400 μamps cm^{-2} are used to determine the voltage-

dependent fluxes (section 1 (i)). Figure 16 (a) shows the resistance, determined in these experiments at the end of each period of potential clamp, plotted against the applied clamping current. Negative values of current refer to a serosal cathode. It is clear that passage of current from serosa to mucosa results in an increased resistance. Conversely, current flow from mucosa to serosa causes a slight decrease in resistance. These effects are similar to current-induced changes observed in frog gall-bladder. Current-induced resistance changes in gall-bladder are due to volume changes in the lateral spaces. These are thought to be generated by water flow resulting from the transport number effect (Wedner and Diamond (1969); Barry and Hope (1969a and b)). As such, they depend upon the ion selectivity of the intestine.

Figure 16 (b) shows the resistance of intestines in the presence of 20mM triaminopyrimidine plotted as a function of the applied clamping current. Tissue resistance is independent of the applied current in the presence of triaminopyrimidine. In particular, passage of current from mucosa to serosa, a condition resulting in low control resistance characterized by dilated lateral spaces, shows resistance values that are no different from tissue resistances when current flow is in the opposite direction. Similarly, control resistance values approach high values when current is passed from serosa to mucosa, this effect is lost in the presence of triaminopyrimidine. Taken together these results suggest that the site of action of triaminopyrimidine is located within the tight-junction as suggested by Moreno (1975). The absence of current-induced resistance-changes in the presence of triaminopyrimidine may be due to the ion selectivity ratio $P_{Cl} : P_{Na}$ being close to unity. In this condition, transport number effects would be absent.

Figure 16

Variation in tissue resistance in flux experiments involving non-zero clamping potentials (see Methods) plotted as a function of the required clamping current. All measurements were made at the end of each period of voltage clamping (twenty minutes after the start of such periods). Resistance is determined from the current and the clamp potential. A negative current refers to a serosal cathode. A = control tissues, B = tissues incubated in the presence of 20mM triaminopyrimidine.


A.
R Ω cm^{-2}
150
100
50
400
200
0
−200
−400
μamps cm^{-2}
B.
R Ω cm^{-2}
150
100
50
400
200
0
-200
-400
μamps cm^{-2}

Current induced resistance-changes will affect the accuracy of the determination of (J_{D13}) paracellular flux components for control tissues. Consideration of the direction of these changes indicate that values of J_{D13} will be slight overestimates.

2. Effect of triaminopyrimidine upon transcellular transport processes

20mM triaminopyrimidine has been shown to decrease paracellular Na permeability. Triaminopyrimidine may, therefore, be a useful tool for defining the paracellular and transcellular components of transepithelial flux. This can only be true if triaminopyrimidine can be shown to have no effect upon transcellular flux.

A decrease in net Na flux, indicative of the rate of active transport by the tissue, has been observed in the presence of triaminopyrimidine (Section 1 (ii) and (iii); Section 3 (ii) (b)). Can this result be taken to indicate changes in cellular integrity due to the presence of triaminopyrimidine?

Three results argue against cellular damage due to triaminopyrimidine:

1. 20mM triaminopyrimidine has no effect upon intracellular cation concentrations nor is there any effect upon the extracellular $[Na]$ (Chapter 3).

2. Galactose-dependent increments in Na flux J_{13} are unaffected by 20mM triaminopyrimidine (see below).

3. Table 7 shows the results of two experiments in which the bidirectional fluxes of D-galactose, together with the tissue/medium galactose accumulation ratio, and the tissue isotope specific activity ratio (R), were determined in the presence and absence of triaminopyrimidine. It can be seen that 20mM triaminopyrimidine has no significant effects upon J_{13}, J_{31}, the accumulation ratio, or the isotope specific activity ratio ($p > 0.1$, 0.1, 0.1, 0.1 respectively.)

Table 7

Effect of 20mM triaminopyrimidine on galactose transport by rabbit ileum. Data are from two experiments (n = 4) for each condition. 2mM galactose was present in both mucosal and serosal solutions (Standard Ringer). Mucosa to serosa galactose flux J_{13} was measured using ^{3}H galactose as tracer, serosa to mucosa flux J_{31} by ^{14}C galactose. Fluxes were averaged from two ½ hour flux periods (see Methods). Errors are expressed as $\pm$ S.E.M.

Table 7

Condition	J_{13}	J_{31}	Ratio of tissue/medium [galactose]	R
2mM galactose	0.468 ± 0.040	0.006 ± 0.001	5.85 ± 0.68	4.79 ± 0.33
2mM galactose + 20mM triaminopyrimidine	0.399 ± 0.050	0.007 ± 0.001	5.33 ± 1.28	3.83 ± 1.22

The decrease in net Na flux observed in the presence of triaminopyrimidine may reflect a decrease in a passive component of net Na flux across the epithelium (see below and also discussion).

3. Bidirectional transepithelial flux measurements

(i) Effects of 2,4,6, triaminopyrimidine

In 140mM Na Ringer, 20mM triaminopyrimidine reduces both mucosa-serosa flux, J_{13} and serosa-mucosa flux, J_{31} compared with control values (see Table 8). The decrease in flux in both directions is highly significant ($p < 0.001$). The triaminopyrimidine-dependent reduction in flux is equivalent to a reduction in the unidirectional m-s Na permeability (P_{13}) from 0.061-0.039 cm hr^{-1}. This reduction in permeability of 0.022 cm hr^{-1} is consistent with the observed reduction in the paracellular shunt permeability to Na described in Section 1.

(ii) Effects of replacement of Ringer Na by choline on the bidirectional Na fluxes

(a) Control tissues In control tissues replacement of Ringer Na with choline causes a reduction in both m-s and s-m Na flux. This decrease in flux is directly proportional to the reduction in Ringer $[Na]$, hence no change in the bidirectional transepithelial Na permeability is observed on reducing Ringer $[Na]$ from 140 to 25mM., (see Table 8). The observed decrease in net Na absorption is also directly proportional to the reduction in Ringer $[Na]$.

(b) Effects of Triaminopyrimidine

Triaminopyrimidine-dependent reduction in bidirectional transepithelial Na flux

As with tissue bathed in Ringer containing 140mM NaCl, triaminopyrimidine also reduces the bidirectional transepithelial fluxes of Na across tissue bathed in Ringer containing 75 and 25mM NaCl.

Table 8

Measured bidirectional mucosa to serosa and serosa to mucosa Na fluxes (J_{13} and J_{31}). All errors are $\pm$ S.E.M. Bidirectional permeabilities were calculated from the relationship $Pij = Jij/_{Ci}$.

Table 8

CONDITION	n	J_{13} μmoles $cm^{-2}hr^{-1}$	P_{13} $cm\ hr^{-1}$	J_{31} μmoles $cm^{-2}hr^{-1}$	P_{31} $cm\ hr^{-1}$	J_{Net} μmoles $cm^{-2}hr^{-1}$
140mM Na (Standard Ringer)	22	8.59 ± 0.47	0.061 ± 0.003	6.76 ± 0.45	0.048 ± 0.003	1.82 ± 0.35
140mM Na + 20mM Triaminopyrimidine	18	5.46 ± 0.28	0.039 ± 0.002	4.61 ± 0.25	0.032 ± 0.002	0.84 ± 0.23
75mM Na	37	4.86 ± 0.22	0.064 ± 0.002	3.81 ± 0.13	0.050 ± 0.002	1.05 ± 0.16
75mM Na + 20mM Triaminopyrimidine	29	3.59 ± 0.13	0.047 ± 0.002	3.01 ± 0.13	0.040 ± 0.002	0.57 ± 0.11
25mM Na	11	1.47 ± 0.24	0.059 ± 0.009	0.95 ± 0.08	0.038 ± 0.003	0.52 ± 0.18
25mM Na + 20mM Triaminopyrimidine	11	1.33 ± 0.10	0.053 ± 0.004	0.97 ± 0.05	0.039 ± 0.002	0.36 ± 0.07
140mM Na + 0.1mM Ouabain	6	6.02 ± 0.48	0.046 ± 0.003	5.93 ± 0.06	0.042 ± 0.004	0.32 ± 0.31
140mM Na + 0.1mM Ouabain + 20mM Triaminopyrimidine	6	4.82 ± 0.34	0.034 ± 0.002	4.81 ± 0.26	0.034 ± 0.002	0.02 ± 0.14
140mM Na Ringer + 20mM galactose	11	10.16 ± 0.58	0.072 ± 0.004	7.15 ± 0.49	0.051 ± 0.003	3.01 ± 0.33
140mM Na Ringer + 20mM galactose + 0.1mM Ouabain	5	6.01 ± 0.96	0.043 ± 0.006	5.88 ± 0.81	0.042 ± 0.005	0.24 ± 0.30
140 Na Ringer + 20mM Triaminopyrimidine + 20mM Galactose	13	7.21 ± 0.47	0.051 ± 0.003	4.12 ± 0.23	0.029 ± 0.001	3.08 ± 0.41
140 Na Ringer + 20mM Triaminopyrimidine + 20mM Galactose + 0.1mM Ouabain	5	5.69 ± 0.18	0.041 ± 0.001	5.37 ± 0.20	0.0383 ± 0.001	0.32 ± 0.07

Table 9

CONDITION	$P_{13(Control)} - P_{13(TAP)}$ cm hr^{-1}	$P_{31(Control)} - P_{31(TAP)}$ cm hr^{-1}
140mM Na + 0.1mM ouabain	0.012 ± 0.003 (10)	0.012 ± 0.004 (10)
75mM Na	0.0169 ± 0.002(5) (64)	0.011 ± 0.001 (38)
25mM Na	0.005 ± 0.003(5) (20)	0.007 ± 0.003 (20)
140mM Na	0.022 ± 0.002 (38)	0.015 ± 0.002(5) (38)

Triaminopyrimidine-dependent reductions in bidirectional permeabilities. The number of degrees of freedom is given by the figures in parentheses.

However, the extent of this reduction varies with Ringer [Na]. This is shown in Table 9. It can be seen that the triaminopyrimidine-dependent reduction in both m-s and s-m Na permeability falls as Ringer [Na] is reduced from 140 to 25mM. With Ringer [Na] at 140 or 75mM the triaminopyrimidine-dependent reduction in both m-s and s-m transepithelial Na fluxes are highly significant ($p < 0.001$). However, with Ringer [Na] = 25mM, a small non-significant reduction in transepithelial Na flux is obtained in the presence of triaminopyrimidine. The difference between the triaminopyrimidine-dependent reduction in transepithelial Na permeability at a Ringer [Na] of 140mM, compared with the smaller triaminopyrimidine-dependent reduction at a Ringer [Na] of 25mM is highly significant ($p < 0.001$).

These results indicate that there is a Na-dependent variable resistance within the paracellular pathway in series with the tight-junction (see Discussion).

Table 9 also shows that the triaminopyrimidine-dependent reduction in m-s (1-3) and s-m (3-1) Na fluxes at Ringer Na concentrations of 140mM and 75mM are not equal. The reduction in m-s flux is significantly larger than the reduction in s-m flux ($p < 0.05$ for both cases). When Ringer [Na] is reduced to 25mM, no significant difference in the reduction of m-s and s-m bidirectional permeabilities due to triaminopyrimidine is seen ($p > 0.1$).

The asymmetric action of triaminopyrimidine is also evident in the triaminopyrimidine-dependent reduction in net Na flux (J_{net}) (Table 8 and Section 1). A significant reduction of J_{net} due to triaminopyrimidine occurs both at 140mM and 75mM Ringer [Na] ($p < 0.05$, $p < 0.05$ respectively). No significant decrease in J_{net} at 25mM Ringer [Na] in the presence of triaminopyrimidine is seen ($p > 0.4$).

These results suggest that triaminopyrimidine may block a portion of net flux that is paracellular (see Discussion).

Effect of variation of Ringer Na on the bidirectional transepithelial Na flux in the presence of 20mM triaminopyrimidine

On reducing Ringer [Na] with 20mM triaminopyrimidine present, there is a progressive increase in m-s Na permeability (P_{1-3}) (Table 8) ($p<0.001$). This progressive increase in permeability indicates that the triaminopyrimidine insensitive (transcellular) flux is a saturable process whose existance in control tissue is obscured by concurrent changes in the permeability of the paracellular pathway on varying Ringer [Na]. The Km for triaminopyrimidine m-s flux is 357 ± 43mM (S.D.) and the Vm is 20.2 ± 8.4 (S.D.) μmoles cm^{-2} hr^{-1}. There is no significant change in s-m Na permeability on reducing Ringer [Na] from 140 to 25mM.

(iii) Effects of 0.1mM ouabain on the bidirectional transepithelial Na flux

(a) Control tissue 0.1mM ouabain reduces m-s Na flux compared with untreated tissue ($p<0.02$). A slight reduction is also seen in the mean s-m Na flux following addition of 0.1mM ouabain, however, the reduction is not significant ($p>0.3$). With ouabain present the net Na absorption does not differ significantly from zero ($p>0.6$) (Table 8).

(b) Triaminopyrimidine treated tissue As with control tissue, ouabain abolishes the net flux across tissue in the presence of 20mM triaminopyrimidine by reducing Na flux J_{13} without significantly affecting Na flux J_{31}. The extent of the triaminopyrimidine-dependent reduction in bidirectional Na flux is significantly less ($p<0.01$) in the presence of ouabain than in its absence (Table 9). This result indicates that ouabain has a similar effect to that of reducing Ringer [Na] on the triaminopyrimidine-sensitive (shunt) conductance.

In the presence of 0.1mM ouabain there is a symmetrical reduction in bidirectional (m-s and s-m) Na permeability, due to triaminopyrimidine. This result contrasts with the asymmetric action of triaminopyrimidine in control tissues (Section 3 (ii) (b)).

(iv) Effects of D-galactose on the bidirectional transepithelial Na fluxes

(a) Control tissues As has previously been shown on numerous occasions (Schultz and Curran (1970)) addition of galactose to the Ringer solution bathing isolated small intestine, increases m-s Na flux. As can be seen in Figure 17 (a) this increase in Na flux J_{13} is a saturable function of Ringer [galactose]. The Km for the galactose-dependent increase in flux in 1.64 $\pm$ 0.35mM (S.D.) and the Vm is 2.08 $\pm$ 0.47 (S.D.) μmoles cm^{-2} hr^{-1}. Galactose also slightly stimulates s-m Na flux in control tissues (see also Table 3). A similar effect of sugars on s-m Na flux in guinea pig intestine has been reported (Binder et al (1972)).

(b) Effects of 0.1mM ouabain on the galactose-dependent increase in bidirectional Na flux As was shown above ouabain reduces both the m-s and s-m Na fluxes. It can be seen from Table 8 that ouabain also abolishes the galactose-dependent increments in both m-s and s-m flux.

(c) Effects of 20mM triaminopyrimidine In the presence of 20mM triaminopyrimidine, the absolute levels of bidirectional transepithelial Na fluxes are reduced, but triaminopyrimidine has no effect on the galactose-dependent increment in m-s Na flux (see Figure 17 (b)). This result is consistent with the view that triaminopyrimidine acts exclusively to reduce the Na permeability at the tight-junction.

In contrast with the effect of galactose in control tissue, galactose significantly decreases s-m Na flux when triaminopyrimidine

Figure 17

The effect of D-galactose (0 to 20mM) upon the bidirectional fluxes J_{13} and J_{31} under voltage clamp (P.D. = 0) for (a) control tissues; (b) tissues incubated in the presence of 20mM triaminopyrimidine. Fluxes are averaged from two separate flux periods of ½ hour (see Methods). Error bars denote the standard error of the mean for each data point. The number of data points for each level of Ringer galactose used is shown above the x-axis.

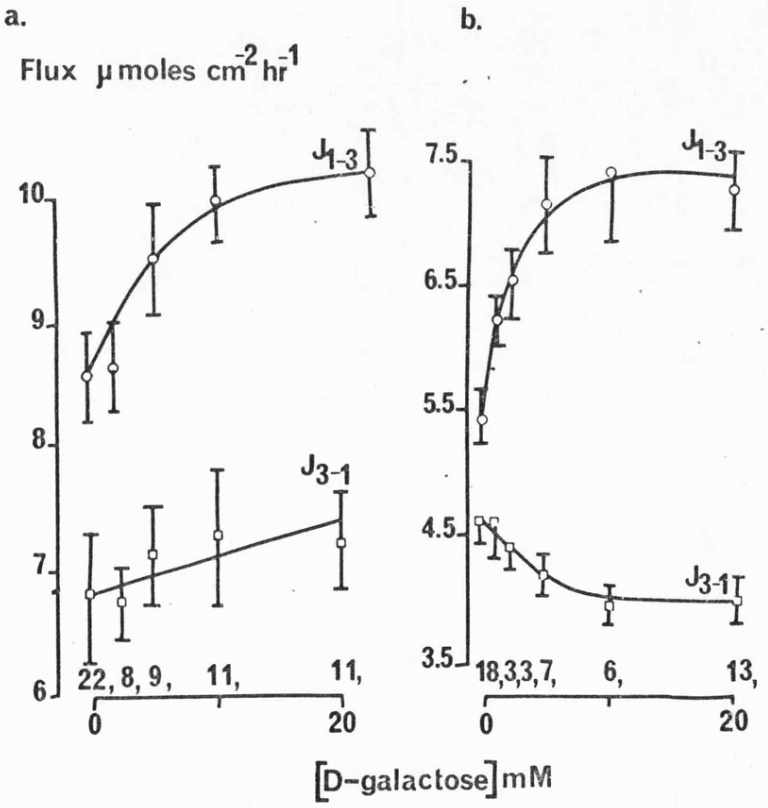
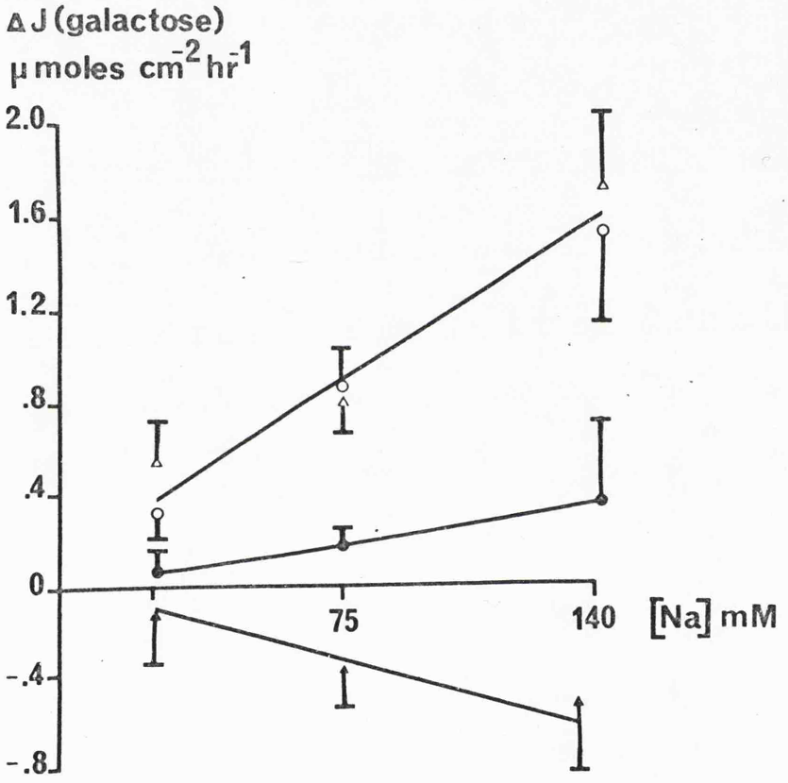

a.
b.
Flux μmoles cm^{-2} hr^{-1}
J_{1-3}
J_{3-1}
10
9
8
7
6
7.5
6.5
5.5
4.5
3.5
22, 8, 9, 11, 11,
18,3,3,7, 6, 13,
0
20
[D-galactose] mM

Figure 18

The effect of Ringer $[Na]$ variation upon the galactose-dependent change in mucosal to serosal Na flux J_{13} (open circles and triangles) and the serosal to mucosal Na flux J_{31} (closed circles and triangles). (△ ▲) denotes tissues incubated + 20mM triaminopyrimidine, (○ ●) are control tissues. Error bars denote the standard error of the difference.


ΔJ (galactose)
μ moles cm⁻² hr⁻¹
2.0
1.6
1.2
.8
.4
0
-.4
-.8
75
140
[Na] mM

is present ($p < 0.01$) (1-way analysis of variance). This result is similar to the sugar-dependent reduction in s-m Na flux noted previously by Quay and Armstrong (1969) in bull-frog small-intestine.

As with control tissue 0.1mM ouabain reduces m-s flux in the presence of 20mM triaminopyrimidine ($p < 0.001$). Since ouabain abolishes the galactose-dependent decrease in s-m Na flux seen with triaminopyrimidine present, ouabain effectively increases s-m Na flux in this condition, (Table 8). Thus with triaminopyrimidine present, the action of ouabain in abolishing net Na flux is revealed to be due both to a reduction in m-s Na flux and an increase in s-m Na flux. These actions strongly resemble the reciprocal effects of ouabain on bidirectional transepithelial galactose flux described by Naftalin and Curran (1974) and by Naftalin and Holman (1974).

(d) Effects of replacement of Ringer Na with choline on galactose dependent bidirectional transepithelial Na fluxes. Figure 18 shows the galactose-dependent changes in m-s and s-m flux following addition of 20mM galactose to the Ringer containing 25, 75 and 140mM Na in both the presence and absence of 20mM triaminopyrimidine. The galactose-dependent increment in m-s Na flux in both the presence and absence of 20mM triaminopyrimidine is identical and increases in direct proportion to the Ringer $[Na]$. The small galactose-dependent increase in s-m Na flux is lost on reducing the Ringer Na to 25mM. With 25mM triaminopyrimidine present the galactose-dependent decrease in s-m Na flux is also lost on reducing Ringer $[Na]$ from 140 to 25mM.

4. The tissue isotope specific activity ratio of $^{24}Na : {}^{22}Na$ (R)

The tissue isotope specific activity ratio of $^{24}Na : {}^{22}Na$ originating from the mucosal and serosal solutions respectively, is 0.669 ± 0.057 (Table 10) for control tissues. Hence the steady-state distribution of Na within the tissue is characterized by a relatively

Table 10

The tissue ratio of ^{24}Na : ^{22}Na. Errors are expressed as $\pm$ S.E.M. Figures in parentheses are the number of separate determinations. + indicates the presence of 20mM triaminopyrimidine.

Table 10

CONDITIONS		CONTROL	20mM GALACTOSE	OUABAIN 10^{-4}M
140mM Na		0.669 ± 0.057 (22)	0.880 ± 0.100 (13)	0.980 ± 0.013 (6)
	+	0.540 ± 0.041 (18)	0.735 ± 0.030 (11)	0.792 ± 0.160 (6)
75mM Na		0.770 ± 0.048 (37)	0.950 ± 0.060 (29)	0.890 ± 0.06 (4)
	+	0.574 ± 0.042 (29)	0.650 ± 0.040 (16)	0.910 ± 0.050 (4)
25mM Na		0.668 ± 0.061 (11)	0.652 ± 0.091 (20)	1.140 ± 0.070 (3)
	+	0.588 ± 0.043 (11)	0.633 ± 0.029 (20)	0.69 ± 0.04 (3)

greater proportion of Na originating from the serosal solution. Partial replacement of Ringer Na by choline has no significant effect upon the ratio (R).

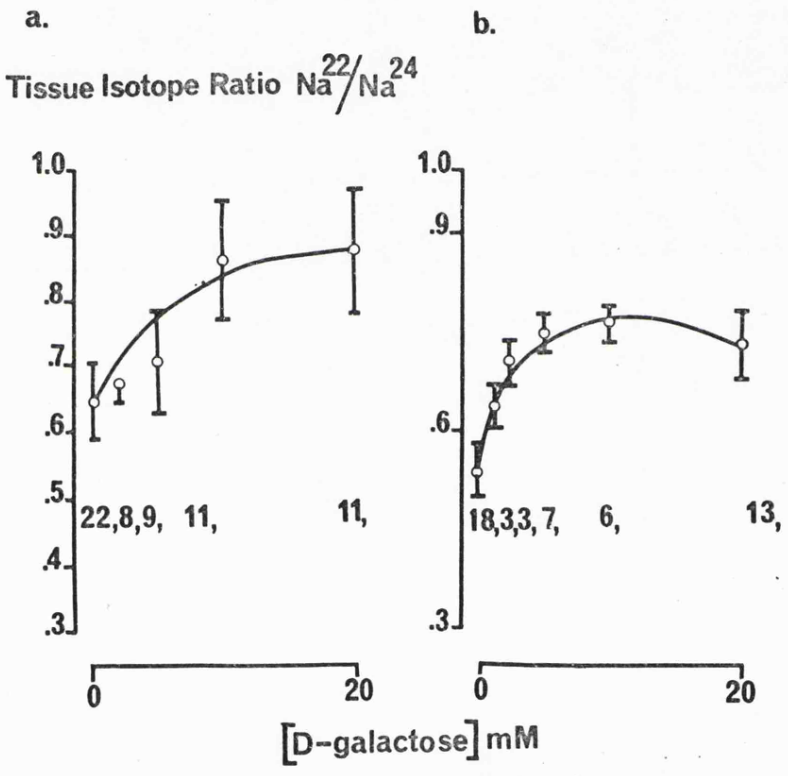
Triaminopyrimidine (20mM) reduces the amount of Na originating from the mucosal solution compared with control values at all Na concentrations tested ($p<0.05$, $p<0.001$, $p>0.1$ for 140, 75 and 25mM Na respectively). Since triaminopyrimidine is known to reduce m-s flux by reducing the Na permeability of the paracellular pathway (Sections 1 and 2), the reduction in the tissue isotope ratio may be concluded to be associated with this effect.

0.1mM ouabain increases the ratio R towards unity for all experimental conditions tested. That is to say proportionately more isotope originates from the mucosal solution in the presence of ouabain. In control Ringer (140mM Na) the ratio R increases from 0.669 to 0.980 following addition of ouabain. The difference between these two values is statistically significant ($p<0.02$). A similar effect of ouabain on tissue ion content has been reported for toad urinary bladder epithelial cells (MacKnight et al (1975a,b). In the presence of triaminopyrimidine a significant increase in R following addition of ouabain is also observed compared with control values ($p<0.05$).

D-galactose increases the proportion of Na originating from the mucosal solution within the tissue both in the presence and absence of triaminopyrimidine. This increase is a saturable function of Ringer galactose (Figures 19 (a) and (b)). Replacement of Ringer Na by choline reduces the galactose-dependent increment in the tissue isotope ratio (Table 10)

Figure 19

The effect of galactose (0 to 20mM) upon the isotope specific activity ratio (Na^{24} : Na^{22}) within the tissue fluid following incubation to determine bidirectional fluxes for (a) control tissues (b) tissues incubated in the presence of triaminopyrimidine. The numbers of data points for each level of Ringer [galactose] is shown above the x-axis.


a.
b.
Tissue Isotope Ratio Na^{22}/Na^{24}
1.0
.9
.8
.7
.6
.5
.4
.3
22,8,9, 11, 11,
18,3,3,7, 6, 13,
0
20
[D-galactose] mM

5. The distribution of the tissue isotope ratio within the extracellular and intracellular tissue compartments

The measurements of the tissue isotope specific activity ratio (R) are made from whole tissue determinations. This section is designed to show these measurements (and hence the unidirectional flux calculations) are not subject to error due to inhomogeneity of the distribution of ^{24}Na to ^{22}Na within the tissue. Experimentally four tissue compartments for Na can be identified (see Methods).

Table 11 shows the results of the analysis of control tissues, tissues incubated in the presence of ouabain, and tissues incubated in the presence of 20mM galactose. With control tissue no effect of washing on the ratio R is observed in either whole tissue ($p > 0.5$), submucosal layers ($p > 0.9$) or epithelial scrapings ($p > 0.6$). Similarly, no significant difference exists between the ratio R in whole tissue and submucosal layers ($p > 0.2$) or whole tissue and epithelial scrapings ($p > 0.2$), or between epithelial scrapings and submucosal layers ($p > 0.6$). Identical effects are seen for galactose and ouabain-treated tissues, since no significant effects of washing or scraping are seen.

These results support the validity of the method of determining unidirectional flux calculations in the case of Na ions, since it is evident that this preparation of rabbit ileum may be treated kinetically as a single compartment.

Table 11

		Unwashed	½ hour choline wash
WHOLE TISSUE	CONTROL	0.802 ± 0.04	0.851 ± 0.076
	0.1mM ouabain	0.95 ± 0.08	0.913 ± 0.090
	20mM galactose	0.905 ± 0.09	0.892 ± 0.097
SUBMUCOSAL LAYERS	CONTROL	0.892 ± 0.065	0.900 ± 0.075
	0.1mM ouabain	1.06 ± 0.15	0.880 ± 0.060
	20mM galactose	1.05 ± 0.102	0.791 ± 0.048
MUCOSAL SCRAPINGS	CONTROL	0.866 ± 0.029	0.898 ± 0.08
	0.1mM ouabain	1.13 ± 0.140	1.04 ± 0.134
	20mM galactose	0.815 ± 0.08	0.915 ± 0.096

The tissue distribution of ^{24}Na : ^{22}Na within the extracellular and cellular compartments of rabbit ileal tissue. Results are all expressed as the mean of nine determinations from three experiments.

C. Discussion

1. Bidirectional Na flux through the paracellular pathway

(i) The effects of triaminopyrimidine on tissue bathed in Ringer

The shunt permeability to Na calculated from the functional relationship between unidirectional transepithelial Na flux and the applied transepithelial p.d. is 0.038 cm hr^{-1}, this is similar to the values obtained by others (Frizzell and Schultz (1972); Munck and Schultz (1974); Desjeux et al (1974)). It is evident that 20mM triaminopyrimidine reduces the permeability of the paracellular route since it abolishes the p.d.-dependent net and unidirectional (m-s) Na fluxes, and reduces the NaCl dilution potential, the Na-choline biionic potential and the total tissue electrical conductance (see Section 1 of Results).

The effects of triaminopyrimidine are all consistent with the view proposed by Moreno (1974,1975a,b) that triaminopyrimidine acts by blocking the cation selective sites within the tight junction between the mucosal borders of the epithelial cells.

However, even with 20mM triaminopyrimidine present, a small residual shunt permeability to Na remains; this is calculated to be $\sim$0.017 cm hr^{-1}. The value of transintestinal Cl permeability is 0.019 cm hr^{-1} (Frizzell and Schultz (1972)). These values may indicate that there is a free-solution shunt pathway across ileum. This point is extensively discussed in Chapter 6.

When the [Na] in the Ringer is reduced by replacement with choline or when 0.1mM ouabain is present in the Ringer, the triaminopyrimidine-dependent reductions in both P_{13} and P_{31} are decreased (see Table 9). As discussed in the introduction the overall permeability P_t of the shunt pathway is the resultant of two resistances in series, the tight junction, tj, and the lateral space, ls,

hence: $1/P_t = 1/P_{tj} + 1/P_{ls}$ (Desjeux et al (1974)). If the lateral space, by virtue of its ability to change its shape, is considered as a variable resistor, then when the lateral space is wide open (low resistance)a large increase in the resistance of the tight junction following addition of triaminopyrimidine will cause a greater reduction in the total permeability of the shunt pathway than when the lateral space is closed (high resistance). Huss and Marsh (1975) have recently described a modified version of Diamond and Bossert's (1967) standing gradient hypothesis which is consistent with the above views. Huss and Marsh's modification makes the assumption that the basal-lateral membrane forms a distensible compartment which responds to the hydraulic pressure of fluid within the space. Hydraulic pressure may be generated in transporting tissue by fluid entry into the space caused by the osmotic pressure gradients across the basal-lateral border and tight junction.

Reducing the Na-pump activity at the basal-lateral border by decreasing Ringer $[Na]$ or by ouabain addition, reduces the hypertonicity of Na sequestered in the space (see Chapter 3), this will reduce the distension of the basal-lateral membranes and thus reduce the permeability of the lateral space.

(ii) Effects of D-galactose on the shunt permeability to Na

Galactose increases both m-s and s-m Na fluxes. When triaminopyrimidine is added to the Ringer, no reduction in the galactose-dependent increments in m-s Na flux is observed; however, there is a small, though significant, galactose-dependent decrease in s-m flux. Similar reductions in s-m Na flux in response to sugars (but in the absence of triaminopyrimidine) have been noted in bull-frog intestine (Quay and Armstrong (1969)). It may be concluded that the galactose-dependent increments in m-s Na flux are due to increased

transcellular Na movement which is insensitive to the action of triaminopyrimidine. The galactose-dependent increase in s-m Na flux in the absence of triaminopyrimidine may result from increased Na flux via the paracellular shunt pathway following enlargement of the intercellular spaces resulting from increases in the extracellular hypertonicity (see Chapter 3). This conclusion is supported by the observation that the galactose-dependent increases in s-m Na flux are abolished by ouabain.

Similar effects of saline-loading on Na flux have been obtained in necturus kidney tubules (Boulpaep (1972)) and rat small intestine (Humphreys and Early (1971)). Saline loading causes an increased shunt conductance, an increased inulin permeability and a decreased net absorption of Na due to increased s-m Na flux. Lateral space distension may give rise to increased tight-junction permeability (Humphreys and Early (1971)). Increased junctional permeability is not, however, theoretically required to explain the effects of saline loading (see Chapter 1).

Binder, Powell and Curran (1972) have shown that glucose, galactose and 3-O-methyl glucose all increase s-m Na flux in isolated guinea-pig intestine. However, Schultz and Curran (1970) found no sugar or amino acid -dependent increase in s-m Na flux in rabbit ileum. The observed galactose-dependent increments in s-m Na permeability reflect similar and concurrent increases is s-m galactose (Naftalin and Curran (1974)), 3-O methyl glucose and β methyl glucoside permeability seen at high concentrations of these sugars (Holman and Naftalin (1976)

(iii) Evidence for asymmetric passive Na via the shunt pathway

In Table 9 it may be seen that the decrease in m-s Na flux following addition of 20mM triaminopyrimidine is significantly greater than the decrease in s-m Na flux. Thus, triaminopyrimidine

causes a significant reduction in net Na absorption. Since triaminopyrimidine is without effect on

a) the distribution of Na and K between the cells and the external solution (see Chapter 3)

b) the galactose-dependent increase in transcellular Na movement (see above)

c) the transport and accumulation of galactose (see above),

it may be inferred that triaminopyrimidine affects only the passive permeability of the shunt to Na without affecting cell metabolism or cell membrane permeability.

Why should triaminopyrimidine reduce m-s Na flux more than s-m Na flux in actively transporting tissue? As suggested in Chapter 1, a possible explanation for these findings is that in actively transporting tissue the osmotic pressure gradient existing across the tight-junction, due to hypertonic saline contained within the lateral intercellular space, causes both Na and water to be dragged from the mucosal solution via the tight-junction. Additionally, fluid is drawn from the cell across the basal-lateral border. Triaminopyrimidine blocks Na movement through the tight-junction and so will reduce the osmotically induced net flow of Na and fluid across the tight-junction.

In support of this explanation of the passive asymmetric flux of Na across the tight-junction, it can be seen that the triaminopyrimidine-sensitive reduction in m-s and s-m Na flux is symmetrical when 0.1mM ouabain is present in the Ringer, or when Ringer $[Na]$ is reduced to 25mM. In both these conditions it has been shown (see Chapter 3) that the fluid within the lateral intercellular space is approximately isotonic, hence no osmotically induced drag of Na is expected.

The magnitude of the osmotically-induced passive net flux of Na across the tight-junction is $\sim$ 1 μmole cm^{-2} hr^{-1}. This comprises the greater part of the total net flux of Na across the tissue in control conditions.

A model describing the generation of passive net fluxes of Na has been described by Schafer *et al*/(1975) and used to explain the asymmetric shunt flux of solutes across the necturus proximal tubule. The driving force for the net flow of Na is considered to be the transtubular concentration differences of Cl and HCO_3 coupled to the existance of different reflexion coefficients for HCO_3 and Cl at the tight-junction. A similar mechanism may contribute to the passive net flux of Na demonstrated here.

The results shown in Figure 17(b) indicate that in the presence of triaminopyrimidine the s-m Na flux progressively decreases as Ringer [galactose] is raised. Since this interaction is apparent only when triaminopyrimidine is present the process retarding Na flux must occur at some site other than the tight-junction.

2. Transcellular Na flux

As triaminopyrimidine markedly reduces the paracellular component of transepithelial Na flux, bidirectional transepithelial Na flux determinations in the presence of triaminopyrimidine provide a basis for the estimation of the transcellular flux components. However, even with triaminopyrimidine present, a small residual passive permeability to Na remains (π 0.017 cm hr^{-1}), this may lead to overestimation of transcellular flux.

(i) Unidirectional Na fluxes across the mucosal boundary

(a) Controls The calculated influx and efflux J_{12} and J_{21} of Na across the mucosal border of tissue bathed in Ringer are 7.97 and 7.13 μmoles cm^{-2} hr^{-1} respectively (Table 12). These values agree

Table 12

Calculated unidirectional Na fluxes across the mucosal and serosal borders in the presence of 20mM triaminopyrimidine. All errors are expressed as the S.E.M.

μ moles cm^{-2} h^{-1}

CONDITION	n	J_{12}	J_{21}	J_{23}	J_{32}
140mM Na Ringer	18	7.97 ± 0.42	7.13 ± 0.46	15.46 ± 1.44	14.60 ± 1.33
140 Na Ringer + 0.1mM Ouabain	5	8.62 ± 0.52	8.61 ± 0.45	10.90 ± 1.26	10.80 ± 1.19
140 Na Ringer + 20mM D-galactose	13	10.15 ± 0.53	7.07 ± 0.32	17.29 ± 1.87	14.02 ± 1.63
140 Na Ringer + 20mM D-galactose + 0.1mM Ouabain	5	8.49 ± 0.50	8.62 ± 0.89	11.42 ± 0.43	11.55 ± 0.62
75mM Na Ringer	29	5.31 ± 0.21	4.74 ± 0.22	10.76 ± 0.69	10.19 ± 0.67
25mM Na Ringer	11	1.91 ± 0.13	1.55 ± 0.10	3.74 ± 0.38	3.38 ± 0.31

reasonably well with those obtained by direct estimation of Na influx across the mucosal border of rabbit ileum after correction for a shunt component of Na influx (Schultz et al (1967)).

(b) Effects of replacing Ringer Na by choline and of 0.1mM ouabain on the unidirectional Na fluxes across the mucosal border

The effects of reducing Ringer [Na] and of 0.1mM ouabain on the calculated unidirectional Na fluxes across the mucosal borders are shown in Table 12. As expected, both influx J_{12} and efflux J_{21} of Na are reduced as Ringer [Na] is reduced from 140 to 25mM. After correction of the transepithelial fluxes for the residual passive component remaining with 20mM triaminopyrimidine present, a hyperbolic relationship between Ringer [Na] and Na influx across the brush-border emerges. The apparent Km of Na for this transport process is 295mM whilst the Vm is 17.6 μmoles cm^{-2} hr^{-1}. Curran et al (1967) previously found a linear relationship between Na influx and Na concentrations. The present data are, however, similar to those of Nellans et al (1974). It is observed that with 0.1mM ouabain present there is no significant reduction in Na influx ($p > 0.5$) but the steady-state efflux of Na from the tissue into the mucosal solution is raised. These results coincide with those obtained in several previous studies. For example, Chez et al (1967) who measured Na influx directly without correcting for the shunt permeability, found no effect of ouabain on Na influx either. Since there is an increase in the intracellular [Na] with ouabain present (see Chapter 3) the results showing increased Na efflux are as expected.

(c) Effects of D-galactose The effects of galactose on the unidirectional Na fluxes across the mucosal border are shown in Figure 20. Influx J_{12} increases as a hyperbolic function of Ringer galactose . The Km is 1.2 $\pm$ 0.32 mM (S.D.) and the Vm is

2.71 ± 0.69 μmoles cm^{-2} hr^{-1}. This result is substantially the same as observed previously by Goldner et al (1969) who measured Na influx across the mucosal border directly.

The unidirectional efflux of Na, J_{21}, in the presence of 20mM triaminopyrimidine is not raised by galactose. Indeed a small (but non-significant) decrease is observed in efflux when Ringer [galactose] is raised from 0 to 20mM. Since galactose increases intracellular [Na] (see Chapter 3) and reduces the electrical potential difference across the mucosal border by 10mV (Rose and Schultz (1971)), Na exit should be increased independently of any possible additional acceleration due to a galactose-dependent Na efflux component predicted by the Na-gradient hypothesis (Curran et al(1970)). Solvent drag resulting from net fluid flow across the brush-border as described by Koefoed-Johnsen and Ussing (1953) could account for the discrepancy between the observed efflux and that predicted on the basis of the electro-chemical gradient.

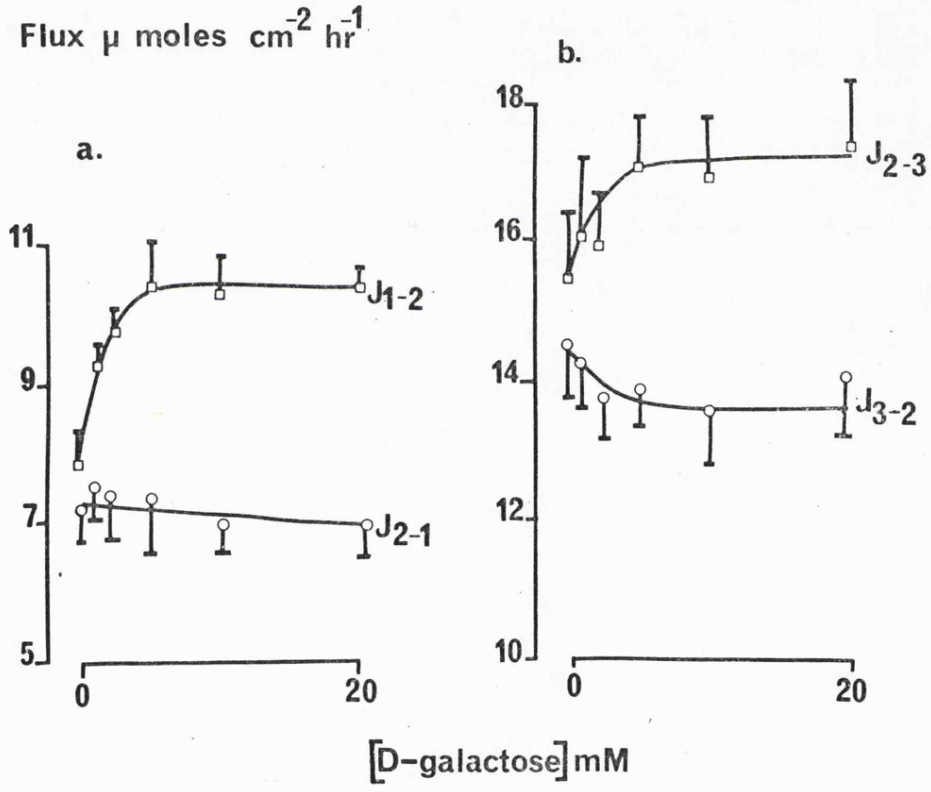
The present data indicating a lack of stimulation of Na exit by galactose apparently contradicts other studies which demonstrate an increased Na efflux in the presence of cell to mucosal solution gradients of alanine and 3-O-methyl glucose (Curran et al (1970); Goldner et al (1972)). Two important experimental differences exist between the present results and those of Curran et al and Goldner et al:

1. The present study eliminates the possible complication of flux through the shunt pathway by the use of triaminopyrimidine.

2. Studies of Na efflux across the mucosal boundary in the presence of cell to mucosal solution gradients of alanine and 3-O-methyl glucose were made in the presence of 0.1mM ouabain.

Figure 20

The effects of galactose (0 to 20mM) upon the calculated unidirectional Na fluxes across (a) the mucosal boundary and (b) the serosal boundary. All data shown are for triaminopyrimidine-treated tissues. Error bars denote the standard error of the mean in each instance.


Flux μ moles cm^{-2} hr^{-1}
a.
11
9
7
5
J_{1-2}
J_{2-1}
0
20
b.
18
16
14
12
10
J_{2-3}
J_{3-2}
0
20
[D-galactose] mM

(d) Stoichiometry of Na/galactose flux across the brush-border

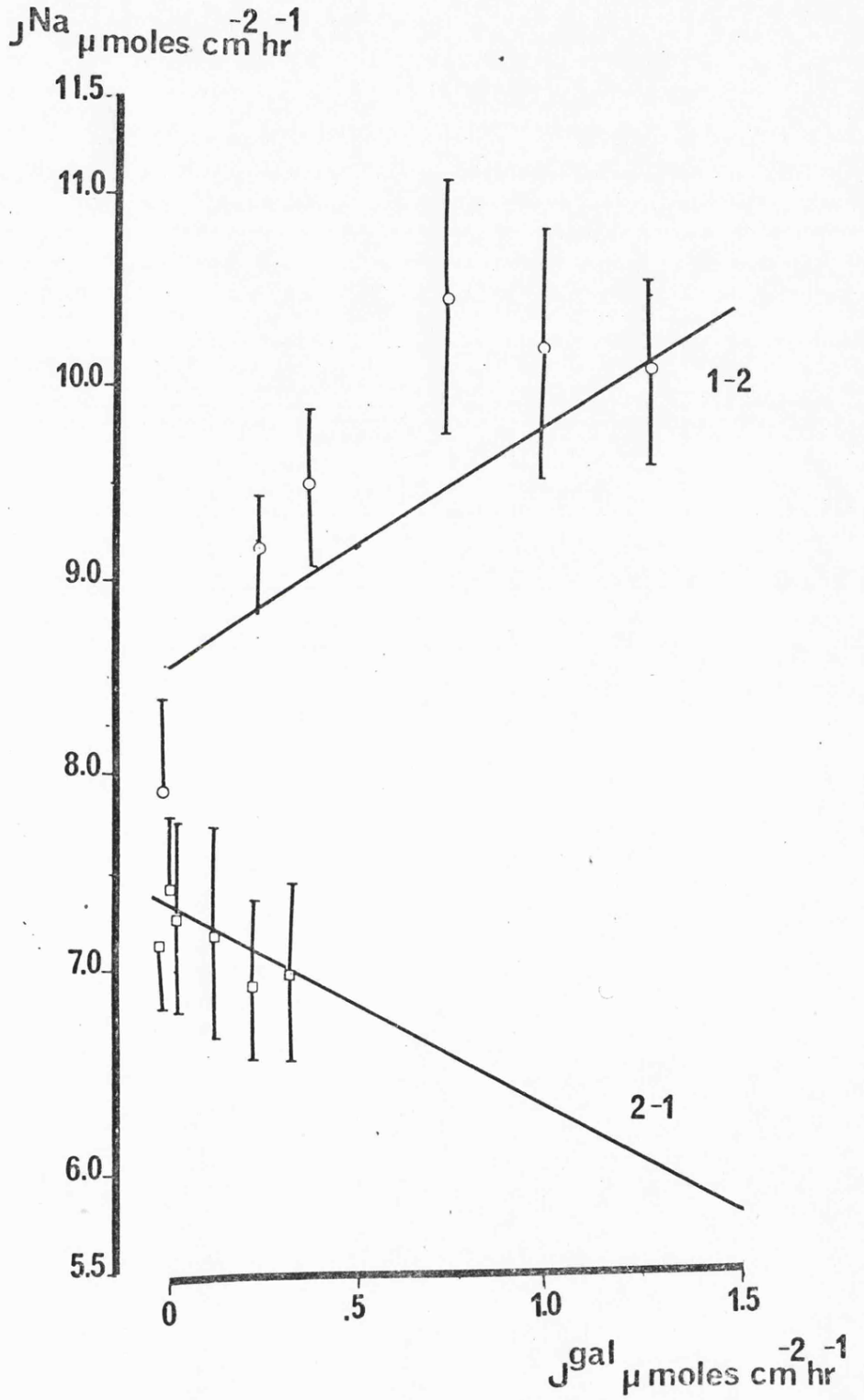
Goldner et al (1969) calculated the stoichiometry of Na and 3-O-methyl glucose interaction within the brush-border of rabbit ileum by simultaneously measuring labelled sugar and Na uptake. Whilst it is feasible using the flux method of this present study to measure bidirectional Na and sugar fluxes with quadruple label counting procedures, it was considered that the counting errors thereby introduced would outweigh any advantage gained from simultaneous flux measurement. An alternative approach has been adopted here. Figure 21 shows the relationship between Na influx (present data) and sugar influx (data obtained by interpolation of the Michaelis-Menten kinetic parameters of galactose influx in 140mM NaCl Ringer obtained previously (Naftalin and Curran (1974)). Although the galactose flux measurements were obtained in open-circuit condition using the same methods, no effects of short-circuiting upon galactose fluxes have been noted (Schultz and Curran (1970)).

The regression line between the increment in Na influx on raising Ringer [galactose] from 0 to 20mM and the increment in galactose influx over the same range has a slope of 1.55 ± 0.56 (SE). Thus the stoichiometry of Na/galactose interaction for flux across rabbit ileum is not significantly different from that obtained by Goldner et al (1969) for Na/3-O-methyl glucose interaction.

On plotting the change of Na exit flux across the mucosal border obtained on raising Ringer [galactose] from 0 to 20mM against the galactose exit flux J_{21} (gal) on changing Ringer [galactose] over the same range, a slope of -1.03 ± 0.59 (SE) is obtained for the regression line of this function. This differs significantly from the slope obtained for the influx stoichiometry ($p < 0.025$) and is contrary to the predictions of the Na gradient hypothesis which

Figure 21

The relationship between the mucosa to cell flux J_{12} (O) of Na and galactose (and for cell to mucosa flux J_{21} ■). Data for galactose fluxes were taken from Naftalin and Curran (1974) corrected for a diffusional component of 0.015 cm hr^{-1}. The regression line for influx (J_{12}Na vs J_{12} galactose) gives J_{Na} = 8.55 + 1.55 J galactose r = 0.88). The regression line for cell to mucosa flux (J_{21}^{Na} vs J_{21} galactose) gives J_{Na} = 7.33 - 1.03 J galactose r = 0.79). Error bars denote the standard error of the Na fluxes.


J^{Na} μ moles cm^{-2} hr^{-1}
11.5
11.0
10.0
9.0
8.0
7.0
6.0
5.5
1-2
2-1
0
.5
1.0
1.5
J^{gal} μ moles cm^{-2} hr^{-1}

implies that the sugar-Na interaction should be similar for both entry and exit across the brush-border (Curran et al (1970)).

(ii) Unidirectional Na fluxes across the serosal border J_{23} and J_{32}

(a) Controls The calculated exit, J_{23} and entry flux J_{32} of Na across the serosal border are 16.74 and 15.90 μmoles cm^{-2} hr^{-1} respectively. These fluxes greatly exceed those obtained by Schultz et al (1967) who estimated J_{23} and J_{32} to be 4 and 1 μmole cm^{-2} hr^{-1}, for data corrected for the shunt fluxes of Na. The difference between the results obtained here and those of Schultz et al is too large to be discounted on the basis of a residual Na shunt permeability in the presence of triaminopyrimidine. Three independently obtained measurements indicate the existance of a large entry flux into the cell across the serosal membranes. These are:

a) the tissue isotope specific activity ratio R, for Na is less than unity indicating that proportionally more isotope within the tissue fluid originates from the serosal fluid than the mucosal fluid,

b) the permeability of the paracellular route (0.038 cm h^{-1}) is significantly less than the total s-m Na permeability in control tissue (0.048 cm h^{-1}),

c) triaminopyrimidine reduces s-m Na permeability only to 0.032 cm h^{-1} this value is incompatible with s-m Na being entirely mediated by the paracellular route as suggested by Frizzell and Schultz (1972).

However, these findings do support the conclusion of Desjeux, Tai and Curran (1974) based on studies of p.d.-dependent s-m Na flux measurements, that there is a significant s-m transcellular flux.

(b) Effects of NaCl replacement by choline chloride and of 0.1mM ouabain added to Ringer on the unidirectional Na fluxes across the serosal border Reduction of Ringer [Na] to 25mM reduces the calculated exit and entry fluxes of Na across the serodal border to 3.74 and

3.38 μmoles cm^{-2} h^{-1} respectively (Table 12). Ouabain reduces both entry and exit flux of Na across the serosal border at all levels of [Na] tested in the range 25-140mM. These marked effects of ouabain on serosal Na flux contrast with the small effect of ouabain in the absence of galactose, on the unidirectional Na fluxes across the mucosal border. These effects of ouabain are entirely consistent with the view that the Na-pump is situated at the serosal border. It can be observed in Table 12 that as well as a ouabain-sensitive active Na transport process situated at the serosal border, there are also both ouabain-sensitive and ouabain-insensitive exchange components to unidirectional Na flux across this border. Since ouabain reduces the hypertonicity of the fluid within the lateral intercellular space and hence, the distension of the space and its accessibility to the serosal bathing solution, it is possible that the ouabain-sensitive Na-exchange flux simply results from an unfolding of the basal-lateral membranes when the tissue Na-pump is activiated. This explanation has been previously proposed to account for the ouabain-sensitive component of galactose exchange flux across the serosal membrane (Naftalin and Curran (1974)). However, further investigation of this process is required in order to define the flux components more exactly.

(c) Effects of galactose on the Na fluxes across the serosal membrane Raising Ringer [galactose] increases the exit flux of Na from cells across the basal-lateral border as a hyperbolic function of Ringer [galactose] (Figure 20 (b)). This result provides support for the view that the tissue Na-pump activity is stimulated by Ringer galactose. This activation of the Na-pump probably results from the increased entry of Na across the mucosal border with subsequent rises in the intracellular [Na]. Consistent with this view, is the finding that ouabain abolishes the galactose-dependent stimulation of Na exit

flux J_{23} across the serosal border (Table 12).

As Ringer [galactose] is raised, there is a slight decrease in the Na entry flux across the serosal border (Figure 20). A possible factor contributing to this apparent decrease in Na entry from the serosal fluid is that the increased Na concentration within the extracellular space competes with Na coming from the serosal bathing solution for transport sites in the serosal border. Other factors such as an increase in the unstirred layer effects or solvent drag effects within the lateral intercellular space may also play a part in the retardation of Na entry across the serosal border.

3. The convective-diffusion model of sugar transport by the small intestine

A new model has been described for sugar transport in the small intestine which is based on evidence concerning unidirectional sugar fluxes across both the mucosal and serosal membranes, (Naftalin and Holman (1974); Holman and Naftalin (1975a, b)). It was proposed that the asymmetric transport of sugar across the brush-border arises because of convective-diffusion of sugar via aqueous pores in the brush-border. The force causing the convective flow of fluid across the brush-border and serosal borders is sustained by the osmotic pressure gradient across the basal-lateral border of the epithelial cells. Sugar accumulation within the intestinal epithelial cells is caused by reflection of the sugar at their basal-lateral membranes.

Na was thought to interact with the sugar transport system at two stages. At the brush-border Na modifies the membrane permeability to sugar; this may be due to a change in membrane pore structure thus affecting sugar diffusivity. At the serosal border Na activates the tissue Na-pump which in turn generates the hypertonic salt solution within the lateral spaces.

Several of the present findings are consistent with this model for sugar transport.

1. Galactose increases the Na influx across the brush-border. The stoichiometry between Na entry and galactose entry is approximately 1:1.

2. Na efflux across the basal-lateral border is stimulated by galactose. This is due to stumulation of the Na-K ATPase following an increase in intracellular [Na] (see Chapter 3).

3. Na efflux across the mucosal boundary in the presence of triaminopyrimidine decreases in the presence of galactose. Since it has been shown that intracellular [Na] increases in the presence of galactose, and since it is known that the membrane potential across the brush-border is depolarized by actively transported sugars (Rose and Schultz (1971)) an increased Na efflux would be expected. Intracellular galactose accumulation should also be expected to stimulate Na efflux on the basis of the Na-gradient hypothesis. The finding that Na efflux <u>decreases</u> is indicative of an additional vectorial force across the brush-border membrane which retards Na efflux. A likely force is the flow of water through aqueous membrane pores.

4. In the presence of 0.1mM ouabain no galactose-dependent change in Na flux is seen across either the mucosal or basal-lateral membranes. Ouabain has been shown to abolish the hypertonic Na concentration within the lateral spaces (see Chaper 3).

CHAPTER FIVE

THE MEASUREMENT OF UNIDIRECTIONAL Cl FLUXES ACROSS THE MUCOSAL AND SEROSAL BORDERS OF RABBIT ILEUM

A. Introduction

The existence of active Cl transport (absorption) in rabbit ileum *in vitro* has been demonstrated by several workers (Dietz and Field (1973), Field, Fromm and McColl (1971), Nellans, Frizzell and Schultz (1974)). Earlier contradictory reports that no net Cl absorption occurred *in vitro* may be attributed (Schultz and Curran (1974) to:

1. the absence of muscle stripping procedures
2. inter-animal variation due to hormonal and intracellular factors *in vivo*
3. the absence of two experimentally convenient radioactive isotopic tracers for Cl (thus rendering simultaneous measurements of transmural bidirectional fluxes impossible).

It has been demonstrated that ^{82}Br may be used as a tracer for Cl flux, if appropriate correction factors are applied, both in frog skin (Tomlinson and Wood (1972)) and toad bladder (Wood and Tomlinson (1974)). Thus, simultaneous bidirectional flux measurements are possible by the use of ^{36}Cl together with ^{82}Br.

It was considered worthwhile to attempt to apply the technique of Tomlinson and Wood to the measurement of bidirectional Cl fluxes in rabbit ileum *in vitro*. Coupled with an estimate of the isotope specific activity ratio within the tissue, it is possible to calculate the unidirectional fluxes of Cl across the mucosal and serosal borders of rabbit ileum (Naftalin and Curran (1974), see also METHODS).

No significant extracellular shunt component of transmural Cl flux exists compared with transcellular flux (Frizzell and Schultz (1972)); this contrasts with the case of Na where a significant part of transmural Na flux traverses the tissue by an extracellular cation-selective pathway.

Schultz, Zalusky and Gass (1964) have examined the effect of glucose on bidirectional Cl fluxes in short-circuited rabbit ileum using ^{36}Cl as tracer. They concluded that glucose has no effect on Cl flux. Glucose stimulated Na transport accounted for the short-circuit current across this tissue. Barry, Smyth and Wright (1965) examined the relationship between sugar evoked short-circuit current and net Na flux in rat jejenum. Agreement between short-circuit current and net Na flux was found with glucose but not with galactose and 3-O methyl glucose. Taylor, Wright, Schultz and Curran (1968) examined these findings in more detail. These workers demonstrated that glucose and galactose had no effect on Cl flux in rabbit and rat ileum and that the short-circuit current was equal to net Na flux. In rat and rabbit jejenum the effect of glucose was similar to that found in ileum, galactose, however, stimulated a net Cl movement from serosa to mucosa. Munck (1972) confirmed (using rat jejenum) the finding that galactose stimulated a net s-m movement of Cl. Proline had a similar effect to galactose on Cl flux in this preparation. Binder, Powell and Curran (1972) have examined the effects of sugars on ion flux in guinea-pig ileum. Glucose stimulated a neutral s-m movement of NaCl. Galactose stimulated s-m movement, but this was without effect on the short-circuit current and, therefore, at the expense of HCO_3 flux. 3-O methyl glucose was without effect on ion flux.

A possible relationship between unidirectional Cl movement and galactose fluxes in rabbit ileum is re-examined using the new methods

available. Also of interest are the possible effects of 2,4,6, triaminopyrimidine which blocks paracellular cation conductance (Chapter 4) and is used as a basis for transcellular Na flux measurements.

B. RESULTS

1. The use of ^{82}Br as a tracer for Cl movement

Table 13 shows Cl bidirectional fluxes determined in experiments in which ^{36}Cl and ^{82}Br were used simultaneously to estimate Cl flux. It can be seen that flux calculated from ^{82}Br as tracer is consistently larger than flux determined using ^{36}Cl as tracer for both mucosa to serosa flux J_{13} ($p < 0.1 > 0.05$) and for serosa to mucosa flux J_{31} ($p < 0.05$). This result is similar to that found by Wood and Tomlinson (1974) and is consistent with the K_i for Br inhibition of Cl flux across the brush-border being smaller than the K_m for Cl influx. (Frizzell, Nellans, Rose, Markscheid-Kaspi, Schultz (1973)). The ratio of ^{82}Br : ^{36}Cl calculated fluxes did not differ significantly between m-s (J_{13}) and s-m (J_{31}) fluxes ($p > 0.2$).

Tissue concentrations of Cl (uncorrected for extracellular space) do not differ whether estimated by ^{36}Cl or ^{82}Br ($p > 0.5$).

Since a small shunt component of transmural Cl flux exists, it is necessary to demonstrate that the relative permeability of ^{82}Br-calculated Cl flux for this pathway is no different from the ^{36}Cl-calculated Cl flux. Table 14 shows the results of an experiment in which mucosa to serosa Cl flux J_{13} was measured simultaneously by ^{36}Cl and ^{82}Br tracers as a function of the applied potential difference. If ^{82}Br permeation was largely via the extracellular (shunt) pathway, variation of the applied potential should cause large changes in transmural flux (see Frizzell and Schultz (1972) and Methods). It is clear that no large potential-sensitive component for ^{82}Br or ^{36}Cl-calculated flux

TABLE 13

^{36}Cl and ^{82}Br-calculated Cl fluxes determined simultaneously in control tissues together with steady-state tissue of Cl calculated from the ^{36}Cl and ^{82}Br distribution ratios. All errors are expressed as ± S.E.M.

	n	Isotope		Ratio of
		^{36}Cl	^{82}Br	$^{82}Br/^{36}Cl$
mucosa to serosa Cl flux J_{13} (μmoles cm^{-2} hr^{-1})	6	9.326 ±0.449	10.815 ± 0.696	1.15 ±0.03
serosa to mucosa Cl flux J_{31} (μmoles cm^{-2} hr^{-1})	6	6.228 ±0.384	7.546 ±0.374	1.21 ±0.03
Tissue Cl (m.equiv/litre tissue H_2O)	12	37.40 ± 5.31	35.91 ± 4.42	1.01 ±0.03

TABLE 14

Ratios of ^{36}Cl : ^{82}Br mucosa to serosa fluxes determined simultaneously in control tissues at 3 levels of clamping potential. All errors are expressed as S.E.M. Data pooled from 3 tissues. Fluxes are expressed as μmoles cm^{-2} hr^{-1}.

Trans-epithelial potential (mv.)	n	Isotope		Ratio of
		^{36}Cl	^{82}Br	$^{82}Br/^{36}Cl$
0	3	10.72 ± 0.35	13.90 ± 0.70	1.29 ± 0.09
+10 (serosal anode)	3	11.40 ± 0.56	14.17 ± 1.18	1.24 ± 0.06
-10 (serosal cathode)	3	9.32 ± 0.70	12.14 ± 0.84	1.30 ± 0.07

exists. Indeed, the ratio of ^{82}Br / ^{36}Cl-calculated fluxes is invariant as the potential is varied. This result demonstrates that the relative permeabilities of ^{82}Br / ^{36}Cl-calculated Cl fluxes are identical for both the paracellular and cellular routes of transmural flux.

The small variation in m-s Cl flux (J_{13}) as a function of the applied potential difference is entirely consistent with the notion that Cl flux is largely transcellular and that the shunt pathway has a low permeability to Cl. (Frizzell and Schultz (1972), Munck and Schultz (1974)).

^{82}Br may be used as an adequate tracer for Cl provided that the appropriate correction factors are used to correct ^{82}Br-calculated Cl fluxes. An additional precaution in the use of ^{82}Br as a tracer for Cl was routinely applied; this involved undertaking experiments in which the experimental conditions were run at least in duplicate, thus allowing estimations of m-s flux (J_{13}) by both ^{82}Br and ^{36}Cl. Large variation from the control relationship between ^{82}Br and ^{36}Cl-calculated Cl flux could, therefore, be detected if present. No such variation was observed in all experimental conditions performed.

2. Bidirectional flux measurements/Residual flux calculations

(i) Controls

The pooled values of bidirectional Cl fluxes in control Ringer's solutions give m-s Cl flux (J_{13}) as 8.33 μmoles cm^{-2} hr^{-1} and s-m flux (J_{31}) as 6.55 μmoles cm^{-2} hr^{-1} (Table 15). The net Cl flux is 1.78 μmoles cm^{-2} hr^{-1}; this value is significantly different from zero ($p < 0.001$). Net Cl flux does not differ from values at control net Na flux estimated in a separate experimental series ($p > 0.5$) (see Chapter 4). The short-circuit current (SCC) for control tissues is significantly different from zero ($p < 0.001$) and is negative (serosal

TABLE 15

Bidirectional flux measurements using ^{36}Cl and ^{82}Br as tracers for Cl flux. Short-circuit current (SCC) measurements were pooled from data covering the period of flux measurement (20 to 80 minutes following introduction of isotopes. Net Na flux measurements are as given in Chapter 4. Residual flux $J_r = SCC - (J^{Na}_{net} - J^{Cl}_{net})$. Permeability $P_{ij} = J_{ij}/C_i$ where C_i is the concentration of Cl in the appropriate bathing solution. All errors are expressed as $\pm$ S.E.M. Figures in parentheses give the number of data points for net Na fluxes and SCC determinations.

TABLE 15

	n	J_{13} μmoles cm^{-2} hr^{-1}	P_{13} cm hr^{-1}	J_{31} μmoles cm^{-2} hr^{-1}	P_{31} cm hr^{-1}	J_{net} μmoles cm^{-2} hr^{-1}	J^{Na}_{net} μmoles cm^{-2} hr^{-1}	SCC μmoles cm^{-2} hr^{-1}	Residual Flux J_r μmoles cm^{-2} hr^{-1}
Standard NaCl Ringer	23	8.33 ± 0.42	0.057 ± 0.003	6.55 ± 0.44	0.045 ± 0.003	1.78 ± 0.33	1.82 ± 0.35 (22)	0.88 ± 0.07 (315)	0.84 ± 0.14 (360)
Standard NaCl Ringer + 20mM triaminopyrimidine	15	8.43 ± 0.63	0.058 ± 0.004	7.24 ± 0.48	0.049 ± 0.003	1.19 ± 0.37	0.84 ± 0.23 (18)	0.75 ± 0.09 (321)	1.09 ± 0.16 (354)
Standard NaCl Ringer + 20mM galactose	20	8.05 ± 0.49	0.550 ± 0.003	6.19 ± 0.47	0.043 ± 0.003	1.85 ± 0.55	3.01 ± 0.33 (11)	3.41 ± 0.45 (217)	2.25 ± 1.05 (248)
Standard NaCl Ringer + 20mM galactose + 20mM trieminopyrimidine	12	6.31 ± 0.28	0.043 ± 0.002	5.68 ± 0.45	0.039 ± 0.003	0.63 ± 0.52	3.08 ± 0.43 (13)	3.03 ± 0.36 (175)	0.58 ± 0.86 (190)
Standard NaCl Ringer + 0.1mM ouabain	11	5.54 ± 0.43	0.038 ± 0.003	5.67 ± 0.53	0.039 ± 0.004	-0.12 ± 0.44	0.32 ± 0.31 (6)	0.24 ± 0.21 (112)	-0.20 ± 0.52 (129)
Standard NaCl Ringer + 0.1mM ouabain + 20mM triaminopyrimidine	15	6.34 ± 0.54	0.043 ± 0.003	5.44 ± 0.59	0.042 ± 0.004	0.40 ± 0.44	0.02 ± 0.14 (6)	0.27 ± 0.12 (84)	0.46 ± 0.38 (105)
Standard NaCl Ringer + 0.1mM ouabain + 20mM galactose	5	6.11 ± 1.50	0.042 ± 0.010	5.95 ± 0.71	0.041 ± 0.005	0.16 ± 0.84	0.24 ± 0.30 (5)	0.38 ± 0.14 (104)	0.30 ± 0.55 (114)
Standard NaCl Ringer + 0.1mM ouabain + 20mM triaminopyrimidine + 20mM galactose	7	5.31 ± 0.60	0.037 ± 0.004	5.64 ± 1.13	0.039 ± 0.008	0.33 ± 0.94	0.32 ± 0.07 (5)	0.27 ± 0.10 (98)	-0.38 ± 0.57 (110)

cathode). The flux equivalent, for a univalent ion, of the SCC is 0.82 μmoles cm^{-2} hr^{-1}. This may represent the net flux of a cation from mucosa to serosa or of an anion from serosa to mucosa. Since the sum of net Na and Cl fluxes is $\simeq 0$ the residual current Jr (Jr = SCC - (J^{Na}_{net} - J^{Cl}_{net}) may represent the net secretion of HCO_3 (serosal to mucosal movement). Two points are worthy of note in regard to the residual current:

a) lactate fluxes, H and OH fluxes may constitute part of the residual fluxes. Dietz and Field (1973) have demonstrated, however, that HCO_3 fluxes account for the greater part of the residual flux

b) residual flux measurements are the result of 5 separate experimental determinations made from 2 experimental series and are therefore subject to large standard errors.

The value for residual current determined in these experiments is consistent with the range of residual current values determined by Field and his co-workers (1971a, b, 1973) of 1.0 - 3.0 μmoles cm^{-2} hr^{-1}. Dietz and Field (1973) estimated net HCO_3 flux directly by acidification and back-titration to be $\sim$ 1.9 μmoles cm^{-2} hr^{-1}. A similar value has been obtained by Frizzell et al (1974).

(ii) <u>The effect of 20mM triaminopyrimidine</u>

Table 15 shows the effect of 20mM triaminopyrimidine on bidirectional Cl fluxes. M-s flux (J_{13}) is unaffected by triaminopyrimidine ($p > 0.9$). S-m flux (J_{31}) is slightly increased compared with control values; this increase is not statistically significant ($p > 0.2$). Triaminopyrimidine results in a small reduction in net Cl flux ($p > 0.2$). This change parallels the significant change in net Na flux observed in the presence of triaminopyrimidine (Chapter 4) and may indicate that the postulated passive movement of Na via the paracellular pathway consists, in part, of neutral NaCl movement.

In Chapter 4 a decrease in SCC was recorded in the presence of triaminopyrimidine. In measurements of SCC averaged over the period of flux measurement (Table 15) no change due to triaminopyrimidine was observed ($p > 0.2$). The difference between these two results is likely to result from decreased sensitivity of short-circuit current measurements on incubation due to a decline in tissue conductance and tissue viability.

No change in the residual flux in the presence of triaminopyrimidine is observed ($p > 0.2$).

The small effects of triaminopyrimidine on Cl fluxes lend support to the conclusion made in Chapters 3 and 4 that triaminopyrimidine has no effect upon transcellular ion transport and that its sole locus of action is the cation-selective site within the tight-junction between the epithelial cells.

(iii) The effects of galactose

(a) Controls D-galactose (20mM) has no significant effect upon the bidirectional Cl fluxes in control Ringer's (Table 15) ($p > 0.5$, $p > 0.5$ for m-s (J_{13}) and s-m (J_{31}) Cl fluxes respectively). These results are in agreement with the findings of Schultz et al (1964). It is, however, evident that the SCC in the presence of galactose cannot be fully accounted for by the net Na and Cl fluxes. The residual current is 2.25 μmoles cm^{-2} hr^{-1}. This is larger than the residual current in control tissues. The increased residual current may be indicative of an increased HCO_3 secretion (see Discussion). The problems associated with residual flux measurements may be emphasized by reference to the large error involved in this determination. The increase in residual flux, though large, is non-significant ($p > 0.1$).

(b) The effects of D-galactose in the presence of triaminopyrimidine 20mM galactose in the presence of triaminopyrimidine reduces m-s Cl flux (J_{13}) compared with control tissues plus triaminopyrimidine

alone ($p < 0.005$) (Table 15). There is no significant change in s-m Cl flux in this condition compared with controls ($p > 0.2$). Net Cl flux is reduced by galactose to 0.63 μmoles cm^{-2} hr^{-1} in the presence of triaminopyrimidine. This value differs from net Cl flux measured in the presence of galactose alone ($p > 0.05 < 0.1$). Net Na flux accounts for the greater part of the SCC in the galactose/triaminopyrimidine condition. This contrasts with control tissues where net Na flux does not contribute to the SCC. The residual flux in tissues treated with both galactose and triaminopyrimidine is 0.58 μmoles cm^{-2} hr^{-1}. The difference between this value of residual flux and residual flux in the presence of galactose alone is large but not statistically significant ($p > 0.2$).

The reason for the decrease in m-s Cl flux is obscure. The interrelations between net Na and Cl flows and the residual current in this condition resemble <u>in vivo</u> relationships (Hubel (1967, 1969); Turnberg et al (1970a,b)).

(iv) <u>The effects of 0.1mM ouabain</u>

(a) <u>Controls</u> Net Cl flux is abolished by 0.1mM ouabain. This is primarily due to a reduction in m-s Cl flux (J_{13}). J_{13} in the presence of ouabain is statistically different from m-s flux (J_{13}) for controls ($p < 0.001$). A small, though non-significant, reduction in s-m Cl flux (J_{31}) is seen in the presence of ouabain compared with controls. These effects resemble the effect of ouabain on bidirectional Na fluxes (Chapter 4). The short-circuit current and residual current are both reduced by 0.1mM ouabain compared with controls ($p < 0.001$, $p < 0.01$ respectively), indeed these values do not differ significantly from zero ($p > 0.5$, $p > 0.5$ respectively).

The net flux of Cl, Na (and the residual current) are, therefore, dependent on the activity of the Na-K ATPase.

The presence of 20mM triaminopyrimidine does not substantially alter the effect of ouabain compared with control tissues.

(b) Tissues + 20mM galactose 0.1mM ouabain abolishes the galactose-dependent decrease in Cl flux seen in the presence of triaminopyrimidine. In addition, the 'stimulation' of residual current by galactose is not observed in ouabain-poisoned tissues.

3. The isotope specific activity ratio within the tissue (R)

The pooled values for the isotope specific activity ratio are shown in Table 16. The ratio for controls is 0.869 ± 0.099. This indicates a slight bias to isotope originating from the serosal solution within the tissue fluid. 20mM galactose does not significantly change the isotope ratio within the tissue ($p > 0.9$). Triaminopyrimidine in the presence and absence of galactose results in a small non-significant increase in the tissue isotope ratio ($p > 0.2$, 0.5 respectively). Tissue incubation with 0.1mM ouabain results in non-significant increments in R for controls ($p > 0.2$) tissues plus triaminopyrimidine ($p > 0.5$) and tissues plus galactose ($p > 0.5$). A decrease in R due to ouabain is observed in tissues incubated in the presence of galactose together with triaminopyrimidine. This is not significant compared with tissues minus ouabain. ($p > 0.2$).

Since tissue isotope specific activity ratios were determined from the whole tissue it was necessary to show that no inhomogeneity in the ratio within the tissue existed (see Methods and Section B5 Chapter 4). Table 17 shows the results of the analysis for control tissues. No significant effect of washing (i.e. removal of extracellular cation) is observed in whole tissue ($p > 0.2$). epithelial (mucosal) scrapings ($p > 0.5$) or submucosal layers ($p > 0.5$). Additionally, no effect of scraping is seen between whole tissue and submucosal layers ($p > 0.5$), whole tissue and mucosal scrapings ($p > 0.5$) and mucosal scrapings and submucosal layers ($p > 0.5$). These results demonstrate that there is no inhomogeneity in the ratio R within the tissue and that the tissue may be treated as a single compartment in relation to Cl flux.

TABLE 16

The tissue isotope specific activity ratio of $^{36}Cl/^{82}Br$ (or $^{82}Br/^{36}Cl$) (R). Errors are expressed as the S.E.M. Figures in parentheses are the number of separate determinations.

CONDITION	CONTROL	+ 0.1mM ouabain
Standard NaCl Ringer	0.869 ± 0.099 (22)	1.110 ± 0.226 (11)
Standard NaCl Ringer + 20mM triaminopyrimidine	1.120 ± 0.192 (16)	1.30 ± 0.211 (15)
Standard NaCl Ringer + 20mM galactose	0.875 ± 0.199 (20)	1.063 ± 0.163 (5)
Standard NaCl Ringer + 20mM galactose + 20mM triaminopyrimidine	0.998 ± 0.139 (12)	0.773 ± 0.060 (7)

TABLE 17

Tissue distribution of the isotope specific activity ratio (R) for $^{36}Cl/^{82}Br$ within the extracellular and cellular compartments. Results are the mean of 3 replicates from a single experiment. Errors are expressed ± S.D. ^{36}Cl is used as a tracer for Cl from the mucosal solution and ^{82}Br as a tracer for Cl from the serosal solution in this experiment.

	Unwashed tissue	½hr isotonic mannitol wash
Whole tissue	0.843 ± 0.029	1.220 ± 0.410
Epithelial scrapings	1.180 ± 0.279	1.660 ± 0.284
Submucosal layers	0.885 ± 0.062	0.970 ± 0.190

C. DISCUSSION

1. Bidirectional Cl flux measurements

The ability of mammalian ileum to absorb Cl was first established by Ingraham and Vischer (1936). Uptake of Cl was followed from loops of intestine in dog and Cl impoverishment noted. Measurement of the electrical potential difference and the Cl concentration difference between lumen and blood concurrent with measurements of net Cl movement allowed the conclusion to be made (Curran and Solomon (1957) *in vivo* rat ileum; Kinney and Code (1964) *in vivo* dog ileum; and Turnberg et al (1970) *in vivo* human ileum) that Cl absorption was active (i.e. against the prevailing electrochemical potential difference).

Measurement of net Cl absorption under voltage clamp *in vitro* was not a consistent observation of earlier studies (Schultz, Zalusky and Gass (1964); Clarkson and Toole (1964)). Improved *in vitro* methodology (e.g. stripping of serosal muscle layers) resolved this apparent anomaly and net Cl absorption has been established to occur *in vitro* (Dietz and Field (1973)).

Inherent variability in net Cl measurement due to the use of single-label isotopic flux measurements using ^{36}Cl has been reduced in the present series of experiments by the use of ^{82}Br as a tracer for Cl thus rendering bidirectional isotopic flux experiments possible (using ^{36}Cl together with ^{82}Br). The results obtained in Section 1 of Results indicate that ^{82}Br may be used in this way providing appropriate correction is made for the larger flux estimated by ^{82}Br. The possibility that ^{82}Br flux does not mimic the bulk Cl flux in all experimental conditions tested must, however, be considered. To this end ^{82}Br was used alternately as a tracer of both mucosal to serosal and serosal to mucosal fluxes; this allows separate estimates of m-s flux by both ^{36}Cl and ^{82}Br and provides internal experimental controls to the use of ^{82}Br.

The existence of net Cl absorption (m-s flux) for *in vitro* ileum stripped of its muscle layers is confirmed in Results. The absolute magnitude of this net flux and the bidirectional transepithelial Cl fluxes estimated by using the dual-label technique are similar to those obtained by single-label measurements using ^{36}Cl alone (Field, Fromm and McColl (1971) and Sheerin and Field (1975). This result provides additional support for the validity of the use of ^{82}Br as a tracer for Cl movement in ileum.

20mM triaminopyrimidine has no significant effect upon the bidirectional Cl fluxes. This result is in agreement with the data from diffusion/biionic potentials obtained in Chapter 4. A small (though non-significant) decrease in J_{net} for Cl is observed; this may be associated with the decrease in J_{net} for Na observed in the presence of triaminopyrimidine. The triaminopyrimidine sensitive component of net Na flux may result from a passive process driven by osmotic water flow through the tight-junctions (Chapter 4). This process should be electrogenic due to unequal sieving of Na and Cl through the cation-selective tight-junctions (Smyth and Wright (1966)). In accordance with this view, the triaminopyrimidine-sensitive component of net Na flux is greater than the triaminopyrimidine-sensitive component of net Cl flux. The existence of high permeability of the tight-junction to osmotic water flow and to diffusional flows of small low-molecular weight solutes is now generally recognized in 'leaky' epithelia (Frizzel and Schultz (1972), Huss and Marsh (1975), Sackin and Boulpaep (1975)). Berry and Boulpaep (1975) have demonstrated solute-solvent coupling within the tight-junctions of necturus kidney proximal tubule.

20mM galactose has no effect on net Cl flux or upon the bidirectional Cl fluxes. This result is in agreement with previous work (Schultz, Zalusky and Gass (1964), Taylor, Wright, Schultz and Curran (1968)).

Triaminopyrimidine causes a reduction in m-s Cl flux (J_{13}) and net Cl flux in the presence of 20mM galactose. The basis for this effect is obscure.

0.1mM ouabain reduces net Cl flux to zero in all four experimental conditions. This is primarily due to a reduction in m-s Cl flux. This result demonstrates a dependence of Cl movement on Na-K ATPase activity.

2. Residual fluxes

The use of net Cl flux measurements and short-circuit current measurements together with previous estimates of net Na flux (Chapter 4) provides a crude basis for estimating the residual flux. Residual fluxes, in rabbit ileum, may be largely attributable to a net HCO_3 flux, (Dietz and Field (1973), Frizzell, Markscheid-Kapsi and Schultz (1974)). The present value for the residual flux ($\approx$1.0 μmoles cm^{-2} hr^{-1}) agrees with the values obtained by Field and co-workers for *in vitro* intestine.

The nature of HCO_3 fluxes in ileum are ill-understood. Direct measurements of HCO_3 fluxes by ^{14}C tracer are difficult due to exchange of ^{14}C tracer with gaseous CO_2. Indeed, high HCO_3 permeabilities for ileum measured by this method are probably due to this effect (Powell, Binder and Curran (1973)). HCO_3 flux measurements, in the traditional sense, are probably impossible, since net HCO_3 movement most probably involves loss from an intracellular pool formed by oxidative metabolism (Frizzell et al (1974); Carlinsky and Lew (1970); Lew (1970)).

20mM triaminopyrimidine has no effect upon the residual flux. 20mM galactose results in a large, though non-significant, increase in the residual current. Since it is known that:

(a) no significant metabolism of galactose occurs under the experimental conditions used in the present study (Naftalin and Curran (1974)),

(b) rabbit ileum in vitro is not limited metabolically (Frizzell et al (1974)),

then the increased residual currents may indicate an increased HCO_3 production due to increased oxidative metabolism.

This result is consistent with increased energy demands due to stimulation of the Na-K ATPase by galactose (Chapter 4). Similar results concerning metabolism have been reported in other epithelia upon stimulation of Na-K pump activity (Martin and Diamond (1966)). Further duscusion of this point is not warranted due to the large errors associated with residual flux measurements.

3. Unidirectional flux calculations

Table 18 shows the calculated values for unidirectional fluxes across the mucosal and basolateral cell boundaries.

Cl influx J_{12} across the mucosal boundary is 14.25 μmoles cm^{-2} hr^{-1}. This value is in reasonable agreement with values of influx determined directly (Nellans, Frizzelland Schultz (1973)). It is pertinent to note that altough paracellular permeability is low (Frizzell and Schultz (1972)) a portion of the calculated flux must represent flux through the paracellular pathway. A permeability of 0.018 cm hr^{-1} for extracellular Cl permeability would yield a value of 2.61 μmoles cm^{-2} hr^{-1}. This diffusional component can, however, only be a small fraction of the total flux.

Efflux J_{21} across the mucosal boundary is calculated to be 12.47 μmoles cm^{-2} hr^{-1}. The flux ratio J_{12}/J_{21} for the mucosal boundary is, therefore, 1.14. For a value of intracellular $[Cl]$ of 58mM (Frizzellet al (1973)) and a mucosal membrance potential of -36mV (Rose and Schultz (1971)) the theoretical Ussing flux ratio is 0.61 The discrepancy between these two values may implicate an active process in Cl influx. This conclusion is in agreement with the data of Frizzellet al (1973).

TABLE 18

Calculated values for unidirectional Cl fluxes across the mucosal and basal-lateral boundaries of rabbit ileum. All errors are expressed as the standard error of the mean. Fluxes are expressed as μmoles cm^{-2} hr^{-1}.

TABLE 18

	n	J_{12}	J_{21}	J_{23}	J_{32}
140mM Na Ringer (Standard Ringer)	23	14.25 ± 1.12	12.47 ± 1.16	19.84 ± 1.12	18.06 ± 1.12
140mM Na Ringer + 0.1mM ouabain	11	10.99 ± 0.68	11.12 ± 0.58	12.75*** ± 1.83	12.88** ± 2.01
140mM Na Ringer + 20mM galactose	20	13.74 ± 1.55	11.89 ± 1.70	20.86 ± 1.67	19.00 ± 1.59
140mM Na Ringer + 20mM galactose + 0.1mM ouabain	5	12.27 ± 1.43	12.11 ± 0.93	12.62* ± 4.20	12.46* ± 3.36
140mM Na Ringer + 20mM triaminopyrimidine	15	16.53 ± 2.18	15.34 ± 2.08	15.95 ± 1.25	14.76 ± 1.17
140mM Na Ringer + 20mM triaminopyrimidine + 0.1mM ouabain	15	14.09 ± 1.03	13.66 ± 1.05	11.21*** ± 1.23	10.81*** ± 1.35
140mM Na Ringer + 20mM triaminopyrimidine + 20mM galactose	20	12.04 ± 1.11	11.40 ± 1.20	13.92*** ± 0.75	13.28** ± 0.95
140mM Na Ringer + 20mM triaminopyrimidine + 20mM galactose + 0.1mM ouabain	7	9.66 ± 1.25	9.99 ± 1.77	12.17*** ± 1.33	12.50*** ± 1.95

Significantly different from controls (Standard Ringer)

*$p < 0.05$
**$p < 0.01$
***$p < 0.005$

The calculated values for Cl fluxes across the basal-lateral border indicate that the bidirectional fluxes are large (19.84 and 18.06 μmoles cm^{-2} hr^{-1} for J_{23} and J_{32} respectively). Recently, Cremaschi and Henin (1975) have measured the ^{36}Cl uptake from the serosal solution into rabbit gall-bladder. They conclude that the basal-lateral border of gall-bladder epithelial cells is impermeable to Cl. In contrast with rabbit gall-bladder, the basal-lateral membrane of intestine is freely permeable to Cl. A portion of the serosal Cl flux may represent Cl/Cl exchange. Zeuthen and Monge (1975) have reported that Cl is present in high concentrations in the lateral intercellular space (300mM). The observed flux ratio for the serosal border (J_{23}/J_{32}) is 1.09; assuming an extracellular $[Cl]$ of 300mM, the predicted theoretical ratio is 0.79. Hence, Cl may be actively extruded across the serosal (basal-lateral) membrane. Assuming that the extracellular $[Cl]$ was 145mM, Frizzell et al (1973) concluded that Cl was passively distributed across the basal-lateral membrane.

Neither galactose nor triaminopyrimidine alone have any significant effect on unidirectional Cl fluxes.

0.1mM ouabain has no significant effect upon bidirectional influx (J_{12}) and efflux (J_{21}) across the mucosal boundary. Frizzell et al (1973) reported that metabolic inhibitors such as cyanide and iodoacetate had no appreciable effect on influx (J_{12}). Ouabain, however, does cause a large reduction in the calculated bidirectional fluxes across the serosal (basal-lateral) boundary, (J_{23} and J_{32}). This effect is similar to that observed with Na fluxes (Chapter 4). Net Cl and net Na fluxes are reduced to zero. Thus net Cl flux may simply be passively coupled to net Na flow. The bidirectional decrease in Cl flux across the serosal boundary may be related to lateral space collapse, thus reducing the effective membrane area for ionic permeation. Similar effects are seen with sugar fluxes

(Naftalin and Curran (1974)).

The significant change in m-s Cl flux observed in the presence of triaminopyrimidine together with galactose is due primarily to a reduction in unidirectional flux across the serosal membrane, (Table 18). As has previously been stated, the nature of this effect is obscure.

CHAPTER SIX

The mechanism of action of theophylline upon Na and Cl fluxes: the effects of 2,4,6, triaminopyrimidine

A. Introduction

Previous investigations of the effect of secretagogues such as theophylline, cholera toxin and c-AMP upon intestinal ion transport have concentrated upon the measurement of the transintestinal bidirectional (mucosa to serosa, and serosa to mucosa) fluxes of both Na and Cl (Sheerin and Field (1975); De Jonge (1975); Powell, Binder and Curran (1973); Al-Awquati, Cameron and Greenough (1973); Field (1971)).

Measurements of unidirectional Na and Cl influx across the mucosal boundary in the presence of secretagogues have been made (Frizzell et al (1973); Nellans et al (1973, 1975)). These studies have not convincingly separated influx across the mucosal membrane from influx across the tight-junction. Ion movements through the shunt-pathway are of prime importance with regard to transepithelial Na movements. A change in tissue conductance, indicative of change in the paracellular pathway has been reported in the presence of theophylline and cholera toxin (Powell(1974); Nellans et al (1974)).

The use of the methods to measure unidirectional Na and Cl fluxes across the mucosal and basal-lateral borders of rabbit ileum should allow a greater insight into the mechanisms involved in theophylline-mediated secretion in rabbit ileum. The use of triaminopyrimidine allows a definitive test to be made of the importance of the para-cellular pathway to Na movement in the secretory state.

B. Results

1. The effects of theophylline on passive ionic permeability

(i) Ouabain-poisoned tissues

(a) Mannitol dilution and biionic potentials Powell (1974) has reported that theophylline decreases transepithelial electrical conductance across rabbit ileum. In ouabain-poisoned tissues, however, he observed a theophylline-dependent increase in Cl permeability. The change in Cl permeability was unaccompanied by changes in Na permeability.

Table 19 shows the biionic and mannitol dilution potentials for controls and tissues incubated in the presence of 5mM theophylline*. It is clear that there is a significant reduction in the mannitol dilution potential with theophylline present compared with control values minus theophylline ($p < 0.001$). That the reduction in the mannitol dilution potential due to theophylline is due to an increased Cl permeability is evident from the following observations: a) the K : Na and b) the choline : Na biionic potentials do not differ from control values ($p > 0.3$, $p > 0.4$ respectively). A more direct test as to increased Cl permeability may be made from the SO_4 : Cl biionic potential. This is significantly increased in the presence of theophylline compared with control values ($p > 0.001$). Since SO_4 is considered to be an impermeant substitute for Cl (Nellans, Frizzell and Schultz (1974)) this result can only be due to increased Cl permeability. These results confirm those of Powell (1974).

The observation that theophylline changes Cl permeability without any change in Na permeability suggests that there exists, in ileum, separate channels conferring Na and Cl permeability to the tissue.

*all solutions contain 0.1mM ouabain (see Methods)

Table 19

Biionic and mannitol dilution potentials for rabbit ileum in the presence of 0.1mM ouabain following a preincubation period of twenty minutes. All ion substitutions were made in the mucosal bathing solution. All solutions contained the normal buffer (10mM $KHCO_3$, 0.4mM K_2PO_4, 2.4mM HPO_4 gassed with 95% O_2, 5% CO_2). All replacements were made isosmotically. SO_4 substitutions involved addition of mannitol to maintain isotonicity. All errors are expressed as the standard error of the mean. n gives the number of separate determinations for each condition. All potentials are expressed relative to the mucosal solution (mv).

Table 19

Condition/Ion Pair	Mannitol	K : Na	choline : Na	SO_4 : Cl
Control	-14.02 ± 0.39	+4.72 ± 0.31	-21.64 ± 1.45	+ 6.04 ± 0.47
5mM theophylline	- 9.52 ± 0.59	+3.60 ± 0.62	-19.89 ± 1.09	+11.00 ± 0.75
20mM triaminopyrimidine	- 5.67 ± 0.38	+2.99 ± 0.56	-11.90 ± 1.57	+ 5.63 ± 0.98
5mM theophylline 20mM triaminopyrimidine	- 2.86 ± 0.41	+3.94 ± 0.86	- 9.86 ± 0.70	+ 9.80 ± 0.50
n	6	3	6	6

Further support for this view is derived from the action of triaminopyrimidine. 20mM triaminopyrimidine significantly reduces the diffusion potential of NaCl into mannitol compared with control values ($p < 0.001$) (Table 19). This, however, is due to a specific reduction in Na permeability (as is evident from the K : Na, choline : Na and SO_4 : Cl biionic potentials). No change in Cl permeability is seen. Similar data have already been presented (Chapter 4).

If triaminopyrimidine and theophylline have separate loci of action, the two drugs together should elicit responses that are no different from their action alone in the case of biionic potentials and which are additive in the case of mannitol dilution potentials. 20mM triaminopyrimidine and 5mM theophylline cause a reduction in the mannitol dilution potential which is significantly lower than potentials evoked either in the presence of theophylline alone ($p < 0.001$) or triaminopyrimidine alone ($p < 0.001$). The choline : Na biionic potential in the presence of both triaminopyrimidine and theophylline is no different from the biionic potential in the presence of triaminopyrimidine alone ($p > 0.2$). Similarly, the SO_4 : Cl biionic potential in the presence of triaminopyrimidine and theophylline is not significantly different from the biionic potential for theophylline alone ($p > 0.1$).

These results are consistent with the view previously stated, that there are separate channels for Na and Cl permeation that may be separated pharmacologically by triaminopyrimidine and theophylline. Also, the indication of a residual Na permeability in the presence of triaminopyrimidine (see Chapter 4) and the existence of Cl permeability in control tissues minus theophylline suggests the existence of a third pathway. This may be a free-solution 'leakage' pathway as

exists in rabbit gall-bladder (Barry et al (1971); Moreno and Diamond (1974); Moreno (1975a,b)).

(b) Concentration dependence of theophylline action on Cl permeability Figure 22 (a) shows the mannitol dilution potentials for controls and tissues incubated in the presence of increasing concentrations of theophylline. The reduction in the mannitol dilution potential at 5mM theophylline is no different from that at 10mM. A reduction in the dilution potential of the observed magnitude (5mM theophylline) is consistent with an increase in Cl permeability of 0.09 cm hr^{-1}. (Thus overall Cl permeability increases from a value of 0.015 cm hr^{-1} to 0.024 cm hr^{-1}. Calculations were made using the Goldman-Hodgkin-Katz equation assuming P_{Na} = 0.038 cm hr^{-1} and P_K = 0.04 cm hr^{-1}).

The percentage change in Cl permeability due to theophylline (expressed in relation to the maximum change obtained) is plotted against the theophylline concentration in Figure 22 (b). The increment in Cl permeability follows saturation kinetics. The calculated K_M for this relationship is 0.89 $\pm$ 0.14 (SE) mM.

(ii) Resistance (conductance) determinations in non-poisoned tissues

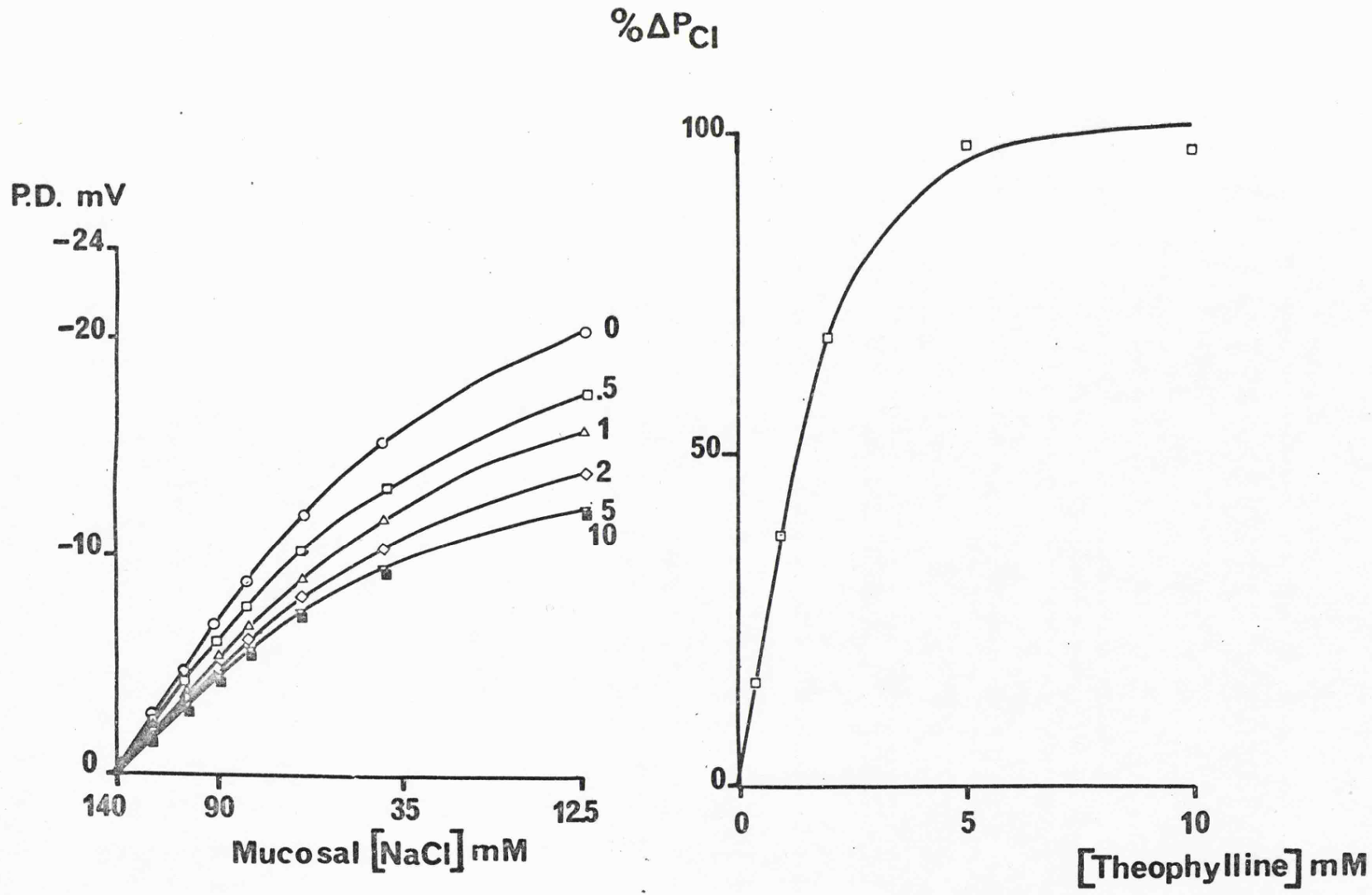
(a) The effects of 10mM theophylline The conductance of intestine (i.e. 1/Resistance) is equal to the sum of the partial ionic conductances of all ions contained in the bathing Ringer solutions (Frizzell and Schultz (1972)). Hence the finding (Powell (1974)) that the partial ionic conductance of Cl is increased (see also above) whereas total conductance decreases, is anomalous An important experimental difference between these two measurements is that Cl conductivity is measured in ouabain-poisoned tissues, whereas total conductance is measured in actively transporting tissues; hence the

Figure 22 (a)

The effect of theophylline (0 to 10mM) upon the transepithelial potential differences obtained by mucosal dilution of Ringer [NaCl] by isosmotic mannitol replacement. All potentials are expressed relative to the mucosal solution. All points are the mean results of six tissues from three experiments. Error bars (± S.E.M.) lie within the points and are omitted. Theophylline was added to all solutions.

Figure 22 (b)

The relative change in Cl permeability (expressed as a percentage of the maximal response) plotted as a function of the theophylline concentration in the bathing solutions.


%ΔP_Cl
P.D. mV
-24
-20
-10
0
0
.5
1
2
5
10
140
90
35
125
Mucosal [NaCl] mM
100
50
0
0
5
10
[Theophylline] mM

anomaly may arise from processes dependent on active transport. Nellans et al (1974) have demonstrated that the change in conductance in non-poisoned tissues is time-dependent.

Figure 23 (a) shows tissue resistance for control tissues and tissues incubated in the presence of 10mM theophylline measured as a function of the incubation time. At the start of the incubation period control tissue resistance is 56.0 $\pm$ 5.6 (SE) Ωcm^{-2}. In contrast, tissue resistance in the presence of 10mM theophylline is 39.8 $\pm$ 7.1 (SE) Ωcm^{-2}. The difference between these values is significant ($p < 0.05$). A decreased resistance (increased conductance) is consistent with the increase in Cl permeability observed in ouabain-poisoned tissue (see above).

Control tissue resistance falls rapidly in the first 20 minutes of incubation (56 to 34 Ωcm^{-2}); between 20 and 80 minutes a further slow decline to 25 Ωcm^{-2} is evident. In the presence of theophylline an <u>increase</u> in resistance to 49 Ωcm^{-2} is observed during the first 20 minutes of incubation. This increase is sustained for 30 minutes, after which period a slow decline is observed, similar to that observed for control tissues over an equivalent period. Hence, apart from the initial resistance measurement (t = 0), tissue resistance with theophylline is consistently greater than control resistance over the whole of this period. These results are in essential agreement with those of Nellans et al (1974).

The increase in tissue resistance following addition of theophylline may be associated with a decrease in the width and volume of the lateral intercellular space (Dibona, Chen and Sharp (1974)). A decrease in the extracellular space in the presence of 10mM theophylline has been noted (see Chapter 3).

Figure 23

Variation in tissue transepithelial resistance as a function of the incubation time. Resistance determinations were made from measurements of the open-circuit potential and the short-circuit current (see Methods).

Figure 23 (a)

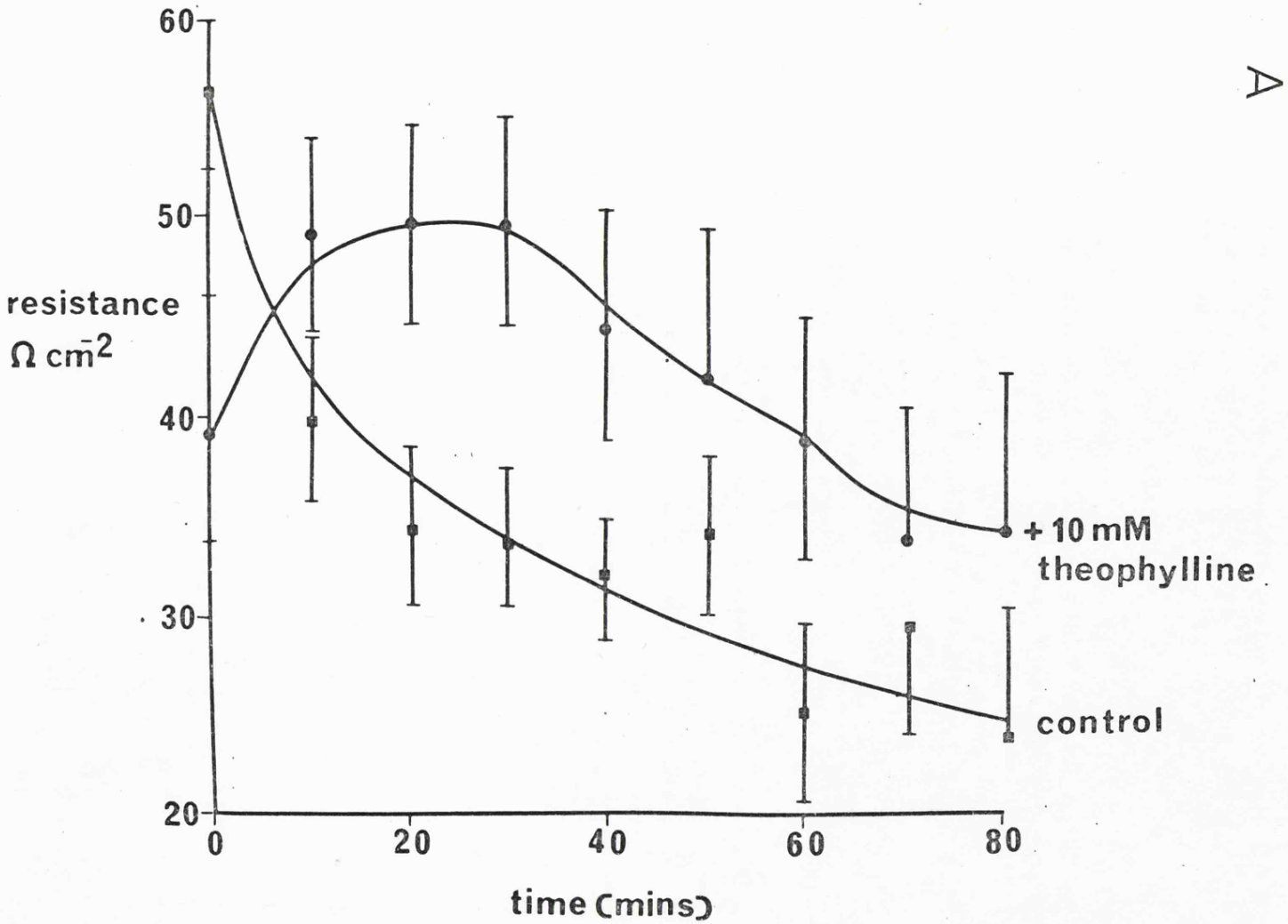
The effect of 10mM theophylline in control tissues. Data are pooled from 32 tissues for controls and from 17 tissues for theophylline-treated tissues. Points and error bars represent the mean $\pm$ S.E.M. for each point.

Figure 23 (b)

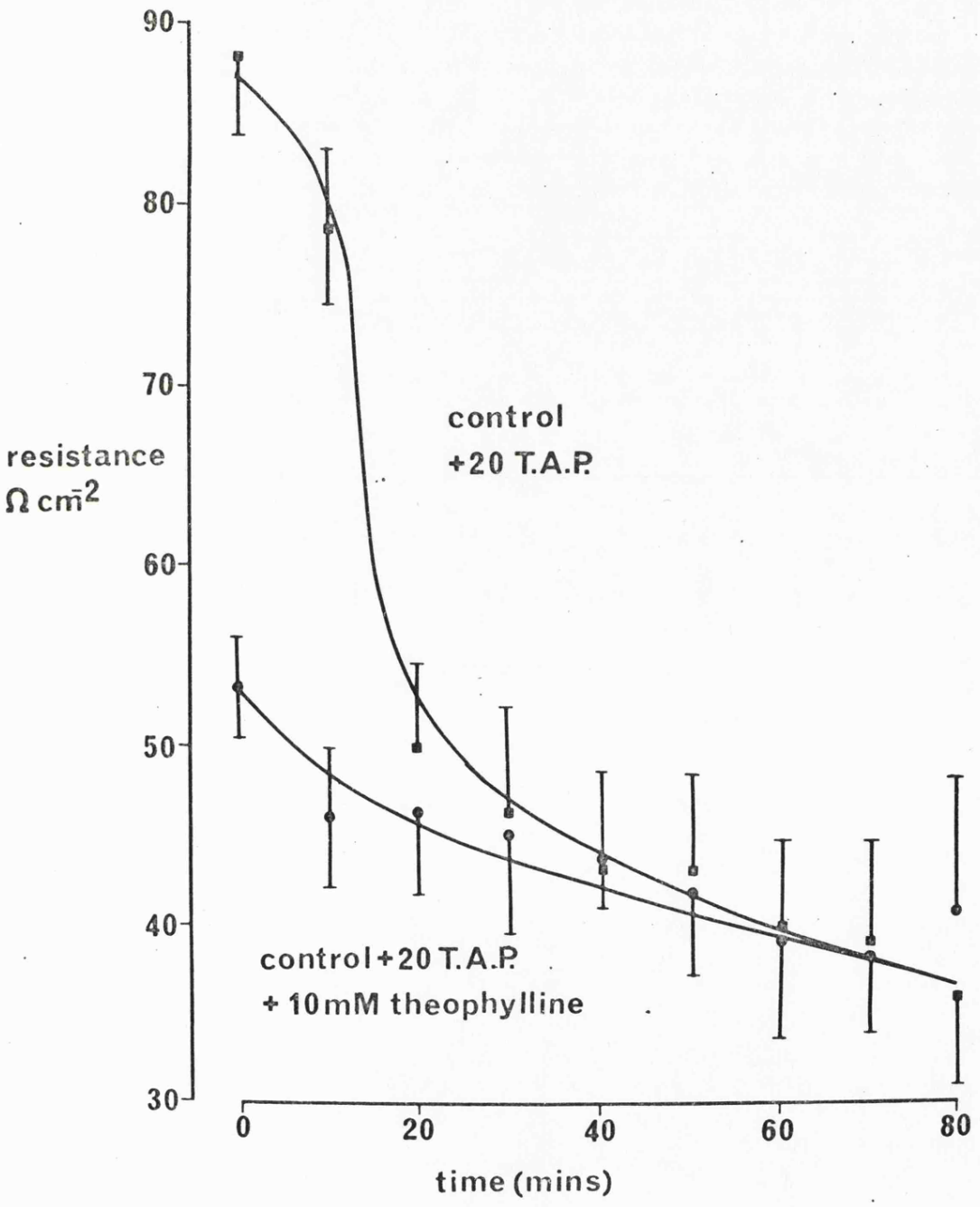
The effect of 10mM theophylline in the presence of 20mM triaminopyrimidine. Data are pooled from 22 tissues for triaminopyrimidine-treated tissues and from 15 tissues for tissues treated with both theophylline and triaminopyrimidine.

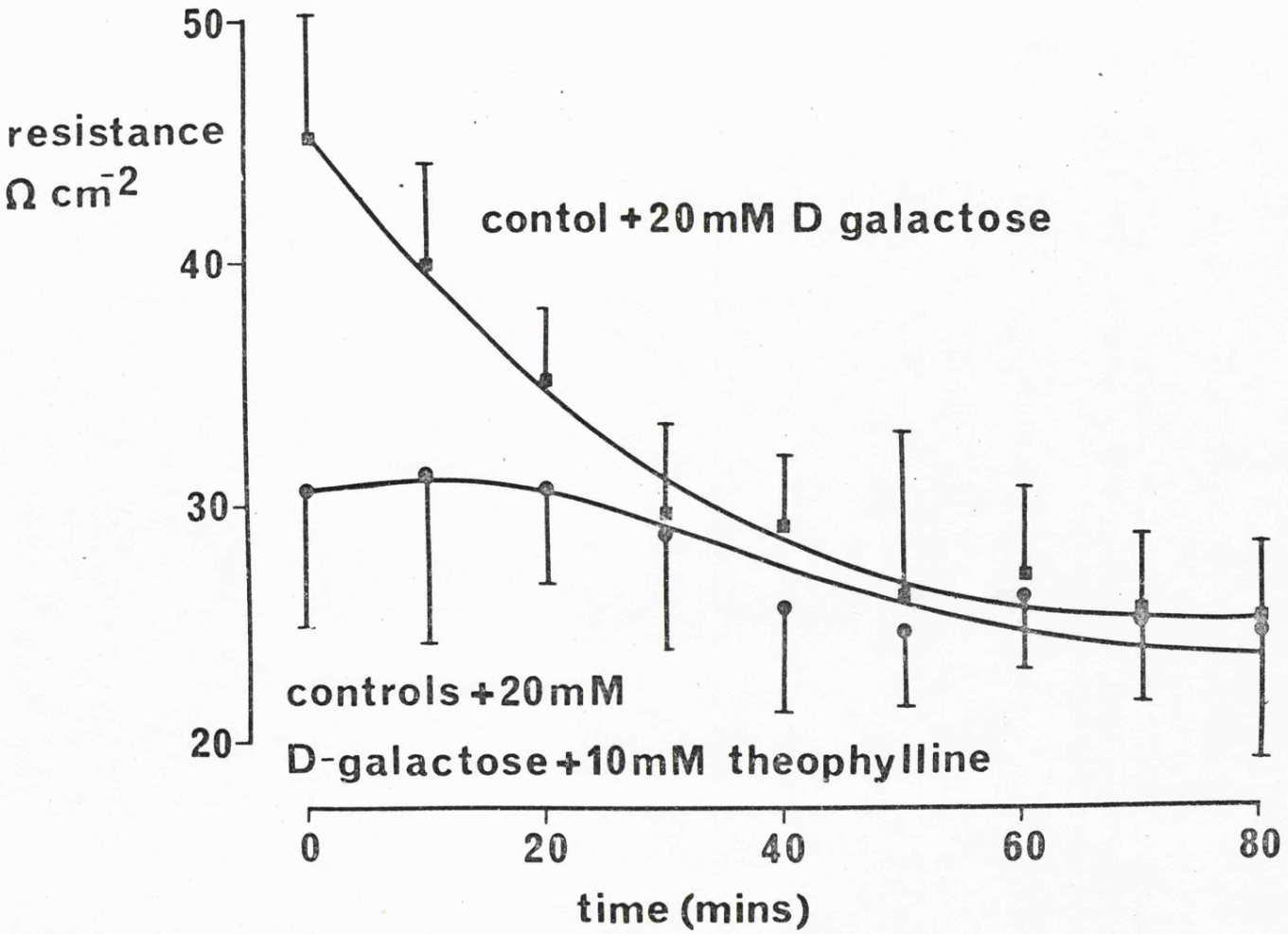
Figure 23 (c)

The effect of 10mM theophylline in the presence of 20mM galactose. Data are pooled from 22 tissues for tissues treated with galactose alsone and from 18 tissues for tissues treated with both galactose and theophylline.


A
60
50
40
30
20
resistance
Ω cm⁻²
+ 10 mM
theophylline
control
0
20
40
60
80
time (mins)

B


90
80
70
60
50
40
30
resistance
Ω cm⁻²
control
+20 T.A.P.
control+20 T.A.P.
+10mM theophylline
0
20
40
60
80
time (mins)


50
40
30
20
resistance
Ω cm⁻²
contol +20mM D galactose
controls +20mM
D-galactose +10mM theophylline
0
20
40
60
80
time (mins)

(b) Effects of 20mM triaminopyrimidine Increased paracellular resistance, independent of changes in lateral space resistance, may be attained following addition of triaminopyrimidine (see Chapter 4) which blocks Na conductance via the tight-junctions.

Figure 23 (b) shows the effect of triaminopyrimidine upon the time-dependent changes in transepithelial resistance.

At zero time triaminopyrimidine increases transepithelial resistance to 88 $\pm$ 6 Ωcm^{-2}. This value is significantly larger than control resistance at zero time ($p < 0.001$). 10mM theophylline together with triaminopyrimidine decreases tissue resistance (to 53.9 $\pm$ 4.1 Ωcm^{-2}) as compared with tissues in the presence of triaminopyrimidine alone ($p < 0.001$). These effects of theophylline and triaminopyrimidine indicate a decrease in resistance when paracellular conductance is blocked. Hence the increase in Cl conductance observed previously may be due to an increased transcellular Cl movement.

In the presence of triaminopyrimidine alone a rapid decline in tissue resistance (between 0 to 20 minutes) is followed by a period of slow decline. This is similar to the time course for resistance change in control tissues. In the presence of both 10mM theophylline and triaminopyrimidine the time course of resistance change is similar to tissues minus theophylline. This result contrasts with the effects of theophylline in control tissues where an increase in tissue resistance is seen over the first 30 minutes of incubation.

Consideration of the paracellular pathway as two series elements, namely the tight-junction and the lateral intercellular spaces, provides a plausible mechanism for these effects. Variation in the lateral space resistance will be obscured if the resistance of the tight-junction is markedly increased.

A convenient statistical method of comparison of these complex changes in tissue resistance as a function of time, theophylline and triaminopyrimidine is afforded by an analysis of variance (3-way design). Table 20 (1) summarizes the results of this analysis.

Both factors C (time) and A (triaminopyrimidine) cause significant change (variance) in tissue resistance when averaged over the whole set of results ($p < 0.001$, $p < 0.025$ respectively).

The tendency for all resistance values to decrease (i.e. the main effect C) is ill-understood. Barry et al (1971) and Moreno and Diamond (1974, 1975) have described the development of a free solution shunt pathway in gall-bladder follwing dissection. A similar situation may exist in rabbit intestine in vitro.

The effect of factor B (theophylline) is non-significant ($p > 0.25$). The strength, however, of a factorial arrangement of results in an analysis of variance lies not in tests of the main effects (i.e. of A, B, and C) but in the interactions between these effects (i.e. AB, AC, BC ABC interactions). The interactive term AB is significant ($p < 0.025$), that is to say, the effect of triaminopyrimidine is significantly modified by theophylline. The nature of this effect has been discussed (see above). An interaction that will also contribute to the AB term is the modification of the theophylline response by triaminopyrimidine (see above). The interaction terms AC and BC indicate that time significantly modifies both the theophylline and triaminopyrimidine response ($p < 0.001$, $p < 0.001$ respectively).

It is clear that more than one effect will contribute to the variation in the theophylline response with time. For instance, the biphasic nature of tissue resistance in the presence of theophylline alone, the abolition of this biphasic change in the presence of triaminopyrimidine and the general tendency towards lower values of resistance with time.

Table 20

Summary of Analysis of Variance, 3-way design (unweighted means solution), for data of Figures 23 a, b, c. p values were taken from the tabulated distribution of F (Fisher and Yates (1949)).

Table 20

	Source of Variation	Degrees of Freedom	Mean Square	F	p
ANALYSIS 1	20mM triaminopyrimidine (A)	1	2168.58	10.26	< 0.025
	10mM theophylline (B)	1	0.14	0.00	> 0.25
	time (C)	8	845.30	800.74	< 0.001
	AB	1	1792.18	8.48	< 0.025
	AC	8	117.43	111.24	< 0.001
	BC	8	461.06	436.75	< 0.001
	ABC	8	129.20	122.39	< 0.001
	Subjects within groups	8	211.20		
	error	64	1.05		
ANALYSIS 2	20mM galactose (A)	1	7402.79	-1.79	> 0.25
	10mM theophylline (B)	1	3979.88	-0.96	> 0.25
	time (C)	8	535.60	3.26	< 0.005
	AB	1	5441.09	-1.31	> 0.25
	AC	8	102.39	0.62	> 0.25
	BC	8	126.08	0.77	> 0.25
	ABC	8	58.70	0.36	> 0.25
	Subjects within groups	8	-4141.39		
	error	56	164.09		

Similar considerations apply to the modification of the triaminopyrimidine response with time. Thus triaminopyrimidine results in an initial increase in resistance in the presence of theophylline but this is subsequently reduced. In control tissues a large increase in tissue resistance due to tiraminopyrimidine is seen initially. This is much reduced as incubation proceeds.

The complexity of the resistance changes may be realized by the existance of a highly significant ($p < 0.001$) three factor interaction (ABC) i.e. time significantly modifies the AB interactive term.

(c) Effects of 20mM D-galactose Closure of the lateral intercellular spaces may result in time-dependent changes (increases) in transepithelial resistance (see above). Procedures that cause lateral space dilation should, therefore, reverse the observed resistance changes. 20mM galactose has been shown to reverse the theophylline-dependent decrease in extracellular space (see Chapter 3).

Figure 23 (c) shows the transepithelial resistance changes when 20mM galactose was present in the bathing Ringer both in the presence and absence of theophylline. Resistance at zero time for tissues in the presence of 20mM galactose alone is $45.2 \pm 6.9 \Omega cm^{-2}$. This value does not differ significantly from control values ($p > 0.1$). The change in resistance with respect to incubation time is similar to controls.

The presence of theophylline in addition to 20mM galactose reduces transepithelial resistance at zero time to $30.2(5) \pm 7.6 \Omega cm^{-2}$. This value does not differ significantly from tissues incubated in the presence of 20mM galactose alone, ($p > 0.1$); but is significantly different from control tissues ($p < 0.05$). No increase in transepithelial resistance due to theophylline is seen at 10, 20 or 30 minutes compared with resistance at zero time. This contrasts with the

effect of theophylline in tissues incubated without galactose (see above). Furthermore, values for transepithelial resistance in the presence of galactose and theophylline (Figure 23 (c)) are consistently lower over the whole period of incubation than tissue resistance in the presence of theophylline alone (Figure 23 (a)). These results are consistent with the view that the increased transepithelial resistance in the presence of theophylline is due to changes in the dimensions of the lateral intercellular spaces.

Table 20 summarizes the analyses of variance in which galactose, theophylline and time are used as the factors A, B and C. 20mM galactose (A) and 10mM theophylline (B) have no significant effect ($p > 0.25$, $p > 0.25$ respectively) when averaged over the whole set of results. This is primarily due to the time-dependent change in transepithelial resistance (factor C) ($p < 0.005$) which obscures the galactose and theophylline dependent changes at longer incubation times. Hence direct comparison of results is more useful in this instance. Interactions AB, BC, AC and ABC are all non-significant.

Section 1: Summary

In ouabain-poisoned tissues

1. 10mM theophylline increases Cl permeability without concurrent change in Na permeability.

2. The effects of theophylline and triaminopyrimidine on mannitol dilution potentials are additive, indicating separate actions.

3. The K_m for the theophylline dependent response on Cl permeability is 0.89mM.

In actively-transporting tissues

1. 10mM theophylline results in a biphasic change in the tissue resistance. An initial decrease is probably the result of increased

Cl permeability whereas a subsequent increase in tissue resistance is probably the result of lateral space collapse.

2. 20mM triaminopyrimidine and 20mM galactose do not effect the initial decrease in resistance but abolish the subsequent increase in resistance.

2. Na fluxes and the tissue isotope ratio R of ^{24}Na : ^{22}Na

(i) Bidirectional transmural Na fluxes: the effects of theophylline/factors affecting the theophylline-dependent changes in bidirectional flux

(a) the effect of theophylline (0 to 10mM) upon the transmural Na fluxes Figure 24 shows the effect of theophylline on mucosa to serosa (J_{13}) and serosa to mucosa (J_{31}) fluxes of Na. It can be seen that increasing Ringer [theophylline] progressively reduces m-s flux (J_{13}) without concurrent change in s-m flux (J_{31}). This result is in accord with the findings of other workers (Field (1971); Field et al (1972); Nellans et al (1974)). The reduction in m-s flux (J_{13}) follows saturation-type kinetics. The K_m for this relationship is 0.27 $\pm$ 0.08 (SE) mM. This value is similar to the apparent K_m for the theophylline-dependent increase in Cl permeability (see Section 1), but is lower than that observed for the theophylline-dependent increase in tissue galactose accumulation at 2mM (Holman and Naftalin (1975b)).

Examination of the pooled data (Table 21) shows that theophylline at a maximal concentration (10mM) significantly reduces m-s flux J_{13} compared with control values ($p < 0.001$). In contrast, 10mM theophylline has no significant effect on s-m flux J_{31} compared with control values ($p > 0.1$). The net Na flux is significantly reduced by theophylline ($p < 0.001$); indeed a negative net flux in the presence of

Figure 24

The effect of theophylline (0 to 10mM) upon the bidirectional m-s (J_{13}) and s-m (J_{31}) fluxes of Na. Net Na flux is also shown. The results are the mean of at least two tissues at each theophylline concentration from two separate experiments.

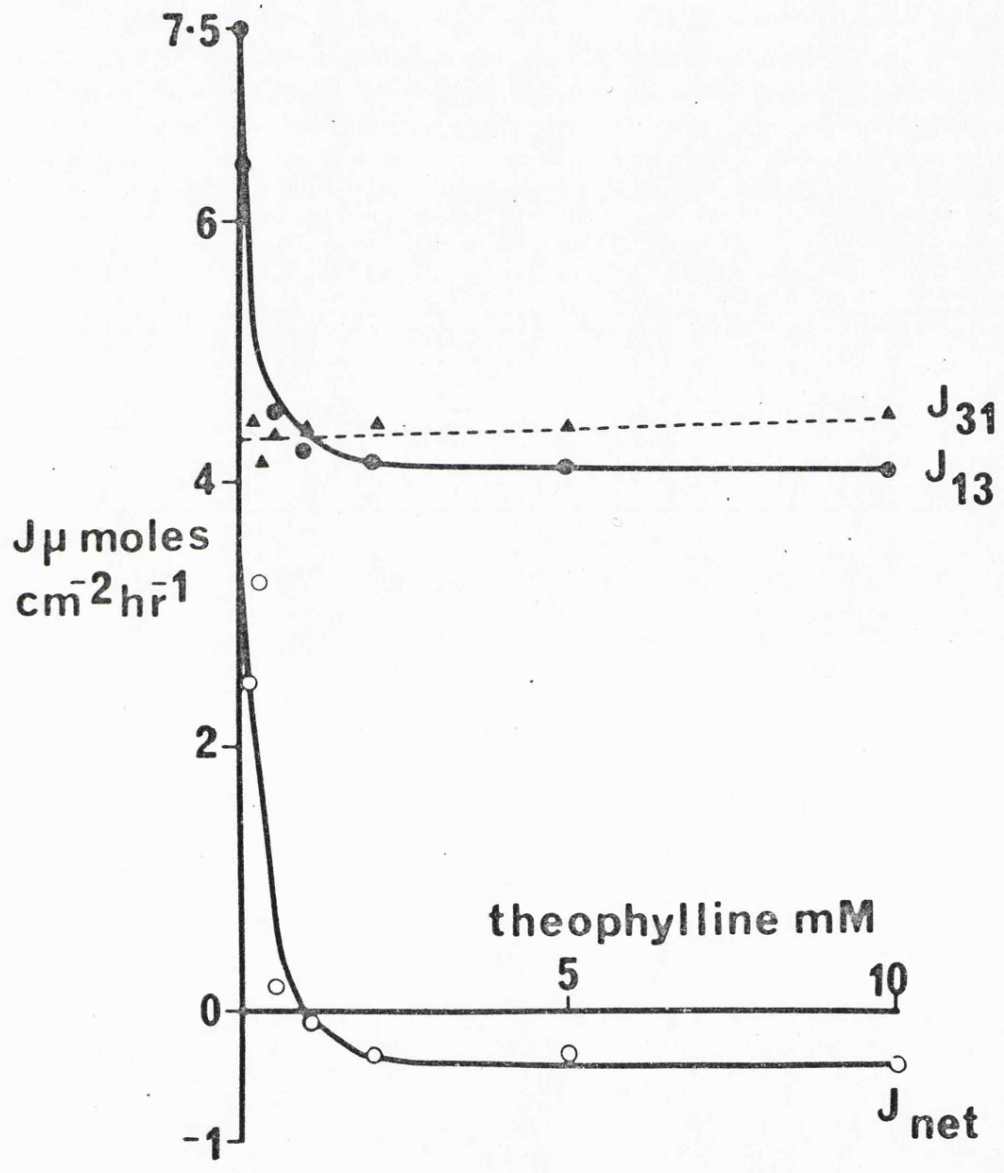

7·5
6
4
2
0
-1
Jμ moles
cm⁻²hr⁻¹
J31
J13
theophylline mM
5
10
J net

Table 21

Theophylline-dependent changes in the measured bidirectional Na fluxes (J_{13}, J_{31}). Net Na flux (J_{net})is also shown. All errors are expressed as the S.E.M. $P_{ij} = J_{ij}/C_{ij}$ where C_{ij} = bathing media Na concentration from the ith compartment. All fluxes are expressed as μmoles cm^{-2} hr^{-1}.

Table 21

Condition	n	J_{13}	P_{13}	J_{31}	P_{31}	J_{net}
Standard Ringer	22	8.59 ± 0.47	0.614 ± 0.003	6.76 ± 0.45	0.048 ± 0.003	1.82 ± 0.35
+ 10mM theophylline	34	5.54 ± 0.36	0.039 ± 0.002(5)	6.02 ± 0.31	0.043 ± 0.002	-0.47 ± 0.15
+ 20mM galactose	11	10.16 ± 0.58	0.073 ± 0.004	7.15 ± 0.49	0.051 ± 0.003	3.01 ± 0.33
+ 20mM galactose/ 10mM theophylline	13	9.42 ± 0.85	0.067 ± 0.006	7.24 ± 0.75	0.051 ± 0.005	2.18 ± 0.33
75mM Na Ringer	17	4.86 ± 0.22	0.064 ± 0.003	3.80 ± 0.13	0.050 ± 0.016	1.05 ± 0.16
75mM Na 10mM theophylline	7	4.39 ± 0.78	0.058 ± 0.010	4.21(5) ± 0.52	0.056 ± 0.007	0.17(5) ± 0.28
25mM Na Ringer	11	1.47 ± 0.24	0.059 ± 0.008	0.95 ± 0.77	0.038 ± 0.003	0.52 ± 0.18
25mM Na 10mM theophylline	7	1.81 ± 0.11	0.072 ± 0.004	1.34 ± 0.10	0.054 ± 0.004	0.47 ± 0.13(5)
Standard Ringer + 0.1mM ouabain	3	6.02 ± 0.48	0.043 ± 0.003	5.93 ± 0.04	0.037 ± 0.004	0.09 ± 0.31
0.1mM ouabain + 10mM theophylline	4	5.52 ± 0.77	0.039 ± 0.005	4.88 ± 0.05	0.034 ± 0.003	0.64 ± 0.47
Standard Ringer + 20mM triaminopyrimidine	18	5.46 ± 0.28	0.039 ± 0.008	4.61 ± 0.25	0.032 ± 0.016	0.84 ± 0.23
10mM theophylline + 20mM triaminopyrimidine	18	6.79 ± 0.51	0.048 ± 0.003	5.90 ± 0.30	0.042 ± 0.002	0.90 ± 0.32

theophylline is observed. This result may indicate the existance of a transcellular Na flux from serosa to mucosa. The magnitude of the net flux plus theophylline does not, however, differ significantly from zero ($p > 0.2$).

(b) Effects of 20mM triaminopyrimidine

The mode of action of triaminopyrimidine has been discussed previously (see Chapter 4). Table 21 and Figure 25 show the effect of triaminopyrimidine upon the theophylline-dependent change in transepithelial Na flux.

20mM triaminopyrimidine increases mucosa to serosa flux (J_{13}) in the presence of theophylline from 5.54 to 6.79 μmoles cm^{-2} hr^{-1}. This result is statistically significant ($p < 0.05$). No signigicant change in serosa to mucosa flux (J_{31}) in the presence of theophylline due to triaminopyrimidine is observed ($p > 0.5$). Net Na flux (J_{net}) in the presence of theophylline is -0.47 μmoles cm^{-2} hr^{-1}, this is reversed to +0.89 moles cm^{-2} hr^{-1} in the presence of triaminopyrimidine. Net flux in the presence of both theophylline and triaminopyrimidine does not differ from net flux in the presence of triaminopyrimidine alone ($p > 0.5$).

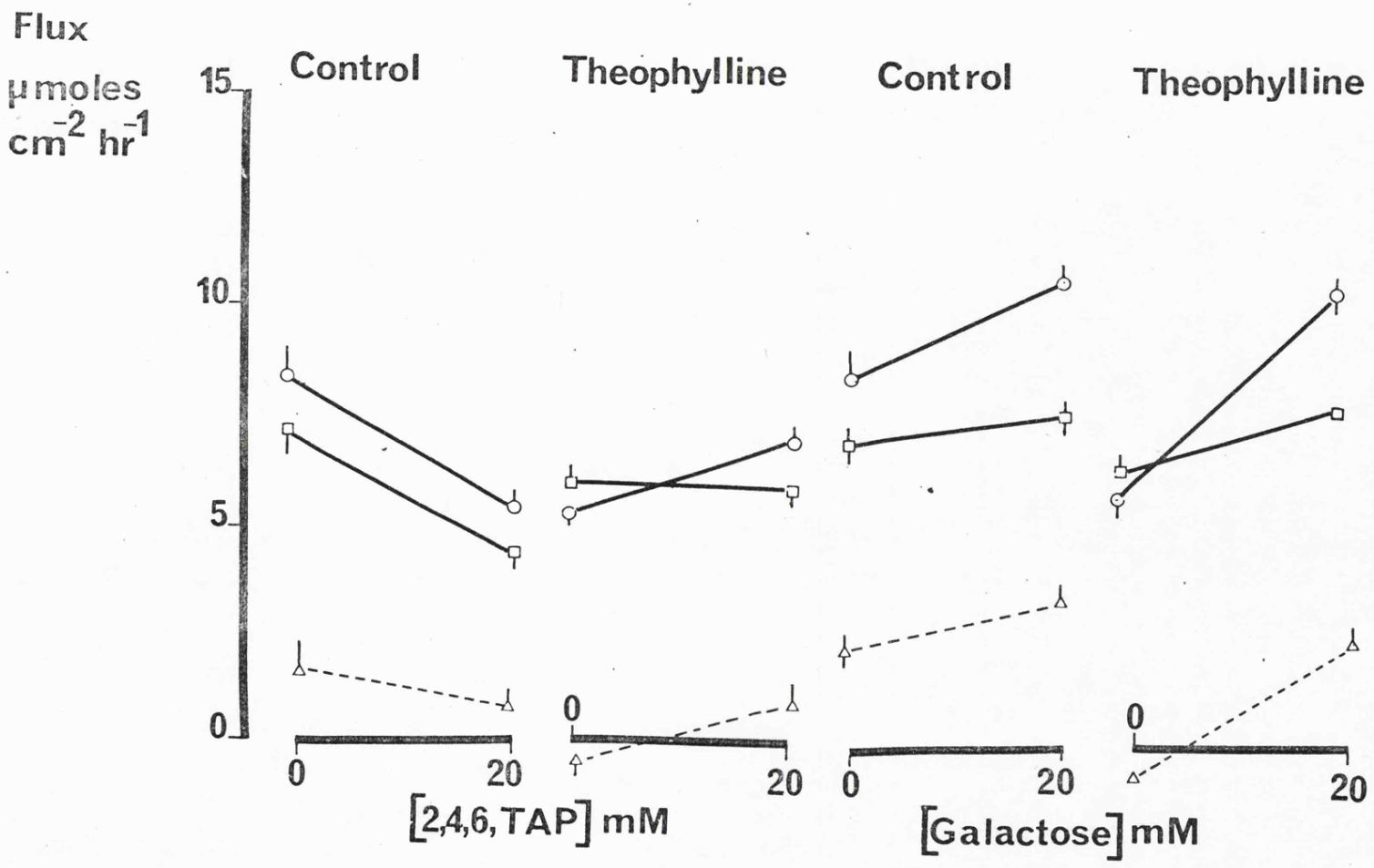
The effects of triaminopyrimidine in the presence of theophylline contrast with the effects of triaminopyrimidine on control tissues where triaminopyrimidine causes a bidirectional decrease in Na flux.

Since triaminopyrimidine blocks Na movements through the tight-junction, the increase in m-s Na flux (J_{13}) due to triaminopyrimidine in the presence of theophylline suggests that the decrease in m-s flux due to theophylline alone results from a recirculation of isotope from the cell into the mucosal solution. The route for this reflux of Na is via the tight-junctions.

Triaminopyrimidine has little effect on s-m flux (J_{31}) in the presence of theophylline, this implies that the major pathway for

Figure 25

A summary of the effects of 20mM triaminopyrimidine and of 20mM galactose in the presence and absence of theophylline upon the transmural bidirectional fluxes of Na. Net Na flux is also shown. Error bars denote the S.E.M. for each point (see text and Table 21 for further details).


Flux
μmoles
cm⁻² hr⁻¹
15
10
5
0
Control
Theophylline
Control
Theophylline
0
20
0
20
0
20
0
20
[2,4,6,TAP] mM
[Galactose] mM

Na permeation from serosa to mucosa is transcellular.

Comparison of the bidirectional Na fluxes in the presence and absence of theophylline but in the presence of triaminopyrimidine, reveals that theophylline causes an increase in both m-s (J_{13}) and s-m (J_{31}) fluxes. It may, therefore, be concluded that theophylline increases transcellular Na permeability in the presence of triaminopyrimidine.

(c) Effects of 20mM galactose

Field (1971) has demonstrated that glucose absorption is unaffected by theophylline as judged by measurements of the glucose-stimulated short-circuit current. Similarly, Holman and Naftalin (1975 b) have shown that in Na-depleted intestine (75mM Na Ringer) the mucosal asymmetry to galactose is unaffected by theophylline. At high Ringer galactose concentrations (>5mM) these authors also demonstrated that 5mM theophylline had no effect upon the net flux of galactose.

It is of interest to determine the effects of galactose upon Na fluxes in the presence of theophylline since galactose reverses the theophylline-dependent change in transepithelial resistance (see Results Section 1).

Table 21 and Figure 25 show that there is a galactose-dependent stimulation of net Na absorption both in the presence and absence of 10mM theophylline. Net Na absorption in the presence of galactose alone is larger than net Na absorption in the presence of both theophylline and galactose. The galactose-dependent stimulation of net Na flux is larger in theophylline-treated tissues than in control tissues. These differences are not, however, statistically significant. The present results are, therefore, in agreement with those of Field (1971) for glucose.

Table 21 indicates that there is a change in passive tissue permeability due to galactose in theophylline-treated tissue; s-m Na flux (J_{31}) increases from 6.02 moles cm^{-2} hr^{-1} to 7.24 μmoles cm^{-2} hr^{-1}. Also, the change in m-s Na flux due to galactose is larger in theophylline-treated tissues than in controls. A change in passive tissue permeability to Na in this situation may reflect the increase in tissue water, extracellular space and electrical conductance outlined previously. These effects are likely to result from lateral space dilation due to the sugar-stimulated net Na flux. This effect has been considered previously in relation to tissues in the absence of theophylline (see Chapter 4).

(d) Effects of Ringer Na replacement by choline/effects of 0.1mM ouabain

Table 21 shows the effect of reducing Ringer [Na] by isosmotic replacement with choline upon the theophylline-dependent change in transmural Na fluxes.

Reduction in Ringer [Na] to 75mM reduces both bidirectional fluxes in the presence of theophylline compared with theophylline-treated tissues in Ringer containing 140mM Na. This change in Na flux is not proportional to the Ringer Na concentration for either m-s (J_{13}) or s-m (J_{31}) fluxes.

M-s Na permeability (P_{13}) in the presence of theophylline increases from 0.039 cm hr^{-1} at 140mM Na to 0.072 cm hr^{-1} at 25mM Na. This difference is highly significant ($p < 0.001$). A similar change in Na permeability to that observed on variation of Ringer [Na] in the presence of theophylline has previously been described (i.e. the effect of Ringer Na replacement in the presence of triaminopyrimidine (see Chapter 4)). The change in Na permeability in the presence of triaminopyrimidine was taken to indicate the existence of a saturable

process. The effect of triaminopyrimidine on Na fluxes in the presence of theophylline suggests that the low m-s permeability to Na seen in the presence of theophylline in 140mM Na Ringer is due to a recycling of Na ions across the mucosal boundary via the tight-junction. This obviates a kinetic analysis of the Michaelis-Menton type. However, it may be concluded that significant reflux of Na through the tight-junctions occurs only at Ringer Na concentrations in excess of 25mM.

10mM theophylline results in an increased tissue permeability to Na in low Na Ringer (25mM). P_{13} and P_{31} are both increased in the presence of theophylline compared with control tissues incubated minus theophylline. This result implies an increased transcellular permeability. A similar result was obtained with theophylline in the presence of triaminopyrimidine (see above).

S-m Na permeability in the presence of theophylline increases from 0.043 cm hr^{-1} in a Ringer containing 140mM Na to 0.054 cm hr^{-1} in a 25mM Na Ringer. This difference is statistically significant ($p < 0.05$) and may indicate the existence of a saturable process of low affinity for Na.

Net Na flux (J_{net}) in the presence of theophylline changes from net secretion in a Ringer containing 140mM Na to net absorption in a 25mM Na Ringer (Table 21). The difference between these two values of net Na flux is statistically significant ($p < 0.025$). Net Na flux in a 25mM Na containing Ringer does not differ, whether in the presence or absence of theophylline ($p > 0.5$). It may be concluded that the theophylline-dependent change in net Na flux is dependent on Ringer $[Na]$ in a similar fashion to the reduction in flux J_{13} through 'reflux'.

0.1mM ouabain abolishes the theophylline-dependent change in net Na flux in a Ringer containing 140mM Na. Values for J_{13}, J_{31} and J_{net}

in the presence of ouabain do not differ significantly whether in the presence or absence of theophylline ($p > 0.5, > 0.2, > 0.4$, for J_{13}, J_{31} and J_{net} respectively).

This result with ouabain differs from the findings of Powell, Binder and Curran (1972) who found that net secretion of Na in guinea-pig ileum was insensitive to ouabain. The overall level of secretion in guinea-pig ileum is higher than that in rabbit ileum.

(e) Effect of Ringer Cl-replacement by SO_4

Table 22 shows the effect of Cl-substitution upon the bidirectional and net fluxes of Na in the presence and absence of 10mM theophylline.

Cl-substitution by an impermeant anion such as SO_4 is thought to lead to a reduction in m-s Na flux due to the inhibition of a coupled neutral pathway for NaCl movement in control tissues (Nellans et al (1973, 1974)).

In the present experiments Cl-free SO_4 media reduce m-s Na flux compared with Cl-Ringer controls (145mM Cl). The difference between these two values of flux is statistically significant ($p < 0.05$). Concurrent with the reduction in m-s Na flux there is also a reduction in s-m Na flux (J_{31}) in SO_4 media, ($p < 0.001$). Net Na flux in Cl-free media does not differ significantly from net Na flux in control conditions ($p > 0.4$). These results suggest that paracellular Na permeability is reduced in SO_4 media. No effect on transcellular Na movement is evident since net Na flux is independent of the Ringer Cl content.

In Ringer containing high [Cl] (145mM and 80mM) 10mM theophylline causes a significant reduction in m-s Na flux ($p < 0.001$ for 145mM and $p < 0.001$ for 80mM Cl Ringer). In contrast, there is no theophylline-dependent reduction in m-s Na flux (J_{13}) in 10mM Cl and Cl-free Ringer

Table 22

Effect of Ringer $[Cl]$ substitution by SO_4 (made isosmotic by addition of mannitol) upon the bidirectional Na fluxes both in the presence (+) and absence (-) of theophylline. Ringer $[Na]$ and $[HCO_3]$ are held constant at 140 and 10mM respectively. The number of tissues used is given in parentheses. All errors are expressed S.E.M.

Table 22

Ringer Cl mM		n	J_{13} μmoles cm^{-2} hr^{-1}	J_{31} μmoles cm^{-2} hr^{-1}	J_{net} μmoles cm^{-2} hr^{-1}
0*	-	5	6.42 ± 0.73	4.03 ± 0.38	2.38 ± 0.48
	+	6	7.10 ± 0.85	6.04 ± 0.79	1.03 ± 0.36
10	-	4	6.88 ± 0.46	5.90 ± 0.34	0.98 ± 0.43
	+	4	6.32 ± 0.94	6.80 ± 0.96	-0.47 ± 0.25
80	-	5	8.48 ± 0.77	7.13 ± 1.17	1.38 ± 0.43
	+	4	4.53 ± 0.88	4.94 ± 0.97	-0.41 ± 0.15
140	-	22	8.59 ± 0.47	6.76 ± 0.45	1.82 ± 0.35
	+	34	5.54 ± 0.36	6.02 ± 0.36	-0.47 ± 0.15

*Addition of isotopes altered [Cl] in the bathing Ringer by ~ 0.2mM. A 1:10 dilution of isotopes in NaCl was made with the Cl-free SO_4 Ringer prior to use.

Table 23

The effect of 10mM theophylline on the tissue specific activity ratio of ^{24}Na : ^{22}Na within the tissue fluid. Data were pooled from measurements following 80 minutes incubation (see Methods). Errors are expressed as $\pm$ S.E.M.

Table 23

Condition	n	Control	n	+ 10mM Theophylline
Standard Ringer	22	0.67 ± 0.05	34	0.93 ± 0.03
Standard Ringer + 20mM galactose	11	0.78 ± 0.09	13	1.14 ± 0.18
25mM Na Ringer	11	0.67 ± 0.06	7	0.93 ± 0.19
Standard Ringer + 0.1mM ouabain	3	0.98 ± 0.11	4	0.79 ± 0.18
Standard Ringer + 20mM triaminopyrimidine	18	0.54 ± 0.04	18	0.81 ± 0.14
Cl-free SO_4 Ringer	5	0.63(5) ± 0.20	6	0.86 ± 0.23

($p > 0.1$, $p > 0.4$ for zero and 10mM Cl respectively). These results suggest that the theophylline effect on Na flux requires the presence of a mobile anion.

This conclusion can also be inferred from the effect of theophylline on the net fluxes of Na. 10mM theophylline reduces net Na flux compared with controls minus theophylline at all Ringer Cl concentrations. In Cl-free SO_4 media, however, the reduction in net Na flux due to theophylline is less than that observed in 145mM Cl-Ringer ($p < 0.05$). Furthermore, net Na flux in theophylline treated intestine in SO_4 media does not differ significantly from net Na flux in control media ($p > 0.1$) in the absence of theophylline.

The residual effect of theophylline on net Na flux in 'Cl-free' SO_4 media may be a consequence of a small concentration of Cl (a) from leakage from the tissue, (b) from addition of isotope. Alternatively, the presence of HCO_3 (10mM) in the Ringer solution may supply the necessary amount of mobile anion.

(ii) <u>The tissue isotope specific activity ratio (R) for Na</u>

(a) <u>Effect of 10mM theophylline</u> 10mM theophylline significantly increases the tissue isotope specific activity ratio (R) compared with controls minus theophylline ($p < 0.025$) (Table 23). A greater proportion of isotope thus originates from the mucosal bathing solution in theophylline-treated intestine. This result is the reverse of that expected if the sole action of theophylline upon transcellular Na flux was an inhibition of an influx pathway at the mucosal boundary as envisaged by Nellans et al (1973). This increase in R implies that the theophylline dependent decrease in mucosal permeability is exceeded by a decrease in serosal permeability.

(b) Modifiers of theophylline action

20mM triaminopyrimidine 20mM triaminopyrimidine reverses the theophylline change in Na flux (see above). Table 23 indicates that theophylline increases R even in the presence of triaminopyrimidine ($p < 0.05$).

The effect of Na replacement by choline and of 0.1mM ouabain Table 23 shows that replacement of Ringer Na by choline has no statistically significant effect upon the tissue ration R in the presence or absence of 10mM theophylline compared with the respective ratios for control tissues ($p > 0.5$, $p > 0.5$ respectively).

In the presence of 0.1mM ouabain there is no theophylline-dependent increase in the tissue ratio R for tissues incubated in the Standard Ringer solution ($p > 0.5$).

The effect of D-galactose There is an increase in the isotope ratio R due to theophylline in the presence of 20mM galactose (Table 23). This increase is not, however, statistically significant ($p > 0.1$). 20mM galactose reverses the theophylline-dependent change in mucosal to serosal and net fluxes of Na.

The effect of Cl-replacement by SO_4 There is a small, though non-significant increase in the tissue ratio R due to theophylline in zero Cl - SO_4 media ($p > 0.4$). A requirement for a mobile anion for the theophylline effect on m-s Na flux has already been demonstrated (Section 2 (i) (e)).

Section 2: Summary of the effects of theophylline on Na fluxes and the tissue ratio R

1. 10mM theophylline reduces net absorption of Na primarily by a reduction in m-s (J_{13}) flux. 10mM theophylline increases the proportion of isotope originating within the tissue from the mucosal solution.

2. The change in Na flux elicited by theophylline is reversed by 20mM triaminopyrimidine. A theophylline-dependent increase in trans-cellular permeability for Na is evident in triaminopyrimidine-treated tissues. Triaminopyrimidine does not affect the increase in tissue ratio R due to theophylline.

3. The theophylline-dependent decrease in m-s flux is abolished in low (25mM) Na Ringer. The increment in tissue ratio R due to theophylline is unaffected by Na replacement.

4. 0.1mM ouabain abolishes the theophylline-dependent change in Na fluxes and the increment in tissue ratio R.

5. The reduction in m-s Na flux (J_{13}) due to theophylline shows a requirement for Cl. In low or 'zero' Cl Ringer solutions no theophylline-dependent reduction in m-s Na flux is observed. The theophylline-dependent increase in the tissue ratio R is reduced but not abolished.

3. Cl fluxes and the tissue isotope specific activity ratio R of $^{36}Cl : {}^{82}Br$

(i) Bidirectional transmural Cl fluxes: the effects of theophylline

(a) The effects of 10mM theophylline Examination of the pooled data (Table 24) shows that 10mM theophylline significantly reduces m-s Cl flux (J_{13}) compared with control values ($p < 0.001$). There is also a theophylline-dependent increase in s-m flux (J_{31}). The increase in s-m flux due to theophylline is not statistically significant ($p > 0.2$). Net Cl absorption in controls is changed to net Cl secretion by 10mM theophylline. The magnitude of this Cl secretion in the presence of theophylline is -1.51 μmoles cm^{-2} hr^{-1}. This value differs significantly from zero ($p < 0.01$) and is significantly larger than the value for net Na secretions due to theophylline recorded in a separate experimental series ($p < 0.01$).

Table 24

Theophylline-dependent changes in the measured bidirectional (J_{13}, J_{31}) and net fluxes (J_{net}) of Cl. All errors are expressed as S.E.M. $P_{ij} = J_{ij}/C_{ij}$ where C_{ij} = concentration of Cl in the ith compartment. The Ringer is of standard composition (see Methods)

Net Na fluxes were determined in a separate experimental series. Short-circuit current (S.C.C.) measurements were averaged from the two experimental series taking values of S.C.C. over the whole period of flux measurement (20 - 80 minutes).

J_r = S.C.C. - ($J_{net_{Na}} - J_{net_{Cl}}$).

Table 24

	n	J_{13} moles cm^{-2} hr^{-1}	P_{13} cm hr^{-1}	J_{31} moles cm^{-2} hr^{-1}	P_{31} cm hr^{-1}	J_{net} moles cm^{-2} hr^{-1}	J_{net} Na moles cm^{-2} hr^{-1}	S.C.C. moles cm^{-2} hr^{-1}	J_r moles cm^{-2} hr^{-1}
Standard Ringer Control	23	8.33 ± 0.42	0.057 ± 0.003	6.55 ± 0.44	0.045 ± 0.003	+1.78 ± 0.34	1.82 ± 0.35 (22)	0.88 ± 0.07 (315)	0.84 ± 0.14 (360)
+ 10mM theophylline	11	5.97 ± 0.57	0.041 ± 0.004	7.48 ± 0.53	0.051 ± 0.003	-1.51 ± 0.46	-0.47 ± 0.15 (34)	2.32 ± 0.23 (360)	1.28 ± 0.28 (395)
+ 20mM triaminopyrimidine	15	8.43 ± 0.64	0.058 ± 0.004	7.24 ± 0.48	0.049 ± 0.003	+1.19 ± 0.37	0.84 ± 0.23 (18)	0.75 ± 0.09 (264)	1.09 ± 0.16 (297)
+ 20mM triaminopyrimidine + 10mM throphylline	5	7.65 ± 0.61	0.527 ± 0.004	9.67 ± 0.69	0.066 ± 0.005	-2.02 ± 0.67	0.90 ± 0.32 (18)	1.31 ± 0.19 (184)	1.01 ± 0.12 (207)
+0.1mM ouabain	11	5.54 ± 0.43	0.038 ± 0.003	5.67 ± 0.53	0.039 ± 0.003	-0.12 ± 0.44	0.32 ± 0.31 (6)	0.24 ± 0.21 (136)	-0.20 ± 0.56 (153)
+0.1mM ouabain + 10mM theophylline	7	6.87 ± 1.20	0.047 ± 0.008	5.94 ± 0.71	0.041 ± 0.004	0.92 ± 0.94	0.64 ± 0.47 (4)	0.30 ± 0.15 (88)	0.58 ± 0.31 (99)

These results are in agreement with previous findings of the effect of theophylline upon bidirectional Cl fluxes, namely that theophylline causes net Cl secretion due to a decrease in m-s Cl flux (J_{13}) (Field (1971); Nellans et al (1974); Powell, Farris and Carbonetto (1974)).

A range of effects of theophylline upon s-m Cl flux (J_{31}) has been reported (Sheerin and Field (1975)). These range from no effect of theophylline to a significant increase in s-m Cl flux. The present pooled data show a small, non-significant, increase in s-m Cl flux due to theophylline. Individual experiments showed instances of a larger stimulation of s-m Cl flux.

10mM theophylline has no effect upon the residual current compared with control values minus theophylline ($p > 0.2$) (Table 24). This finding is in agreement with previous workers (Sheerin and Field (1975); Dietz and Field (1973)).

(b) The effect of 20mM triaminopyrimidine upon the theophylline-dependent response The effect of 20mM triaminopyrimidine upon bidirectional Cl fluxes is of considerable interest in the light of the triaminopyrimidine-dependent reversal of the theophylline-dependent decrease in m-s (J_{13}) and net fluxes of Na (Section 2(i)b).

Table 24 shows that the change in net Cl secretion due to the presence of theophylline is not reversed by triaminopyrimidine. Net Cl secretion in the presence of theophylline and triaminopyrimidine is no different from net Cl secretion in the presence of theophylline alone ($p > 0.5$).

(c) Effect of Ringer Na substitution by isosmotic replacement with choline Replacement of Ringer Na by choline is thought to lead to the observed reduction in m-s Cl flux by the inhibition of a neutral influx pathway for NaCl located on the brush-border membrane

(Frizzell et al (1973); Nellans et al (1973, 1974)). The effect of Cl-substitution by SO_4 on Na fluxes has been examined (see Section 2(i) e. Table 25 shows the effect of Ringer Na replacement by choline upon the bidirectional Cl fluxes in the presence and absence of 10mM theophylline.

In tissues incubated in the absence of theophylline complete replacement of Ringer Na by choline leads to a reduction in net Cl flux. Indeed, net Cl flux in this condition does not differ significantly from zero ($p > 0.5$). The reduction in net Cl flux in Na-free media is primarily due to a reduction in m-s Cl flux (J_{13}). No change is evident in s-m Cl flux in Na-free media compared with control tissues. These findings are thus in agreement with previous workers (Nellans et al (1974); Binder et al (1973)).

Net Cl flux is progressively reduced by Ringer Na substitution in control tissues. At all levels of Ringer [Na] tested, the reduction in net Cl flux results primarily from a reduction in m-s Cl flux (J_{13}). Net Na flux shows a concurrent reduction to the fall in net Cl flux as Ringer [Na] is reduced; this suggests that net Na and Cl flows are coupled.

The presence of 10mM theophylline reverses net Cl flux from absorption to secretion at Ringer [Na] concentrations of 140, 75 and 25mM Na (Table 25). The change in net Cl flux due to theophylline is statistically significant at both 140 and 75mM Na ($p < 0.001$, $p < 0.001$ respectively). The change in net Cl flux in the 25mM Na buffer is not significant ($p < 0.1 > 0.05$). The theophylline-dependent change in net Cl flux is abolished in zero Na choline Ringer. Net Cl flux in this condition does not differ whether in the presence or absence of theophylline ($p > 0.5$).

Table 25

The effect of Ringer Na substitution by replacement with choline upon the bidirectional and net fluxes of Cl both in the presence (+) and absence (-) of 10mM theophylline. Pooled data are expressed as the mean $\pm$ S.E.M. n = number of separate tissues. Ringer $[Cl]$ and $[HCO_3]$ were held constant at 145 and 10mM respectively.

Table 25

Ringer [Na] mM		n	J_{13} μmoles cm^{-2} hr^{-1}	J_{31} μmoles cm^{-2} hr^{-1}	J_{net} μmoles cm^{-2} hr^{-1}
140	-	23	8.33 ± 0.42	6.55 ± 0.44	1.78 ± 0.34
	+	11	5.97 ± 0.57	7.48 ± 0.53	-1.51 ± 0.46
75	-	8	6.55 ± 0.59	5.40 ± 0.56	1.15 ± 0.59
	+	4	6.28 ± 0.43	9.43 ± 0.33	-3.14 ± 0.59
25	-	4	6.13 ± 0.89	5.73 ± 0.80	0.39 ± 0.36
	+	4	7.02 ± 1.12	8.90 ± 1.37	-1.88 ± 0.98
0	-	5	7.35 ± 0.55	6.91 ± 0.65	0.43 ± 0.75
	+	3	8.70 ± 1.32	8.03 ± 1.90	0.67 ± 0.77

This effect of Na-free choline media in abolishing the response of Cl flux to theophylline is similar to the effect of 0.1mM ouabain (see below). These results support the view that active secretion of Cl is dependent upon a functioning Na-K ATPase. Powell et al (1974) and Sheerin and Field (1975) have observed that the increment in short-circuit current due to theophylline is dependent upon the medium Na concentration.

(d) Effect of 0.1mM ouabain Table 24 shows that in the presence of 0.1mM ouabain there are no theophylline-dependent changes in Cl flux. M-s (J_{13}), s-m (J_{31}) and net Cl fluxes are no different, whether in the presence or absence of theophylline ($p > 0.4$, $p > 0.5$, $p > 0.5$ respectively).

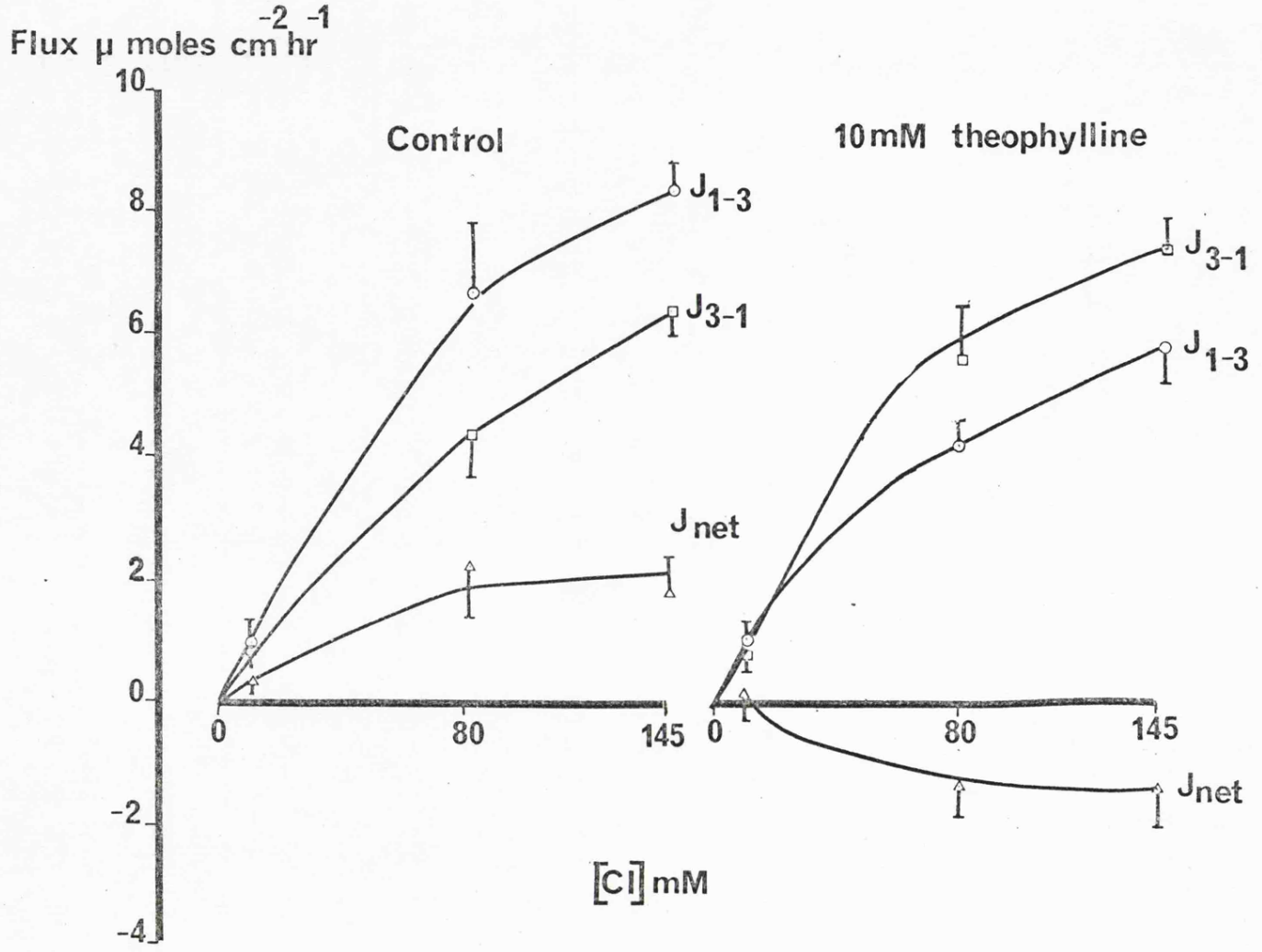
(e) Effect of Ringer Cl replacement by SO_4 Figure 26 shows the effect of variation in Ringer [Cl] upon the bidirectional and net fluxes of Cl in the presence and absence of 10mM theophylline. Ringer [Na] and [HCO_3] were held constant at 140mM and 10mM respectively.

In control tissues increasing Ringer [Cl] from 0 to 145mM results in a saturable increase in m-s (J_{13}), s-m (J_{31}) and net Cl fluxes. The increment in net Cl flux is maximal by 80mM Cl. Correction of the bidirectional Cl fluxes (J_{13} and J_{31}) for a shunt (extracellular) component of flux (P_{Cl} = 0.018 cm hr^{-1}. See Section 1 Chapter 6) gives K_M values for J_{13} and J_{31} for controls of 54 $\pm$ 21 (S.D.)mM and 27 $\pm$ 10 (S.D.) mM respectively. The corresponding V_{max} values are 11.56 $\pm$ 4.98 (S.D.) μmoles cm^{-2} hr^{-1} and 5.17 $\pm$ 2.20 (S.D.) μmoles cm^{-2} hr^{-1} respectively. These kinetic parameters may represent the sum of more than one saturable process for transcellular Cl flux. This point has been discussed in detail (Frizzell et al (1973)).

In the presence of 10mM theophylline the direction of net Cl flux is changed to secretion at 140 and 80mM Cl. A small, though

Figure 26

The effect of Ringer $[Cl]$ variation by isosmotic replacement with SO_4 and mannitol upon the bidirectional and net fluxes of Cl in the presence and absence of 10mM theophylline. Results are pooled from all available experiments. Errors are expressed as $\pm$ S.E.M. n = 23, 4, 4, for 145, 80 and 10mM Cl for controls, and n = 11, 8, 8, for 140, 80 and 10mM for tissues incubated in the presence of 10mM theophylline.


Flux μ moles cm -2 hr -1
10
8
6
4
2
0
-2
-4
Control
J1-3
J3-1
J net
0
80
145
10 mM theophylline
J3-1
J1-3
0
80
145
Jnet
[Cl] mM

non-significant change is seen in Ringer containing 10mM Cl due to theophylline ($p > 0.5$). The change in net Cl flux is primarily due to a decrease in m-s Cl flux both at Ringer [Cl] of 145 and 80mM (see above). Calculation of the kinetic parameters for the saturable increase in Cl flux as a function of the [Cl] of the bathing Ringer indicate a change due to theophylline. The K_m values for J_{13} and J_{31} in the presence of 10mM theophylline are 21 ± 12 (S.D.) mM and 65 ± 27 (S.D.) mM respectively. The values for V_{max} for J_{13} and J_{31} are 4.38 ± 2.53 (S,D,) and 10.47 ± 4.52 (S.D.) μmoles cm^{-2} hr^{-1} respectively. A correction for a diffusion Cl permeability of 0.018 cm hr^{-1} was made. A larger correction for the diffusional component was not made since the increased Cl permeability in the presence of theophylline may represent a transcellular route for Cl permeation (see Discussion).

(ii) <u>The tissue isotope specific activity ratio R of ^{36}Cl : ^{82}Br</u> *

(a) <u>The effect of 10mM theophylline</u> The effect of 10mM theophylline upon the tissue ratio R is shown in Table 26. 10mM theophylline significantly increases R compared with control values ($p < 0.005$). A greater proportion of isotope, therefore, originates from the mucosal bathing solution in the presence of 10mM theophylline. This effect may be related to the increase in passive tissue Cl permeability deduced from the mannitol dilution potentials (Results Section 1) and is similar to the increase in ratio R seen for Na (the absolute increase in R for Cl is, however, larger). An increased proportion of isotope originating from the mucosal solution is the reverse of the situation predicted if the sole effect of theophylline were the inhibition of a neutral influx mechanism at the brush-border.

*or of ^{82}Br : ^{36}Cl (see Chapter 5)

Table 26

The tissue isotope specific activity ratio of ^{36}Cl : ^{82}Br (or ^{82}Br : ^{36}Cl) for the experimental conditions tested. Errors are expressed as $\pm$ S.E.M. n = number of determinations (see Methods).

Table 26

Condition	n	Control	n	10mM Theophylline
Standard NaCl Ringer	22	0.87 ± 0.09	11	1.64 ± 0.23
Standard Ringer + 20mM triaminopyrimidine	16	1.12 ± 0.19	5	1.70 ± 0.10
Standard Ringer + 0.1mM ouabain	11	1.11 ± 0.22	7	1.65 ± 0.20
75mM Na Ringer	8	0.72 ± 0.07	4	1.20 ± 0.09
Zero Na Ringer	6	1.00 ± 0.12	4	1.03 ± 0.29
80mM Cl Ringer	4	0.65 ± 0.15	8	1.40 ± 0.14
10mM Cl Ringer	4	1.18 ± 0.15	8	1.32 ± 0.14

(b) The effect of triaminopyrimidine 20mM triaminopyrimidine has no significant effect upon the ratio R in the presence ($p > 0.9$) or absence ($p > 0.4$) of 10mM theophylline. 20mM triaminopyrimidine has no effect on net Cl flux in the presence of theophylline.

(c) Effects of 0.1mM ouabain In the presence of 0.1mM ouabain there is an increase in the ratio R due to 10mM theophylline. This is not, however, statistically significant ($p > 0.1$). Values for R in the presence of ouabain do not differ from controls for non-poisoned tissues. The increase in Cl permeability measured in Section 1 occurred in ouabain-poisoned tissues.

(d) Effects of Ringer Na replacement Partial Na replacement has no effect on the tissue ratio R for tissues incubated in the absence of theophylline. At 75mM Na the increment in R due to theophylline is reduced. The increase over controls is, however, still statistically significant. In zero Na media there is a small non-significant increase in the ratio R in the absence of theophylline ($p > 0.5$). No theophylline-dependent increase is seen in zero Na media.

(e) Effect of Ringer Cl replacement A reduction in Ringer $[Cl]$ to 80mM has no significant effect on the tissue ratio in the presence or absence of theophylline compared with tissues incubated in a 145mM Cl Ringer ($p > 0.4$, $p > 0.5$ respectively). A reduction in Ringer $[Cl]$ to 10mM reduces the theophylline-dependent increase in the ratio R; indeed, there is no significant difference between the values of R in the presence or absence of theophylline ($p > 0.5$).

Section 3: Summary of the effects of theophylline on Cl fluxes and the tissue ratio R

1. 10mM theophylline reverses net absorption of Cl to secretion primarily by a reduction in m-s (J_{13}) flux. 10mM theophylline increases the proportion of isotope originating within the tissue from the mucosal solution.

2. 20mM triaminopyrimidine has no effect upon net Cl secretion in the presence of theophylline. 20mM triaminopyrimidine has no effect upon the tissue ratio R in the presence or absence of theophylline.

3. Na deprivation (zero choline) abolishes the net absorption of Cl in control tissues minus theophylline and prevents the theophylline-dependent changes in bidirectional and net Cl fluxes. There is no theophylline-dependent change of the tissue ratio R in zero-Na Ringer.

4. 0.1mM ouabain abolishes the theophylline-dependent change in Cl fluxes and decreases the effect of theophylline on the tissue ratio R.

5. Reduction of Ringer [Cl] to 80mM has no effect upon the theophylline-dependent change in net Cl flux, m-s Cl flux, or tissue ratio R. A further reduction in Ringer [Cl] to 10mM abolishes the significant change in Cl flux and tissue ratio R.

C. Discussion

1. Theophylline-dependent changes in passive ion permeability

The action of triaminopyrimidine and theophylline in ouabain-poisoned tissues suggest that ionic permeation through ileum is mediated by three separate pathways (Results Section 1). These are:

1. A cationic-selective pathway that is inhibited by triaminopyrimidine.
2. An anionic-selective pathway that is stimulated by theophylline.
3. A neutral or 'leakage' pathway that is affected by neither triaminopyrimidine nor theophylline.

The existence of multiple pathways for ionic permeation requires a re-examination of present concepts concerning the nature of the cation-selective pathways of ileum (Frizzell and Schultz (1972); Munck and Schultz (1974)). In particular, a leakage pathway in parallel to a cation-selective pathway will distort the ion selectivity sequences deduced for ileum towards those characteristic of free solution mobilities. Hence, P_{Cl}, the chloride permeability, will be considerably less for the cation-selective channels than previously envisaged. This suggests that the anionic field within the pore is of greater intensity than previously envisaged (see Schultz and Curran (1974)). Further work will be necessary to clarify this issue.

The question arises as to the anatomical location of the 'leakage' and anion-selective pathways in ileum. Since ileum is normally cation-selective (Frizzell and Schultz (1972); Clarkson (1967); Smyth and Wright (1966)) the cation channels are probably located at the junctions (tight-junctions) between the epithelial cells. Work in other epithelia (e.g. necturus gall-bladder, Fromter (1972); Fromter and Diamond (1972)) has shown that most of the

transepithelial current flow occurs via the junctions between the epithelial cells.

Three possible locations exist for a leakage (free-solution shunt) pathway; namely regions of cell exfoliation at the villous tips (Clarkson (1967)); damaged tissue at the edges of the Ussing Chamber windows (Helman and Miller (1973)); or leaky non-selective ion channels between the epithelial cells (Moreno (1975a). No choice may be made between these alternatives on the basis of present experimental evidence. As pointed out by Moreno, these three alternatives may be tested experimentally. Variation in the radius of the Ussing Chamber window will yield higher or lower proportions of damaged/undamaged tissue; the presence of a leak pathway due to edge-damage will result in tissue resistances that are proportional to the radius of the exposed tissue. Resistance values for intestine are, however, low and in practice this test is difficult. If the leakage pathway were due to modified junctional properties then Na and Cl leak permeabilities should decrease in experimental conditions designed to collapse the lateral intercellular spaces (e.g. current passage or hypertonic mucosal mannitol). Moreno (1975) has deduced that in frog gall-bladder the ionic leakage pathway probably comprises non-selective junctional seals between the epithelial cells.

The location of the anion-selective channels stimulated by theophylline is probably not the cation-selective junctional seals between the epithelial cells since (a) theophylline has no effect upon Na permeability deduced from dilution potential measurement,and (b) the effect of theophylline is seen in triaminopyrimidine-treated tissues. It is reasonable to conclude that the theophylline-dependent increase in Cl permeability is due to the development of a trans-cellular Cl-shunt pathway.

Cuthbert and Painter (1968) have shown an increase in Cl-conductance across the outer barrier of frog skin using micro-electrode measurements. This is analogous to the increase in Cl-conductance across the mucosal boundary postulated in the present instance.

The activation of Cl-permeability is not the sole effect of theophylline upon passive ion permeability. In non-poisoned tissues theophylline results in a time-dependent change in transepithelial resistance.

The initial decrease in tissue resistance due to theophylline is unmodified by triaminopyrimidine or by galactose, and probably results from the increase in transcellular Cl permeability. The action of theophylline in affecting transcellular Cl permeability is rapid and indicates a direct action on membrane sites. The rapid action of theophylline is in agreement with work on the effect of theophylline on the short-circuit current (Sheerin and Field (1975); Field (1971)). This effect is similar to exogenously applied c-AMP (Field (1971)) and is consistent with a rapid-rise of intracellular c-AMP levels in the presence of 10mM theophylline (Nellans et al (1974)). The rapid onset of action of theophylline contrasts with that of cholera enterotoxin (Powell et al (1973)).

Subsequent to the initial fall in tissue resistance in the presence of theophylline, tissue resistance with theophylline increases to values greater than those for tissues incubated in the absence of theophylline. An increased tissue resistance in the presence of theophylline, cholera toxin and c-AMP has previously been reported by several workers (Powell et al (1973); Powell (1974); Nellans et al (1974) and Field (1971)).

10mM theophylline is known to cause a reduction in tissue water (Chapter 3 and also Holman and Naftalin (1975b))and in tissue extracellular space (Chapter 3). Dibona, Chen and Sharp (1974) have shown that theophylline mediated secretion is accompanied by a decrease in the width and volume of the lateral spaces. Klipstein et al (1975) have reported similar changes in gross morphology in rat small intestine on treatment with klebsiella pneumoniae enterotoxin, an agent that also produces net secretion of salt and water.

The theophylline-dependent increase in tissue resistance is not evident in tissues treated with 20mM triaminopyrimidine. Triaminopyrimidine decreases tissue conductance by blockage of paracellular Na conductance at the locus of the tight-junctions (Chapter 4).

Similarly, no theophylline-dependent change in tissue conductance is seen in the presence of 20mM galactose. 20mM galactose may dilate the lateral intercellular spaces in rabbit ileum. This may be inferred since the tissue water and tissue extracellular space increase in the presence of 20mM galactose. Also 20mM galactose reverses the theophylline-dependent decrease in tissue water and extracellular space (Chapter 3).

Taken together, these results suggest that the delayed conductance decrease associated with theophylline is due to the closure of the lateral spaces which act as a variable resistance in series with the cation-selective channels formed by the tight-junctions. Changes in paracellular permeability due to lateral space closure will primarily affect cation permeability.

The primary action of theophylline is, therefore, the formation of a transcellular anion-selective pathway. Increased tissue resistance, resulting probably from lateral space collapse is subsequent to this primary event.

2. Unidirectional flux calculations

It has previously been shown that the unidirectional Na fluxes across the mucosal and basal-lateral cell membranes may be calculated from groupings of two or three independent measurements, namely, m-s Na flux (J_{13}), s-m Na flux (J_{31}) and the tissue isotope specific activity ratio (R) (see Methods and Chapters 4 and 5). Correction of the bidirectional fluxes for the shunt permeability to Na may be made by the use of triaminopyrimidine which blocks the cation-selective channels in ileum (Moreno (1975) and Chapter 4).

In the presence of theophylline, triaminopyrimidine paradoxically increases m-s Na flux (J_{13}) but is without effect on s-m Na flux (J_{31}). It is clear, therefore, that paracellular Na permeability is closely related to the theophylline-dependent change in Na flux. Simple corrections for the paracellular component of flux are, therefore, impossible.

For this reason, unidirectional flux calculations are uncorrected for Na flux through the paracellular pathway. The effect of a change in diffusional flux through an extracellular pathway on the unidirectional fluxes is simply to increase or decrease the absolute magnitude of all four unidirectional fluxes. This is illustrated by the effect of 20mM triaminopyrimidine on control tissues in the absence of theophylline.

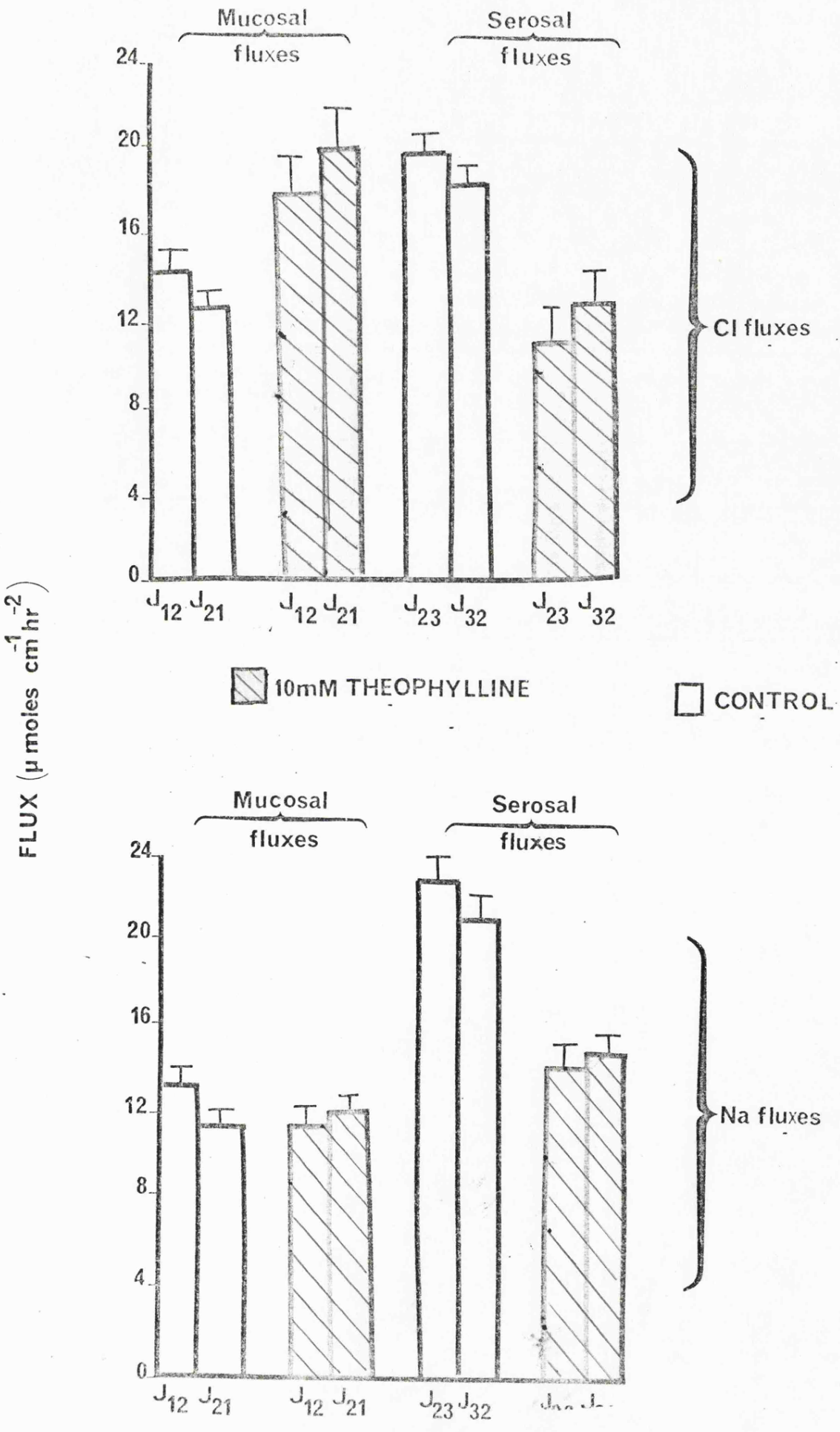
(i) The effect of 10mM theophylline on the unidirectional fluxes of Na and Cl across the mucosal and serosal border of rabbit ileum

Figure 27 shows the calculated unidirectional fluxes of Na and Cl across both cellular borders of the intestinal epithelial cell.

It can be seen that 10mM theophylline causes a significant increase in both unidirectional fluxes across the mucosal membrane compared with Cl fluxes in control tissues. Influx (J_{12}) increases

Figure 27

Calculated unidirectional Na and Cl fluxes across the mucosal and serosal (basal-lateral) boundaries in the presence and absence of 10mM theophylline. Error bars denote ± S.E.M.


Mucosal fluxes
Serosal fluxes
24
20
16
12
8
4
0
J_{12} J_{21} J_{12} J_{21} J_{23} J_{32} J_{23} J_{32}
Cl fluxes
FLUX (μ moles cm^{-1} hr^{-2})
10mM THEOPHYLLINE
CONTROL
Mucosal fluxes
Serosal fluxes
24
20
16
12
8
4
0
J_{12} J_{21} J_{12} J_{21} J_{23} J_{32}
Na fluxes

from 14.25 to 17.94 μmoles cm^{-2} hr^{-1} ($p < 0.001$), whilst efflux across the mucosal border increases from 12.47 to 19.45 μmoles cm^{-2} hr^{-1} ($p < 0.001$). This result correlates with the increase in Cl permeability deduced from mannitol dilution potentials (Section 1) and to the decrease in total tissue resistance seen in theophylline-treated tissues on mounting in the flux chambers.

This result is contradictory to previous measurements of unidirectional Cl influx across the brush-border (Frizzell et al (1973); Nellans et al (1973)). The difference between these two findings lies in the method of measurement. To measure a true unidirectional influx it must be established that no significant back-flux of isotope occurs. The rapid action of theophylline on resistance (section 1) and the stimulation of unidirectional fluxes J_{12} and J_{21} by theophylline suggest that a significant back-flux of isotope will occur in the experimental conditions of Frizzell et al (1973) and Nellans et al (1973). Hence the stimulation of Cl flux across the mucosal boundary observed in the present study predicts that an apparent inhibition of influx should occur in unidirectional influx measurements.

The increase in mucosal Cl fluxes is inferred to be the primary action of theophylline (see above).

Examination of the unidirectional Na fluxes across the mucosal boundary show that influx J_{12} is reduced by theophylline from 13.11 to 11.42 μmoles cm^{-2} hr^{-1}. No change in back-flux across the mucosal boundary occurs in the presence of theophylline. These results are in complete accord with the unidirectional influx measurements of Na made by other workers (Nellans et al (1973)). However, it may be concluded (from the action of triaminopyrimidine upon bidirectional Na fluxes in the presence of theophylline) that this apparent reduction in influx is due to an increased back-flux of

isotope primarily through the tight-junctions (Results Section 2(i)b). Examination of unidirectional fluxes across the mucosal border in the presence of 20mM triaminopyrimidine (Figure 28) lend support to this view. In the presence of 20mM triaminopyrimidine, theophylline increases both unidirectional fluxes across the mucosal boundary: J_{12} is increased from 7.97 to 11.50 μmoles cm^{-2} hr^{-1} ($p < 0.001$) and J_{21} is increased from 7.13 to 10.60 μmoles cm^{-2} hr^{-1} ($p < 0.001$). Hence, in the absence of the major route for recirculation of Na, no theophylline-dependent decrease in flux J_{12} is observed. The increased unidirectional Na fluxes across the mucosal border in the presence of theophylline suggest that theophylline may also have a direct effect on mucosal Na permeability.

If the formation of anion-selective channels within the mucosal membrane is the primary action of theophylline, how then is a reflux of Na ions through the tight-junction stimulated? An associated question concerns the driving force for net ion flows in the presence of theophylline.

(ii) A model for secretion

The neutral flows of electrolyte across a composite artificial ion-exchange membrane composed of parallel cation and anion selective elements have been experimentally measured (Neihof and Sollner (1955)). In the presence of a salt concentration difference across this membrane-type the permeability of the composite membrane for ion movement is greatly enhanced compared with the permeability of each of the separate ion-exchange membranes alone. Kedem and Katchalsky (1963) have developed the theoretical aspects of composite ion-exchange membranes. The enhanced salt permeability of the composite membrane results from a circulation of electric current through the membrane array (Diagram 1). In a membrane

Diagram 1

Neutral diffusion of NaCl across a heterogeneous membrane composed of separate anion and cation selective elements will result in a circulation of electric current through the composite membrane. This results in enhanced net movement of NaCl.

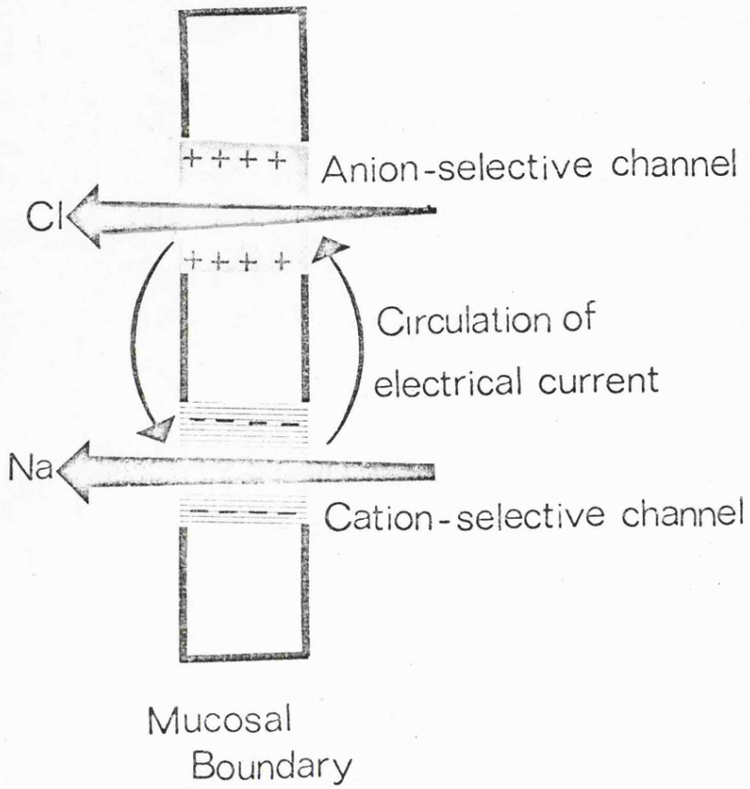
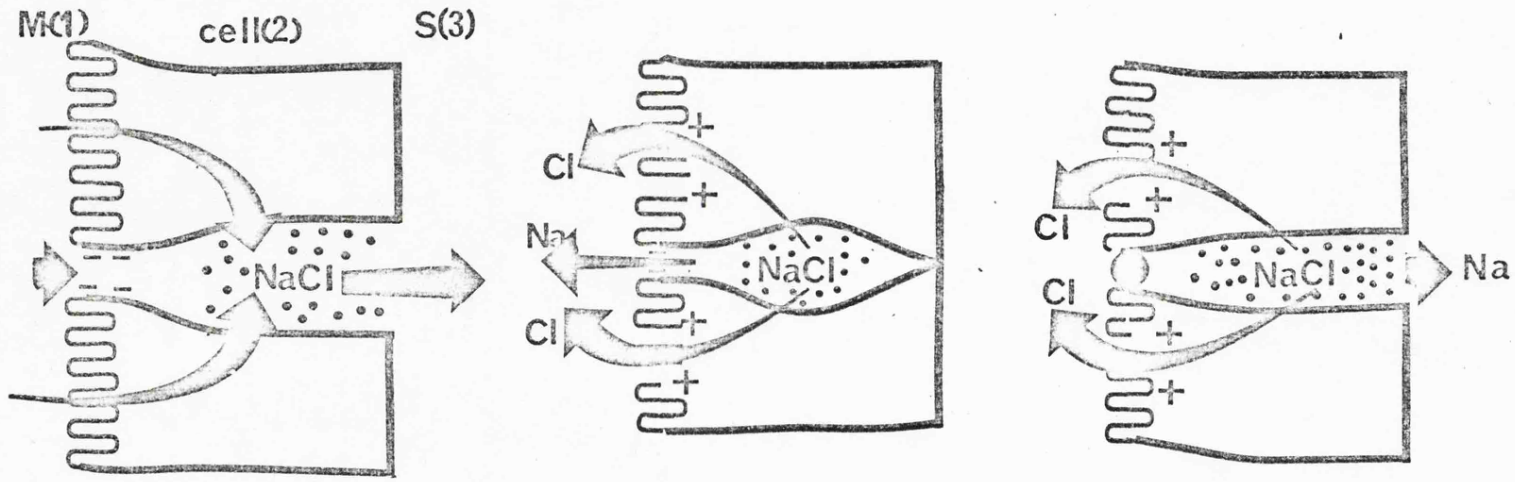

++++
Anion-selective channel
Cl
++++
Circulation of
electrical current
Na
Cation-selective channel
Mucosal
Boundary

Diagram 2

The theophylline-dependent increase in Cl-permeability allows a net diffusion of Na and Cl across the mucosal boundary from regions of hypertonic NaCl contained within the lateral spaces. The anion-selective channels may be located at the mucosal membrane or may be closely associated with the cation-selective tight-junctions. Triaminopyrimidine reverses the direction of net Na movement in the presence of theophylline by blocking Na passage through the cation-selective tight-junctions.



CONTROL

THEOPHYLLINE

THEOPHYLLINE
+
TRIAMINOPYRIMIDINE

bathed unilaterally with KCl the electric current is generated by the net diffusive movement of both K and Cl across the membrane in the same direction.

In rabbit ileum, treated with theophylline, there exists a composite membrane type composed of cation-selective channels (the tight-junctions) and anion-selective channels (formed by the action of theophylline). Passive diffusive flux through the tight-junction will therefore, be enhanced by theophylline. The presence of a hypertonic salt solution sequestered in the lateral space (Chapter 3; Zeuthen and Monge (1975)) may provide the necessary driving force for net secretion of Na and Cl across the mucosal boundary (Diagram 2).

Strict coupling of Na and Cl flows requires an equality of potential difference across both cation and anion-selective elements (Kedem and Katchalsky (1963)). This condition is unlikely to be met in the present experimental circumstances. For this reason, net Cl flux is of greater magnitude than net Na flux in theophylline-treated tissues. The existence of a transcellular pump mechanism for Cl need not be involved, but may neither be excluded.

(iii) <u>The relationship between secretion and the unidirectional ion fluxes across the serosal boundary</u>

Figure 27 shows the effect of 10mM theophylline upon the unidirectional ion fluxes across the serosal boundary for both Na and Cl. It is clear that for both Na and Cl unidirectional fluxes there is a large decrease in the presence of 10mM theophylline. This effect is probably related to the collapse of the lateral spaces. This will effectively reduce the ionic fluxes across the serosal membrane by reducing the area of serosal membrane avaliable for flux. Similar effects have been observed for unidirectional galactose fluxes across the serosal membrane (Holman and Naftalin (1975b)).

A collapse of the lateral spaces in the presence of theophylline has been inferred from the resistance measurements (Results Section 1). This view is supported by tissue wet weight determinations, and by extracellular space determinations. The change in resistance due to lateral space collapse occurs after the supposed change in Cl- conductance. Hence, lateral space closure must be viewed as a result of, and not a cause of, secretion.

Huss and Marsh (1975) have recently proposed a modification of Diamond and Bossert's (1967) model for NaCl and water flows (see Chapter 1). They consider that the lateral space forms a distensible compartment in which the hydrostatic pressures within the space will determine its dimensions. A net secretion of solute and water will create a negative hydrostatic pressure within the lateral spaces that will lead to their collapse.

Collapse of the lateral spaces is not concurrent with a reduction of lateral space hypertonicity (see Chapter 3).

(iv) Evidence supporting the composite membrane model for secretion

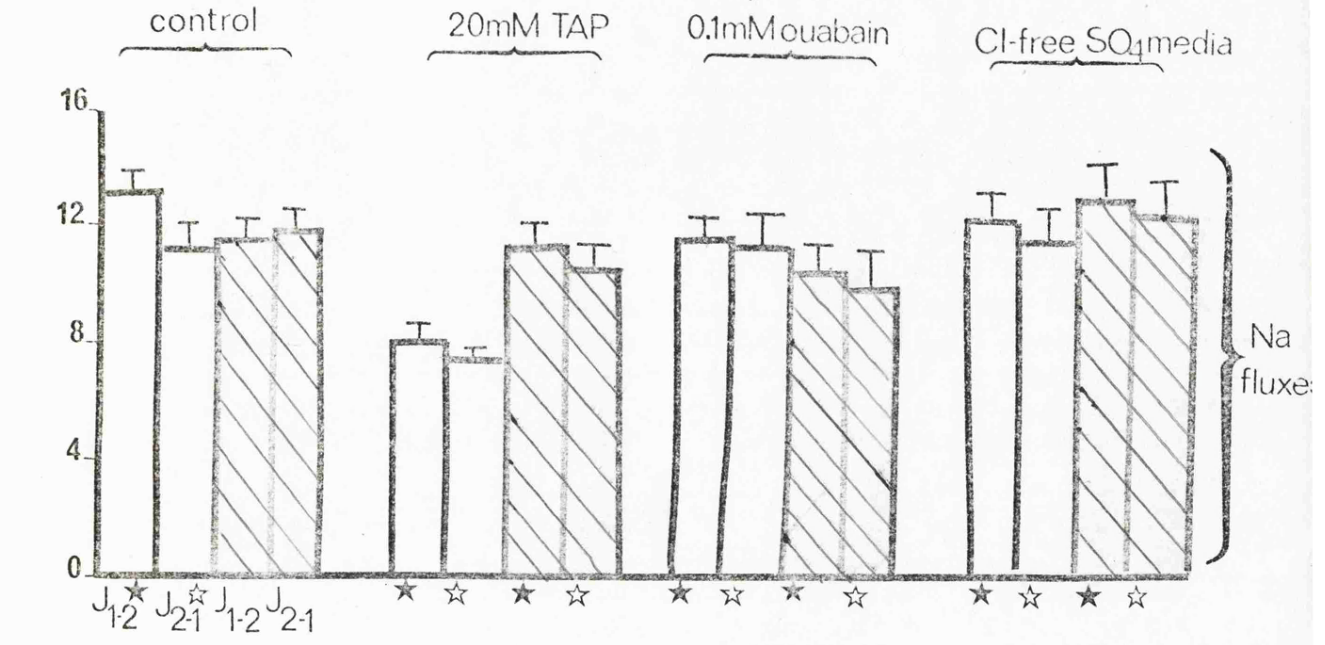
(a) The effect of 0.1mM ouabain If the driving force for net secretion of Na and Cl is the hypertonic salt solution sequestered in the lateral spaces, secretion should be abolished if Na pump activity is inhibited. It has previously been shown that 0.1mM ouabain abolishes extracellular hypertonicity (Chapter 3). The net secretion of both Na and Cl due to theophylline is abolished by 0.1mM ouabain, (Results sections 2 and 3).

Figure 28 shows that there is a theophylline-dependent increase in Cl exchange across the mucosal boundary, even in the presence of 0.1mM ouabain. This result is consistent with the increased

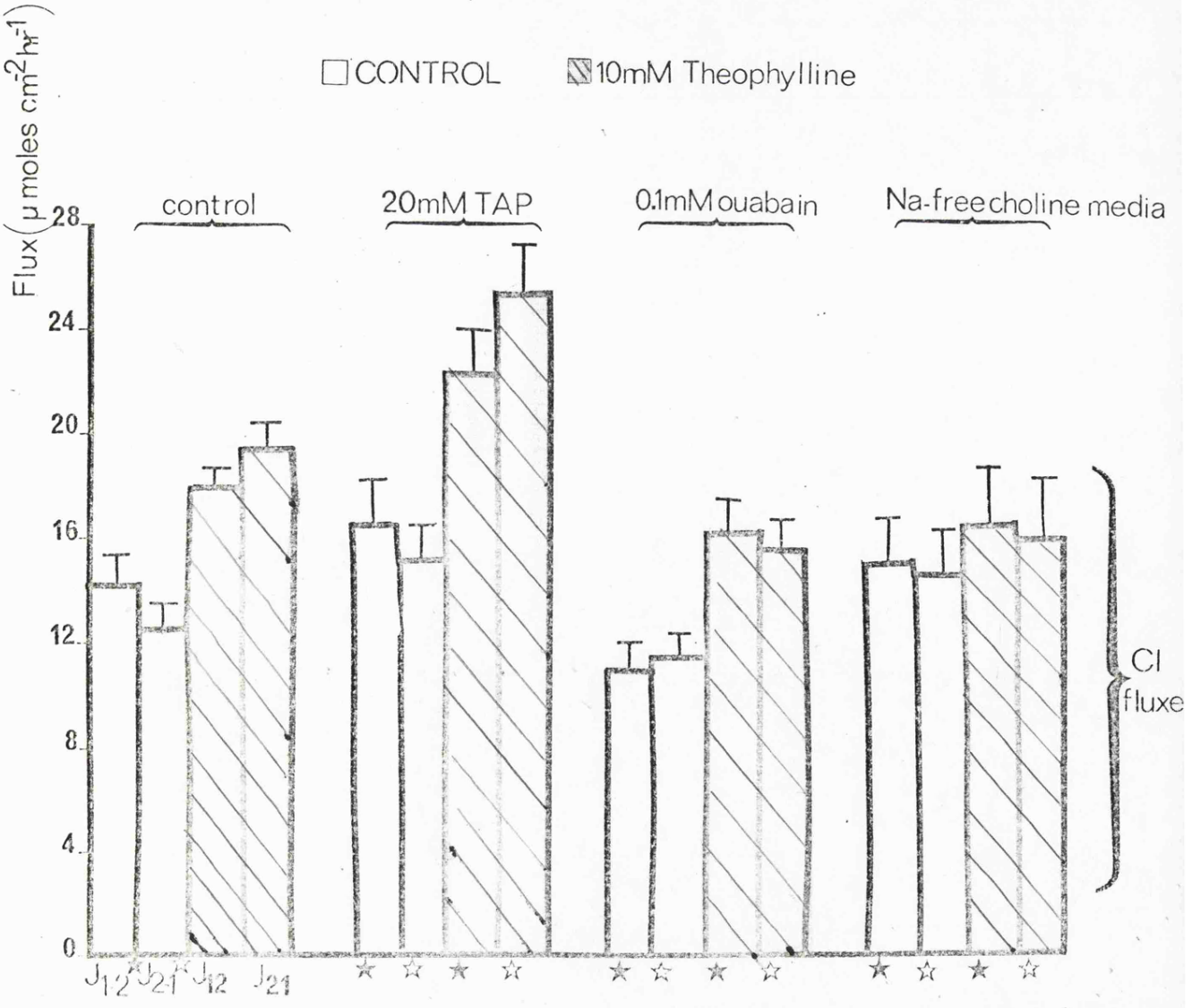
Figure 28

The effect of theophylline on the unidirectional fluxes of Na and Cl across the mucosal boundary for controls, tissues + 20mM triamino-pyrimidine, 0.1mM ouabain, Cl-free SO_4 media (Na fluxes) and Na-free choline media (Cl fluxes). Error bars denote $\pm$ S.E.M.

The effect of theophylline on the unidirectional fluxes across the mucosal boundary



□ CONTROL ▨ 10mM Theophylline



Cl-permeability deduced from electrical measurements in the presence of ouabain (Results: section 1).

No theophylline-dependent decrease in unidirectional Na influx across the mucosal boundary is expected, nor indeed observed, in the presence of 0.1mM ouabain (Figure 28).

(b) Effect of Cl-replacement by SO_4 on Na fluxes Figure 28 shows the effect of theophylline on the unidirectional Na fluxes across the mucosal boundary in Cl-free SO_4 media. No significant change in either of the unidirectional Na fluxes occurs. A reduced change in net Na flux due to theophylline is seen in SO_4 media (Section 2).

It is apparent that the change in Na fluxes in the presence of theophylline across the mucosal boundary requires the presence of a mobile anion.

(c) Effect of Na substitution by choline on Cl-fluxes Figure 28 shows that theophylline has no effect on the net or exchange Cl fluxes across the mucosal membrane in the absence of the mobile cation.

These findings are thus consistent with the model that has been proposed for theophylline-induced secretion, since Na-pumping provides the concentration gradient for net ion movements, whilst enhanced Cl-permeability is ultimately dependent upon the presence of a mobile counterion.

(d) Effect of Ringer [Na] variation on Na fluxes The theophylline-dependent change in net Na flux is abolished at low Na Ringer concentrations (25mM). Since there is no significant extracellular hypertonicity in 25mM Na containing Ringer (Chapter 3) this result lends supportive evidence to the mechanism of secretion that has been proposed. 10mM theophylline causes a small stimulation of unidirectional fluxes across the mucosal membrane (from 2.12 to 2.89 μmoles cm^{-2} hr^{-1} for J_{12} ($p > 0.1$) and from 1.64 to 2.52 μmoles cm^{-2} hr^{-1} for J_{21} ($p < 0.05$). This effect on mucosal Na fluxes is similar to the

effect of theophylline observed in a 140mM Na Ringer containing triaminopyrimidine.

(e) Effect of Ringer [Cl] variation on Cl fluxes Net Cl secretion if driven by a hypertonic solution of NaCl within the lateral spaces should be sensitive to Ringer [Cl] variation. Reduction of Ringer [Cl] to 80mM by SO_4 does not reduce net Cl flux (Results section 3). A further reduction in Ringer [Cl] to 10mM abolishes net Cl secretion. A pump mechanism involved in Cl secretion should show net Cl secretion even at low [Cl]. However, the presence of 10mM HCO_3 in the Ringer may be of importance in this respect.

(f) The effect of triaminopyrimidine Triaminopyrimidine reduces Na movement across the tight-junction. The action of triaminopyrimidine in reversing the direction of net Na movement in the presence of theophylline is strong evidence for a passive mode of coupling. Increased Cl-movement enhances Na exit across the mucosal boundary via the tight-junction in the presence of theophylline (see above).

The route of serosa to mucosa flux involves two components, the transcellular and extracellular pathways. A transcellular component of s-m Na flux is evident in theophylline-treated tissue since there exists a triaminopyrimidine insensitive flux component that cannot be accounted for on the basis of a residual Na permeability in the presence of triaminopyrimidine (see also Chapter 4). However, extracellular Na movements are of prime importance in the theophylline induced reversal of net Na flux across the mucosal boundary.

Triaminopyrimidine has no effect on net Cl movement. A reduction in net Cl movement might have been expected on the basis of the model for secretion that has been proposed.

The existence of a secretory Cl pump that is stimulated by theophylline may not be excluded on the basis of present evidence. A transcellular secretory pump mechanism for organic anions such as para-amino hippurate gives rise to net secretion of Na and water in kidney proximal tubule (Grantham (1976)).

(g) The effect of 20mM galactose 20mM galactose stimulates a net absorption of Na both in the presence and absence of 10mM theophylline. This result implies a separation of the absorptive and secretory modes of ion transport in ileum. De Jonge (1975) has presented evidence that suggests a functional separation of absorptive and secretory modes is not achieved by two entirely separate epithelial cell populations. Instead, the secretory and absorptive modes are confined to the same population of cells. It is likely that net secretion of Na is achieved by an enhanced exit of Na across the tight-junction (see above). Stimulation of net Na absorption in control tissues is unaffected by triaminopyrimidine (Chapter 4); this suggests that the route of galactose stimulated Na absorption is transcellular.

The epithelial cell population is thus capable of both absorption and secretion simultaneously. Net transport is roughly the difference between these two processes. Field (1971) has previously suggested this on the basis of short-circuit current measurements.

Table 27 shows the effect of 20mM galactose on the calculated unidirectional Na fluxes across the mucosal and serosal boundaries in the presence and absence of 10mM theophylline. 10mM theophylline has no effect on the Na fluxes across the mucosal boundary in tissues incubated in the presence of 20mM galactose. Similarly, there are no statistically significant differences between the Na fluxes across the serosal boundary in the presence of galactose whether in the presence

or absence of 10mM theophylline. 20mM galactose does, however, cause a significant increase in Na fluxes across the serosal boundary in the presence of 10mM theophylline. This effect is most probably related to dilation of the lateral spaces due to transport of sugar Na and water. Galactose has previously been shown to reverse the theophylline-dependent decrease in tissue water and extracellular space (Chapter 3).

(v) Relation to other studies on secretion

The transition between absorption and secretion in intestine results from a simultaneous decrease in the mucosal to serosal fluxes of Na and Cl (Field (1971); Field et al (1972); Al-Awquati et al (1971); Powell et al (1973); Nellans et al (1974)). These findings are duplicated in the present study (Results: sections 2 and 3).

Nellans et al (1974), Frizzell et al (1973) and Nellans et al (1973) have developed the concept of a neutral influx process, the inhibition of which leads to secretion. These workers have presented evidence to support this claim, that is based on the measurement of a 'unidirectional' influx across the brush-border. Measurements of a single flux such as influx across the mucosal boundary fail to incorporate the possible effects of other unidirectional fluxes upon Na movements through the paracellular pathway.

The use of triaminopyrimidine and of techniques designed to measure all four unidirectional fluxes have shown that the apparent inhibition of ion flux across the mucosal boundary is due to a rapid reflux of isotope via the tight-junctions for Na and via another pathway in the mucosal membrane for Cl.

On the basis of this data, and data identifying the major effect of theophylline as a development of an anion-selective pathway across

Table 27 The effect of 20mM galactose on the calculated unidirectional Na fluxes in the presence and absence of 10mM theophylline

Condition	n	Mucosal fluxes μmoles cm^{-2} hr^{-1}		Serosal fluxes μmoles cm^{-2} hr^{-1}	
		J_{12}	J_{21}	J_{23}	J_{32}
Control	22	13.11 ± 0.84	11.28 ± 0.84	23.18 ± 1.65	21.35 ± 1.65
10mM theophylline	34	11.42 ± 1.28	11.88 ± 1.31	14.43 ± 1.15	14.90 ± 1.07
20mM galactose	11	15.48 ± 1.24	12.47 ± 1.18	26.47 ± 2.69	23.46 ± 2.60
10mM theophylline/20mM galactose	13	18.55 ± 3.27	16.36 ± 3.17	21.75 ± 2.09	19.56 ± 2.20

ileum, a new model for secretion is proposed that is a composite membrane model composed of anion and cation-selective elements. The driving force for net ion flows is the hypertonic NaCl solution contained within the lateral spaces that is dependent upon Na-K ATPase activity.

The neutral influx model of Schultz and his co-workers, besides explaining secretion, also satisfactorily explains the effect of Cl-replacement upon net Na fluxes and the effect of Na replacement on net Cl fluxes in small intestine, (Nellans et al (1974); Quay and Armstrong (1969) and Turnberg et al (1970)), large intestine (Binder and Rawlins (1973)).

An alternative mechanism for coupling of net Cl to net Na flows is a passive diffusion of Cl with respect to a transmembrane electrical potential difference. A transmembrane potential may be generated by an electrogenic Na pump (Thomas (1972)) located at the basal-lateral cell membrane. Inhibition of the elctrogenic Na pump by replacement of Na by choline would inhibit net Cl movement. Replacement of Cl by an impermeant anion such as SO_4 would lead to an enhanced potential due to electrogenic Na pumping which would reduce, but not abolish, net Na flux.

Nellans et al (1974) have pointed out that ion replacement experiments are usually carried out in the short-circuit condition. This would seem to exclude the possibility of electrogenic coupling. Turnberg et al (1970) report that the change in electrical potential on ion replacement of Na by choline or of Cl by SO_4 is insufficient to generate the change in ion flux that was experimentally observed. Electrical coupling between net Na and Cl flows may be attained even in the absence of transepithelial P.D. changes, (Keynes (1969)).

A trans-serosal potential difference of large magnitude could easily be attenuated by the cable properties of the basal-lateral membrane and by the high conductance shunt pathway (Frizzell and Schultz (1972)).

A passive mode of coupling between net Na and Cl absorption is supported by the parallelism between these two fluxes in Na-depleted and ouabain poisoned tissues. The existence of a coupled neutral influx process at the brush-border membrane may thus be questioned.

CHAPTER SEVEN

CONCLUDING REMARKS

The work described in this thesis has involved the measuremant of transcellular and extracellular ion movements in ileum. Since the extracellular ion pathway is cation-selective, extracellular Na movements are of greater importance than extracellular movements of Cl. Extracellular Na movements have been assessed by the use of the agent 2,4,6, triaminopyrimidine which blocks Na passage through the extracellular pathway.

Previous workers (Frizzell and Schultz (1972); Desjeux et al (1974)) who have studied ion movements through the extracellular (paracellular)route conclude that ion movement adheres strictly to the laws of electrodiffusion; that is to say, the paracellular pathway behaves as a leak or shunt. The magnitude of the conductance of the paracellular pathway relative to that of the transcellular pathway ensures that active transcellular movements of solute, in particular of Na, are effectively short-circuited;that is, passive diffusional leakage back through the shunt reduces net transintestinal transport. Quantitatively speaking, the size of the leakage pathway controls the magnitude of the Ussing flux ratio for the epithelium (Kedem and Essig (1965)).

In high-resistance epithelia such as frog skin, there is only a small diffusional passive leak through the shunt pathway. High Ussing flux ratios, and large ion concentration differences are experimentally observed across frog skin. The existance of a high-conductance shunt pathway in ileum is in apparent contradiction to the absorptive role of ileum. What functions may, therefore, be ascribed to the shunt pathway in ileum?

Hill (1975a and b) has emphasized the apparent discrepancy between the osmotic permeability of biological cell membranes and that needed in epithelia for adequate functioning of a standing-gradient model for isotonic water absorption. The extracellular shunt pathway in ileum is probably freely permeable to water (see Chapter 1) and thus may serve as a major route of transintestinal water movement in response to lateral space hypertonicity. Huss and Marsh (1975) have demonstrated theoretically that tight-junctional osmotic permeability may control the rate of formation and osmolarity of the absorbate in leaky epithelia. A high-conductance shunt pathway may, therefore, allow greater values of net water absorption to be attained. Net fluid transport is characteristically greater in leaky epithelia such as intestine and gall-bladder, compared with high-resistance epithelia such as frog skin (House (1974)).

Assessment of the importance of tight-junctional water permeability in determining transepithelial water movements requires a direct measure of both transcellular and junctional water permeabilities. This measurement has yet to be made in ileum. A possible development is the use of triaminopyrimidine to separate paracellular and transcellular water-movements. Moreno (1974) has suggested that triaminopyrimidine binding to the tight-junction ligands physically occludes the junctional pore. Leak pathways upon which triaminopyrimidine has no action have, however, been demonstrated (Chapters 4 and 6).

The existance of bulk water flow across the tight-junction in response to an osmotic pressure gradient is indicated by the results that show an asymmetric action of triaminopyrimidine upon bidirectional Na fluxes under voltage clamp (Chapter 4). Triaminopyrimidine

reduces m-s Na flux (J_{13}) more than s-m Na flux (J_{31}) in the Standard Ringer's solution. Solute-drag due to the osmotic flow of water across the tight-junction thus creates a net flux of Na into the lateral space. Triaminopyrimidine has been shown to abolish a proportion of net Na flux in control tissues. These results provide experimental evidence for the operation of 'solute-amplification' by the paracellular pathway (Diamond (1974)). Active pumping of salt into the lateral spaces results in an extra net flux of Na across the tight-junction. The mechanism of solute-amplification is maximal when the Staverman (reflexion) coefficient (σ) for salt at the tight junction is 0.5 (Diamond (1974)). Over half of the net flux of Na in ileum in control tissues may be attributed to this effect (Chapter 4).

Hendrix and Bayless (1970) have stressed the importance of ileum as a secretory organ. Apart from pathological conditions, secretion of salt and water into the ileal lumen may serve the physiologically important function of maintaining the lumenal contents hydrated, thus facilitating nutrient absorption and passage of material into the colon (Hendrix and Bayless (1970); Schultz et al (1974)). The action of triaminopyrimidine in reversing the theophylline-dependent secretion of Na clearly implicates the shunt pathway in the secretory process (Chapter 6). Indeed, the reversal of net Na movement in the presence of secretagogues such as theophylline results from an enhanced permeability of the tight-junction to Na. Enhanced junctional permeability to Na is a consequence of electric current circulation through the mucosal boundary due to net diffusion of Na and Cl through separate ion-selective channels (Neihof and Sollner (1955); Kedem and Katchalsky (1963); Chapter 6).

The route of transintestinal water flow in the secretory state may be primarily via the extracellular pathway. Since extracellular

hypertonicity is unaffected by theophylline (Chapter 3) the direction of net water flow would thus occur against the prevailing concentration gradient of Na. The rate of osmotic water flow J_v across a membrane in response to a salt concentration difference ΔC is given by the equation:

$$J_v = \sigma L_p \Delta C$$

where L_p is the osmotic water permeability of the tight-junction and σ is the reflexion coefficient of salt for the membrane. A negative reflexion coefficient for salt across the tight-junction in the presence of theophylline could theoretically account for net water flow from the hypertonic lateral spaces to the mucosal solution. Kedem and Katchalsky (1963) have demonstrated that a consequence of electrical current circulation through a composite membrane array is a negative reflexion coefficent of the membrane for salt. The hypertonic NaCl solution within the lateral spaces is thus the driving force for the secretion of water into the intestinal lumen.

As is the case for absorption no definitive data on the route of water flow (transcellular or paracellular ?) exists for the secretory state. Lifson et al (1972) studied absorption and cholera-induced secretion in dog ileum. These authors suggest that the pathways of water movement are different in the absorptive and secretory states. Since changes in the extracellular pathway are known to occur in the secretory state (Chapters 3 and 6) the data of Lifson et al do not necessarily show a change in the pathway for water flow. The use of triaminopyrimidine may be of value in defining the route of trans-intestinal water flow in the secretory state.

Measurements of the bidirectional transcellular ion fluxes together with an estimate of the ratio of isotope specific activities

within the tissue fluid have provided the basis for the calculation of the unidirectional ion fluxes across the mucosal and serosal boundaries.

Although direct measurements of ion influx across the mucosal boundary have previously been made (see Schultz and Curran (1974)), little information existed concerning ion flux across the basal-lateral aspect of the epithelial cells. Estimates of ion flux across this membrane had led to the conclusion that ion flux was completely rectified with respect to Na; s-m Na flux was regarded as being entirely due to movement through the paracellular pathway (Frizzell and Schultz (1972); Schultz and Curran (1974)).

Lindemann and Pring (1969) were the first to suggest that the hypertonic NaCl solution contained within the lateral spaces may modify the intracellular ion concentrations within the epithelial cell. The present estimates of the unidirectional Na and Cl fluxes across the basal-lateral membrane show a large exchange component for both Na and Cl (Chapters 4 and 5). This result is entirely consistent with the presence of a hypertonic salt solution contained within the lateral spaces coupled with the existance of a high-capacity Na-K ATPase located at the basal-lateral membrane.

A major question concerning the transcellular movement of Na and Cl in control tissues is the nature of the coupling between the net movements of Na and Cl. This question has yet to be resolved in other leaky epithelia such as gall-bladder (Diamond (1974)). Coupling may be due to a neutral influx process as envisaged by Nellans, Frizzell and Schultz (1973); alternatively, coupling may be electrogenic as envisaged by Keynes (1969). The present data showing the dependence of net Na and Cl fluxes upon a functional Na-K ATPase, the dependence of net Cl movement on the presence of Na, and the partial

inhibition of net Na movement in the absence of a mobile anion, clearly do not differentiate between the two alternatives. The resolution of this question awaits further experimental evidence.

The existance of an active pump mechanism for serosa to mucosa Cl movement in the secretory state need not be invoked (Chapter 6). Instead, Cl movement is driven by the concentration difference for Cl existing between the lateral spaces and the mucosal solution. Agents such as theophylline probably act in such a way as to form anion-selective channels across the mucosal boundary. A question of significance is, therefore, the possible mode of action of ethacrynic acid which was presumed to inhibit the secretory Cl pump (Al-Awquati et al (1974)).

Al-Awquati et al suggested that an inhibitory action of ethacrynic acid upon the NA-K ATPase could not be excluded. A decrease in the rate of active Na pumping would reduce extracellular hypertonicity, so reducing the driving force for net salt secretion. An alternative possibility for the mode of action of ethacrynic acid is suggested by the work of Cousin and Motais (1976) and Motais and Cousin (1976) upon anion permeability in ox red blood cells. These authors have shown that diuretics such as furosemide, other sulphonamides and ethacrynic acid inhibit Cl transport in a competitive fashion. There exists the possibility that ethacrynic acid inhibits secretion in ileum by blocking Cl-diffusion across the mucosal boundary. Indeed, Cousin and Motais (1976) have suggested that the diuretic action of these drugs may be mediated by their direct action on Cl-permeability. Acetazolamide reduces net Cl absorption in ileum but does not effect theophylline induced Cl secretion (Nellans et al (1975)). Acetazolamide does not affect Cl transport in ox red-cells (Cousin and Motais (1976)).

Clearly more work is needed to define the actions of acetazolamide and ethacrynic acid upon intestine. A possible development is to test the action of these drugs upon the theophylline-dependent changes in dilution and biionic potentials (see Chapters 2 and 6).

The present work does not attempt to incorporate the effects of Ringer $[HCO_3]$, pH and PCO_2 variation on the model that has been proposed for secretion. Sheerin and Field (1975) have examined inter-relationships between Na, Cl and HCO_3 transport in rabbit ileum. These authors conclude that net absorption of both Na and Cl is controlled by serosal pH and HCO_3 concentration. Clearly future work must pay attention to these factors in the control of ion flux in intestine.

A final question concerns the mechanism of stimulus-secretion coupling in small intestine. Although there is abundant evidence to suggest that cyclic-AMP is the central mediator of secretion (Al-Awquati et al (1974); Field (1971); Nellans, Frizzell and Schultz (1974)) the mode of action of c-AMP in increasing Cl-permeability is unknown. Increased intracellular levels of Ca have been implicated in the majority of cases where activation by c-AMP has been studied (Rasmussen (1970)). Perhaps Ca plays an important role in the activation of Cl-permeability by c-AMP.

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*Obtained by cross-reference

N.L. SIMMONS. Ph.D. Thesis. 1976.

SUMMARY

Parallel pathways for ion movement exist in rabbit ileum. A high-conductance cation-selective extracellular pathway parallels the low-conductance transcellular route. The extracellular pathway comprises a series array of lateral cell space and tight-junction. Aspects of ion flux through these two pathways have been studied.

The cation content of strips of rabbit ileum has been measured. The [Na] in the tissue extracellular (inulin) space was calculated to be 40mM hypertonic to the bathing medium (140mM Na). This result provides evidence that the lateral intercellular spaces of rabbit ileum are 80 m.osmoles hypertonic to the bathing medium.

2,4,6, triaminopyrimidine reduces the Na conductance of the extracellular pathway and has been used as a means of separating transcellular from extracellular Na movements.

A portion of net Na flux in control tissues is due to asymmetry generated in the extracellular pathway; this may result from an osmotic pressure gradient across the tight-junction due to lateral-space hypertonicity.

Measurement of the tissue isotope specific activity ratio together with the measurement of bidirectional transcellular Na or Cl fluxes allows calculation of the four unidirectional ion fluxes across the mucosal and serosal boundaries of the transcellular pathway. The effects of D-galactose, a sugar actively transported by ileum, upon transcellular unidirectional ion fluxes has been examined. The coupling between galactose and Na fluxes is discussed in relation to models for active sugar transport.

The effect of the secretagogue, theophylline, upon Na and Cl fluxes was tested. Triaminopyrimidine reverses the theophylline-stimulated secretion of Na, indicating that Na secretion occurs via

the tight-junctions. Theophylline causes an increased passive permeability of the mucosal border to Cl. Net secretion of NaCl in the presence of theophylline may result from passive movement of NaCl from the hypertonic lateral space to the mucosal solution.