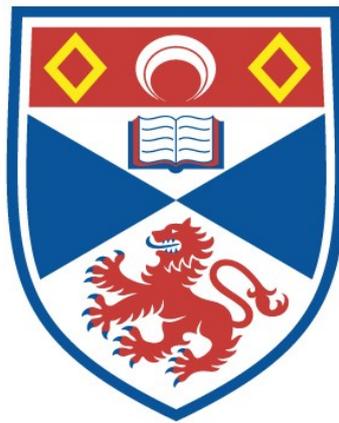


PASSIVE POTASSIUM FLUXES IN CULTURAL HELA
AND MDCK CELLS

David Robert Tivey

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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PASSIVE POTASSIUM FLUXES IN CULTURED
HELA AND MDCK CELLS.

A THESIS SUBMITTED TO THE UNIVERSITY OF ST. ANDREWS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
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To the memory of Walter Tivey, my father, who gave encouragement and support throughout my education.

ACKNOWLEDGEMENTS

It is my pleasant duty to thank Dr. N.L. Simmons for the supervision and encouragement throughout this study. I would also like to thank Mr I. Laurie and Mrs. C. Voy for their technical assistance and Miss J. Necel for the professional typing of the tables presented in this thesis. Thanks are also extended to the S.E.R.C. for providing the financial support which I received during this study.

I must also thank my family and in particular my mother for the endless encouragement throughout my university career. Finally, and most of all, I must thank my wife, Jill, who endured my moods during the preparation of this thesis. She has also read and re-read this work many times as well as providing moral support at all times.

ACADEMIC RECORD

I matriculated at the University of Bradford in October 1978, and graduated with the degree B.Sc. Hons. (2nd class, upper division) in Medical Science in June 1982. I then matriculated as a research student in the Department of Physiology and Pharmacology, University of St. Andrews in October 1982.

CERTIFICATE

I hereby certify that David Robert Tivey has spent eleven terms engaged in research work under my direction and that he has fulfilled the conditions appropriate for the degree of Doctor of Philosophy, and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology of the University of St. Andrews under the supervision of Dr. N.L. Simmons.

ABSTRACT

The cultured cell lines HeLa (human cervical carcinoma) and MDCK (renal epithelial) possess a trans-membrane K^+ transport that can be pharmacologically divided into ouabain-sensitive, diuretic-sensitive and ouabain- and diuretic-insensitive components. The K^+ transport of primary interest in this thesis is that sensitive to "loop" diuretic. Analysis of the cation and anion dependencies and "loop" diuretic sensitivity of this K^+ transport indicates that it is mediated via a $Na^+ K^+ Cl^-$ "cotransporter" as has already been reported in other cell types.

Cell shrinkage stimulated the diuretic-sensitive K^+ transport in both HeLa and MDCK cells. Analysis of the $Na^+ K^+$ and Cl^- dependency of this K^+ flux demonstrated that this stimulation is principally due to an increase in the maximal velocity (V_{max}) of this K^+ transport. This stimulated K^+ flux was not regulated by cAMP nor was the response modified by elevated cellular cAMP or by low extracellular Ca^{2+} (+ EGTA) media. The increased maximal velocity for K^+ transport cannot simply be considered to be an absolute increase in the number of $Na^+ K^+ Cl^-$ "cotransport" units in the membrane. However, a possible regulation of the $Na^+ K^+ Cl^-$ "cotransport" turnover rate (molecules/site.second) may be involved.

In this study the $Na^+ K^+ Cl^-$ "cotransport" of both HeLa and MDCK cells was inhibited in metabolically depleted cells (metabolic inhibition induced by the limited-metabolisable sugar 2 deoxy-D-glucose). This effect may be considered a secondary reponse of cell swelling however, since exposure of metabolically depleted cells to hyperosmolar media re-activates the $Na^+ K^+ Cl^-$ "cotransport" in both HeLa and MDCK cells.

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NOTE:

Throughout this thesis the monovalent ions Na^+ , K^+ , Rb^+ and Cl^- are represented by Na, K, Rb and Cl respectively. Similarly the divalent ions Ca^{2+} , Mg^{2+} and Ba^{2+} are represented by Ca, Mg and Ba respectively.

CHAPTER 1

GENERAL INTRODUCTION

The trans-membrane transport of permeant ions and nutrients is important for the physiology of the cell; for example, the regulation of the cell volume (see reviews: MacKnight and Leaf, 1977; Kregenow, 1977; Cala, 1983; Rink, 1984), the propagation of nerve impulses (Hille, 1984), amino acid and sugar transport (see Young and Ellory, 1977; Guidotti, Borghetti and Gazzola, 1978; Crane, 1983; Hudson and Schultz, 1984), and epithelial absorption and secretion (see Frizzell, Field and Schultz, 1979; Petersen and Maruyama, 1984).

The membrane transport of interest in this thesis is that of the permeant ions Na^+ , K^+ and Cl^- , with particular emphasis on the transport of K^+ . These ions may be transported through the membrane by one of three processes classified by Stein (1967): passive diffusion, where the ions pass through the membrane in a random manner; facilitated diffusion, in which the ions are transported across the membrane through "pores" or molecular carriers down their electrochemical gradient; and active transport, whereby an ion species may be moved against its concentration gradient by the expenditure of energy i.e. the high energy phosphate bonds of ATP.

In many pioneering studies on the mechanisms of ion transport (for example, trans-membrane K^+ transport), the use of a convenient model system, such as the red blood cell, where there is control of both internal and external environments, has been of crucial importance. It is often the case that identification of discrete membrane transport systems in such models precedes a knowledge of their wider distribution in other tissues and the general importance of such systems to cell function.

Three principal ion pathways for K permeation have been identified in human erythrocytes (Sachs, 1971; Beauge and Adragna, 1971; Dunham, Stewart and Ellory, 1980; Chipperfield, 1980, 1981 and 1985), Ehrlich ascites cells (Tupper, 1975; Mills and Tupper, 1975; Bakker-Grunwald, 1978) and in the cultured cell lines, HeLa, chick heart cells, BC3 H1 cells, MDCK cells (Aiton, Chipperfield, Lamb, Ogden and Simmons, 1981) and LLC PK1 cells (Brown and Murer, 1985). These are: the ouabain-sensitive Na K Pump; the "loop" diuretic-sensitive Na K Cl "cotransport" system(s) (* see footnote at the end of chapter 1); and the ouabain- and "loop" diuretic-insensitive pathways. In this present work, the ouabain-insensitive pathways are of primary interest, with particular attention being given to the "loop" diuretic-sensitive component of the K fluxes of the cultured cell lines HeLa and MDCK.

The Na K pump is the primary membrane transport pathway which maintains the Na and K gradients across the cell membrane. This membrane transport pathway has been extensively studied with this work being reviewed by Glynn and Karlish (1975), Schwartz, Lindenmayer and Allen (1975), Skou (1975), Cavieres (1977) and Jorgensen (1980). The early work of Shaw (1955) demonstrated that the K influx of erythrocytes was dependent upon the external K concentration and could be described by saturable and non-saturable components of this flux. Glynn (1956) demonstrated that starvation of human erythrocytes reduced the magnitude of the saturable K influx and also that a portion of the Na efflux was dependent upon the external K concentration. With the half maximal activation of the K influx and Na efflux near 2 mM, this infers a link between the Na and K fluxes through the Na K pump, with a stoichiometry of 3 Na (efflux) and 2 K (influx) determined by the net fluxes of Na and K after cold storage

of human erythrocytes (Post and Jolly, 1957), which has been confirmed by Garrahan and Glynn (1967).

Cardiotonic steroids, and in particular ouabain, have been shown to inhibit the Na K pump in a highly specific and an essentially irreversible fashion in ouabain-sensitive cell types (Beauge and Adragna, 1971; Glynn and Karlish, 1975; Forbush, 1985). For Na K pump inhibition to occur, Hoffman (1966) demonstrated that strophanthidin must be applied to the cell externally, since the incorporation of strophanthidin into resealed red cell ghosts was seen to be ineffective on the Na efflux, thereby confirming the ouabain micro-injection studies in the squid giant axon of Caldwell and Keynes (1959). These studies indicate that the site for ouabain inhibition of the Na K pump is at the extracellular aspect of the plasma membrane. Although the absolute sensitivity for the Na K pump inhibition by ouabain varies with different tissues and certainly between species, the use of cardiac glycosides in determining Na K pump-mediated fluxes is of key importance (Willis and Ellory, 1983; Levenson, 1985).

Skou (1957) demonstrated that an ATPase of the crab nerve membrane requires not only Mg for activation, but also Na and K. Similar ATPase activities have also been shown to exist in human erythrocyte ghosts and part of the ATPase activity is inhibited by cardiac glycosides (Post, Merritt, Kingsolving and Albright, 1960; Dunham and Glynn, 1961) with half maximal concentration for the activation of the ATPase activity by Na and K being similar to those for the activation of flux by external K and internal Na. These studies are consistent with the hypothesis that the Na K ATPase is the molecular entity associated with the ouabain-sensitive Na and K transport. The mechanism of the Na K ATPase and the protein structure are reviewed by Jorgensen (1982), and can be modelled to a

series of operational steps of the Na K ATPase, which probably exists as a dimer in the membrane (see Kyte, 1981). The Na K pump has been demonstrated in almost all mammalian cells (for references see Schuurmans Steckhoven and Bonting, 1981).

As discussed above, ouabain is now regarded as a specific inhibitor of ion movements through the Na K pump. It is possible to attribute fluxes to being ouabain-sensitive (mediated through the Na K pump) or ouabain-insensitive, this latter representing ion fluxes through other membrane transport systems (see Beauge and Lew 1977).

From the early studies of Glynn (1956) it was apparent that the passive Na and K fluxes of human erythrocytes did not behave as expected for independent leak pathways down their electrochemical gradients, and since these observations much interest has been focussed upon these fluxes. Several passive Na K fluxes, not requiring ATP hydrolysis, are known to occur via the Na K pump, since different modes of ion translocation may be mediated by the Na K pump. This latter mediates substantial K K exchange and to a lesser extent Na Na exchange and K Na reversal fluxes (for references see Glynn and Karlish, 1975). It should be stressed that ascribing these fluxes to being mediated through the Na K pump arises from their sensitivity to cardiac-glycosides.

The passive, ouabain-insensitive K flux of the human erythrocyte exhibits a saturable dependence upon the external K in the presence of external Na (Glynn 1957; Garrahan and Glynn, 1967; Beauge and Adragna, 1971; Wiley and Cooper, 1974). The ouabain-resistant K influx of the human erythrocyte has been demonstrated to be sensitive to inhibition

by the diuretic furosemide and to the removal of the external Na; with Na-free conditions reducing the ouabain-insensitive K influx (Garrahan and Glynn, 1967; Beauge and Adragna, 1971; Wiley and Cooper, 1974). Thus the "passive" ouabain-insensitive K fluxes of the human erythrocyte may be subdivided into furosemide-sensitive, Na⁺-dependent and furosemide-insensitive, Na⁺-independent components (see above). Comparison of the ouabain-resistant, Na⁺-dependent K influx with the K efflux from erythrocytes under similar experimental conditions (for references see Beauge and Lew, 1977) strongly suggested a 1:1 K exchange which is Na dependent. However, this may not be considered to be an obligatory exchange flux since the K efflux sensitive to furosemide is independent of the external K (Wiley and Cooper, 1974).

With regard to the Na influx of human erythrocytes in ouabain-poisoned cells, a saturable component has been observed (Glynn, 1957; Wieth, 1970; Garrahan and Glynn, 1967; Wiley and Cooper, 1974). Studies by Sachs (1970) on the human erythrocyte's Na influx in media free of ouabain, but with sufficient external K to inhibit Na⁺ Na exchange through the Na⁺ pump, exhibited a saturable component of Na⁺ influx. These data indicate that the saturable component of the Na influx of the red cell may not be attributed to a ouabain-induced flux. The ouabain-insensitive Na⁺ influx of the erythrocyte may be considered to be independent of the intracellular Na concentration (see Beauge and Lew, 1977).

There is strong evidence to suggest that the ouabain-insensitive saturable component of the Na efflux is dependent upon the intracellular Na concentration of erythrocytes of human (Sachs, 1971; Dunn, 1973; Garay and Garrahan, 1973; Beauge, 1975) and rats (Beauge

and Ortiz, 1973). The dependence of the Na efflux upon the external Na concentration observed when using Mg as the Na substitute (see Beauge and Lew, 1977) was not seen when other Na replacements were used, e.g. choline (Garrahan and Glynn, 1967; Dunn 1973; Rettori and Lenoir, 1972). This reduction of the Na efflux resulting from the substitution of Mg for Na as the principal extracellular cation may be considered to be a direct inhibitory action of the external Mg rather than the effect of the removal of Na (Rettori and Lenoir, 1972). This effect of extracellular Mg upon the Na and K movements has been confirmed in the human erythrocyte by Ellory, Flatman and Stewart (1983).

In erythrocytes a significant proportion of the ouabain-insensitive Na fluxes were inhibited by the diuretics furosemide and ethacrynic acid (Lubowitz and Whittam, 1969; Dunn, 1970 and 1973; Sachs, 1971; Wiley and Cooper, 1974). This effect of furosemide and ethacrynic acid on the Na efflux was described by Dunn (1970) and Lubowitz and Whittam (1969) as a Na Na exchange. However, the data described above suggest that the unidirectional Na⁺ influx and efflux are not mediated through an obligatory exchange pathway (see discussion Wiley and Cooper, 1974). The dependence of the ouabain-resistant, diuretic-sensitive Na influx on the external K concentration as demonstrated by Wiley and Cooper (1974) are similar to the previous reports of the K stimulation of the ouabain-insensitive Na influx in the human erythrocyte by Garrahan and Glynn, (1967); Wieth, (1970); Sachs, (1971). These data reflect the Na dependence of the ouabain-resistant, diuretic-sensitive K influx in these cells (see above). Wiley and Cooper (1974) described the interdependence of the Na and K movements in the human erythrocyte as a "furosemide-sensitive Na K cotransport pathway".

This Na K "cotransport" in the human erythrocyte described by Wiley and Cooper (1974) has been substantiated by Dunham et al. (1980) and Chipperfield (1980, 1981 and 1985) who have also shown the diuretic-sensitive Na and K fluxes to be dependent upon Cl with only Br being effective as a partial replacement for Cl. Similar Cl dependences of the Na K "cotransport" have also been demonstrated in the avian erythrocyte (Schmidt and McManus, 1977 a-c; McManus and Schmidt, 1978). However, trans-membrane transport of Cl through the furosemide-sensitive Na K "cotransport" of the human erythrocyte has not been shown (see Ellory, Dunham, Logue and Stewart, 1982). In addition to the inhibition of this Na K Cl -dependent "cotransport" by the diuretic furosemide, this membrane transport system is also sensitive to other "loop" diuretics e.g. bumetanide and piretanide (Ellory and Stewart, 1982). Similar sensitivities to these diuretics have been observed in the avian erythrocyte (Palfrey, Feit and Greengard, 1980).

Diuretic-sensitive "cotransport" systems (Na + Cl ; K + Cl ; Na + K + Cl) have been described in many different tissue types; e.g. many epithelia, red cells (human, avian), thick ascending limb of the loop of Henle, Ehrlich ascites cells, nerve and muscle (for references see Ellory et al., 1982; Palfrey and Rao, 1983). These differences in the ion requirements of the various diuretic-sensitive "cotransport" systems may be partly due to the lack of comprehensive examination of the Na K and Cl dependences; e.g. the Cl transport in the thick ascending limb of the loop of Henle was first described by Burg and Green (1973) as being Na -dependent, but was later established to be a coupled Na + Cl transport (Greger, 1981) which was subsequently shown to be K -dependent (Greger and Schlatter,

1981). Stoichiometry of the Na K Cl for the diuretic-sensitive "cotransport" has been elucidated in avian erythrocytes (Haas, Schmidt and McManus, 1982), Ehrlich ascites cells (Geck, Pietrzyk, Burckhardt, Pfeiffer and Heinz, 1980; Geck, Heinz and Pfeiffer, 1981), and the MDCK cell line (McRoberts, Erlinger, Rindler and Saier, 1982) and may be considered to be $1\text{Na}^+ : 1\text{K}^+ : 2\text{Cl}^-$ "cotransport" systems. However, the stoichiometry does vary between tissue types e.g. the cation-coupled chloride influx of the squid axon, which is sensitive to furosemide, exhibits a complex stoichiometry of $2\text{Na}^+ : 1\text{K}^+ : 3\text{Cl}^-$ (Russell, 1983). In these cell types, the diuretic-sensitive Na K Cl⁻ "cotransport" system(s) may be considered to be electroneutral.

Caution must be exercised in using the furosemide sensitivity of ion fluxes as the sole criterion for ascribing these fluxes to a Na K Cl "cotransport" system(s), because furosemide may inhibit a variety of other ion transport systems, including the Na K pump but in particular the band 3 Cl/HCO₃ exchanger (Brazy and Gunn, 1976; Ellory et al., 1982; Palfrey and Rao, 1983). NaCl and KCl transport may be indirectly coupled through Na/H or K/H and Cl/HCO₃ exchangers as demonstrated in the small intestine (Liedtke and Hopfer, 1982) and the Amphiuma erythrocyte (see Cala, 1983 and 1985) but these would be sensitive to inhibition by amiloride and the disulphonic stilbenes. Thus the criteria of Cl dependence and inhibition by the diuretics bumetanide, piretanide and furosemide as described by Palfrey and Rao (1983) should be used in determining whether a diuretic-sensitive flux is mediated through a "cotransport" pathway, as discussed in chapters 3 and 5.

The "second pump" proposed by Hoffman and Kregenow (1966) to account for the ouabain-insensitive Na transport of the human red

cell was consolidated by Wiley and Cooper (1974) who demonstrated the Na K "cotransport" to be ouabain-insensitive but diuretic-sensitive. Several groups have suggested that the phosphorylated forms of the Na K ATPase present in the red cell membrane may mediate the ouabain-insensitive cation transport (for references, Beauge and Lew, 1977). However, a diuretic-sensitive Na K "cotransport" in either the presence or absence of ouabain could not be demonstrated in reconstituted proteoliposomes prepared from purified pig kidney Na K ATPase, although ouabain-sensitive fluxes were confirmed to be present (Karlisch, Ellory and Lew, 1981). These results support the rejection of the hypothesis that altered forms of the Na K pump mediate the Na K "cotransport".

The physiological function of the Na K Cl "cotransport" system(s) is well established for epithelia, and has been demonstrated to be involved in either net salt absorption or secretion (see Frizzell, Field and Schultz, 1979; Handler, 1983; Petersen and Maruyama, 1984). However, in non-epithelial cells the precise function is unclear, although the Na K Cl "cotransport" system(s) have been implicated in the maintenance and/or regulation of cell volume (see Cala, 1983 and 1985). A detailed review will be presented in chapters 3 and 5.

Footnote:

The term diuretic-sensitive in relation to the Na K Cl "cotransport" system(s) indicates the sensitivity to the three diuretics, bumetanide, piretanide and furosemide.

The general term Na K Cl "cotransport" will be used in this present work when the stoichiometry of the diuretic-sensitive "cotransport" is not specified.

CHAPTER 2

MATERIALS AND METHODS.

MATERIALS

Cell culture:

Tissue culture grade materials were purchased from either Flow Laboratories (P.O. Box 17, 2nd Avenue, Industrial Estate, Irvine, Ayrshire), or Gibco Ltd. (Trident House, P.O. Box 35, Renfrew Road, Paisley, Renfrewshire). Disposable plastic ware was supplied by Sterlin Ltd. (Fletham, Middlesex, TW14 8BR) and Nunclon (through Gibco Ltd.).

Cultured cell lines used in this present work were: (a) Madin Darby Canine Kidney (MDCK), serial passage No. 67-76, source Flow Laboratories, 1979; (b) HeLa, supplied by Flow Laboratories, 1974.

Radiochemicals:

Radionucleotides ^{22}Na , ^{86}Rb , ^{36}Cl , 5,5-dimethyl (2- ^{14}C) oxazolidine-2,4-dione (DMO), 3-o-methyl-D-(^3H)glucose and (^{14}C)methyl triphenyl phosphonium iodide (TPMP) were purchased from Amersham International p.l.c. (Amersham, Buckinghamshire, HP7 9LL). However, ^{43}K was obtained from the Medical Research Council's Cyclotron Unit (Hammersmith Hospital, Ducane Road, London W12 0HS).

Chemicals and Biochemicals:

Sigma Chemical Company Ltd. (Poole, Dorset, BH17 7NH) supplied the cardiac glycoside Ouabain, Calcium ionophore A23187, Potassium ionophore Valinomycin, and the adrenergic agonist Adrenalin. The plant

diterpene Forskolin was purchased from Calibiochem (Thorpe House, King's street, Hereford). Molecular Probes (24750 Lawrence Road, Junction City, Oregon, U.S.A.) supplied the carbocyanine dye DiSC₂(5).

The "loop" diuretics furosemide and piretanide were gifts of Hoechst Pharmaceuticals Ltd. Bumetanide was given by Leo Laboratories Ltd.. Stock solutions of the diuretics (10^{-2} M) were in Tris buffer (10^{-2} M). [³H] bumetanide was a gift of Hoffman-La Roche (Nutley, New Jersey, U.S.A.) and was finally dissolved in ethanol and used directly in Krebs solution in uptake studies.

All other chemicals used were either of Analar grade or the best possible grade available.

METHODS

Cell culture:

MDCK (serial passage No. 67-76) and HeLa were maintained as frozen stocks (below -70°C) in a liquid nitrogen refrigerator. To prevent cell damage via ice crystal formation, the cryopreservative dimethylsulphoxide (DMSO (10% v/v)) was included in cell samples destined for frozen stock. Frozen cells taken from store were warmed rapidly to 37°C and dispersed into glass Roux bottles (sterile) containing culture media. The Roux bottles were then sealed, after gassing with 95% air, 5% CO_2 , and placed in a 37°C room. To remove the DMSO, cells were subjected to a media change 24 hours after thawing and allowed to grow for 5-6 days before being used in experimentation.

Propagation of cells:

After a 5-6 day interval, cells were subcultured according to the following protocol. The media was poured off the culture Roux, the monolayer of cells remaining then being washed with 3ml of Ca and Mg free Earle's Balanced Salt Solution (EBSS), containing trypsin at 0.025%, plus 2mM ethylenediaminetetracetic acid (EDTA) for MDCK. The wash solution was then poured off to be replaced by 5ml of the appropriate trypsin solution. After a suitable incubation period in order to allow the trypsin and the low divalent-cation conditions to break the cell-cell and cell-glass interactions, the monolayer of cells were seen to have become detached from the culture Roux. Neutralisation of the trypsin was achieved by dilution with complete culture media. A single cell suspension was prepared by repeatedly

blasting the cells through a wide bore syringe needle attached to a 10ml sterile syringe. Cell number and volume were determined using a Coulter Counter, model ZF, linked to a Coulter Channelyzer, model C-1000, (Boardman, Huett, Lamb, Newton and Polson, 1974).

To maintain cell lines, continuation Roux seeded at approximately 5×10^6 cells per Roux and containing 150ml complete media appropriate for the cell type were prepared and incubated for 5-6 days. Experiments were performed on cells grown on plastic culture dishes (3,5 and 9cm in diameter or 12 well multi-well plates) after 3-5 days growth depending upon the seeding density of the cells.

Cell number and volume determination:

Cell number and volume were determined by electronic counting and sizing using a Coulter Counter, model ZF, linked to the Coulter Channelyzer, model C-1000, and interfaced to a Sharp personal computer, model MZ 80 K (Boardman et al., 1974). The Coulter Counter was periodically calibrated with commercially prepared standards of known volume, either 4C fixed red cells or Latex microspheres. The Coulter Counter performs these determinations by forcing cells suspended in an electrically conductive liquid through a small aperture with immersion electrodes on either side between which a constant current is maintained. As cells pass through the aperture, a change in the resistance produces a voltage pulse of short duration, the magnitude of which is proportional to the cell size, with a series of pulses being electronically scaled and counted. Data from the Coulter Counter are passed to the channelyzer and plotted, as the cumulative frequency versus the cell size. The resulting curve can then be integrated using the Sharp computer to provide a measure of

the mean cell volume (Boardman et al., 1974) of the analysed sample. The use of trypsinized cell suspensions for electronic cell sizing has been validated against the method of Kletzien, Pariza, Becker and Potter (1975) for determining the intracellular space which utilises the non-metabolisable sugar 3-O-methylglucose. Comparison of the cell water (μl) determined by 3-O-methylglucose equilibration or by electronic cell sizing were in good agreement for both the HeLa and MDCK cell lines (Simmons, 1984; Tivey et al., 1985; see also chapter 5).

Influx determination:

Potassium influx was measured on sub-confluent layers of cells grown on plastic petri-dishes. Sub-confluent layers of cells were used to enhance the access to the cell surface adjacent to the petri-dish. This is particularly important for cell cultures of epithelial origin (MDCK) which retain many epithelial features, e.g. cell-cell tight junctions sealing the apical surface and lateral spaces (Simmons, 1982). An illustration of this problem of access can be seen in alanine uptake by renal epithelial cells (LLC PK1), since the uptake is only associated with cells at the border of confluent monolayers of cells (Sepulveda and Pearson, 1984), alanine uptake being directly visualised by autoradiography.

To determine the influx of potassium, the isotope ^{86}Rb was used, this isotope being equivalent to ^{42}K (Aiton, Chipperfield, Lamb, Ogden and Simmons, 1981; Aiton, Brown, Ogden and Simmons, 1982; Brown, 1983). Prior to experimentation cells were pre-washed in a standard Krebs solution (pre-incubation for 3 hours if influx measurements were

to be directly compared with efflux measurements). ^{86}Rb influxes were determined over a 5 minute interval in both MDCK and HeLa cells, thereby ensuring that influx measurements were made upon the linear portion of the ^{86}Rb accumulation curves for both cell types (Aiton et al., 1981). Termination of ^{86}Rb influx was elicited by x5 (>15secs.) washes with Krebs solution at 4°C . This wash protocol was checked by the loss of the extracellular space marker ^{14}C inulin, and was shown to remove all the ^{14}C inulin from both HeLa and MDCK cell cultures (Aiton et al., 1981 and 1982). Cells were removed from the plates by trypsinization and a single cell suspension prepared by blasting the cells through a wide bore syringe needle. Samples of the cell suspension were taken for cell number, volume and ^{86}Rb determination. Blanks were prepared under identical conditions except that culture dishes were devoid of cells (Boardman et al., 1974; Aiton and Simmons, 1983). The typical retention of radioactivity was 0.001% or less of the loading solution, indicating an adequate wash protocol.

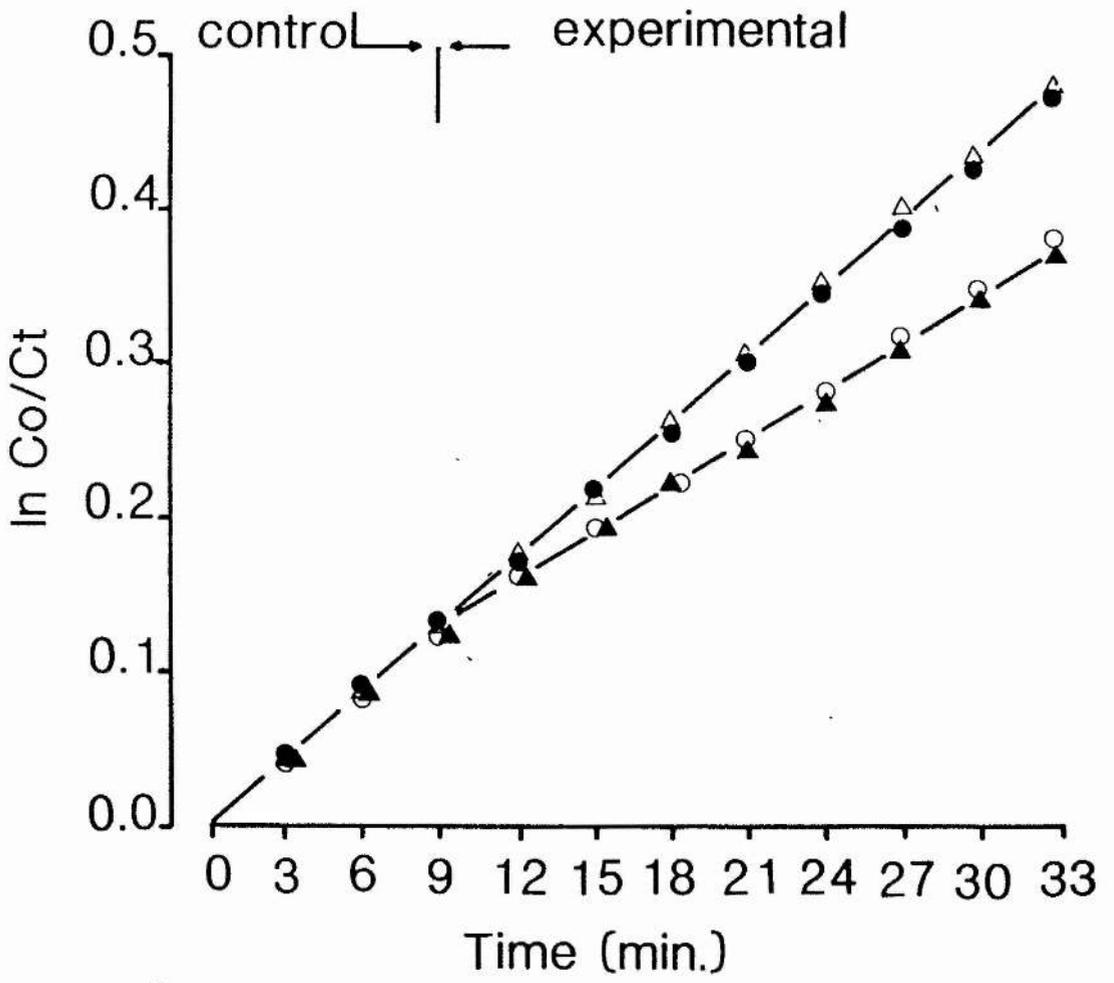
The specific activities of the ^{86}Rb influx solutions were determined from the radioactivity of 100 μl aliquots of the ^{86}Rb flux solutions and the external K concentration measured by flame photometry. Influx results were expressed in relation to 10^6 cells (nmol/ 10^6 cells.min.).

^{86}Rb , ^{22}Na and ^{36}Cl efflux determination:

K, Na and Cl efflux measurements were performed upon sub-confluent cells (see Influx determination). Cells were pre-loaded with the appropriate isotope for 3 hours at 37°C in tris-buffered Krebs solution. ^{86}Rb was used as a tracer for K efflux, which has been judged to be equivalent to ^{43}K efflux in the presence or absence of 0.1 mM furosemide (figure 2.1) and confirms previous reports by Aiton et al. (1981, 1982). To initiate the experiments plates were washed

Figure 2.1

Validation of the use of ^{86}Rb as a measure for K efflux from HeLa cells grown on petri-dishes. Simultaneous determinations of ^{43}K and ^{86}Rb efflux were made as described in text. $\text{Log}_e \text{Co}$ (total cpm in the cells at time zero minutes) / Ct (cpm remaining in the cells at time t minutes) dependence upon time for control ^{43}K and ^{86}Rb (Δ, \bullet) and in the presence of 0.1 mM furosemide (\blacktriangle, \circ) were equivalent. Data are the mean of 3 determinations, errors are omitted for clarity.



rapidly to remove extracellular isotope loading. Effluxes of ^{86}Rb , ^{22}Na and ^{36}Cl were determined by successive addition and collection of aliquots of Krebs solution and the residual radioactivity was collected by a distilled water extraction of the cells. The fractional rate of efflux was calculated as described by Brown and Simmons (1982).

Determination of radioactivity:

^{86}Rb activity (single isotope) was determined by a Packard Tri-carb liquid scintillation spectrometer (model 3255) by the Cerenkov method, and by the B emission of ^{86}Rb in dual isotope experiments, where the second isotope is ^{43}K , in scintillation cocktail for aqueous media (Cocktail-T, Fison Ltd.). ^{22}Na and ^{36}Cl activities were determined by B emission in the liquid scintillation fluid, Cocktail-T (Fisons Ltd.). Activity of ^{43}K samples was determined in a Packard Autogamma counter, corrected for ^{86}Rb activity after the decay of ^{43}K . Single isotopes were determined at optimal gain with wide window settings (no discrimination). All samples, including standards and blanks were prepared identically to eliminate variation due to colour or chemical quenching.

Measurement of Intracellular Na and K Ion Contents.

Total intracellular Na and K of cells were determined in double distilled water extracts, after x5 washes in Na- and K-free isotonic sorbitol solution, pH 7.4 at 4 °C (for verification of wash protocol see influx determination). Na and K concentrations (range 0 - 0.1 mEq/l) of the double distilled water extracts from cells were measured against a combined Na and K standard (0.1 mEq/l) by flame

photometry (Boardman et al., 1974; Aiton and Simmons, 1983). Cell numbers were determined from identical plates in the same cell batch, see above.

Intracellular Chloride:

Cellular Cl was determined isotopically using ^{36}Cl (Cotlove, 1963a) or non-isotopically (Cotlove, 1963b), these two methods being used because the chemical sensitivity of non-isotopic methods available are low. Cells equilibrated with ^{36}Cl for 3 hours at 37°C were washed rapidly with Krebs solution at 4°C (for verification of wash protocol see influx determination); after trypsinization, aliquots were taken for cell number and ^{36}Cl activity determination. Non-isotopic determination of intracellular Cl in either water or acid extracts (0.4N HNO_3 and 40% glacial acetic acid) was carried out using a Buchler Digital Chloridometer. Pooled cell extracts (3 plates, 0.5 ml/plate) were centrifuged to remove cell debris. One ml aliquots were then diluted in 3 ml of distilled water and titrated.

The operating principle of the Buchler Digital Chloridometer is the combination of Ag and Cl in a quantitative reaction forming insoluble AgCl. The instrument is electronically calibrated to 100 mEq/l Cl solutions, but was checked against a 100 mEq/l Cl solution before each operation. The reaction is carried out at a constant rate by the passage of fixed direct current between a pair of silver electrodes immersed in an acid solution (0.1N HNO_3 + 10% glacial acetic acid). As the equilibrium point is reached, current flowing between a single pair of reference electrodes increases. At a preset indicator current, the instrument stops its incremental count and generation of silver ions. Since the generator current is constant,

the total time is directly proportional to the amount of Cl present in a sample vial and the meter displays the relative time in terms of mEq/l Cl. Errors in the determination of Cl are commonly due to inaccurate sample volumes. However, erroneous readings will be given in the presence of iodide, bromide or high protein (Cotlove, Trantham and Bowman, 1958).

Uptake of 5,5-dimethyl[2-¹⁴C] oxazolidine-2,4-dione (DMO):

The intracellular pH may be determined directly by the distribution of the weak acid 5,5-dimethyl -oxazolidine -2,4-dione (DMO) labelled with ¹⁴C between the intracellular and extracellular water. Assuming the membrane to be permeable to the unionized form of the acid, then at steady state the intracellular pH is given by:

$$pH_i = pK_a + \log(T_i/T_e (10^{pH_e - pK_a} + 1) - 1)$$

where pH_i = intracellular pH

pH_e = extracellular pH

T_i = HA + A (total intracellular DMO)

T_e = HA + A (total extracellular DMO)

pKa of DMO = 6.3 (Nuhm and Woitkowitz, 1977)

The protocol for DMO uptake was a modification of that reported by Rindler and Saier (1981). Cells were incubated for 30 minutes in Krebs solution containing 0.2 μ Ci/ml [¹⁴C] DMO and the incubation period was terminated by x5 rapid washes with 4° C Krebs solution. The ¹⁴C activity of trypsinized cell suspensions was determined by liquid

scintillation counting in Cocktail-T (Fisons Ltd.), see determination of radioactivity. Cell numbers were determined from identical plates of the same cell batch (see above). Cellular water was determined by the method reported by Kletzien et al., (1975), which utilises the non-metabolizable sugar, 3-O-methyl-D- [1-³H]glucose as an intracellular space marker (see also Simmons, 1984).

[¹⁴C] Methyl Triphenyl Phosphonium iodide (TPMP) uptake: measurement of membrane potential.

The determination of the membrane potential by means of the distribution of permeant lipophilic ion (TPMP) is based on the passive equilibration of this ion with the potential difference across the membrane. In steady-state the ion accumulation should be a function of the transmembrane potential.

The protocol for TPMP uptake was a modification of that reported by Deutsch, Holian, Holian, Daniele and Wilson (1979). The accumulation of [¹⁴C] TPMP (1.5 - 10 μM) in the presence or absence of tetraphenylboron, TPB, (1 - 3 μM) was studied over a 30 minute period. The uptake of TPMP was terminated by x5 washes in 4° C Krebs solution. Cellular water content was determined by electronic cell sizing (Boardman et al., 1974) and by the use of intracellular space marker 3-O-methyl-D-[1-³H]-glucose (Kletzien et al., 1975; Simmons, 1984).

Further details on the theory of TPMP usage as a monitor of membrane potential are given in the experimental section of Cheng, Haspel, Vallano, Osotimehin and Sonenberg (1980).

Determination of intracellular ATP:

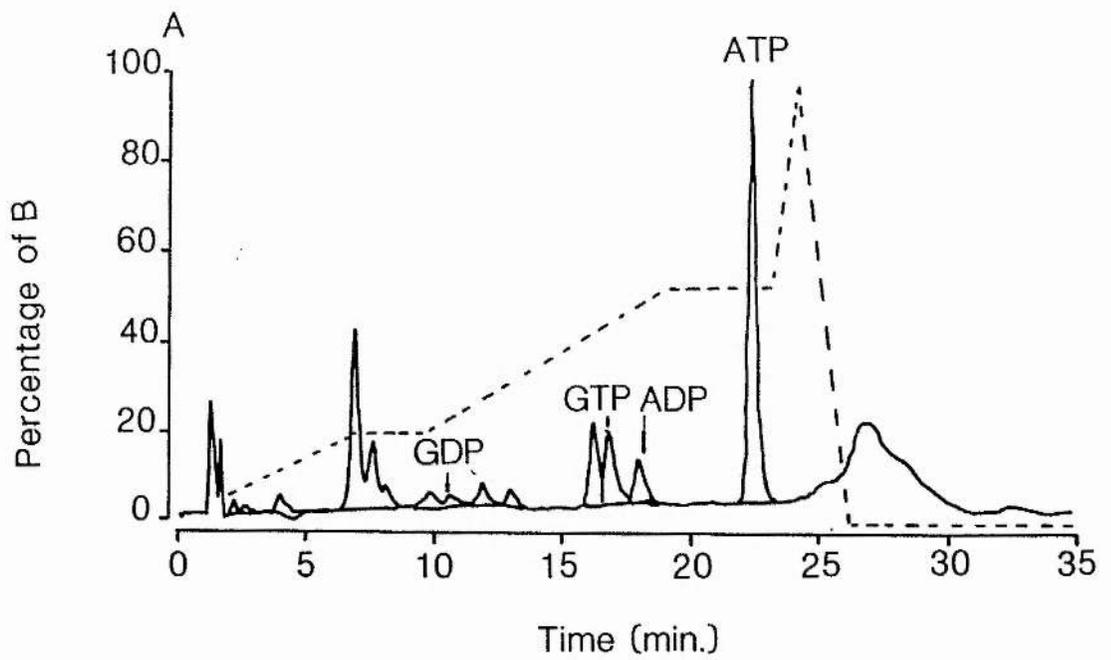
The cellular ATP, ADP, GTP and GDP were determined by reversed phase ion-pair high performance liquid chromatography as described by Aiton, Cramb and Wood (1985). To summarize, chemically-bonded silica support (e.g. O.DS plus aqueous phase) contained butyl $4N^+$. The solutes form ion-pairs with the butyl $4N^+$, whose strength depends upon their degree of ionisation. Solutes forming weak ion-pairs (AMP) are eluted before those solutes forming stronger ion-pairs (ATP). It was necessary to apply a gradient system, with the progressive introduction of an organic modifier (Methanol) into the mobile phase. The ion-pairs and the organic modifier compete for the absorption sites, with the result that the equilibrium of the ion-pairs will be shifted to the mobile phase, thereby effecting elution of the solutes (Pryde and Gilbert, 1979). Example chromatograms for HeLa and MDCK cells are presented in figure 2.2 a,b.

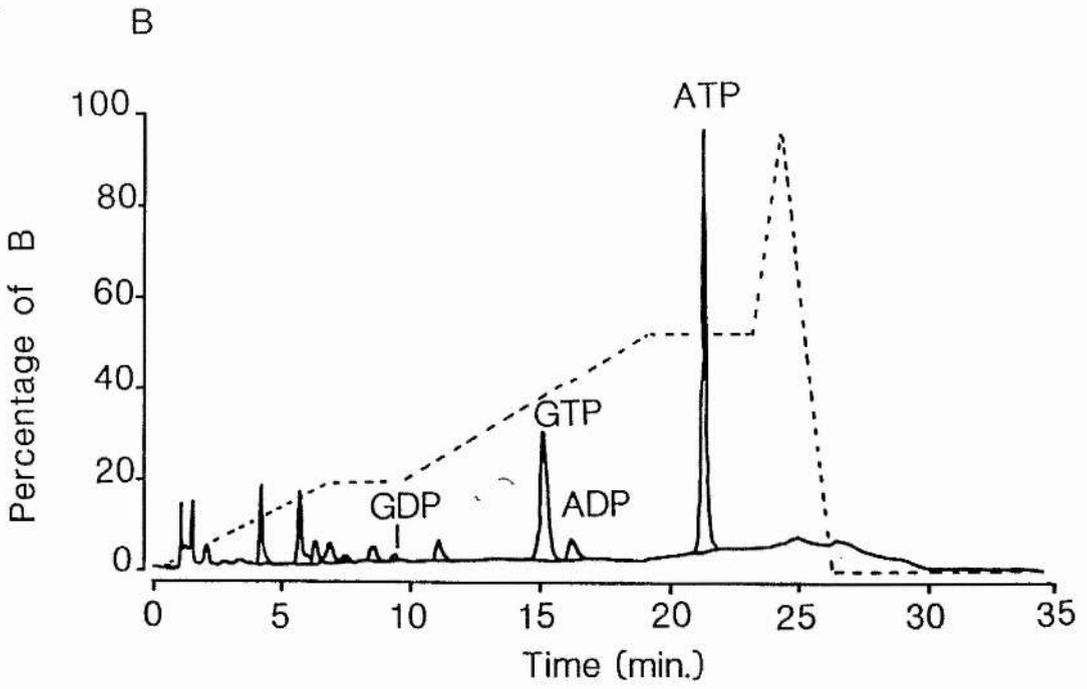
Extraction of biological samples was carried out according to the following protocol:

- a) Incubation in the required conditions i.e Krebs solution of varying composition.
- b) Removal of the incubation media.
- c) Addition of 0.5 ml 6% perchloric acid (PCA) and then incubation at $4^{\circ}C$ for 30 minutes.
- d) Removal of 0.4 ml into tubes containing 65 μ l K_2CO_3 , which were then vortexed
- e) Centrifugation of samples at 3000 rpm for 15 minutes at $4^{\circ}C$ and decantation into new tubes for frozen storage until analysis by hplc (see above).

Figure 2.2 a,b

Control chromatograms for the separation of nucleotides in cell extracts of (a) HeLa and (b) MDCK cells. Positions of ATP, ADP, GTP and GDP are as indicated. Peaks are normalised to the largest and expressed in percentages and represent intracellular nucleotide concentrations in the HeLa and MDCK cells of 4.9 ± 0.2 , 6.8 ± 0.5 (ATP, mmol/l); 0.7 ± 0.1 , 0.6 ± 0.1 (ADP, mmol/l); 8.4 ± 0.6 , 23.1 ± 1.6 (GTP, peak area ($\times 10^6$)/l); 2.0 ± 0.1 , 1.1 ± 0.2 (GDP, peak area ($\times 10^6$)/l) respectively. Dashed line indicates the percent addition of the organic modifier, methanol, to the eluent.





The retention time for ATP, ADP, GTP and GDP were determined by spiking samples with the appropriate nucleotide and were as indicated for HeLa and MDCK cell extracts (figure 2.2 a,b, respectively). Absolute intracellular concentrations were determined from calibration curves for each nucleotide.

Measurement of intracellular cAMP:

MDCK cells were incubated for 5 minutes under isotonic or hypertonic conditions in the presence or absence of 10 μ M Forskolin. Incubations were terminated by the rapid removal of the incubation media followed by submersion in liquid nitrogen. The extraction of intracellular cAMP was carried out according to the method of Rugg and Simmons (1984), i.e. cells were extracted in 0.25 ml 0.2 M HCl for 10 minutes at 4 $^{\circ}$ C, neutralized by 0.2 M NaOH and centrifuged. The supernatant was assayed for cAMP using a competitive binding protein assay (Brown, Albano, Ekins and Sghezi, 1971) and cAMP was expressed in pmole/mg protein. The cell protein was determined in cells of the same batch according to the method of Lowry, Rosebrough, Farr and Randall (1957) using bovine serum albumin as a standard.

[3 H] bumetanide uptake:

Measurements of [3 H] bumetanide were made essentially as described by Rugg, Simmons and Tivey (1985 a,b). [3 H] bumetanide uptake was determined at equilibrium, after a standard incubation time of 15 minutes. To terminate uptake, cultures were repeatedly washed (x5) in 4 $^{\circ}$ C Krebs solution (pH 7.4) to remove extracellular isotope (see influx determination for verification of wash protocol). Remaining isotope was then extracted by the use of trypsin (0.1% v/v) plus 2mM

EDTA in a Ca and Mg free Earle's saline solution. [^3H] activity was measured using a scintillation cocktail for aqueous media (Cocktail T, Fisons Ltd.). All samples, including standards and blanks, were prepared identically to eliminate variation due to chemical or colour quenching. In parallel measurements, the mean cell number and volume were determined from single cell suspensions prepared by trypsinisation (see cell number and volume determination).

Solutions:

Experiments were performed, except where otherwise stated, in a standard Krebs solution containing (in mM): 137, NaCl; 5.4, KCl; 2.8, CaCl_2 ; 1.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3, NaH_2PO_4 ; 12, HCl; 14, Tris base; 11, glucose; supplemented with either 1.0% v/v donor horse serum in MDCK cell experiments or 1.0% v/v new born calf serum in HeLa cell experiments. However, for the [^3H] bumetanide uptake studies serum was 0.02% v/v, this lower concentration being necessary due to the serum binding of "loop" diuretics (Olsen, 1977). The pH of the solutions was 7.4 at 37° C.

Statistics:

Data are represented as the mean \pm the standard deviation (S.D.) and/or the standard error (S.E.). Tests for significant differences between mean values were made by a two-tailed Student's t-test (unpaired mean solution). Kinetic data were fitted to Michaelis-Menten kinetics by linear regression of a Eadie-Hofstee transformation, followed by non-linear regression using the Gauss-Newton approximation to produce the best fit with minimum values for the residual sum of squares (Knack and Rohm, 1981). Estimates of kinetic parameters were

also obtained for the Hill equation by using a linear regression of a form of the Eadie-Hofstree equation, where H is evaluated by the minimum sum of squares from a range of initial values of H (Knack and Rohm, 1981).

CHAPTER 3

OUABAIN-INSENSITIVE K TRANSPORT

INTRODUCTION.

A proportion of the ouabain-insensitive plasma membrane K transport of the HeLa and MDCK cultured cell lines has been shown by Aiton et al., (1981 and 1982), McRoberts et al., (1982), and Rindler et al., (1982) to be inhibited by the "loop" diuretic furosemide. This action of furosemide is similar to that observed for inhibition of (Na + K) "cotransport", which is Cl-dependent in human red blood cells (Wiley and Cooper, 1974; Dunham et al., 1980; Chipperfield, 1980, 1981). Furosemide has also been shown to inhibit the passive, linked Na K and Cl fluxes in a variety of other cell types, such as Ehrlich ascites cells (Geck et al., 1980), smooth muscle (Brading, 1979), shark rectal glands (Eveloff, Kinne, Kinne-Saffran, Murer, Silva, Epstein, Stoff and Kinter, 1978), avian erythrocytes (Palfrey et al., 1980; Palfrey and Rao, 1983) and the renal epithelial cell line LLC-PK1 (Brown and Murer, 1985).

Does this action of furosemide on diverse cell types represent inhibition of a common transport system? The Na K Cl "cotransport" system in many cell types possesses several unique features which may be used to demonstrate the existence of such a transport system (Palfrey and Rao, 1983). Inhibition by "loop" diuretics, furosemide, piretanide and bumetanide and their related compounds, occurs at relatively high affinities (Palfrey and Greengard, 1981; Ellory and Stewart, 1982; Palfrey and Rao, 1983). The greater potency of bumetanide over furosemide (50:1) is characteristic and has been noted in other cell types, such as the avian erythrocyte (Palfrey and Rao, 1983), the thick ascending limb of the loop of Henle (Schlatter, Greger and Weidtko, 1983), and human erythrocytes (Ellory and Stewart,

1982). However, furosemide is known to inhibit other membrane transport systems, e.g. the Na K ATPase (Sachs, 1971; Wiley and Cooper, 1974; Jorgensen, 1980), the band 3 Cl/exchanger of red blood cells (Brazy and Gunn, 1976), and the N-ethyl maleimide (NEM) induced K transport of the erythrocyte (Ellory et al., 1982) but this is at higher concentration (50% inhibition at 2×10^{-4} M, 2×10^{-3} M) than is usual for the inhibition of Na K Cl "cotransport". Separation of the diuretic-sensitive flux components from other transports dependent on anion permeability, or linked fluxes including Na:H, Cl:HCO₃ exchange is possible by their insensitivity to amiloride and the disulphonic stilbenes SITS and DIDS (Aiton et al., 1982; Geck et al., 1980; Chipperfield, 1980; Dunham et al., 1980). The anion specificity for diuretic-sensitive K flux or (Na + K) transport systems observed in various preparations is narrow, only Br having the ability to partially substitute for Cl, whilst nitrate, iodide, acetate, gluconate and methyl sulphate do not substitute or are not transported (Aiton et al., 1981, 1982; Dunham et al., 1980; Palfrey and Greengard, 1981; Geck et al., 1980; McRoberts et al., 1982; Solomon, Silva, Stevens, Epstein, Stoff, Spokes, and Epstein, 1977). These characteristics are sufficient to distinguish Na K Cl cotransport system(s) from other cation and anion membrane transport pathways in most cell types.

Net ion movement mediated by Na K Cl "cotransport" system(s) reflects the balance between inwardly and outwardly directed unidirectional diuretic-sensitive ion transport (Duhm and Goebel, 1984 a,b). Thus net ion movements and apparent ion exchanges are complementary functions of Na K Cl "cotransport" system(s) (Duhm and Goebel, 1984 a,b). The driving forces for net movement via "cotransport" of Na K Cl through the Na K Cl "cotransport" system(s)

is determined by the sum of the chemical gradients for all three participating ions. A role for the Na K Cl "cotransport" system(s) in K homeostasis, in vivo, has been proposed with outwardly and inwardly directed net ion movements, predicted in human and rat erythrocytes respectively (Duhm and Goebel, 1984 a,b). Therefore, the overall direction of net flux is dependent upon the ionic environment.

The primary purpose of this chapter is to define the basic properties of the ouabain-insensitive K flux on HeLa and MDCK cells; to examine the "loop" diuretic-sensitive K transport; and to determine what relationship, if any, exists between these fluxes and the Na + K + Cl "cotransport"-indicated fluxes in other cell types.

RESULTS:

K (^{86}Rb) influx:

The actions of (1 mM) ouabain and (0.1 mM) "loop" diuretic (i.e. furosemide or bumetanide) upon the K (^{86}Rb) influx of the HeLa and MDCK cell lines are shown in table 3.1. Ouabain at 1 mM in these ouabain-sensitive cells (Baker and Willis, 1972; Lamb, Ogden and Simmons, 1981; Aiton et al., 1981 and 1982) will give instantaneous and complete inhibition of the Na K ATPase and therefore reduction of the K (^{86}Rb) influx (the ouabain-sensitive component). As discussed in the introduction, the "loop" diuretics furosemide, piretanide and bumetanide inhibit the Na⁺ K Cl "cotransport" system(s) of a variety of cell types. The inclusion of 0.1 mM bumetanide completely inhibits the Na K Cl "cotransport" of the HeLa and MDCK cells (see below, diuretic potency), significantly reducing the K (^{86}Rb) influx ($P < 0.001$) in these cells (the diuretic-sensitive component).

If the pharmacological agents ouabain and "loop" diuretics have separate and unique actions, it might be expected that their effect would be additive when applied singly and then in conjunction with each other. This is of course dependent on little change occurring in the chemical driving force due to changes in intracellular ionic composition. For the measurements shown in table 3.1 an estimate of the ouabain-sensitive flux may be made in the presence or absence of "loop" diuretic or conversely the "loop"-diuretic sensitive flux may be estimated in the presence or absence of ouabain. Table 3.1 shows that ouabain and "loop" diuretics, when added separately, reduce the K influx, and when applied in conjunction, further reduce the influx in

Table 3.1 K^+ ($^{86}\text{Rb}^+$) influx determined in HeLa and MDCK cell lines over a 5 minute incubation period as described in the methods: 1 mM ouabain and 0.1 mM bumetanide were added as indicated (a)

Cell Type	K^+ ($^{86}\text{Rb}^+$) influx (nmol/ 10^6 cells.min)				Ouabain-sensitive	Bumetanide-sensitive
	(1) Total	(2) + Ouabain	(3) + Ouabain + Bumetanide	(4) + Bumetanide		
HeLa	12.27 \pm 0.10	7.63 \pm 0.32	3.00 \pm 0.05	6.15 \pm 0.75	(1-2) 4.64 \pm 0.35	(1-4) 6.12 \pm 0.66
					(4-3) 3.13 \pm 0.70 ^b	(2-3) 4.61 \pm 0.36 ^c
MDCK	10.11 \pm 0.76	5.96 \pm 0.62	0.57 \pm 0.20	4.27 \pm 0.04	(1-2) 4.14 \pm 0.43	(1-4) 5.83 \pm 0.74
					(4-3) 3.70 \pm 0.24 ^{ns}	(2-3) 5.39 \pm 0.81 ^{ns}

(a) Data are the pooled means of 3 experiments, where 3 replicates were made for each datum. Errors are expressed \pm SD. Significant differences from (1-2) for ouabain-sensitive and (1-4) for bumetanide-sensitive components of K^+ influx by two-tailed Student's t-test. ns = not significant.

- (b) 0.05 < p < 0.1
- (c) p < 0.05

both HeLa and MDCK cells.

The actions of 1 mM ouabain 0.1 mM bumetanide were approximately additive, suggesting independent actions (table 3.1). In the bulk of the experiments performed (table 3.2), (62% (HeLa) - 69% (MDCK)), the ouabain-sensitive component of K (^{86}Rb) influx determined in the presence of 0.1 mM "loop" diuretic did not significantly differ from that determined in the absence of 0.1 mM "loop" diuretic (table 3.2). However, in 29% (HeLa) - 15.4% (MDCK) of experiments, the ouabain-sensitive K (^{86}Rb) influx was significantly ($P < 0.05 - 0.001$) greater in the absence of diuretic, and in 8.1% (HeLa) - 15.4% (MDCK) of experiments, this component of the K (^{86}Rb) influx was significantly greater ($P < 0.05 - 0.001$) in the presence of 0.1 mM bumetanide. For the diuretic-sensitive component of K (^{86}Rb) influx in the HeLa and MDCK cells (table 3.2), 73% (HeLa) - 65% (MDCK) of experiments determining the diuretic-sensitive K (^{86}Rb) influx, the presence or absence of 1mM ouabain did not significantly affect the magnitude of this component, although for 19% (HeLa and MDCK) of experiments the diuretic-sensitive component was markedly greater ($P < 0.05 - 0.001$) in the absence of ouabain, than in its presence. In 8% (HeLa) - 15% (MDCK) of experiments, this trend was reversed with the diuretic-sensitive component being greater in the presence of ouabain ($P < 0.05 - 0.001$). It has previously been noted that prolonged incubation in the presence of ouabain stimulates the magnitude of the diuretic-sensitive component (Aiton, et al., 1982; Aiton and Simmons, 1983). This cannot explain why the ouabain-sensitive component may be larger in the absence of "loop" diuretic than in its presence. These data may indicate that the Na K Cl "cotransport" system mediates a small net (Na) ion movement. The activity of the Na K ATPase is sensitive to changes in internal Na concentration and in human

Table 3.2 Additivity of ouabain-sensitive K^+ ($^{86}\text{Rb}^+$) influx determined in the presence (B-OB) or absence (T-O) of 0.1 mM diuretic and the diuretic-sensitive K^+ ($^{86}\text{Rb}^+$) influx determined in the presence (O-OB) or absence (T-B) of 1.0 mM ouabain

K^+ influx	Cell type	Number of experiments	Percentage of total number of experiments			
			(T-O) = (B-OB)	(T-O) \gg (B-OB) ^a	(T-O) \ll (B-OB) ^a	
Ouabain-sensitive	HeLa	37	62.1%	29.8%	8.1%	
	MDCK	26	69.2%	15.4%	15.4%	
Diuretic-sensitive	HeLa	37	(T-B) 73.0%	(O-OB) ^a 19.0%	(T-B) 8.0%	(O-OB) ^a
	MDCK	26	65.4%	19.2%	15.4%	

(a) Significant difference between the two determinations of ouabain-sensitive and diuretic-sensitive K^+ ($^{86}\text{Rb}^+$) influx. $p < 0.05-0.001$.

erythrocytes half maximal concentration for activation of the Na K pump by intracellular Na being near 20mM in K containing media, (Sachs, 1977). The intracellular Na concentration of HeLa is 14 mM (table 3.6) and MDCK 25 mM (Aiton et al., 1982). Therefore, if "loop" diuretic-sensitive Na K Cl "cotransport" mediates a net uptake of Na, bumetanide should decrease the K flux through the Na K ATPase due to a decrease in intracellular Na concentration. Conversely, if the Na K Cl "cotransport" system was mediating a net loss of Na, bumetanide under these conditions should increase the ouabain-sensitive component of the K flux due to the increased intracellular Na. That the majority of experiments demonstrate additivity between ouabain and "loop" diuretic action on K influx is evidence that little or no net influx is mediated via the "cotransport" flux.

An additional problem to the interpretation advanced above, and already alluded to, is the time-dependency of the ouabain stimulation of the diuretic-sensitive K transport of HeLa and MDCK cells (Aiton et al., 1982; Aiton and Simmons, 1983). Table 3.3 shows the effect of an extended incubation (15 minutes) in the presence of 1 mM ouabain before the determination of the K (^{86}Rb) influx of the MDCK cells. The apparent ouabain-sensitive K (^{86}Rb) influx was not detectable when calculated by subtraction of the K (^{86}Rb) influx observed in cells pre-treated with ouabain from control values. This is due to the increased ouabain-insensitive K (^{86}Rb) influx of the cells, which is predominantly diuretic-sensitive, and is in agreement with Aiton and Simmons (1983). The ouabain stimulation of the diuretic-sensitive K transport was also observed in the K (^{86}Rb) efflux of both the HeLa and MDCK cells (table 3.4). This stimulation was progressive over the time course of the K (^{86}Rb) efflux and was totally inhibited by furosemide at 0.1mM, these results being in agreement with Aiton and Simmons (1983) and Aiton et al. (1982). It should be noted, that at

Table 3.3 Effect of 15 minute preincubation in Krebs in the presence or absence of

1.0 mM ouabain upon the K^+ ($^{86}Rb^+$) fluxes in MDCK cells (a)

Preincubation	K^+ ($^{86}Rb^+$) influx nmol/ 10^6 cells.min	Ouabain- sensitive	Diuretic- sensitive
(1) Krebs	13.21 \pm 0.84	-	-
(2) + Ouabain	7.33 \pm 0.75 ^b	1-2 5.88 \pm 0.64	-
(3) + Bumetanide	0.90 \pm 0.11 ^b	-	2-3 6.43 \pm 0.44
(4) + Bumetanide	6.32 \pm 0.49 ^b	4-3 5.42 \pm 0.28	1-4 6.89 \pm 0.54
(5) + Ouabain	13.25 \pm 1.00 ^{ns}	1-5 -0.04 \pm 0.74	5-6 12.61 \pm 0.64
(6) + Bumetanide	0.64 \pm 0.46 ^b	2-6 6.69 \pm 0.51	

(a) Data are the mean \pm SD of 3 observations. Significance of differences of $K(^{86}Rb^+)$ influxes compared to $K(^{86}Rb^+)$ influx determined in Krebs (1) by Student's t-test. ns = not significant.

(b) $p < 0.001$

Table 3.4 Effect of 1.0 mM ouabain on the fractional rate of K^+ ($^{86}Rb^+$) efflux (X100) from HeLa and MDCK cells at time 6 minutes and 30 minutes exposure. Data are the mean \pm SD of 3 determinations

Cell type	Time	Total	+ 0.1 mM Furosemide	+ 1.0 mM Ouabain	+ Ouabain 0.1 mM + Furosemide 0.1 mM
HeLa	6 min	6.03 \pm 0.19	5.03 \pm 0.33 ^a	5.93 \pm 0.17 ^{ns}	4.00 \pm 0.17 ^c
	30 min	5.30 \pm 0.94	4.23 \pm 0.03 ^{ns}	7.10 \pm 0.25 ^a	3.23 \pm 0.09 ^a
MDCK	6 min	3.70 \pm 0.30	2.3 \pm 0.1 ^b	4.10 \pm 0.50 ^{ns}	2.60 \pm 0.30 ^b
	30 min	3.90 \pm 0.30	2.0 \pm 0.2 ^c	8.2 \pm 1.1 ^b	2.40 \pm 0.20 ^b

Test of significant difference from total efflux

ns = not significant

a 0.02 > p > 0.01

b p < 0.01

c p < 0.001

time 6 minutes, the K (^{86}Rb) efflux from HeLa and MDCK cells, in ouabain-containing media, did not differ significantly from control conditions. Therefore, the determination of the diuretic-sensitive K (^{86}Rb) influx in ouabain-poisoned cells over a 5 minute interval should not be complicated by the secondary effects of ouabain.

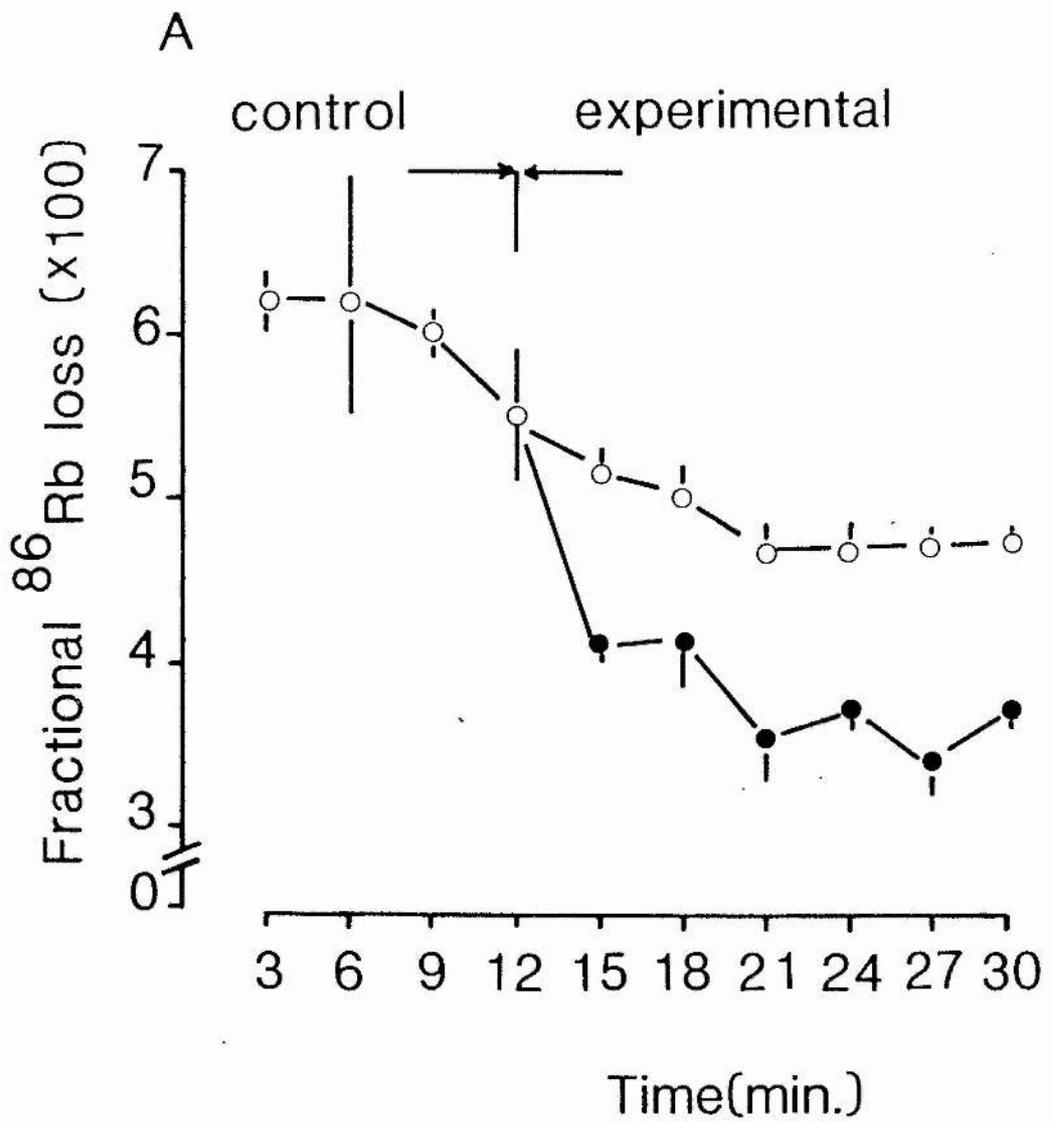
The K influx of HeLa and MDCK cells comprises a ouabain-sensitive, diuretic-sensitive and ouabain- and diuretic-insensitive components. This is in agreement with previous reports (Aiton et al., 1981, 1982), and similar results have been reported for human erythrocytes (Chipperfield, 1980, 1981, 1985; Dunham et al., 1980) turkey erythrocytes (Bakker-Grunwald, 1981) and Ehrlich ascites cells (Bakker-Grunwald, 1978).

K (^{86}Rb) efflux:

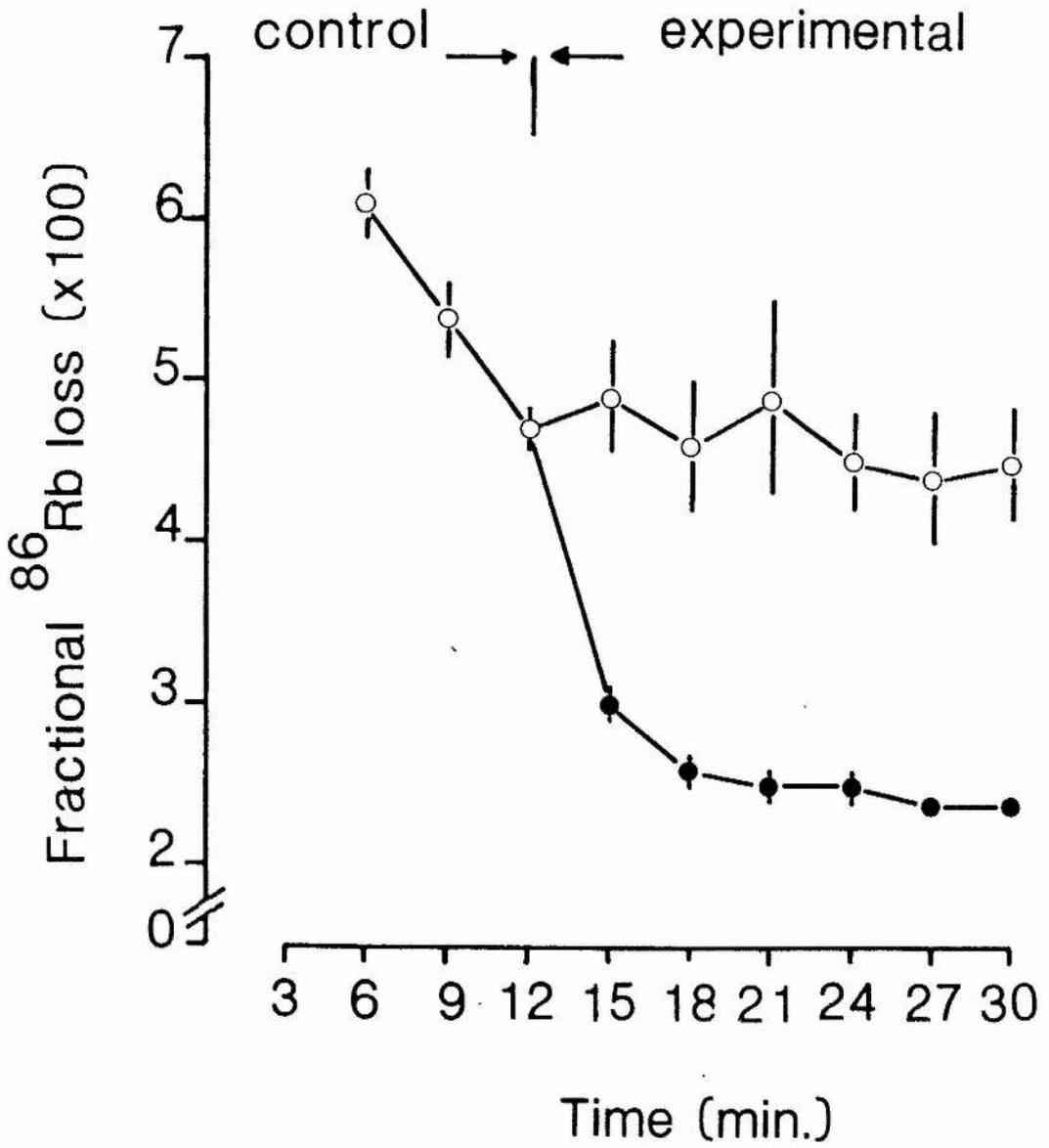
Efflux of K (^{86}Rb) from HeLa and MDCK cells pre-loaded with isotope as described in the methods is shown in figures 3.1a and 3.1b. After a suitable control efflux had been established in a standard Krebs solution, cells were exposed to media containing 0.1 mM diuretic, which in these experiments was furosemide. Inclusion of furosemide produced significant decreases in the fractional rates of the K (^{86}Rb) efflux in both cell lines studied. At the 30 minute interval, the fractional rate constant (x100) of K (^{86}Rb) efflux was reduced from 4.7 ± 0.1 (S.D. n=3) to 2.7 ± 0.15 (S.D. n=3), $P < 0.001$, for HeLa, and in the case of MDCK cells from 4.5 ± 0.6 (S.D. n=3) to 2.4 ± 0.17 (S.D. n=3), $P < 0.01$, indicating that these cells can mediate unidirectional K transport which is diuretic-sensitive. Simultaneous measures of diuretic-sensitive K (^{86}Rb) influx and efflux indicate

Figure 3.1 A,B

K (^{86}Rb) efflux from (A) HeLa and (B) MDCK cells, in standard Krebs solution (O), and the presence of 0.1mM furosemide (●). Data up to time 12 minutes mean \pm S.D., n=6, time 15 minutes onwards mean \pm S.D., n=3.



B



that these fluxes (expressed in $\text{nmol}/10^6$ cells.min.) are equivalent, thus indicating that no net ion transport is occurring through this pathway under these conditions (see results, chapter 5).

Intracellular ion contents.

If the diuretic-sensitive transport of K was mediating substantial net flux, inhibition by diuretics should lead to an alteration in internal cations. The total cation content of the HeLa and MDCK cells was determined in the presence or absence of 0.1 mM diuretic, i.e. furosemide (tables 3.5 and 3.6). After incubations of 0.5 to 3 hours in the presence of 0.1 mM diuretic, the intracellular K and Na content, expressed in $\text{nmol}/10^6$ cells for both HeLa and MDCK cells, did not differ significantly from control cells, under physiological conditions with external media containing 140mM Na and 5.4mM K. The variation in the control cellular cation contents between experiments (tables 3.5 and 3.6) is due to a density-dependent difference in the cell volume (see table 3.15 and fig. 3.15) and is similar to the previous report by Simmons (1984). Therefore, the diuretic-sensitive K (^{86}Rb) fluxes of the HeLa and MDCK cells may be considered to be in balance with little or no net flux under these experimental conditions.

In the presence of ouabain (1mM), the intracellular K of both the HeLa and MDCK cells was significantly reduced, whereas the cellular Na content was significantly increased compared with the control condition, due to the blockade of the Na K pump (tables 3.5 and 3.6). It should be noted that the inclusion of 0.1 mM diuretic in the ouabain-containing Krebs solution produced a small but significant retardation of the loss of cellular K in the HeLa cell line and this

Table 3.5 Effect of 0.1 mM diuretic upon the intracellular Na_i^+ and K_i^+ content of HeLa cells incubated in either Krebs or Krebs plus 1.0 mM ouabain^(a)

Incubation time hours	Diuretic (0.1 mM)	nmol/ 10^6 cells		nmol/ 10^6 cells	
		K_i^+	Na_i^+	K_i^+	Na_i^+
		Krebs		Krebs + 1 mM Ouabain	
1	-	359 ± 4.7	31.0 ± 0.0	-	-
	+	345 ± 18.0 ^{ns}	21.0 ± 4.4 ^c		
1.5	-	226 ± 14.0	35.0 ± 3.0	92.5 ± 5.0 ^d	111 ± 12
	+	231 ± 2.00 ^{ns}	38.3 ± 7.0 ^{ns}	122.0 ± 8.0 ^d	118 ± 8.8 ^{ns}
1.5	-	275 ± 26	20.4 ± 3.2	119.9 ± 8.0	120 ± 4.0
	+	277 ± 20 ^{ns}	18.5 ± 3.0 ^{ns}	169.0 ± 19.1 ^c	116 ± 4.0 ^{ns}
2.0	-	240 ± 9.0	11.8 ± 1.7	78 ± 3.4	128.9 ± 4.0
	+	235 ± 6.4 ^{ns}	13.6 ± 0.0 ^{ns}	122 ± 4.0 ^e	139.9 ± 19.8 ^{ns}
3	-	277 ± 11.2 ^b	35 ± 3.0	42 ± 3.5	114 ± 11.5
	+	313 ± 23.0	34 ± 2.9 ^{ns}	142.7 ± 12.0 ^d	114 ± 8.4 ^{ns}
3	-	343 ± 34	34.5 ± 6.0	-	-
	+	327 ± 13 ^{ns}	24.2 ± 0.0 ^{ns}		

(a) Monolayers of cells were incubated for time indicated at 37°C, pH 7.4. Data are the mean ± SD of 3 observations determined in 6 separate experiments. Significant effect of 0.1 mM diuretic on the intracellular ion content tested by Student's t-test (unpaired two-tailed solution). ns = not significant.

(b) $0.05 < p < 0.1$

(c) $p < 0.02$

(d) $p < 0.01$

(e) $p < 0.001$

Table 3.6 Effect of 0.1 mM diuretic upon the intracellular Na_i^+ and K_i^+ contents of MDCK cells incubated in either Krebs or Krebs plus 1.0 mM ouabain (a)

Incubation time hours	Diuretic (0.1 mM)	nmol/ 10^6 cells			
		Krebs		Krebs + 1.0 mM Ouabain	
		K_i^+	Na_i^+	K_i^+	Na_i^+
0.5	-	242	12	104	38.3
	+	213	9.2	115	44.4
1.0	-	353	20.5	237	127
	+	376	20.5	243	163
1.5	-	279.0	24.7	152.8	109
	+	285.0	25.8	159	111
2.25	-	225.9	17.7	46.3	204.1
	+	277.0	46.0	46.3	201
3.0	-	251	72	73	250
	+	234	60	98	270

(a) Monolayers were incubated for time indicated at 37°C, pH 7.4. Data are the mean \pm SD of 3 observations determined in 5 separate experiments. Significant effect of 0.1 mM diuretic on the intracellular ion content tested by Student's t-test (unpaired two-tailed solution). ns = not significant.

- (b) $p < 0.05$
- (c) $p < 0.01$

loss was not accompanied by an equivalent effect on intracellular Na. This is similar to the observation of Aiton and Simmons (1983). The retardation of the K loss by 0.1 mM diuretic in ouabain-poisoned MDCK cells was only significant in one out of five of experiments performed (table 3.6).

The effect of a two hour incubation in furosemide-containing Krebs solution on the cellular ionic composition of HeLa cells was further investigated (table 3.7). The cellular Na and K concentrations of HeLa cells after two hours' exposure to furosemide did not differ significantly from control cells incubated in standard Krebs solution, thus confirming the result given in tables 3.5 and 3.6. However, in these experiments the Cl concentration was determined by the distribution of ³⁶Cl or coulometry. The inclusion of 0.1 mM furosemide did not result in a significant decrease in the cellular concentration of Cl compared with controls (table 3.7).

Effects of external K upon diuretic-sensitive K efflux.

The demonstration that the diuretic-sensitive fluxes of HeLa and MDCK cells mediate balanced unidirectional fluxes raises the question: does the diuretic-sensitive ion transport represent an obligatory exchange flux? Haas, Schmidt and McManus (1982) have demonstrated that the Na K "cotransport" flux of the duck erythrocyte displays no dependence upon the trans-concentrations of either Na or K. The cation fluxes via the "cotransport" pathway are solely dependent upon the cis-concentrations of Na and K.

Table 3.7 Intracellular concentration of Na⁺, K⁺ and Cl⁻ for HeLa cells after a 2 hour incubation in Krebs solution in the presence or absence of 0.1 mM furosemide^(a)

Experiment	Furosemide	Intracellular ion concentration, mM			
		Na _i ⁺	K _i ⁺	Cl _i ⁻	
				³⁶ Cl ⁻ Determination	Coulometric Determination
A	-	14.4 ± 3.8 (6)	196.1 ± 6.6 (6)	84.7 ± 14.7 (6)	84.5 ± 1.7 (3)
	+	17.7 ± 3.7 ^{ns} (6)	183 ± 16.4 ^{ns} (6)	68.6 ± 13.8 ^{ns} (6)	79.0 ± 4.0 ^{ns} (3)
B	-	16.5 ± 5.1 (4)	172.0 ± 25.7 (6)	58.6 ± 0.9 (3)	50.7 ± 4.9 (3)
	+	23.8 ± 4.6 ^{ns} (6)	175.0 ± 5.0 ^{ns} (6)	60.8 ± 1.4 ^{ns} (3)	61.0 ± 8.6 ^{ns} (3)
		Extracellular ion concentration, mM			
		139.5	5.71	160	

(a) Each datum is the mean ± SD. Number of observations given in (). Significant effect of furosemide upon intracellular ion concentration tested by Student's t-test (unpaired, two-tailed solution). ns = not significant.

Footnote:

For balanced influx and efflux through the Na⁺ K⁺ Cl⁻ 'cotransport' pathway, the sum of chemical gradient (μ_{net}) should = 0

$$\frac{K_o^+}{K_i^+} \cdot \frac{Na_o^+}{Na_i^+} \cdot \frac{Cl_o^-^2}{Cl_i^-^2} = 1 \quad \text{if } \mu_{net} = 0$$

Solution for controls = 1.01 (Exp A) 2.11 (Exp B)
 Solution for furosemide = 1.42 (Exp A) 1.35 (Exp B)

For HeLa cells, the fractional rates ($\times 100$) of diuretic-sensitive K (^{86}Rb) efflux at time 30 minutes in external K concentrations of 1 and 10mM, were 1.01 ± 0.29 and 0.63 ± 0.12 (mean \pm S.E.) respectively and were not significantly different from the K (^{86}Rb) efflux observed in 5mM media, 0.33 ± 0.15 (mean \pm S.E.) (table 3.8). Similarly, for MDCK cells (table 3.9), the diuretic-sensitive K (^{86}Rb) efflux in 0.04mM and 14.5mM K media being 3.17 ± 0.41 (mean \pm S.E.) and 2.04 ± 0.31 (mean \pm S.E.) respectively were not significantly different from the K (^{86}Rb) efflux measured in 5mM K containing media. These results indicate that external K has no pronounced effect upon the K efflux in either the HeLa or MDCK cell lines. The K flux of the MDCK cells under conditions where no net transport of ions occurs, does not represent an obligatory K-K exchange, since reducing the external K to near zero does not inhibit the K efflux*. These data are thus similar to duck red cells and to data from human red blood cells (Haas et al., 1982; Wiley and Cooper, 1974).

*Note in proof.

Recent reports by Canessa, Brugnara, Cusi and Tosteson (1986) and Brugnara, Canessa, Cusi and Tosteson (1986) demonstrate that trans ion concentrations can stimulate and/or inhibit the Na K "cotransport" pathway of human red cells. However, the major portion (75%) of ion fluxes through this transport pathway in this cell type are independent of the trans ion concentrations thereby indicating the non-obligatory exchange nature of the major portion of these diuretic-sensitive ion fluxes. The above reports do not affect the conclusions drawn from data presented in this chapter demonstrating the non-obligatory exchange nature of the diuretic-sensitive K fluxes through the Na K Cl "cotransport" pathway in both HeLa and MDCK cells.

Table 3.8 Effects of varying external K^+ on the K^+ ($^{86}\text{Rb}^+$)
efflux from HeLa cells. Data are the mean \pm SD
of 4 experiments

Fractional rate constant (X100) at t 30 minutes

K_o	Control (1)	+ Furosemide (0.1 mM) (2)	1-2
1.00	4.78 \pm 0.50	3.15 \pm 0.09	1.01 \pm 0.29 ^{ns}
5.19	3.99 \pm 0.17	3.66 \pm 0.19	0.33 \pm 0.15
10.00	4.15 \pm 0.12	3.55 \pm 0.16	0.63 \pm 0.12 ^{ns}

ns = not significantly different from 5.19 K_o^+ values.

Table 3.9 Effects of varying external K^+ on the K^+ ($^{86}Rb^+$) efflux from MDCK cells. Data are the means \pm SD of 3 observations

K_o	Control (1)	+ 0.1 mM Furosemide (2)	1-2
0.04	5.40 \pm 0.62	2.23 \pm 0.35	3.17 \pm 0.41 ^{ns}
5.00	5.47 \pm 0.84	2.67 \pm 0.35	2.80 \pm 0.52
14.50	4.07 \pm 0.40	2.03 \pm 0.35	2.04 \pm 0.31 ^{ns}

ns = not significantly different from 5.0 K_o^+ values

Cation and anion dependence of the ouabain-insensitive K (^{86}Rb) influx.

The dependence of the ouabain-insensitive K (^{86}Rb) influx of HeLa and MDCK cells upon the cation and anion composition of the external incubation media was also examined.

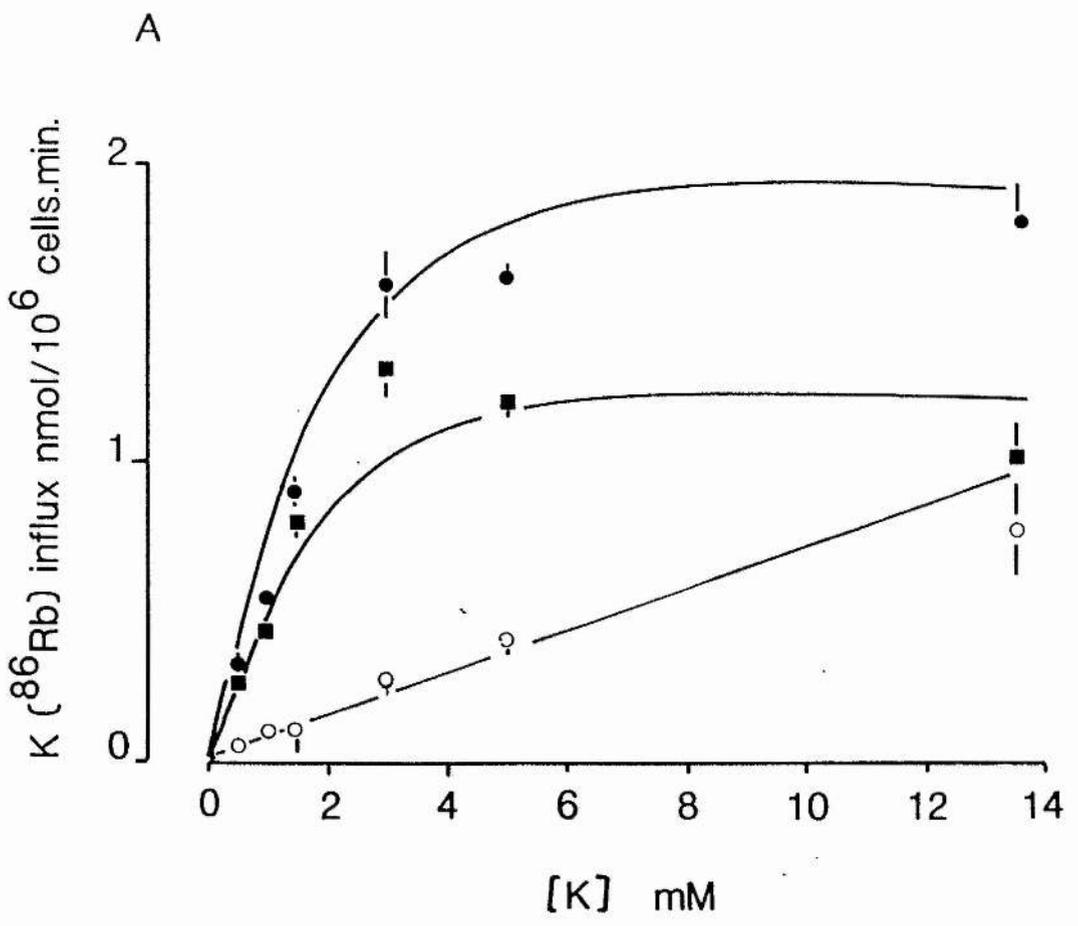
a) K dependence.

Figures 3.2 a and b illustrate the dependence of the ouabain-insensitive K (^{86}Rb) influx of the HeLa and MDCK cells upon the external K concentration. Increasing the external K concentration produced a hyperbolic activation curve for the diuretic-sensitive component of the K (^{86}Rb) influx, saturating at around 3 mM for both HeLa and MDCK cells. These data were fitted to the Michaelis-Menten equation giving an apparent K_m (in mM) for HeLa and MDCK of 2.00 ± 0.49 (S.E.) and 2.46 ± 0.64 (S.E.) and apparent maximal velocity (in $\text{nmol}/10^6$ cells.min.) of influx of 1.69 ± 0.15 (S.E.) and 9.67 ± 1.24 (S.E.), respectively. The ouabain- and diuretic-insensitive K (^{86}Rb) influx increased in an approximately linear fashion with increased extracellular K and is consistent with this component of K (^{86}Rb) representing a transmembrane diffusion. These results are similar to previous reports of Aiton et al., (1981 and 1982) for these cell types.

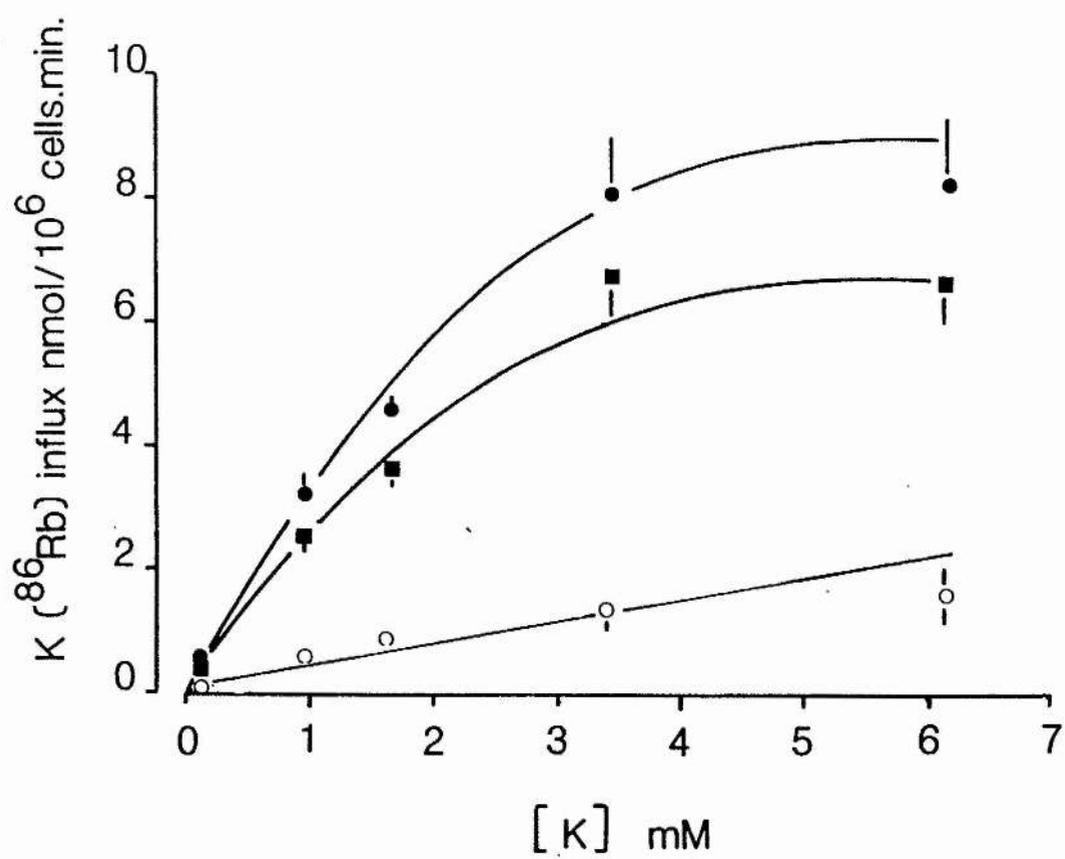
The trans-membrane transport of one ion by the Na K Cl "cotransport" system has been conclusively shown to be affected by the presence of the co-ions to this transport system in the avian

Figure 3.2 A,B

Effect of varying the external K concentration upon the ouabain-insensitive K (^{86}Rb) influx of (A) HeLa and (B) MDCK cells, in the presence (○) or absence (●) of 0.1 mM diuretic. The diuretic-sensitive component (■) is the difference the ouabain-insensitive and ouabain- and diuretic-insensitive K (^{86}Rb) influxes. Diuretic-sensitive data are the best-fit lines to Michaelis-Menten kinetics, fitted as described in the methods. The kinetic constants V_{max} and K_{m} for HeLa and MDCK cells were: 1.69 ± 0.15 , 9.24 ± 1.24 (nmol/ 10^6 cells.min \pm S.E.); 2.00 ± 0.49 , 2.46 ± 0.64 (mM \pm S.E.) respectively. Data are the mean \pm S.D. of 3 determinations, of a representative experiment.



B



erythrocyte and MDCK cells (Palfrey and Rao, 1983; Rindler et al., 1982; McRoberts et al., 1982). Therefore, it was of interest to investigate the effect of varied, but maintained, concentrations of Na and Cl upon the K activation of the diuretic-sensitive K (^{86}Rb) influx of the HeLa cell line.

Figure 3.3 shows data for the K activation of the K (^{86}Rb) influx in varying concentrations of the co-ion Na, with the Na being set near its apparent K_m and x2 apparent K_m (see Na-dependence). As the external Na concentration was decreased from 133 to 72 and 42mM (N-methyl-D-glucamine substitution), the shape and position of the K dependence curve changed, becoming flattened and moving further to the right. These results were fitted to the Michaelis-Menten equation and the apparent K_m (in mM) for K was seen to increase from 1.58 ± 0.25 in 133mM Na to 7.25 ± 1.07 in 42mM Na. This increase can be considered to be a linear function of the the external co-ion Na (figure 3.4). These data indicate a positive cooperation between the Na and K co-ions of the Na K Cl "cotransport" system of HeLa cells. It also important to note the apparent V_m of the K activation which decreases with the increasing co-ion Na. This is in contrast to MDCK cells (Rindler et al., 1982) and avian erythrocytes (Palfrey and Rao, 1983) where the apparent V_m increases or is constant with increased external Na concentration. The reason for these differences between cell types is unclear.

The second co-ion to the system, Cl, was also studied, in order to observe the effect of varying Cl (nitrate substitution) upon the K activation (figure 3.5). Again, the K activation is seen to be a hyperbolic curve, saturating at around 3mM K, when external Cl was 160mM. However, as the external Cl concentration was progressively

Figure 3.3

Activation of the diuretic-sensitive K (^{86}Rb) influx by external K in HeLa cells. Na was varied (N-methyl-D-glucamine substitute) but held constant for the K dependence of K influx at 42 mM (\blacktriangle), 72 mM (O) and 133 mM (\bullet). The curves are the best fit lines of the data to Michaelis-Menten kinetics (see methods). The kinetic constants V_m and K_m were (a) Na = 42mM, 15.84 ± 1.66 (S.D.) nmol/10⁶ cells.min. and 7.25 ± 1.07 mM (S.D.), (b) Na = 72mM, 19.56 ± 1.59 (S.D.) nmol/10⁶ cells.min. and 5.06 ± 0.63 mM, (c) Na = 133mM, 9.91 ± 0.67 nmol/10⁶ cells.min. (S.D.) and 1.58 ± 0.23 mM respectively. Data are the mean \pm S.E. for 3 determinations.

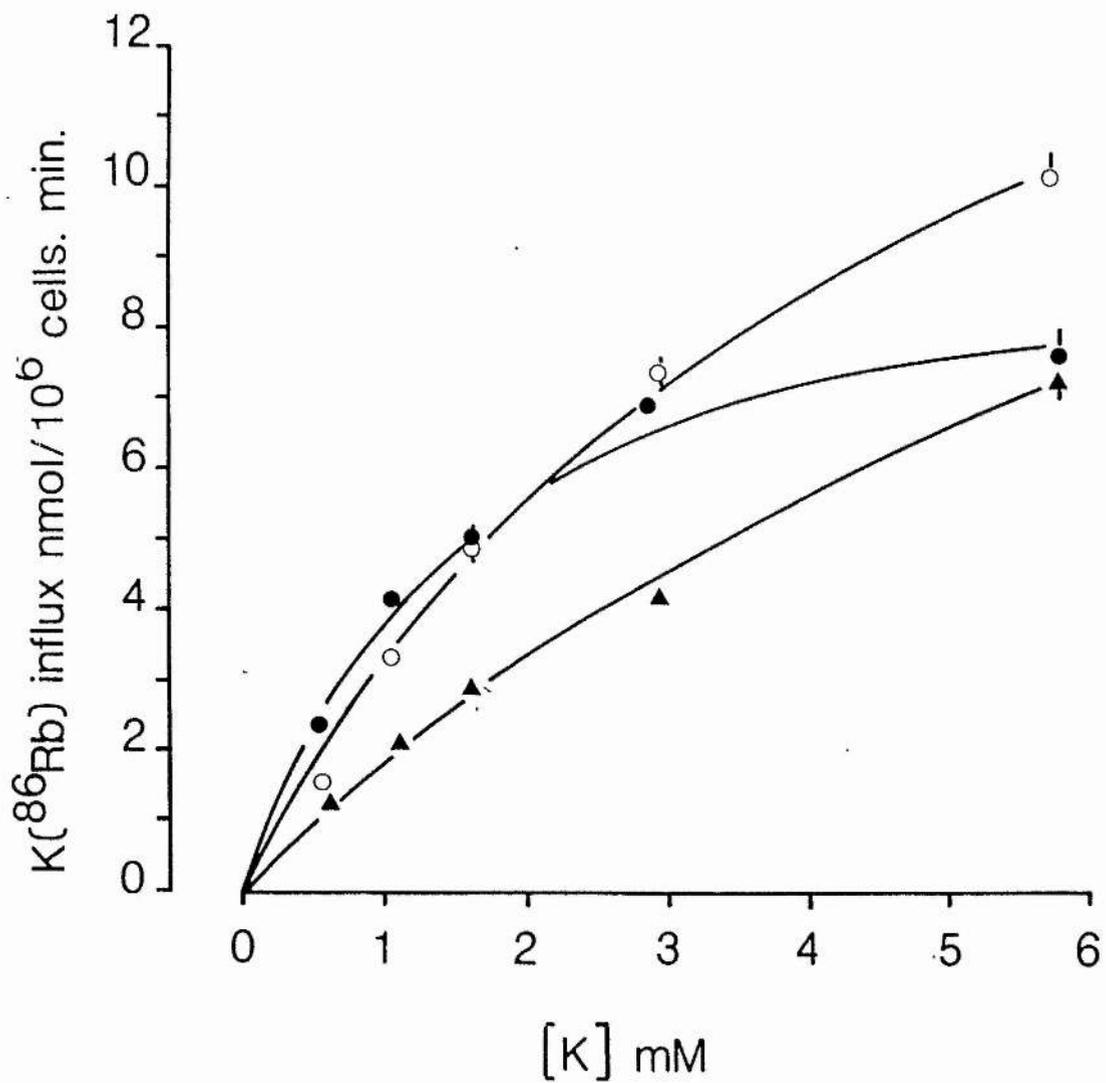


Figure 3.4

Dependence of the apparent K_m for K activation of the diuretic-sensitive K (^{86}Rb) influx of HeLa cells upon the external Na concentration. Data from the Michaelis-Menten fit of the results presented in figure 3.3.

Apparent K_m for K dependence

of K influx (mM)

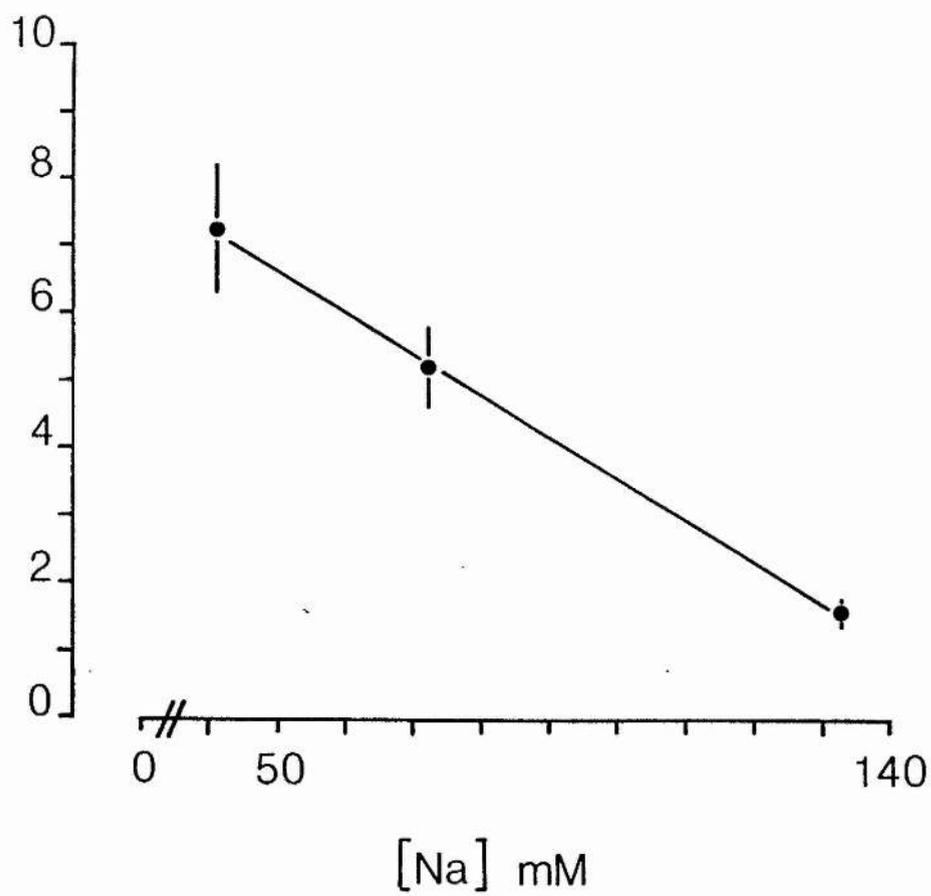
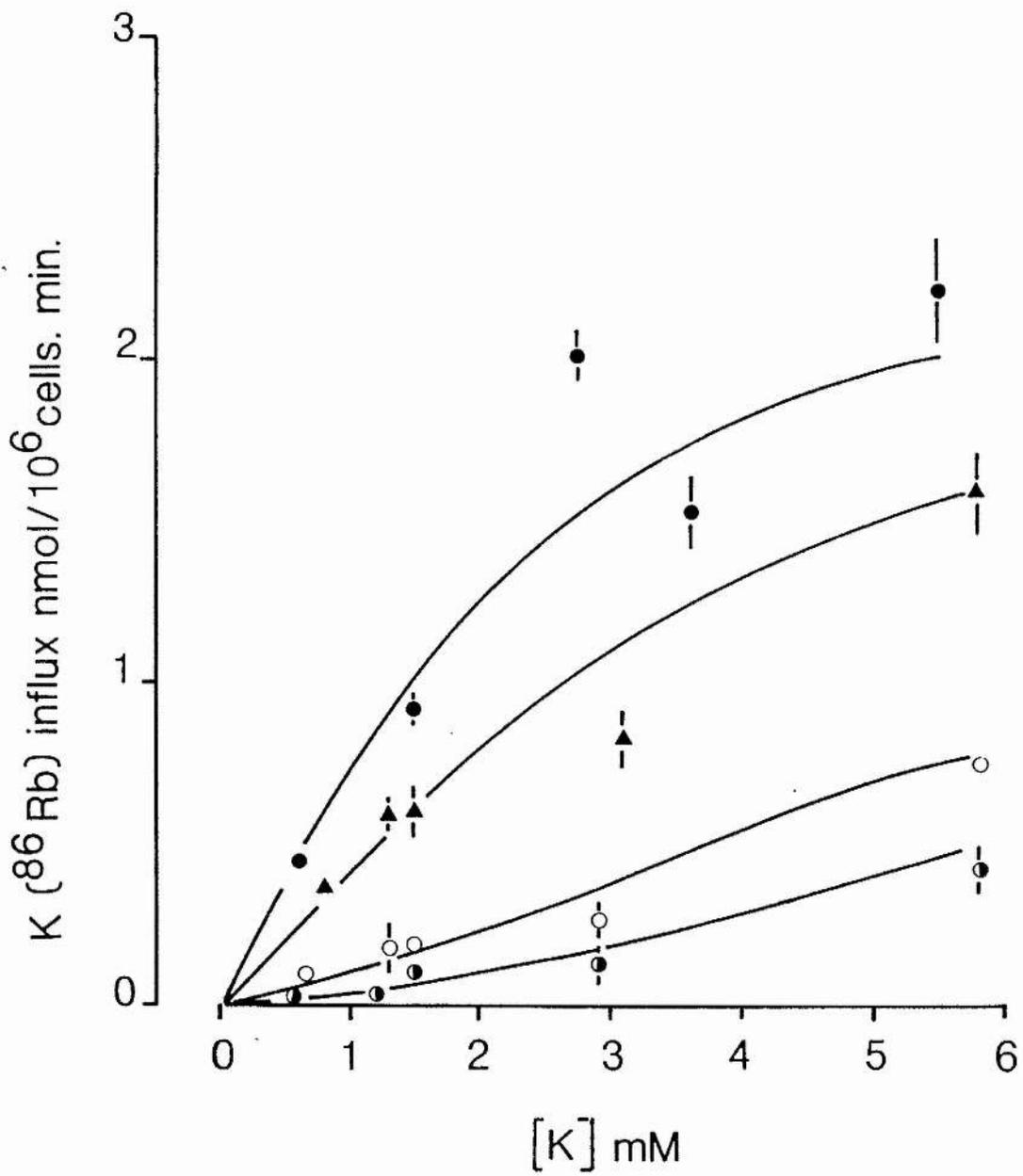


Figure 3.5

Dependence of the diuretic-sensitive K (^{86}Rb) influx upon external K in HeLa cells. Activations by K were performed in varying external Cl concentrations (NO_3^- substitute), (●) 160mM Cl, (▲) 120mM Cl, (○) 80mM Cl, (◐) 40mM Cl. Data are the mean \pm S.D. of 3 determinations.



decreased (nitrate substitution), it was seen that the magnitude of the K (^{86}Rb) influx (in $\text{nmol}/10^6$ cells.min.), at saturating K concentrations, decreased from 2.33 ± 0.34 , $\text{Cl}=160\text{mM}$, to 0.58 ± 0.38 , $\text{Cl}=40\text{mM}$. The concentration of K (in mM) producing 50% activation of the diuretic-sensitive K (^{86}Rb) influx also increased with decreased extracellular Cl concentration. As the external Cl concentration is decreased, the shape of the K activation becomes markedly sigmoidal and non-saturating. The shape of this series of curves is analogous to the series of expected curves obtained in binding studies of substrate to allosteric enzyme systems, where the omission of the allosteric activator or inclusion of an allosteric inhibitor to the enzyme system produces markedly sigmoidal kinetics for substrate binding (Engel, 1977).

b)Na-dependence.

Replacing the medium Na with either CholineCl or N methyl-D-glucamine significantly ($P < 0.001$) reduced the diuretic-sensitive K influx (table 3.10) as previously reported for HeLa and MDCK cells (Aiton et al., 1981, 1982). However, Li as a substitute for Na partially maintained a diuretic-sensitive K (^{86}Rb) influx in both the HeLa and MDCK cells (Aiton et al., 1981, 1982; Rindler et al., 1982). In choline media, the ouabain- and diuretic-insensitive K influx was markedly increased compared to Na media in the HeLa cells (Aiton et al., 1981), a similar result having been observed for MDCK cells (Aiton et al., 1982). This significant ($P < 0.05$ for MDCK; $P < 0.001$ for HeLa) stimulation of the residual component of K flux in choline media was also observed in this present work (table 3.10) and markedly reduced the precision of the determination of diuretic-sensitive K (^{86}Rb) influx in low Na media.

Table 3.10 Dependence of the diuretic-sensitive $K^+(^{86}Rb^+)$ influx of HeLa and MDCK cells upon media Na^+ (a)

Cell type	Major Cation	Na^+	$K^+(^{86}Rb^+)$ influx nmol/ 10^6 cells.min		
			Total	+ Diuretic 0.1 mM	Total - diuretic
HeLa	Na^+	140	4.38 ± 0.36	1.20 ± 0.04	3.18 ± 0.21
	Choline	3	3.79 ± 0.30^{ns}	$3.09 \pm 0.30^{(d)}$	$0.70 \pm 0.24^{(d)}$
	Na	140	4.83 ± 0.50	1.50 ± 0.34	3.77 ± 0.25
	N-methyl-D-glucamine	15	5.12 ± 0.68^{ns}	$3.80 \pm 0.27^{(d)}$	$1.32 \pm 0.42^{(d)}$
MDCK	Na	140	9.92 ± 1.63	0.75 ± 0.26	9.17 ± 0.95
	Choline	2	$2.29 \pm 0.11^{(c)}$	$1.29 \pm 0.17^{(b)}$	$1.00 \pm 0.12^{(d)}$
	Na^+	140	7.48 ± 0.83	0.77 ± 0.48	6.71 ± 0.48
	N-methyl-D-glucamine	15	$4.43 \pm 0.23^{(c)}$	1.21 ± 0.48^{ns}	$3.22 \pm 0.31^{(d)}$

(a) $K^+(^{86}Rb^+)$ influx measured as described in methods: 1 mM ouabain was present in all media and 0.1 mM diuretic as indicated. Results are the mean \pm SD of 3 determinations. Significance of difference from 140 [Na^+] values tested by Student's t-test. ns = not significant.

(b) $p < 0.05$

(c) $p < 0.01$

(d) $p < 0.001$

For this reason, in this present study the ouabain-insensitive K (^{86}Rb) influx measured as a function of external Na was determined in two Na substitutes, CholineCl and N-methyl-D-glucamine, titrated with HCL (Blackstock, Ellory and Stewart, 1985; Lauf, Adragna and Garay, 1984; Reuss and Finn, 1975). The effect of choline replacement on the ouabain- and diuretic-insensitive K influx has not been observed in human erythrocytes (Sachs, 1971; Wiley and Cooper, 1974; Chipperfield, 1980, 1985), but the effect of Na replacement on the diuretic-sensitive component agree.

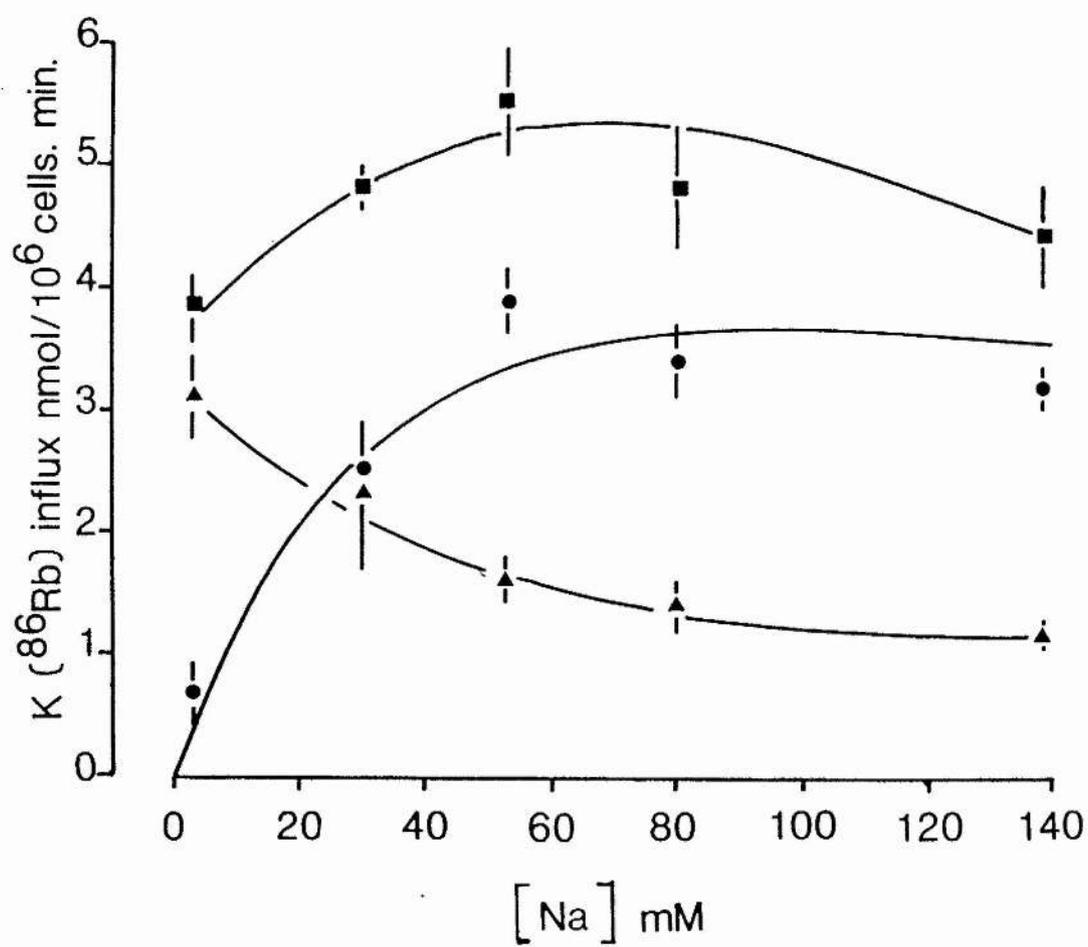
On increasing the extracellular Na concentration from 5 to 140 mM, the ouabain-insensitive K (^{86}Rb) influx was unaltered when Na was replaced by either CholineCl or N methyl-D-glucamine in the HeLa cell line. However, the diuretic-sensitive K (^{86}Rb) influx increased in a hyperbolic and saturable manner as the media Na increased, (figures 3.6 a and b). The apparent half maximal activation (apparent Km) of the diuretic-sensitive K (^{86}Rb) influx was at 9.9 ± 4.8 (S.E.) mM and 25.6 ± 10.6 (S.E.) mM for choline and N methyl-D-glucamine Na substitution respectively. These results are consistent with those previously reported for this cell type (Aiton et al., 1981). Similarly in the MDCK cell line, increasing the media Na produced a hyperbolic activation of the diuretic-sensitive K (^{86}Rb) influx with apparent Km at 18.5 ± 4.6 (S.E.) mM in choline media and 25.0 ± 4.6 (S.E.) mM in N methyl-D-glucamine media (figure 3.7 a,b).

The Na-dependence of the diuretic-sensitive K (^{86}Rb) influx of the HeLa cell line was further investigated in varying concentrations of the co-ion K (figure 3.8). For Na activation (N methyl-D-glucamine substitution) performed in media containing 5.4mM K and 160mM Cl, the K (^{86}Rb) influx increased hyperbolically with increasing external Na as seen in figures 3.6 a and b. The apparent Km and Vm were $30.79 \pm$

Figure 3.6 A,B

Na-dependence of the ouabain-insensitive K (^{86}Rb) influx of HeLa cells. NaCl was replaced isosmotically by (A) cholineCl and (B) N-methyl-D-glucamine in the presence (\blacktriangle) or absence (\blacksquare) of 0.1 mM diuretic. The diuretic-sensitive component (\bullet) is the difference between the ouabain-insensitive and ouabain- and diuretic-insensitive K (^{86}Rb) influxes. Data are the mean \pm S.D. of 3 determinations of a representative experiment.

A



B

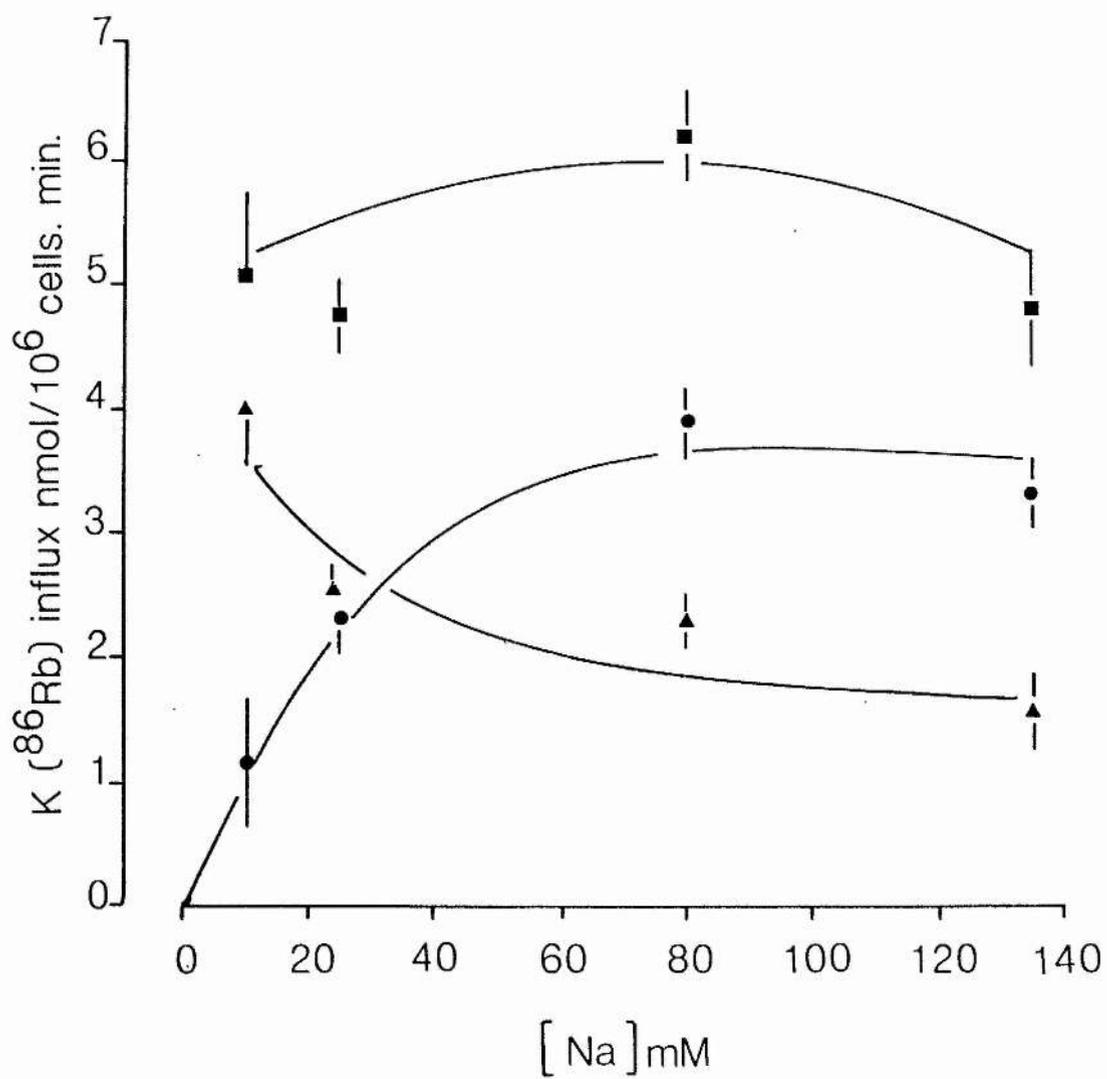
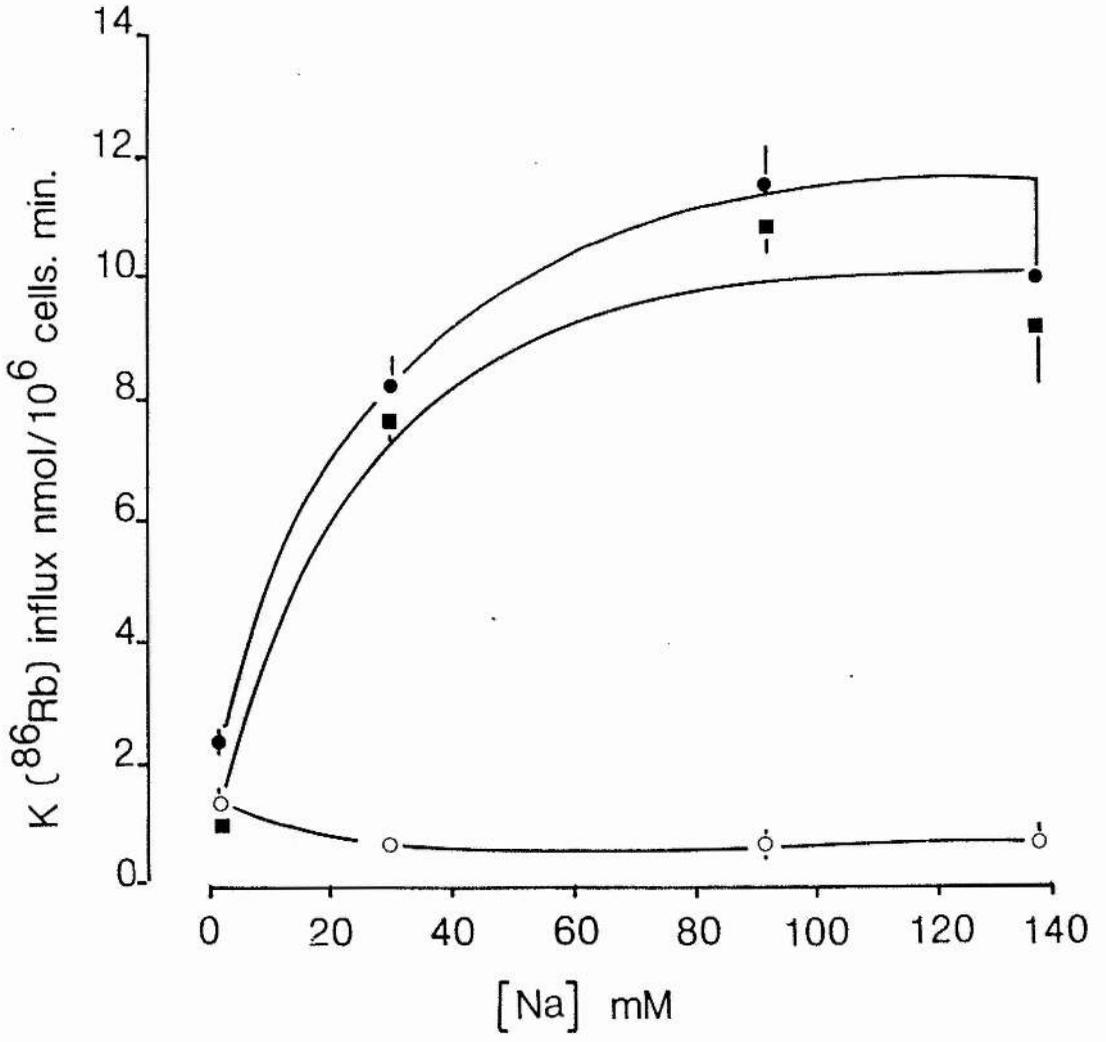


Figure 3.7 A,B

Na-dependence of the ouabain-insensitive K (^{86}Rb) influx of MDCK cells. NaCl was replaced isosmotically by (A) cholineCl and (B) N-methyl-D-glucamine in the presence (O) or absence (●) of 0.1 mM diuretic. The diuretic-sensitive component (■) is the difference between the ouabain-insensitive and ouabain- and diuretic-insensitive K (^{86}Rb) influxes. Data are the mean \pm S.D. of 3 determinations of a representative experiment.

A



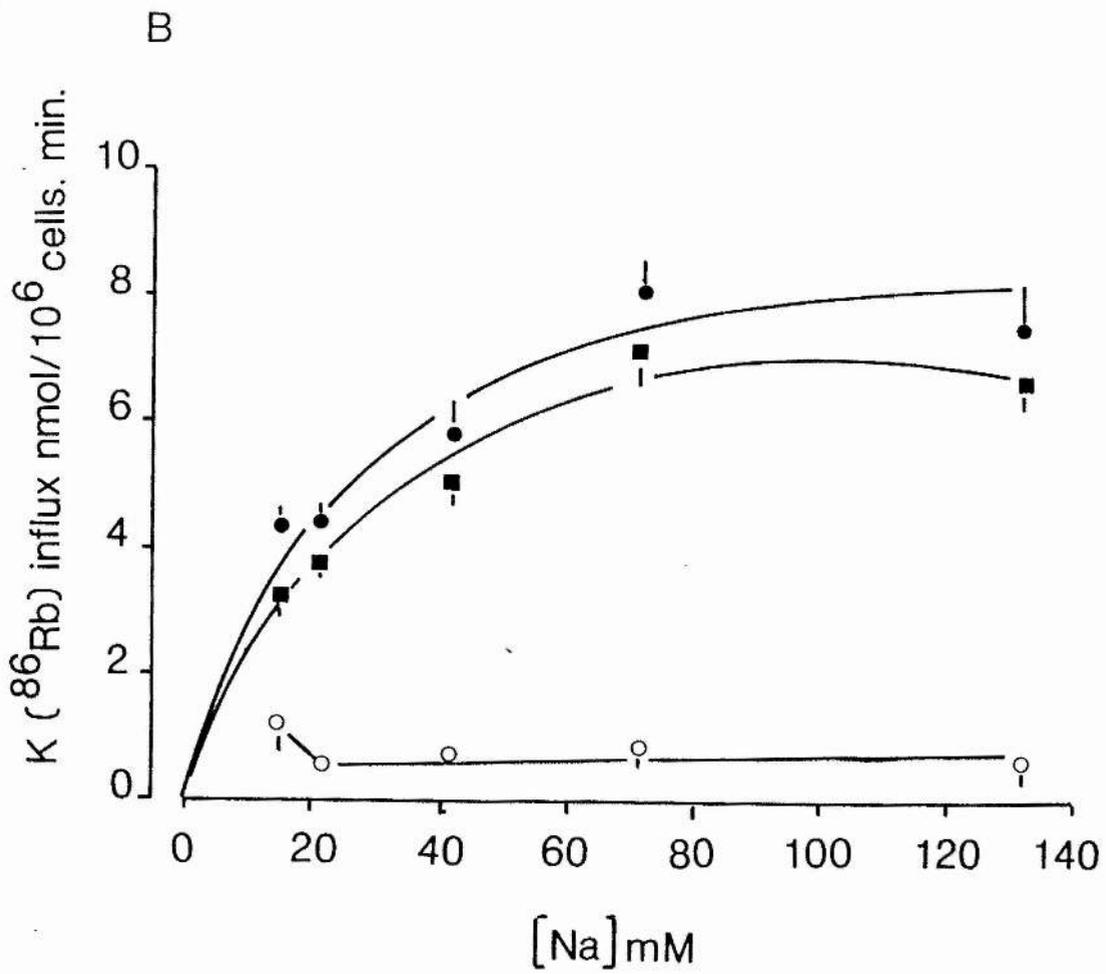
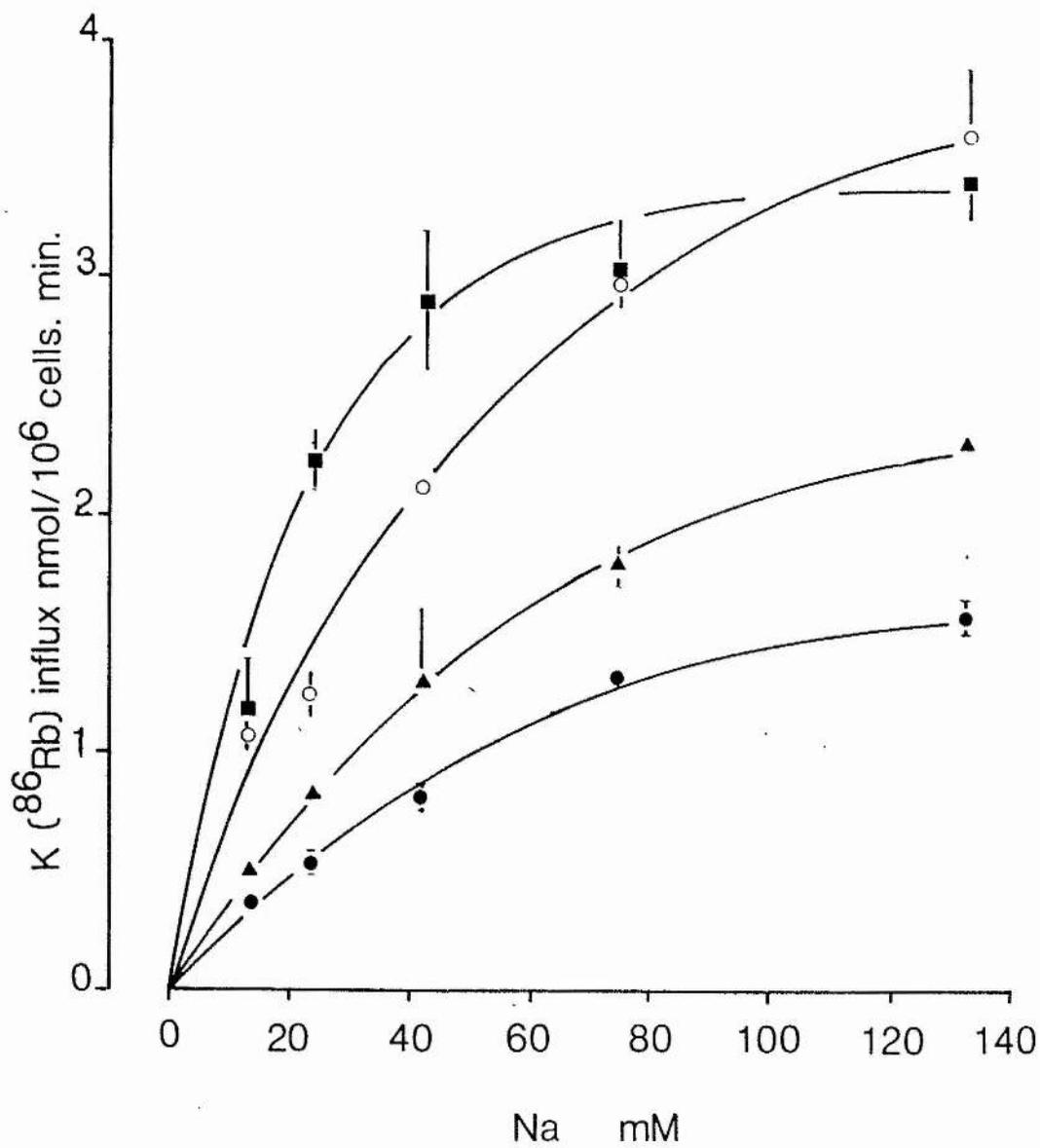


Figure 3.8

Na-dependence of the diuretic-sensitive K (^{86}Rb) influx in HeLa cells. External K was varied but held constant in each activation by Na, (●) 0.8mM K, (▲) 1.45mM K, (○) 2.88mM K, and (■) 5.6mM K. Curves are the best fit to Michaelis-Menten kinetics (see methods). The apparent V_m (nmol/ 10^6 cells.min) and K_m (mM) for the Na-dependence were: 0.8 K, 2.79 ± 0.26 (S.D.) and 97.86 ± 14.80 (S.D.); 1.45 K, 4.27 ± 0.40 (S.D.) and 100.80 ± 15.13 (S.D.); 3.88 K, 5.33 ± 0.53 (S.D.) and 64.13 ± 11.83 (S.D.); 5.6 K, 4.37 ± 0.45 (S.D.) and 30.79 ± 7.72 (S.D.) respectively. Data are the mean \pm S.E. of 3 determinations.



7.72 mM and 4.37 ± 0.45 nmol/ 10^6 cells. min. respectively for these data. Under conditions of reduced external K concentrations, the hyperbolic activations were shifted progressively to the right and flattened. The apparent K_m for Na activation decreased linearly with increasing external K (figure 3.9), indicating the positive co-operativity of K upon Na activation of the diuretic-sensitive K influx, similar to the Na co-operativity on K activation (see K-dependence). These results are in agreement with those obtained in the MDCK cell line (Rindler et al., 1982).

c) Cl-dependence.

The Cl-dependence of the K (^{86}Rb) influx, inhibited by "loop" diuretics in the HeLa cell line, was studied using equimolar substitutions of Cl salts for NO_3^- or Gluconate $^-$ salts (figure 3.10 a and b), as it is now clear that the Cl activation curves of the Na K Cl "cotransport" system are dependent upon the anion substitute used (see Chipperfield, 1984, 1985). The reasons for these differences are as yet unclear, but Cl replacement studies are complicated by replacement of Cl at one or both membrane surfaces, depending upon the penetrability of the replacement anion. Secondary effects of changes in membrane potential and intracellular pH or anions other than Cl could directly inhibit the Na K Cl "cotransport" fluxes are also possible causes of variations between anion replacements.

The reduction of the external Cl to 5mM by NO_3^- substitution (figure 3.10 a) completely abolishes the furosemide-sensitive K (^{86}Rb) influx. Activation of the ouabain-resistant, furosemide-sensitive K (^{86}Rb) influx being a non-saturating upward-sloping curve with an apparent K_m of 90mM for the Cl-dependence. K influx, insensitive to

Figure 3.9

Dependence of the apparent K_m for Na activation of the diuretic-sensitive K (^{86}Rb) influx of HeLa cells upon the external K concentration. Data from the Michaelis-Menten fit of the results presented in figure 3.8.

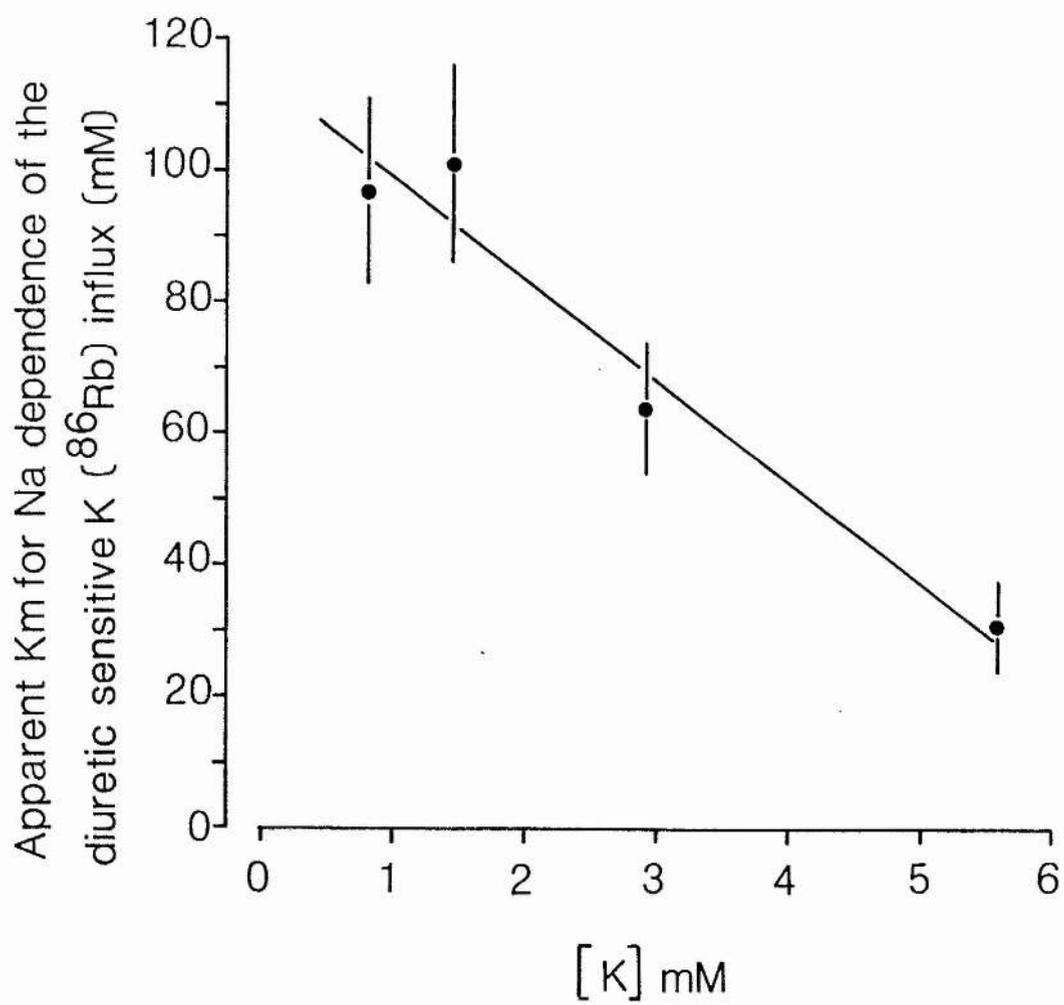
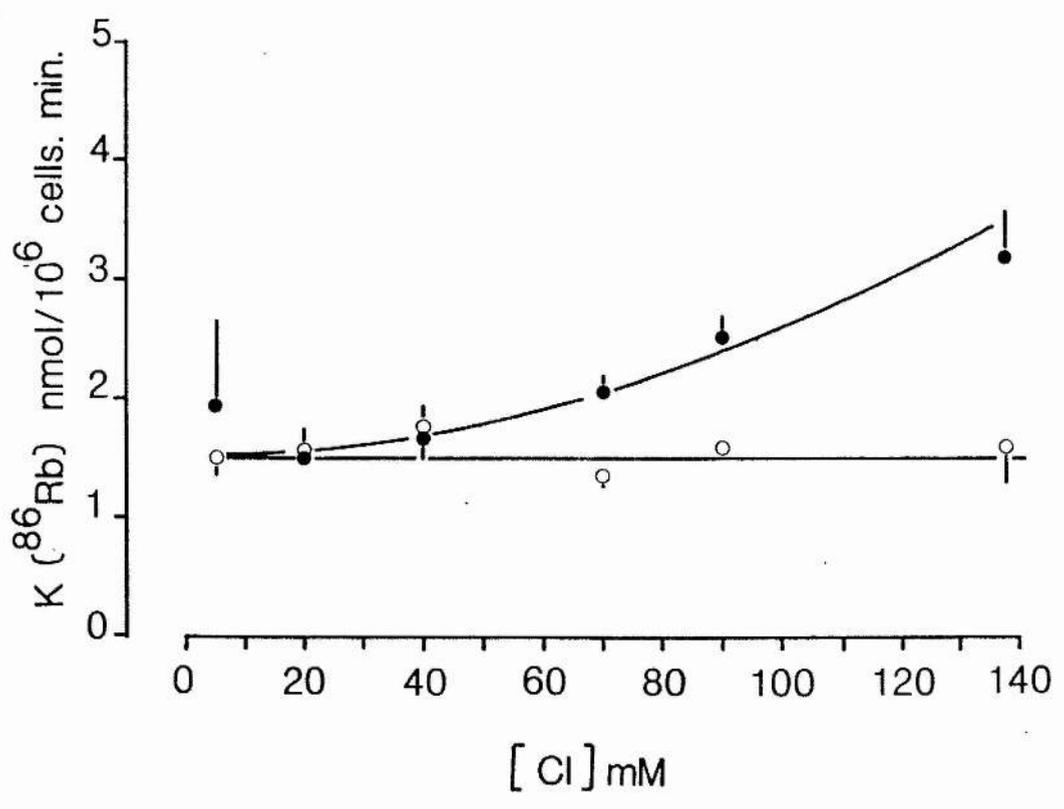
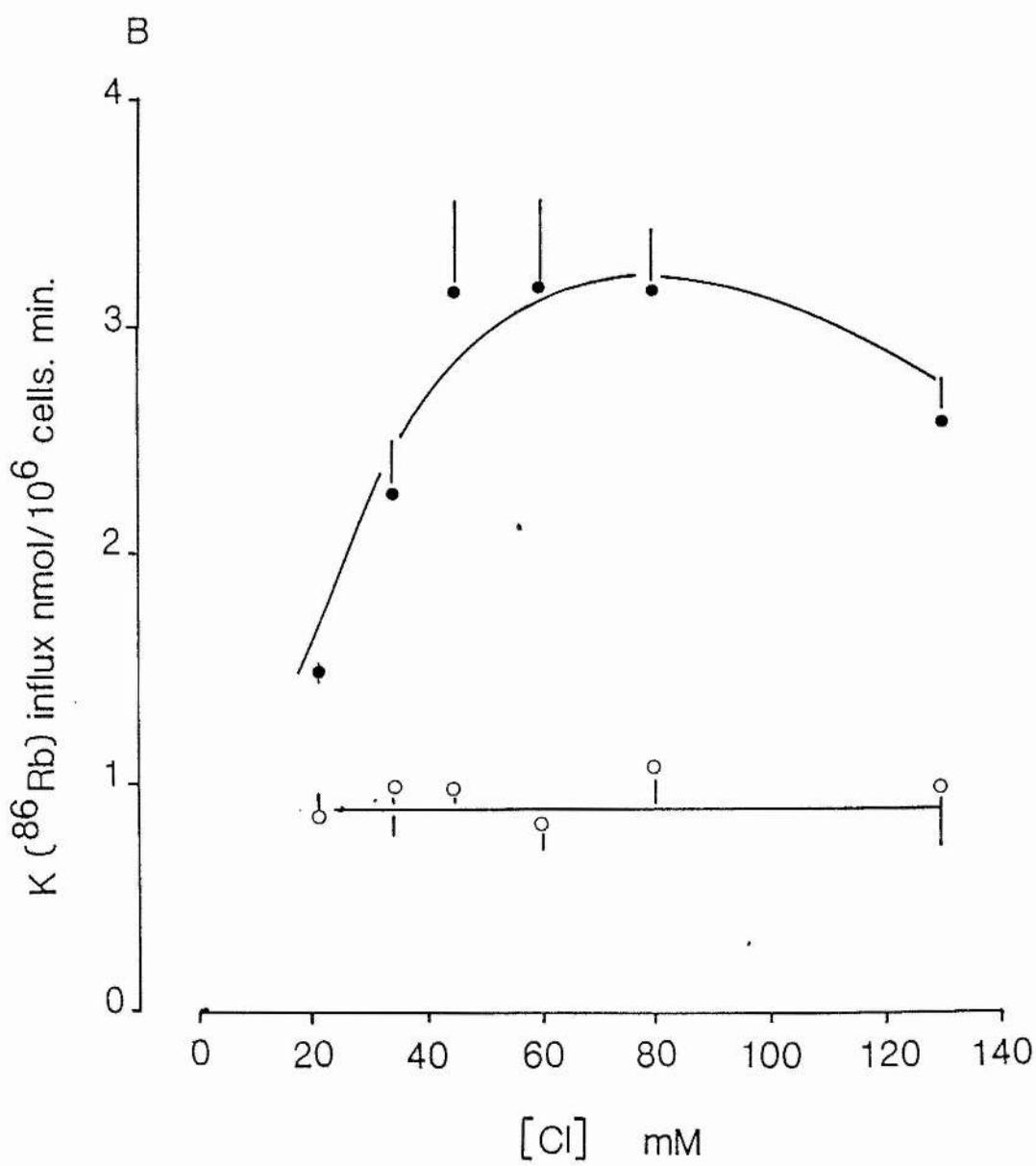


Figure 3.10 A,B

Cl-dependence of the ouabain-insensitive K (^{86}Rb) influx of HeLa cells. NaCl was replaced isosmotically by (A) NaNO_3 or (B) Na gluconate, in the presence (O) or absence (●) of 0.1 mM diuretic. The initial estimates to the Hill equation gave kinetic constants V_{max} , $K_{1/2}$ and Hill coefficient (n) for the Cl-dependence determined in NO_3 and gluconate media of: 1.53 ± 0.26 , 1.52 ± 0.33 (V_{max} , $\text{nmol}/10^6$ cells.min (\pm S.E.)); 90, 30 ($K_{1/2}$); 1.96, 1.96 (n) respectively. Data are the mean \pm S.D. of 3 determination of a representative experiment.

A





both ouabain and furosemide, can be considered to be independent of the external Cl concentration, these results being in good agreement with previous reports for Cl-dependence in the HeLa cell line (Aiton et al., 1981). If the replacement anion is gluconate⁻ (figure 3.10 b), the activation of the diuretic sensitive K (⁸⁶Rb) influx is markedly sigmoidal and is saturable at 60 mM Cl with half maximal activation near 30mM Cl. Again the ouabain- and diuretic-insensitive K (⁸⁶Rb) influx was independent of the external Cl concentration.

Variation of external Cl was attempted without the use of substitute anions, by the use of mannitol and choline Cl (table 3.11). Here the external NaCl was reduced to 32mM (tonicity was maintained by the addition of mannitol), this solution giving a Cl of 50mM. The external Cl was increased by replacing the mannitol with choline Cl. The diuretic-sensitive K (⁸⁶Rb) influx in 160mM Cl media does not differ significantly from that measured in media containing 50mM Cl. This is surprising when compared with the NO₃⁻ replacement of external Cl, where, at a Cl concentration of 50 mM, the diuretic-sensitive K influx was reduced to 10% of K (⁸⁶Rb) influx in a 160mM Kreb's solution.

The anion-dependence of the diuretic-sensitive K (⁸⁶Rb) influx of the MDCK cell line was only studied with the anion substitute NO₃⁻ (figure 3.11). Similar to HeLa cells, replacement of the media Cl completely abolished the diuretic-sensitive component of the K (⁸⁶Rb) influx. Increasing the Cl gave a sigmoidal curve and could be described by a Hill equation, with a Hill coefficient near 2, and 50% activation at external Cl of 100 mM, these results being similar to previous reports of Aiton et al., (1982).

Table 3.11 Effect of reduced Cl^-_o (mannitol replacement) on diuretic-sensitive K^+ influx

Cl^-_o	Mean \pm SD K^+ influx $\text{nmol}/10^6$ cells \cdot min							
	Total	+ Ouabain	+ Ouabain (1 mM) + Diuretic (0.1 mM)	+ Diuretic (0.1 mM)	Quabain-sensitive	Quabain-sensitive	Diuretic-sensitive	Diuretic-sensitive
	(1)	(2)	(3)	(4)	1-2	4-3	1-4	2-3
50 mM	5.96 \pm 0.1	2.65 \pm 0.19	1.53 \pm 0.24	3.46 \pm 0.12	3.31 \pm 0.21	1.94 \pm 0.27	2.50 \pm 0.16	1.13 \pm 0.31
160 mM	5.09 \pm 0.05 ^b	3.25 \pm 0.34 ^{ns}	1.60 \pm 0.63 ^{ns}	2.29 \pm 0.33 ^a	1.84 \pm 0.34 ^a	0.69 \pm 0.71 ^{ns}	2.80 \pm 0.33 ^{ns}	1.90 \pm 0.71 ^{ns}

Test for significant difference from 50 mM Cl^-_o values

ns = not significant

a = $p < 0.01$

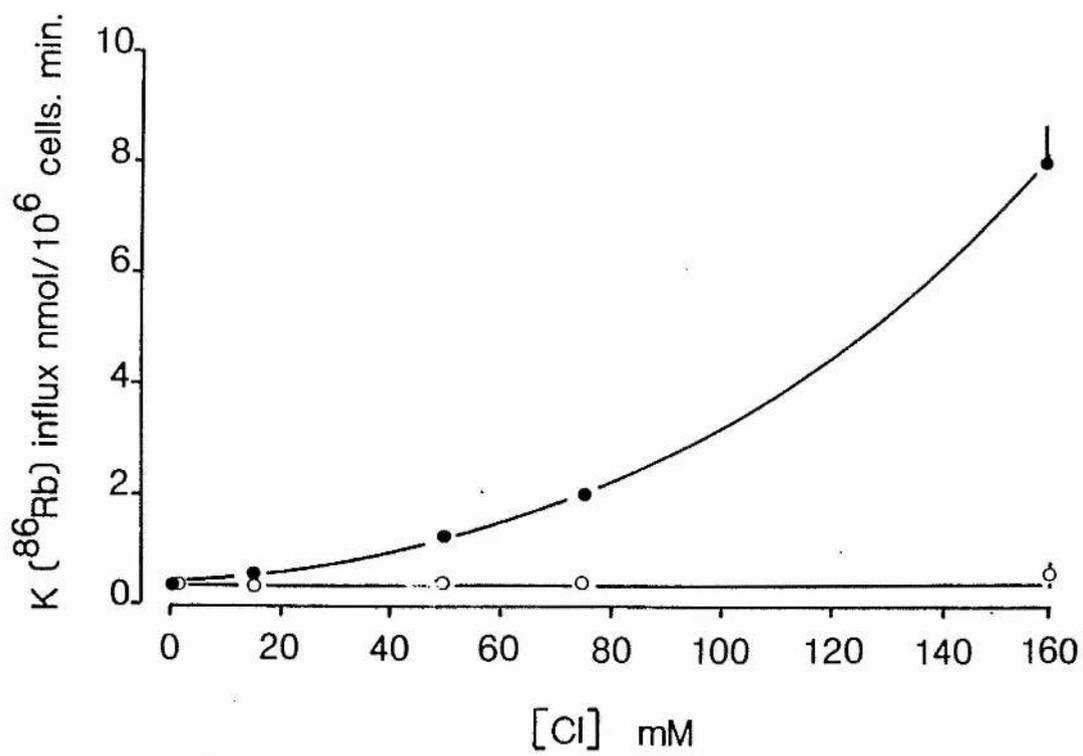
b = $p < 0.001$

Footnote:

External Cl^-_o reduced by reducing the external NaCl to 32 mM NaCl and maintained at this concentration (isotonicity maintained by mannitol addition). Cl^-_o was increased by equimolar replacement of the mannitol with CHCl_3 . In both conditions, external K^+ was maintained at 5.4 mM.

Figure 3.11

Cl-dependence of the ouabain-insensitive K (^{86}Rb) influx of MDCK cells. NaCl was replaced isosmotically by NaNO_3 , in the presence (O) or absence (●) of 0.1 mM diuretic. Data are the mean \pm S.D. of 3 determinations of a representative experiment.



Nature of the inhibitory action of "loop" diuretics on K transport.

a) Diuretic action is due to inhibition of a single transport system.

Due to the possibility that diuretics may inhibit more than one K transport system, experiments designed to test for the additivity of the diuretics bumetanide, piretanide and furosemide at sub-maximal and maximal inhibitory concentrations were performed (tables 3.12 and 3.13). For sub-maximal additions, exposure of MDCK cells to 0.4 μ M bumetanide, 5 μ M piretanide and 10 μ M furosemide inhibited the diuretic-sensitive K influx by 22 - 43% (100% inhibition in the presence of 0.1mM diuretic).

Combinations of the diuretics at sub-maximal concentrations produced greater inhibitions for all conditions (table 3.12). Bumetanide plus piretanide however gave an increase which was not significantly different from the action of piretanide without any addition of bumetanide. The inhibition of a similar fraction of the ouabain-insensitive flux by diuretics (figures 3.12 and 3.13 a,b) is also good evidence for a single locus of action.

At maximal inhibitory concentrations of bumetanide, piretanide and furosemide, the K (86 Rb) influx adjudged to be sensitive to the three diuretics was 9.66 ± 0.38 (mean \pm S.D. n=3) nmol/ 10^6 cells.min. (table 3.13) Exposure of the MDCK cells to the combinations of the diuretics (table 3.13) at their maximal inhibitory concentrations produced no significant increase in the diuretic-sensitive K influx.

Table 3.12 Additivity of Bumetanide (4×10^{-7} M), Piretanide (5×10^{-6} M) and furosemide (10^{-5} M) at sub-maximal concentrations on the inhibition of the K^+ ($^{86}\text{Rb}^+$) influx in ouabain-poisoned MDCK cells^(a)

Diuretic	Diuretic-sensitive K^+ influx	% age inhibition	T-test against ()	Expected inhibition
Bumetanide (1)	2.08 ± 0.30	22	-	
Piretanide (2)	4.73 ± 0.42	49	-	
Furosemide (3)	4.19 ± 0.31	43	-	
Bumetanide + Piretanide	5.41 ± 0.44	56	(1) $p < 0.001$ (2) $p > 0.1$ ns	6.81
Bumetanide + Furosemide	5.56 ± 0.36	58	(1) $p < 0.001$ (3) $p < 0.01$	6.27
Piretanide + Furosemide	6.27 ± 0.33	65	(2) $p < 0.01$ (3) $p < 0.01$	8.93

(a) Data are the mean \pm SD of 3 observations. Significance of difference from () by Student's t-test.

Footnote:

The magnitude of the diuretic-sensitive K^+ influx on the presence of maximal inhibitory concentration 0.1 mM was 9.66 ± 0.38 nmol/ 10^6 cells.min (mean \pm SD, n = 3)

Table 3.13 Additivity of the diuretics, bumetanide (10^{-4} M),
 piretanide (10^{-4} M) and furosemide (10^{-4} M) at
 maximal inhibitory concentrations in ouabain-
 poisoned MDCK cells^(a)

Diuretic	Diuretic-sensitive K ⁺ (⁸⁶ Rb ⁺) influx nmol/10 ⁶ cells.min
Bumetanide	9.93 ± 0.30
Piretanide	9.83 ± 0.30
Furosemide	9.22 ± 0.32
Bumetanide + Piretanide	9.92 ± 0.32
Bumetanide + Furosemide	9.92 ± 0.30
Piretanide + Furosemide	9.86 ± 0.30

(a) Data are the mean ± SD of 3 determinations

Footnote:

Mean maximal diuretic-sensitive K⁺(⁸⁶Rb⁺)
 influx = 9.66 ± 0.38 (mean ± SD, n = 3)

Figure 3.12

Sensitivity of the ouabain-insensitive K (^{86}Rb) influx of HeLa cells to inhibition by "loop" diuretics (●) bumetanide, (○) piretanide, (▲) furosemide. Lines were fitted by eye from linear regression analysis of the data to the Hill equation.

$$\log (V/V_{\max} - V) = n \log S - \log K.$$

Where V_{\max} is the maximal inhibition by diuretics and S the diuretic concentration, n = the Hill coefficient and $K = S_{0.5}$. For bumetanide, $K_i = 0.668 \text{ uM}$ and $n = 1.20 \pm 0.13$; piretanide, $K_i = 1.31 \text{ uM}$ and $n = 0.95 \pm 0.09$; furosemide, $K_i = 6.73 \text{ uM}$ and $n = 0.65 \pm 0.09$. Data are the mean \pm S.D. of 3 determinations.

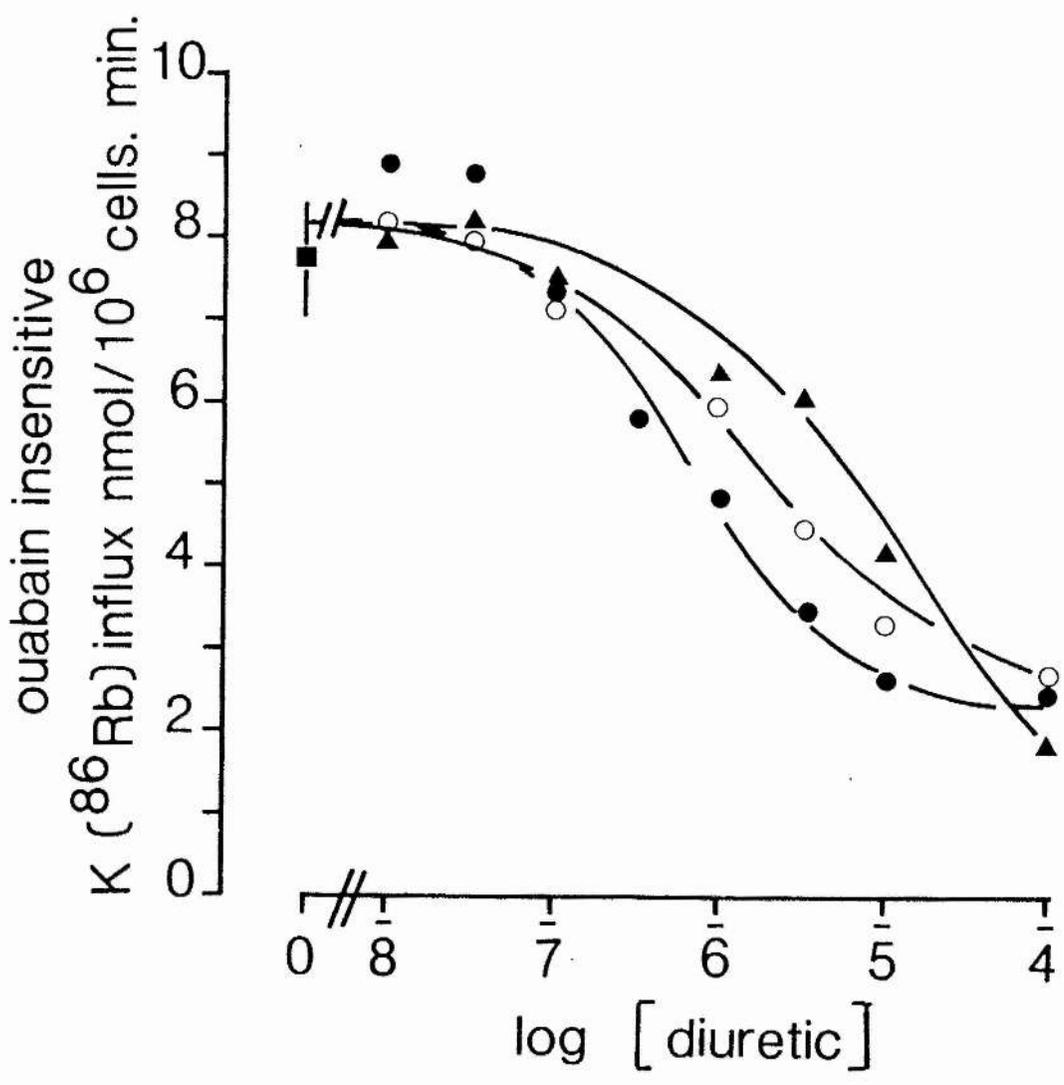
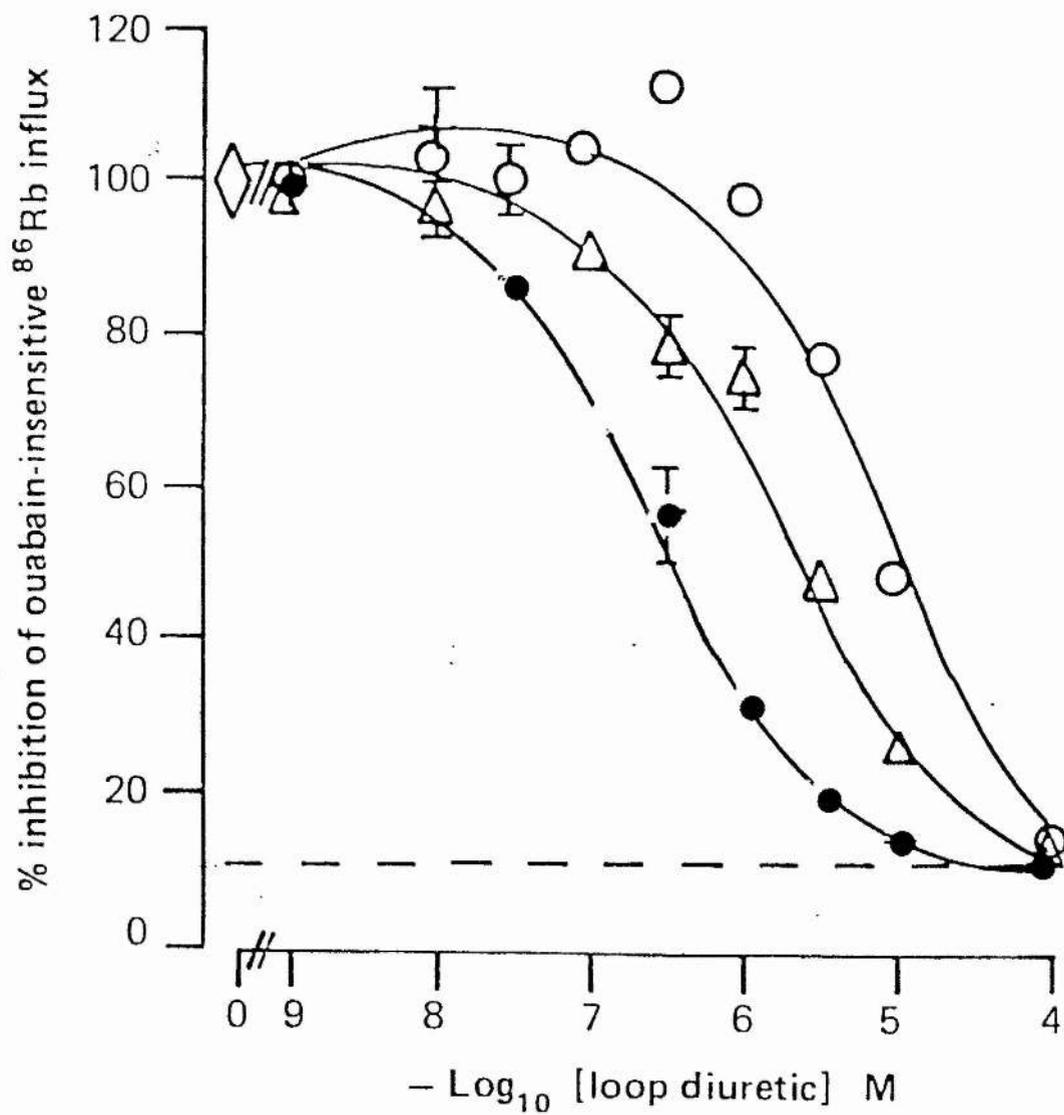


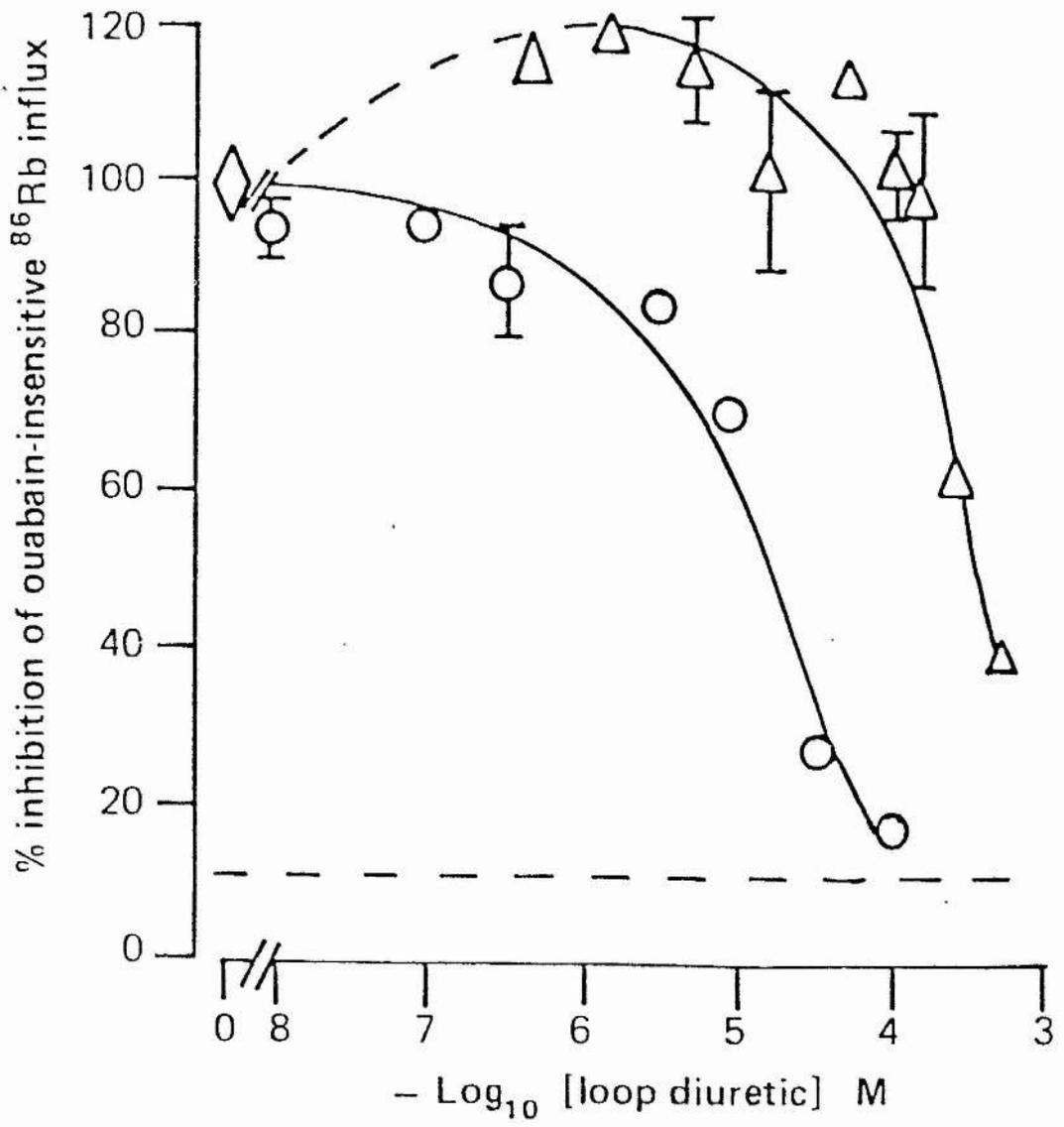
Figure 3.13 A,B

Sensitivity of the ouabain-insensitive K (^{86}Rb) influx of MDCK cells to (A) bumetanide (●), piretanide (Δ) and furosemide (○) and (B) ethacrynic acid (Δ) and ethacrynic acid plus adduct cysteine (○). Data are the mean \pm S.D. of 3 determinations. Lines were fitted by eye, and the data linear regressed to the Hill equation (see legend of figure 3.10). For bumetanide, $K_i = 0.25 \mu\text{M}$; piretanide, $K_i = 2.65 \mu\text{M}$; furosemide, $K_i = 14.80 \mu\text{M}$; ethacrynic acid, $K_i = 318.00 \mu\text{M}$; ethacrynic acid plus the adduct cysteine, $K_i = 7.92 \mu\text{M}$.

A



B



b) Diuretic potency

The relative potency of "loop" diuretic inhibition of the ouabain-insensitive K (^{86}Rb) influx of HeLa cells is shown in figure 3.12. Bumetanide, with half maximal inhibition (apparent K_i) at $0.67\mu\text{M}$, was the most effective inhibitor of the K influx compared with piretanide (apparent K_i $1.31\mu\text{M}$) and furosemide (apparent K_i $6.73\mu\text{M}$). The relative potency of the diuretics, bumetanide > piretanide > furosemide, is similar to their action in the turkey erythrocyte, their natriuretic effect in the dog (Palfrey, Feit and Greengard, 1980), and their potency in human erythrocytes (Ellory and Stewart, 1982). These pharmacological data are regarded by Palfrey and Rao (1983) as a fingerprint for the existence of a Na + K + Cl "cotransport" system.

Identical experiments on MDCK cells are shown in figures 3.13a and 3.13b. The order of potency for the three diuretics is similar to the HeLa data. Bumetanide (apparent K_i of $0.25\mu\text{M}$) is 10.6 times more effective than piretanide (apparent K_i $2.65\mu\text{M}$) and 59 times more potent than furosemide (apparent K_i $14.80\mu\text{M}$). In the isolated ascending limb of the loop of Henle and human erythrocytes, ethacrynic acid has been demonstrated to inhibit Na + K + Cl "cotransport" system(s), the efficacy being considerably increased in the presence of cysteine (Burg and Green, 1973; Ellory and Stewart, 1982) due to the formation of an adduct. This effect is mirrored in the MDCK cell line (figure 3.13b) with the inclusion of equimolar cysteine decreasing the apparent K_i from $318\mu\text{M}$ to $7.92\mu\text{M}$, which is similar to the apparent K_i of piretanide. There is a stimulation of the K influx in low concentrations of ethacrynic acid, possibly due to its action upon -SH groups, similar to N-ethylmaleimide (-SH reducing agent) in sheep red blood cells (Lauf and Theg, 1980). The pharmacological data

are thus consistent with the existence of Na + K + Cl "cotransport" in HeLa and MDCK cells.

c) Effects of external ionic medium upon diuretic inhibition

Haas and McManus (1983) showed that an increase in the external Cl concentration decreased the bumetanide inhibition of "cotransport" (net Rb uptake) in duck red cells at constant levels of bumetanide. Palfrey et al. (1980) have also demonstrated that cations actually enhance bumetanide inhibition in turkey cells by varying the ionic composition of the experimental media. In view of this evidence, the dose-dependent inhibition by bumetanide in MDCK cells and furosemide in HeLa cells was investigated in the ionic conditions described in table 3.14 and figure 3.14

The apparent K_i for bumetanide in MDCK cells determined under the external ionic conditions (a - c) given in the legend of figure 3.14 are within one standard deviation of the mean apparent K_i for bumetanide determined in control media (140mM Na, 5.4mM K and 160mM Cl), i.e. $0.55 \pm 0.26 \mu\text{M}$, the mean \pm S.D. of 3 experiments. The apparent K_i for bumetanide inhibition of K (^{86}Rb) influx determined in a low Cl (NO_3^- substitution) Krebs solution were 0.38 and 1.26 μM in two experiments, and the mean value of 0.82 ± 0.62 (S.D.) is not significantly different for the apparent K_i from bumetanide determined in a standard Krebs solution. The wide range of these results may be due to the small nature of the diuretic-sensitive influx in low external Cl. However, these results do not show the increased efficacy of bumetanide inhibition of "cotransport" in low Cl media observed in duck erythrocytes (Haas and McManus, 1983). Similarly, the apparent K_i of furosemide in HeLa cells (table 3.14) in either low K or Cl media are within one standard deviation of the apparent K_i for

Table 3.14 Effect of varying external ionic composition of the incubation media upon the sensitivity of ouabain-insensitive K^+ ($^{86}Rb^+$) influx of HeLa cells to furosemide (a)

Condition	External ionic composition in mM			Half-maximal inhibitory concentration M
	K^+_o	Na^+_o	Cl^-_o	
A	5.4	140	160	$4.45 \pm 2.79^{(b)}$
B	2.5	140	160	3.81
C	2.5	69	160	23.00
D	5.4	140	80	2.10

(a) K^+_o substituted with mannitol to maintain isotonicity. Na^+ and Cl^- were replaced equimolarly with choline and NO_3^- respectively. app.Ki determined from linear regression of the data fitted to the Hill equation as described for Figure 3.10.

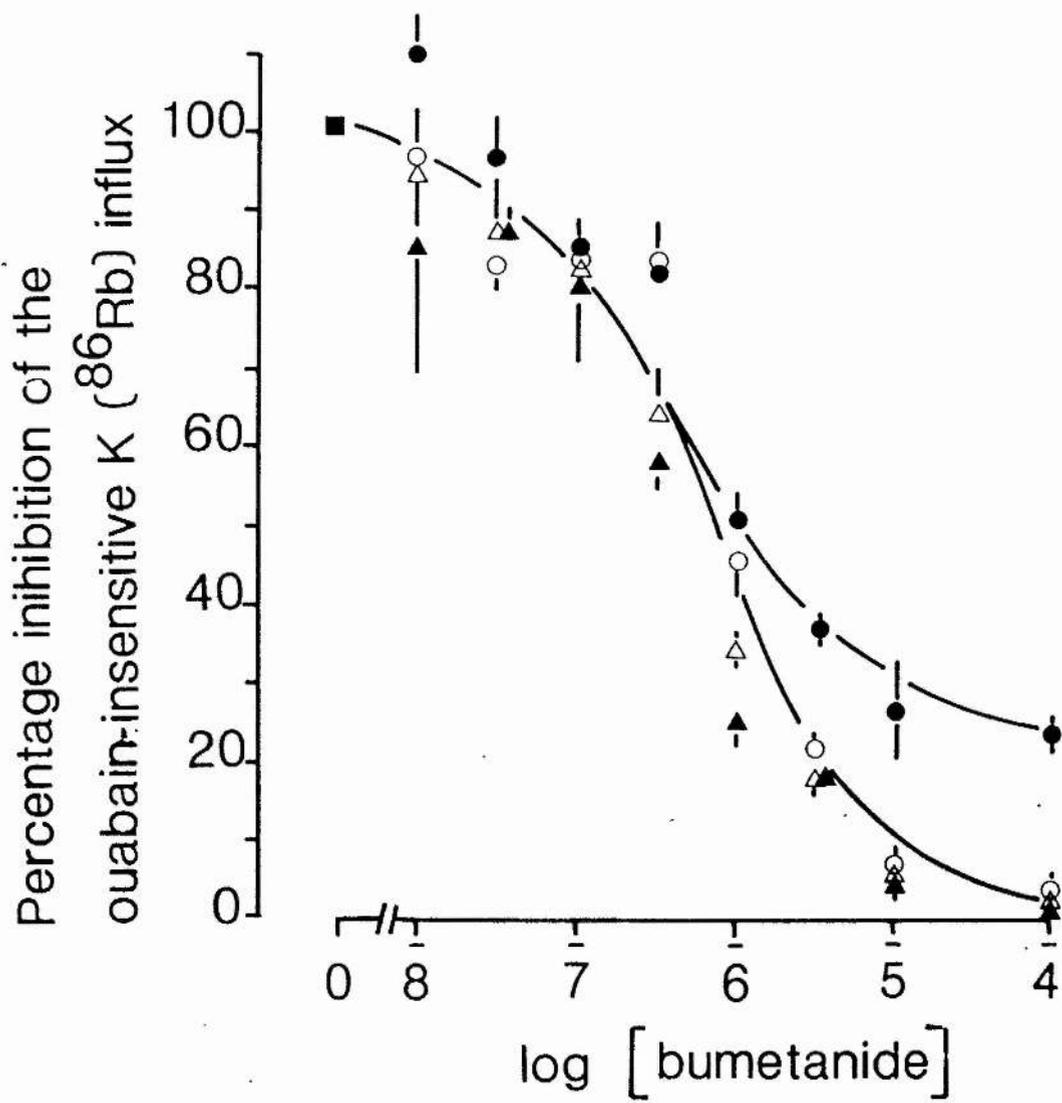
(b) Mean \pm SD value from 3 experiments.

Figure 3.14

Effect of varying the external ionic composition of Krebs solution upon the sensitivity of the ouabain-insensitive K (^{86}Rb) influx of MDCK cells to bumetanide. The external concentrations of Na, K and Cl in mM were as follows: (●) 140, 14.5, 160; (△) 140, 3.8, 160; (○) 60, 3.6, 160; (▲) 140, 3.6, 60. The K_i (μM) for these conditions were 0.62, 0.44, 0.49, 0.38 respectively. Data are the mean \pm S.D. of 3 determinations; lines were fitted by eye. Data has been linear regressed to the Hill equation (see legend figure 3.10).

Footnote.

The mean \pm S.D. ($n=3$ experiments) K_i for bumetanide in MDCK cells in standard Krebs solution (Na 140mM, K 5.4mM, Cl 160mM) is 0.55 ± 0.26 μM .



furosemide determined in control media (140mM Na, 5.4mM K and 160mM Cl), i.e. $5.48 \pm 2.79 \mu\text{M}$, mean \pm S.D. of 3 experiments. Reduction of both the external Na and K (condition c) increased the apparent K_i for furosemide in HeLa from $1.58 \mu\text{M}$ to $23 \mu\text{M}$, in agreement with the results of Palfrey et al. (1980) and this indicates that the "loop" diuretic site of action in HeLa cells is removed from the Na and K sites. Since changes in internal ions under these experimental conditions are neither controlled nor measured, the possibility of secondary effects giving rise to the observed data cannot be excluded at present.

Influence of cell density on K influx.

During the studies on the K transport in the HeLa and MDCK cell lines, it became clear that the absolute magnitude of the K (^{86}Rb) influx varied from experiment to experiment.

The cell volumes of the HeLa and MDCK cells as a function of the cell density are given in figure 3.15 and table 3.15 respectively. A 30 - 50 % reduction in the cell volume was observed in HeLa and MDCK cells when cells passed from low to high density cultures. In the HeLa cell line, a significant negative correlation was observed between the cell volume (μ^3) and the cell number ($r = -0.84$; $P < 0.001$). These data are similar to those reported for the MDCK and T1 (chemically transformed MDCK cells) cells (Erlinger and Saier, 1982).

The total, ouabain-sensitive, diuretic-sensitive and residual components of the K (^{86}Rb) influx expressed in $\text{nmol}/10^6 \text{ cells}\cdot\text{min}$ as a function of the cell number is depicted in figure 3.16 a - d and table 3.16 respectively for HeLa and MDCK cells. For the HeLa cell line, a

Figure 3.15

Cell volume (μm^3) of HeLa cells, determined by electronic cell sizing (see methods), as a function of cell density. HeLa cells were grown for 4 days in B.M.E. media supplemented with 10% newborn calf serum. Cell number variation due to different seeding densities of cultures. Data are the mean \pm S.D. of at least 6 determinations in 13 experiments.

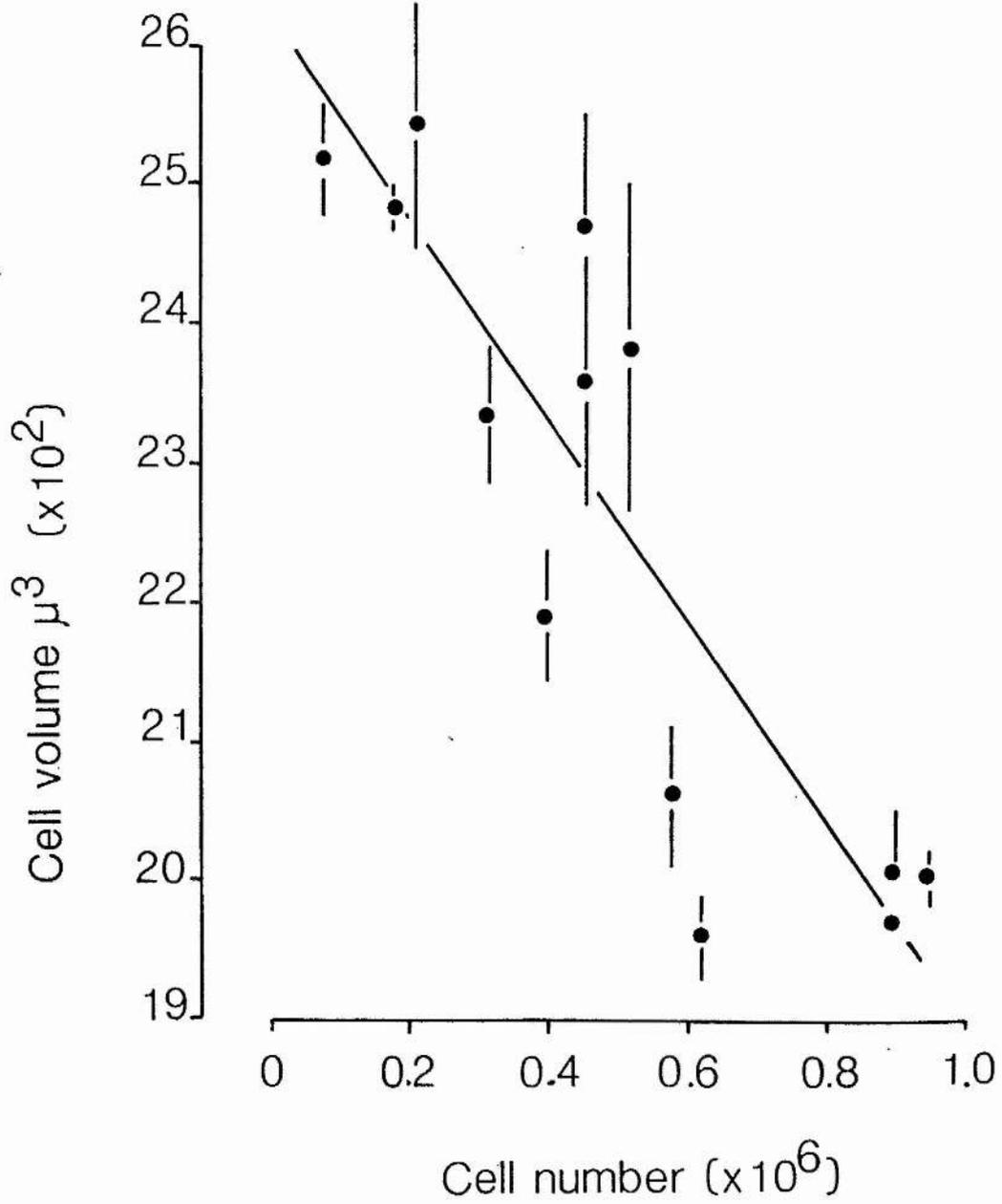


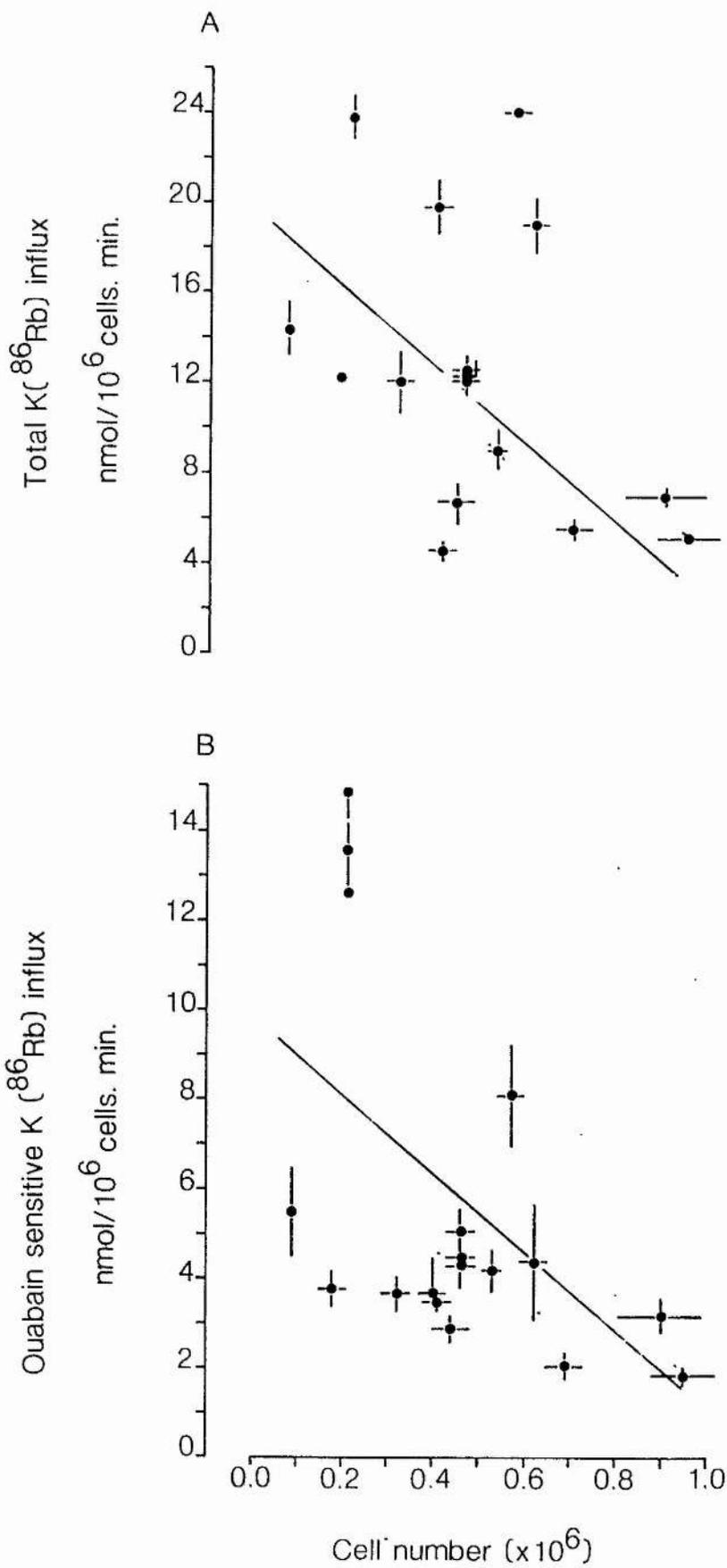
Table 3.15 Effect of cell density upon the magnitude of K^+ ($^{86}\text{Rb}^+$) influx in MDCK cells (a)

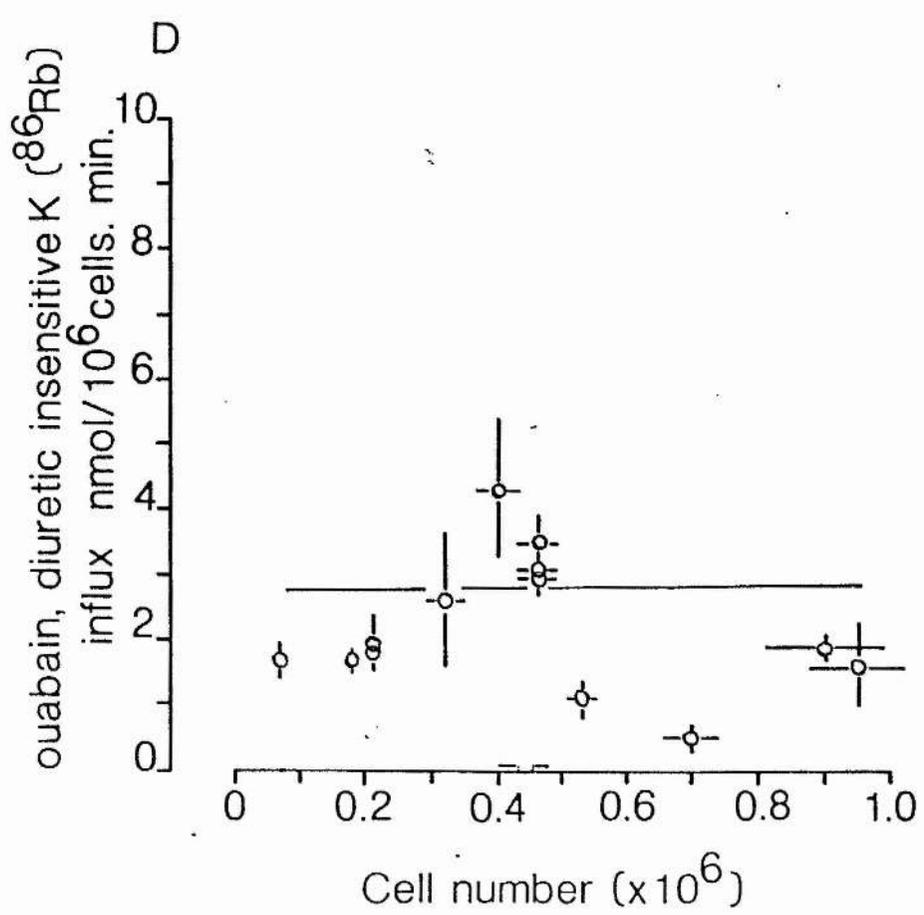
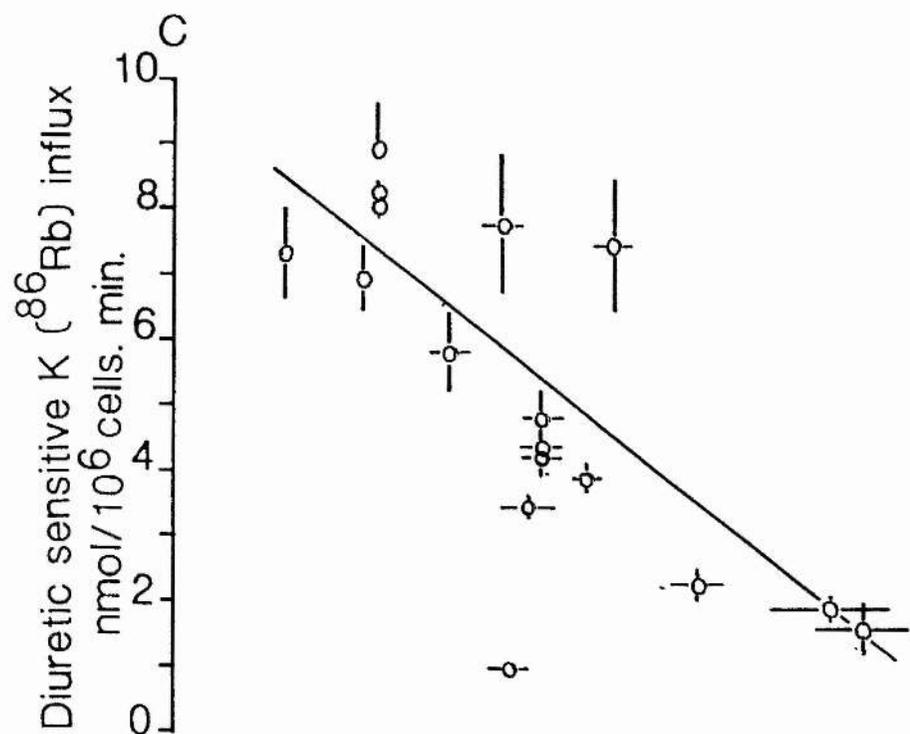
Cell Number (X10 ⁶) n=12	Volume (μ ³) n=12	Total	Ouabain -sensitive	Diuretic -sensitive	Residual
0.14 ± 0.01	2443 ± 73	19.28 ± 1.95	7.09 ± 2.12	11.16 ± 0.91	1.03 ± 0.34
0.41 ± 0.03	2048 ± 116	11.15 ± 0.62	3.14 ± 0.66	7.45 ± 0.22	0.56 ± 0.04
0.69 ± 0.005	1875 ± 55	10.31 ± 0.44	2.06 ± 0.63	7.49 ± 0.43	0.76 ± 0.07
0.89 ± 0.08	1646 ± 53	7.72 ± 0.21	1.77 ± 0.37	4.86 ± 0.31	1.09 ± 0.04
0.78 ± 0.03	1458 ± 38	4.87 ± 0.28	1.59 ± 0.40	1.87 ± 0.29	1.41 ± 0.05

(a) Data are the mean ± SD of 3 determinations except where otherwise stated.

Figure 3.16

Dependence of the K (^{86}Rb) influx upon cell number/plate. All experiments were performed on HeLa cells grown for 4 days on multi-well plates, Nunc, in 3 ml B.M.E. supplemented with newborn calf serum. (A) total K (^{86}Rb) influx, (B) ouabain-sensitive component, (C) diuretic-sensitive component, (D) the ouabain- and diuretic-resistant residual component. Data are the mean \pm S.D., $n=3$, for K (^{86}Rb) influx and the mean \pm S.D., $n=12$, for cell number of individual experiments.





significant negative linear correlation with total K (^{86}Rb) influx was observed with increasing cell number ($r = -0.63$; $P < 0.02$). The decrease in the total K (^{86}Rb) influx was reflected in both the ouabain-sensitive and diuretic-sensitive components of this flux (figures 3.16 b-c) and gave significant negative correlations to the cell number ($r = -0.55$, $P < 0.02$; $r = -0.80$, $P < 0.001$ respectively). The residual component was largely unaffected by the cell density of the HeLa cultures ($r = 0.04$; not significant). Similar results were obtained for the MDCK cell line (table 3.16), with highly significant decreases in the total, ouabain-sensitive and diuretic-sensitive components of the K (^{86}Rb) influx ($P < 0.02 - 0.001$). Again, the residual component is largely unaffected by increased cell density. These data for the dependency of the total K (^{86}Rb) influx of MDCK cells on the cell density are in agreement with the ^{22}Na uptake in this cell type reported by Erlinger and Saier (1982). A caveat to these observations in the MDCK cell line is the retention of many epithelial features; cell-cell tight junctions, baso-lateral location of the Na K ATPase, and Na K Cl "cotransport" system (see Simmons, 1982). Thus, in cell cultures that retain epithelial features, the access to the baso-lateral cell membrane will be restricted in high density compared to low density cultures (Sepulveda and Pearson, 1984).

Does the decrease in the K (^{86}Rb) influx per cell of the HeLa and MDCK cell lines reflect the decrease in the surface area of the cells or is there a real decrease in flux per unit membrane area as cells pass from low to high density cultures? The K influxes expressed as $\text{nmol}/\mu^2 \cdot \text{min}$. are given in table 3.16 for MDCK and HeLa cells. Significant decreases ($P < 0.01 - 0.001$) were observed in the total, ouabain-sensitive and diuretic-sensitive K (^{86}Rb) influx per

Table 3.16 Effect of cell density upon K^+ ($^{86}\text{Rb}^+$) influx expressed relative to (a) cell number and (b) per unit membrane area in HeLa and MDCK cells (c)

Cell type	Cell number (X10 ⁶)	Cell volume (μ^3)	Surface area (μ^2)	Total		Ouabain-sensitive		Diuretic-sensitive		Residual	
				A	B	A	B	A	B	A	B
HeLa	0.21	2542	900	23.58	26.02	12.61	14.00	8.98	9.97	1.88	2.48
	± 0.01	± 144		± 0.95	± 1.10	± 0.92	± 1.00	± 0.77	± 0.86	± 0.31	± 0.28
MDCK	0.95	2017	772	5.09 ^e	6.59 ^e	1.84 ^e	2.38 ^e	1.65 ^e	2.14 ^e	1.60 ^{ns}	2.07 ^{ns}
	± 0.07	± 20		± 0.05	± 0.06	± 0.20	± 0.26	± 0.40	± 0.53	± 0.63	± 0.83
(A) nmol/10 ⁶ cells.min	0.14	2446	876	19.28	22.09	7.09	8.09	11.16	12.74	1.03	1.18
	± 0.01	± 20		± 1.95	± 2.26	± 2.12	± 2.42	± 0.91	± 1.04	± 0.34	± 0.39
(B) nmol/ μ^2 min (X10 ⁻⁹)	0.78	1458	622	4.87 ^e	6.10 ^e	1.59 ^d	1.99 ^d	1.87 ^e	2.35 ^e	1.41 ^{ns}	1.77 ^{ns}
	± 0.03	± 38		± 0.28	± 0.35	± 0.40	± 0.52	± 0.29	± 0.36	± 0.05	± 0.06

(c) Data are the mean \pm SD of 3 observations. Significant differences from low density cultures tested by Student's t-test. ns = not significant.

(d) $p < 0.02$

(e) $p < 0.001$

unit membrane area. Therefore, the negative correlation between the K flux and cell number represents a real decrease in the K flux per unit membrane area in both cell types, and may be due to decreased protein content of the cells in high density cell cultures (Erlinger and Saier, 1982). The biological significance of these observations is unclear, but the monovalent cation transport of Ehrlich ascites cell varies according to the cell cycle, with the Na K ATPase-mediated K transport and furosemide-sensitive K-K exchange being depressed in the early stationary phase of the cell cycle (see review, Boonstra, Mummery, van Zoelen, van der Saag and de Laat, 1982).

DISCUSSION.

The results presented in this chapter demonstrate that the K influx of the cell lines studied, HeLa (human cervical carcinoma) and MDCK (epithelial cell line), can be partitioned into three components on the basis of their differential pharmacological sensitivity. These components are: the ouabain-sensitive, Na K ATPase-mediated K flux; a "loop" diuretic-sensitive pathway; and the "loop" diuretic- and ouabain-insensitive (residual) K influx. This substantiates previous work by Aiton et al., (1981 and 1982) and the present results are comparable to earlier reports in the erythrocyte (Sachs, 1971; Wiley and Cooper, 1974; Dunham et al., 1980; Chipperfield, 1980 and 1981) and the Ehrlich ascites cell (Tupper, 1975; Bakker-Grunwald, 1978). There are also diuretic-sensitive and insensitive-components of the K (⁸⁶Rb) efflux.

The diuretic-sensitive K transport of HeLa and MDCK cells possesses many features in common with the Na K Cl "cotransport" systems described for other cell types (Palfrey and Rao, 1983). A major feature is the inhibition of the Na K Cl "cotransport" system(s) by the "loop" diuretics, bumetanide, piretanide and furosemide (Palfrey and Greengard, 1981). The inhibitory potency of the three "loop" diuretics mentioned above on the diuretic-sensitive K transport of the HeLa and MDCK cell lines has been assessed and data have been presented in this chapter. The order of potency, bumetanide > piretanide > furosemide, in both HeLa and MDCK cells, mirrors their natriuretic activity in dogs (Palfrey et al., 1980) and their inhibitory action on the Na K Cl "cotransport" system(s) in avian and human erythrocytes (Palfrey and Rao, 1983; Palfrey et al., 1980; Ellory and

Stewart, 1982) and MDCK cells (Rindler et al., 1982). The greater efficacy of bumetanide compared with furosemide has also been observed in the thick ascending limb of the loop of Henle (Schlatter, Greger and Weidtko, 1983) and the amphibian cornea (McGahan, Yorio and Bentley, 1977). In the MDCK cell line, the inhibitory potency of another "loop" diuretic, ethacrynic acid, was assessed and exhibited an apparent K_i for the diuretic-sensitive K (^{86}Rb) influx of more than 0.1mM, which, with the use of the adduct cysteine, could be made equipotent to piretanide. A similar differential potency of ethacrynic acid and ethacrynic acid plus cysteine has been demonstrated in the thick ascending limb of the loop of Henle (Burg and Green, 1973), turkey erythrocytes (Palfrey and Greengard, 1981) and human erythrocytes (Ellory and Stewart, 1982). However, there are caveats to the use of the furosemide-sensitivity as the sole criterion for the identification of the "cotransport" pathway, since there are several transport systems inhibited by furosemide, notably the Cl/HCO_3 exchange of human red blood cells (Brazy and Gunn, 1976) and the volume-stimulated KCl transport of sheep erythrocytes (Dunham and Ellory, 1981). However, only high affinity inhibition is limited to the Na K Cl "cotransport" system(s) (Palfrey and Rao, 1983).

In avian erythrocytes, the sensitivity of the "loop" diuretics is dependent upon the ionic composition of the media (Palfrey et al., 1980; Haas and McManus, 1983) and the site at which the "loop" diuretics inhibit the Na K Cl "cotransport" system appears to be removed from the Na and K binding sites (Palfrey et al., 1980) but associated with the Cl binding site (Haas and McManus, 1983). It was therefore felt worthwhile to investigate the possible site of diuretic action in HeLa and MDCK cells. However, in this present work the data are inconsistent with "loop" diuretics (bumetanide and furosemide)

being effective at the Cl site of the Na K Cl "cotransport" observed in either HeLa or MDCK cells. However, in HeLa cells the action of furosemide is removed from the Na and K sites similar to the duck erythrocyte (Palfrey et al., 1980). In MDCK cells, the "loop" diuretics, bumetanide, piretanide and furosemide, do appear to inhibit the Na K Cl "cotransport" at a single locus (see results), and the structural requirements for the inhibitory activity of these agents and related analogs are quite narrow (Palfrey et al., 1980; Palfrey and Greengard, 1981; Ellory and Stewart, 1982).

A further observation of the "cotransport" systems is their anion sensitivity, only the Cl replacement by Br supporting significant diuretic-sensitive fluxes in human erythrocytes (Dunham et al., 1980; Chipperfield, 1980 and 1981), avian erythrocytes (Palfrey and Greengard, 1981), Ehrlich ascites cells (Geck, Heinz, Pietrzyk and Pfeiffer, 1978; Geck, et al., 1981), and the cultured cell lines HeLa and MDCK (Aiton et al., 1981 and 1982; McRoberts et al., 1982). In the present study, the anion dependency of the diuretic-sensitive K (^{86}Rb) influx of HeLa and MDCK cells is confirmed.

The two characteristics discussed above, "loop" diuretic and anion sensitivity, are sufficient to distinguish between coupled Na Cl and Na K Cl "cotransport" systems and putative "parallel exchanger" systems. An instance of the latter has been suggested for net salt transport in the brush border membrane of the small intestine (Liedtke and Hopfer, 1982), where an amiloride-sensitive Na/H antiport and the disulphonic stilbenes- (SITS and DIDS) sensitive Cl/HCO₃ exchange system are operative. These inhibitors have been demonstrated to be ineffective on the Na Cl and Na K Cl "cotransport" systems of avian and human erythrocytes (Palfrey and Greengard, 1981; Dunham et al.,

1980), the thick ascending limb of the loop of Henle (Schlatter et al., 1983) and the cultured cell lines HeLa and MDCK (Aiton et al., 1981 and 1982; McRoberts et al., 1982). On the basis of these criteria, the diuretic-sensitive K (^{86}Rb) influx observed in this present study can be considered to be mediated via a "cotransport" system, thus substantiating previous work by Aiton et al. (1981) in HeLa cells and by Aiton et al. (1982), Rindler et al. (1982) and McRoberts et al. (1982) in MDCK cells.

In this present study, a comprehensive analysis of the Na, K and Cl dependence of the ouabain-insensitive K (^{86}Rb) influx in both HeLa and MDCK cells was performed. The diuretic-sensitive K influx of the HeLa and MDCK cells exhibits a requirement for external Na and K, this dependency being similar to that observed in the human erythrocyte (Wiley and Cooper, 1974; Chipperfield, 1981; Dunham et al., 1980) and substantiating the previous reports for HeLa and MDCK cells (Aiton et al., 1981 and 1982; McRoberts et al., 1982; Rindler et al., 1982). The effect of the replacement of the external Cl with NO_3^- on the diuretic-sensitive K influx is comparable with the earlier work in the human erythrocyte by Chipperfield (1980 and 1981) and Dunham et al., (1980), but the activation curve is dependent upon the Cl replacement used (Chipperfield, 1984) with the sigmoidicity of the Cl activation especially obvious with gluconate replacement of Cl. The indirect evidence for the stoichiometry of the Na K Cl "cotransport" of the MDCK cell (McRoberts et al., 1982) being 1:1:2 is applicable to the HeLa cell "cotransport" system, since the K (^{86}Rb) influx only occurs in the presence of Na and Cl, with apparent Hill coefficients for the Na- and Cl-dependency near unity and 2 respectively, and the system is electroneutral (see chapter 7).

The diuretic-sensitivity of the membrane transport of Na K and Cl ions has been demonstrated for the MDCK cell line (Rindler et al., 1982) and is similar to the coupled Na K 2Cl "cotransport" of Ehrlich ascites cells and duck erythrocytes (Geck et al., 1980; Haas et al., 1982). Thus the diuretic-sensitive K transport of MDCK cells measured in this study may be considered to be Na K 2Cl "cotransport". To date no direct measures of the diuretic-sensitivity of Na and Cl fluxes have been made in the HeLa cell line, so that it is unknown whether the Na- and Cl- dependent K transport through the "cotransport" pathway represents a coupled Na K Cl ion movement (see chapter 4). However, the marked similarity between the Na- and Cl- dependent, diuretic-sensitive K (^{86}Rb) influx of HeLa cells and the K (^{86}Rb) influx of MDCK cells through the Na K 2Cl "cotransport" system is strong prima facie evidence to suggest that Na K Cl "cotransport" occurs in the HeLa cell line, but this does not eliminate the possibility that this K flux is Na- and Cl- dependent and not a coupled transport of all three. The possibility that the operational parameters of the "cotransport" system may vary considerably may not be excluded, since the 1-2 hour incubation of HeLa cells in a 1 mM ouabain solution reveals a proportion of the diuretic-sensitive K efflux to be independent of Na (see results; Aiton and Simmons, 1983).

Irrespective of the degree of coupling of Na K and Cl, it is likely that co-operativity between the ion binding sites will occur, which is indeed true for MDCK cells (Rindler et al., 1982; McRoberts et al., 1982) and the avian erythrocyte (Palfrey and Rao, 1983). Similarly in HeLa cells, the effect of varying external Na on K activation and K on Na activation of the K (^{86}Rb) influx indicates that positive co-operation is occurring between these two ion binding

sites (Segel, 1975), since the apparent K_m of the activating ion decreases with increased co-ion concentration. Flattened hyperbolae of the K and Na activations of the diuretic-sensitive K (^{86}Rb) influx in sub-optimal concentrations of the co-ion may indicate Na and K binding to a heterogenous group of cation sites on the "cotransport" entity (Levitzki, 1980). Interestingly the effect of decreased Cl concentration (NO_3^- substitution) on the K activation of the diuretic-sensitive K (^{86}Rb) influx produced marked sigmoidal kinetics (see results). This series of curves are analogous to the effect of an allosteric inhibitor on the substrate binding to allosteric enzymes (Engel, 1977). Thus NO_3^- may act as an allosteric inhibitor on the "cotransport" system. This may be possible since the activity of the diuretic-sensitive K transport of the HeLa cells is only 10% of controls (external Cl, 160 mM) in 50 mM Cl when NO_3^- is the replacement anion, but in gluconate- or mannitol-replaced media this K flux is near that of controls. This effect may however be due to secondary effects of membrane potential and/or intracellular pH rather than a direct inhibitory action of NO_3^- on the "cotransport" system and requires further elucidation.

The Na K Cl "cotransport" of HeLa and MDCK cells does not appear to mediate a net transfer of ions under physiological conditions, since prolonged incubation in the presence of 0.1 mM diuretic does not affect the Na K and Cl contents of HeLa cells and the Na and K contents of MDCK cells (see also Aiton et al., 1982; Aiton and Simmons, 1983). Therefore in these conditions the sum of the chemical potential gradients should be zero, since Schmidt and McManus (1977 c) and Haas et al., (1982) have demonstrated in avian erythrocytes, that net flux through the Na K Cl "cotransport" is dependent upon the sum of the chemical potential gradients of the ions involved, i.e.,

assuming electroneutrality and a stoichiometry of Na + K + 2Cl, then

$$1) \quad \mu_{net} = \mu_{Na} + \mu_K + 2 \mu_{Cl}$$

$$2) \quad \mu_{net} = RT \cdot \ln \frac{Na_o}{Na_i} + RT \cdot \ln \frac{K_o}{K_i} + 2RT \cdot \ln \frac{Cl_o}{Cl_i}$$

$$3) \quad \mu_{net} = RT \cdot \ln \frac{Na_o}{Na_i} \cdot \frac{K_o}{K_i} \cdot \frac{Cl_o^2}{Cl_i^2}$$

$$4) \quad \text{If } \mu_{net} = 0$$

$$\text{Then } \frac{Na_o}{Na_i} \cdot \frac{K_o}{K_i} \cdot \frac{Cl_o^2}{Cl_i^2} = 1$$

For the HeLa cells the result of equation 4 under physiological conditions was 1.01 and in the presence of 0.1 mM furosemide 1.42 (results table 3.7), thus indicating the system to be at or near chemical equilibrium. However, a small inwardly directed driving force can also be observed under physiological conditions (equation 4 = 2.11 and plus furosemide = 1.35, see results table 3.7). This supports the hypothesis that the greater magnitude of the ouabain-sensitive K (⁸⁶Rb) influx determined in the absence as compared with the presence of bumetanide inhibition of "cotransport", observed in 20-30% of the K (⁸⁶Rb) experiments (see results), may represent a small net Na influx through the "cotransport" system, which thereby activates the Na K ATPase. This effect is in agreement with the model proposed by Geck and co-workers (1981) for ion transport in Ehrlich ascites cells.

In MDCK (epithelial) cells, Na K Cl "cotransport" has been demonstrated to be involved in the catecholamine stimulation of Cl secretion (Brown and Simmons, 1981). Similar inhibitions of Cl

secretion by "loop" diuretics have been observed in various secretory epithelia e.g. trachea, shark rectal gland and cornea (see Palfrey and Rao, 1983). The importance of the Na K Cl "cotransport" system to the physiology of the non-epithelial cell is at present uncertain. However, Na K Cl "cotransport" has been implicated in the volume regulatory response of the avian erythrocyte (Kregenow, 1977; Schmidt and McManus, 1977 a,b,c; Ueberschar and Bakker-Grunwald, 1983), but volume regulation is only observed in either elevated (cell swelling) or reduced (cell shrinkage) external K (Schmidt and McManus, 1977b). Thus in non-epithelial cells, the Na K Cl "cotransport" system may represent a K homeostasis mechanism (Duhm and Goebel, 1984 a,b).

CHAPTER 4

^{22}Na AND ^{36}Cl TRANSPORT IN HELA AND MDCK CELL LINES : DO "LOOP"

DIURETICS INHIBIT?

INTRODUCTION.

In chapter 3 I have demonstrated that in both HeLa and MDCK cells, there is a "loop" diuretic-sensitive portion of the (ouabain-insensitive) passive K flux. As already discussed, the diuretic-sensitive component of K influx exhibits many features in common with the known Na K Cl "cotransport" system of the Ehrlich ascites cell line (Geck et al., 1980) and MDCK cell line (McRoberts et al., 1982) and the Na K "cotransport" system of the human erythrocyte (Wiley and Cooper, 1974), which has been demonstrated to be Cl dependent by Chipperfield (1980) and Dunham et al., (1980).

In order to substantiate the concept of "cotransport" between Na K and Cl ions in the HeLa and MDCK cell lines, a portion of the trans-membrane transport of these three ions should be inhibited by "loop" diuretics. Measurements of the Na influx/efflux and Cl efflux were performed with this goal in mind. Additionally, if it is possible to measure diuretic-sensitive Na K and Cl fluxes an exact determination of the transport stoichiometry is possible as has been demonstrated for the squid axon (Russell, 1983). Other determinations made in various cell types, e.g. duck red cells (Schmidt and McManus, 1977 a-c; Haas et al., 1982), MDCK cells (McRoberts et al., 1982) and Ehrlich ascites cells (Geck et al., 1980), are measurements of net ion uptake through the transport system. Measurement of "cotransport" fluxes under these conditions are more ambiguous (see discussion, Haas et al., 1982), since other conductive elements and electroneutrality considerations may markedly alter or obscure the primary transport (diuretic-sensitive) of interest.

The Ehrlich ascites cell line, which possesses a Na K 2Cl "cotransport" system (see above), has also been studied under steady state conditions by Aull (1981), demonstrating a K-dependent and bumetanide-sensitive inhibition of the ^{36}Cl uptake. However, Hoffmann, Sjöholm and Simonsen (1983) have demonstrated that, in physiological steady state, a Na-dependent, bumetanide-sensitive ^{36}Cl uptake was only detectable in Ehrlich ascites cells exposed to hypertonic media (shrunken cells).

In this chapter, data are presented on the Na and Cl fluxes in the HeLa cell line and Na fluxes in the MDCK cell line and their sensitivity to inhibition by "loop" diuretics.

RESULTS.

Typical time courses for ^{22}Na uptake in HeLa and MDCK cells are given in figures 4.1 and 4.2. The uptake has a half time of 1 to 1.5 minutes and becomes non-linear after 1 minute. The low specific activity of the ^{22}Na in the external medium in these control conditions restricts the reliability of the initial uptake rate measurements expressed as $\text{nmol}/10^6 \text{ cells}\cdot\text{min}$. (figure 4.2). The problems associated with measurements of ^{22}Na influx are intractable; accurate measurements of effects of "loop" diuretics are thus impossible.

^{22}Na efflux was determined from cells pre-incubated to equilibrium prior to experimentation in isotonic (310 mosmol/kg) 140 mM $^{22}\text{NaCl}$, 5.4 mM KCl Krebs solution. Immediately before efflux measurements were made, the extracellular ^{22}Na was removed by rapid (x5) washes in isotope-free Krebs solution. After a control period of two media replacements at 30 second time intervals, the HeLa cells were exposed to a standard Krebs solution with or without 0.1mM furosemide (figure 4.3). The inclusion of furosemide (0.1mM) produced a significant decrease in the fractional loss of ^{22}Na only at time 3.0 minutes, 38.7 ± 6.2 (mean \pm S.D., n=3) in controls and 22.6 ± 6.0 (mean \pm S.D., n=3) in the presence of 0.1mM bumetanide, $0.02 < P < 0.05$. The extreme rapidity of the Na efflux should be noted (half time of efflux ($T_{1/2}$) of 1 to 1.5 minutes), which, coupled with the low internal Na concentration and specific activity of the ^{22}Na , again reduced the precision of the efflux determination. Thus it is difficult to state conclusively that the HeLa cell line exhibits a diuretic-sensitive ^{22}Na efflux under control conditions.

Figure 4.1

Time dependence of ^{22}Na accumulation into HeLa cells. Incubation media contained $1.0 \mu\text{Ci/ml}$ ^{22}Na (140 mM Na, 5.4 mM K). Data are the mean \pm S.D. of 3 determinations.

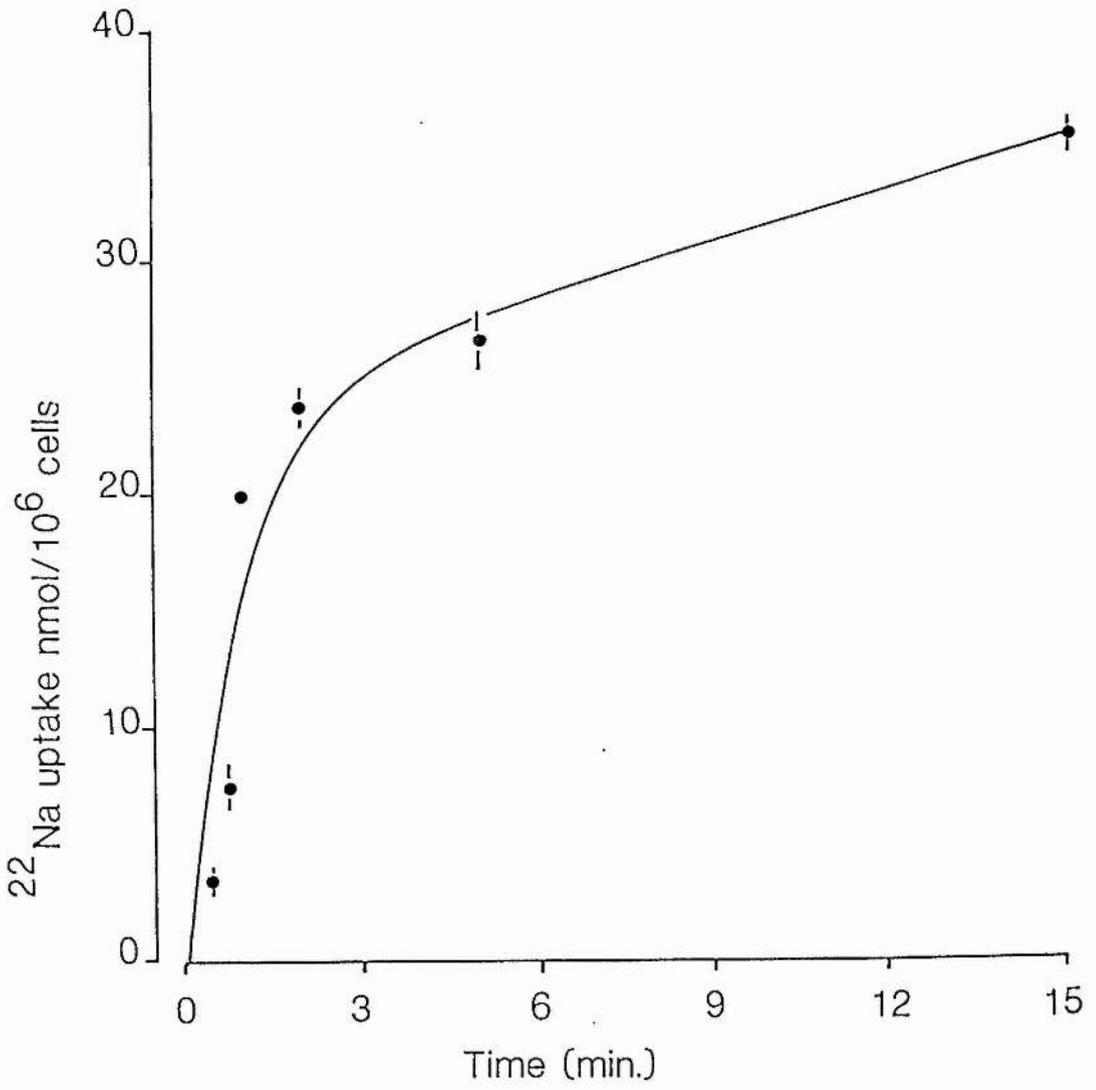


Figure 4.2

Time dependence of ^{22}Na accumulation into MDCK cells. Incubation media contained $1.0 \mu\text{Ci/ml } ^{22}\text{Na}$ (140 mM Na, 5.4 mM K). Data are the mean \pm S.D. of 3 observations.

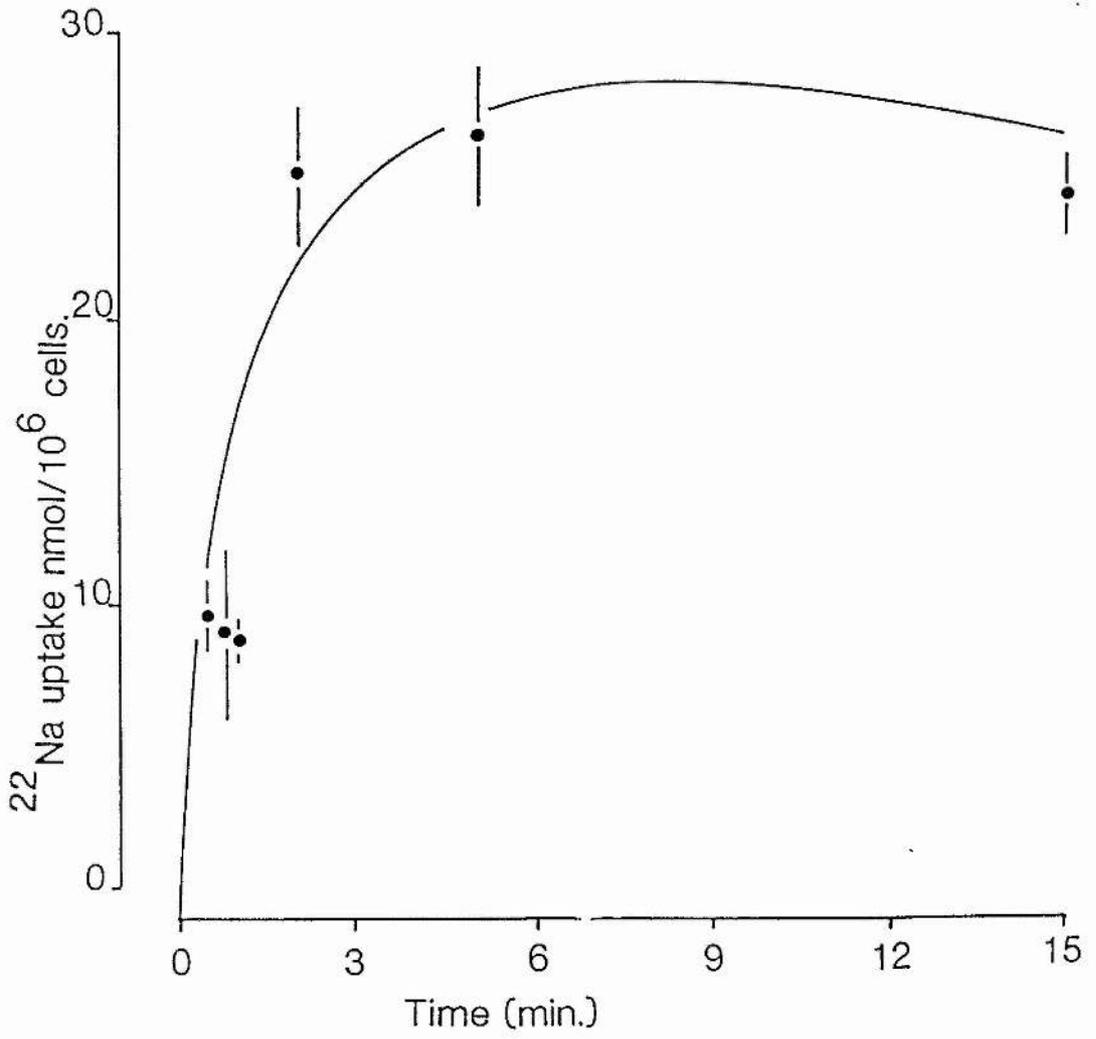
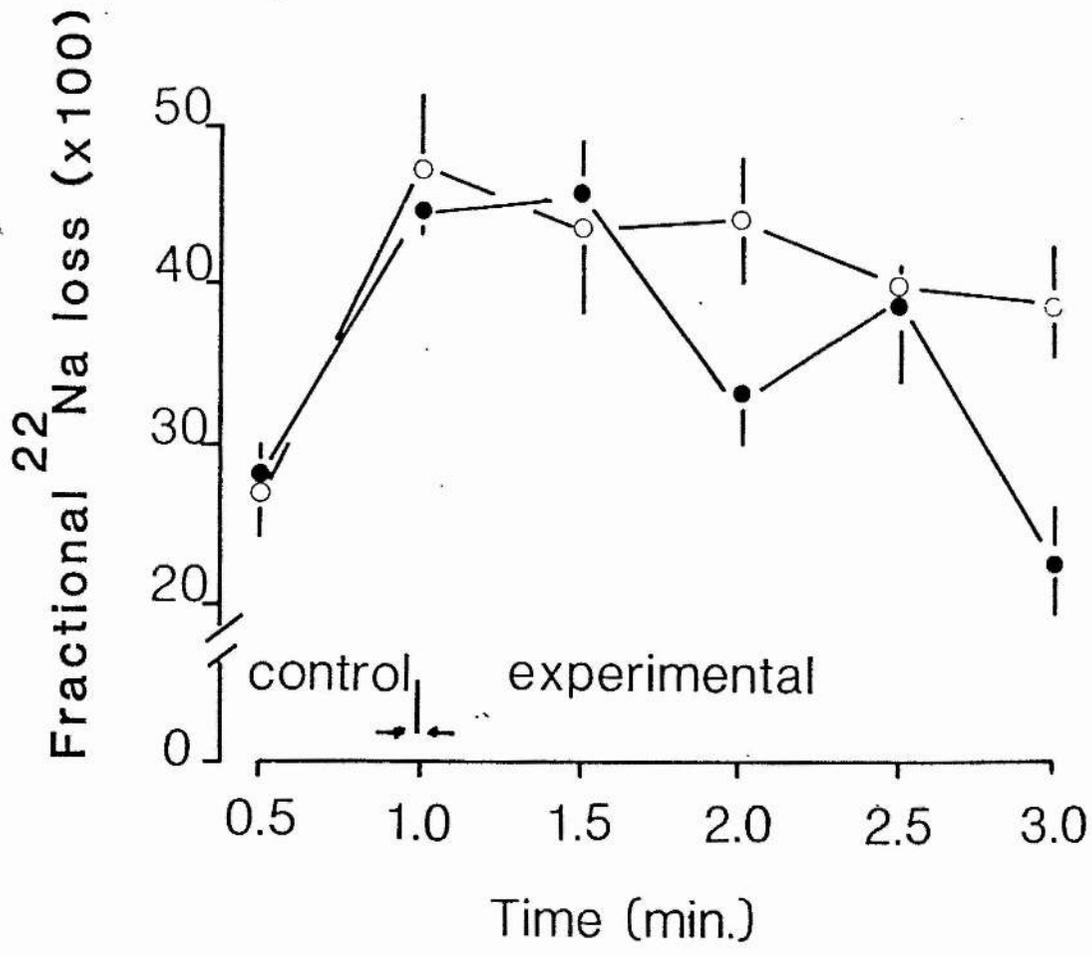


Figure 4.3

^{22}Na efflux from HeLa cells into 140 mM Na and 5.4 mM K media in (O) control cells and (●) plus 0.1 mM furosemide. Cells were loaded at 37° C in the presence of 1.0 $\mu\text{Ci/ml}$ ^{22}Na . Data are the mean \pm S.D. (n=3) of a representative experiment.



It was felt worthwhile to examine ^{22}Na efflux from Na-loaded, ouabain-treated MDCK cells into a standard Krebs solution containing 140mM Na, 5.4mM K (figure 4.4). Amiloride was present during the efflux determination to reduce the background flux (Rindler and Saier, 1981). In the presence of 0.1mM furosemide, no significant effect on the fractional rate of efflux even after a 2.5 minute exposure to the diuretic could be observed. In the Na-loaded condition, intercellular Na - 100mM, the fractional Na loss exceeds K loss 2-3 fold (see chapter 3).

Experiments to determine the ^{36}Cl efflux from HeLa cells were also performed in steady-state cells, 140mM NaCl and 5.4mM KCl (figure 4.5). The efflux protocol was identical to that used for the ^{22}Na efflux measurements. ^{36}Cl efflux was not significantly affected by the inclusion of furosemide in the incubation media. The half time for the ^{36}Cl efflux from HeLa cells was 3 minutes, and the fractional ^{36}Cl efflux exceeds the K loss 6 fold (see chapter 3). In a similar way to the ^{22}Na fluxes, the rapid nature of the ^{36}Cl efflux and low intracellular specific activity of the ^{36}Cl do not facilitate accurate measurements of the effect of diuretics.

Figure 4.4

^{22}Na efflux from MDCK cells into a standard Krebs solution (140 mM Na , 5.4 mM K), (O) control cells and (●) plus 0.1 mM furosemide. Cells were loaded with ^{22}Na at 1.0 $\mu\text{Ci/ml}$ in the presence of 1.0 mM ouabain. Efflux was performed in the presence of 0.1 mM amiloride to reduce background flux. Data are the mean \pm S.D. of 3 observations.

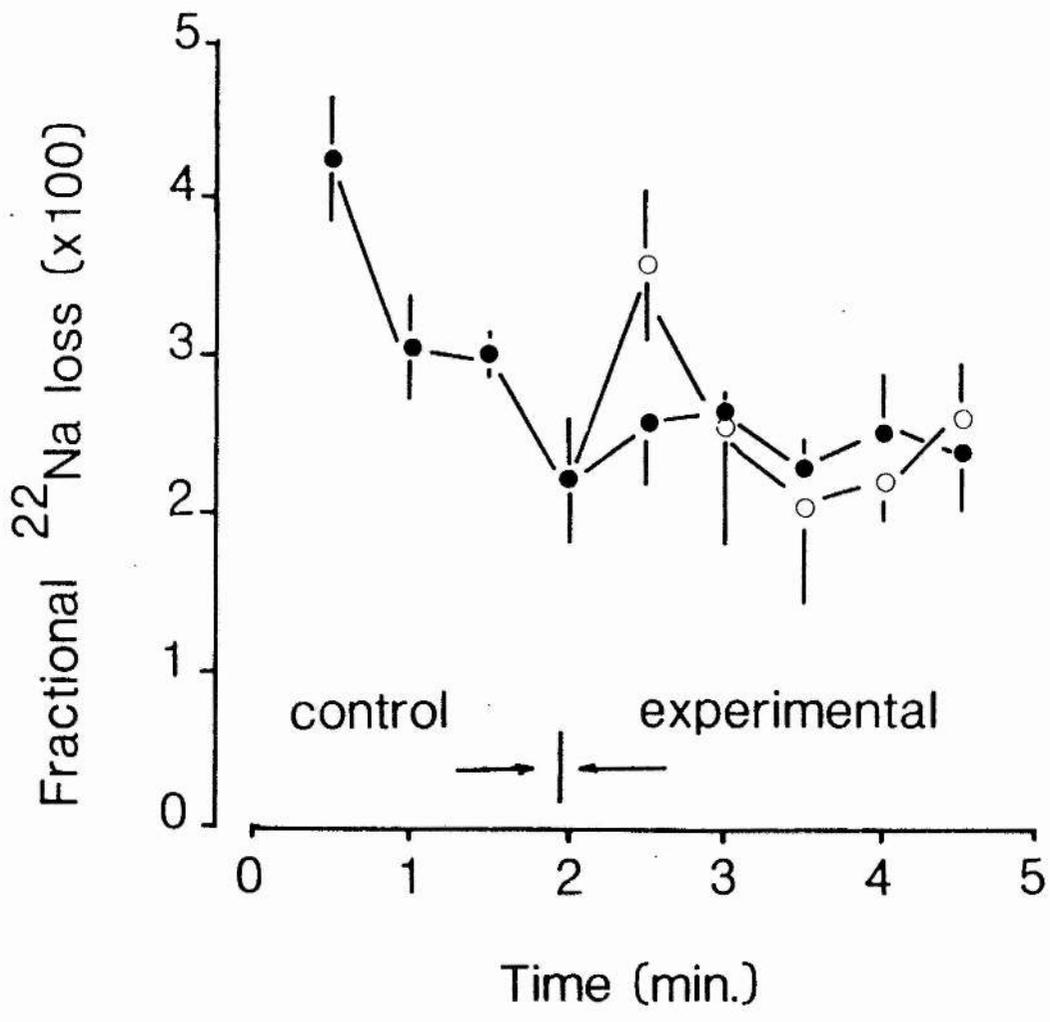
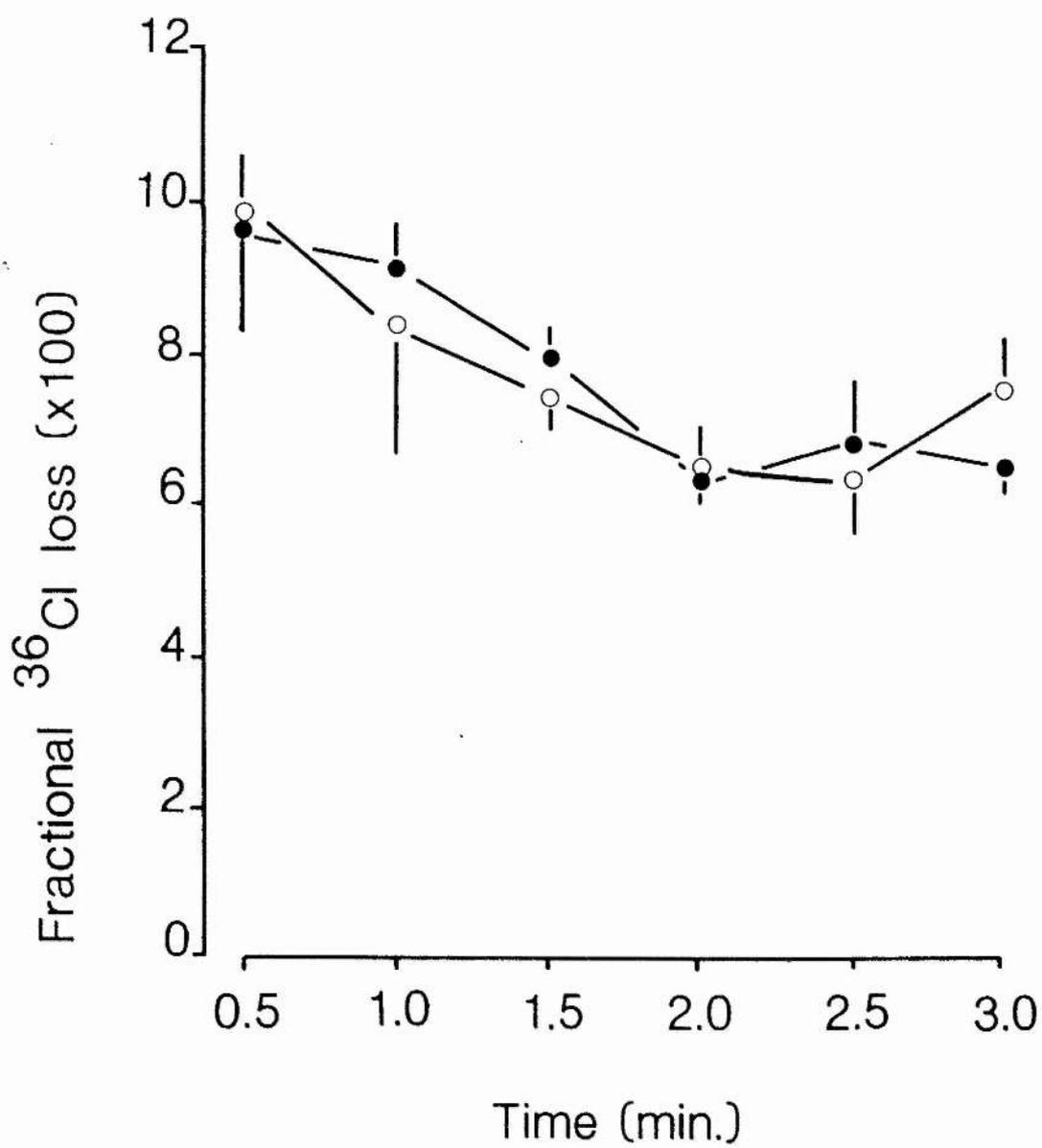


Figure 4.5

^{36}Cl efflux from HeLa cells in a standard Krebs solution (140 mM Na, 5.4 mM K), (●) control (○) plus 0.1 mM furosemide. Cells were loaded with 1.0 $\mu\text{Ci/ml}$ ^{36}Cl . Efflux was performed in the presence of 10 μM DIDS. Data are the mean \pm S.D. of 3 observations of a representative experiment.



DISCUSSION.

The results presented in this chapter underline the difficulties of determining either a Na or Cl unidirectional flux in cells with relatively high permeabilities for both these ions.

For both the HeLa and MDCK cell lines, it is possible to calculate the expected decrease in the fractional loss (x100) due to an inhibition of the "cotransport" system. Assuming that: a) the Na flux is coupled to the K efflux with a stoichiometry of 1:1; b) the diuretic-sensitive K efflux (in nmol/10⁶ cells.min) from HeLa and MDCK cells equals 3.34 ± 0.34 and 1.37 ± 0.16 respectively (see results chapter 3); and c) the intracellular Na content (nmol/10⁶ cells) for control HeLa is 35 ± 2.93 and for MDCK cells exposed to ouabain is 200 ± 4.9, then the decrease in the fractional rate of Na loss required for a 1:1 "cotransport" with K would be 4.77 ± 0.49 (x100, 30sec.⁻¹) in HeLa cells and 0.34 ± 0.04 (x100, 30sec.⁻¹) in MDCK cells. These values are within the standard errors of the control value for the fractional rate of ²²Na efflux observed in both cell lines.

Using the same argument as outlined above, the expected decrease in the fractional loss of ³⁶Cl (assuming intracellular Cl to be 108.5 ± 2.44 nmol/10⁶ cells) would be 1.55 ± 0.15 (x100, 30 sec.⁻¹). This inhibition was not observed and is equivalent to the error in control ³⁶Cl efflux determinations for the HeLa cell line. However, in Ehrlich ascites cells, a Na K 2Cl "cotransport" system has been demonstrated (Geck et al., 1980). Contradictory reports of Aull (1981) and Hoffmann et al. (1983) regarding the inhibition of a unidirectional Cl influx in the Ehrlich ascites cell under steady state conditions illustrate

the need for caution in the interpretation of unidirectional Cl flux data, especially in view of the rapid nature of this flux in the HeLa cell line.

Measurements of net diuretic-sensitive Na K and Cl fluxes in the MDCK cell line are consistent with the concept of "cotransport" with a stoichiometry of 1Na: 1K: 2Cl (McRoberts et al., 1982). But it must be stressed that the measurements of McRoberts et al. (1982) were performed in Na-depleted cells for Na influx and Na-loaded cells for Na efflux and are therefore more likely to represent a net movement of ions than a unidirectional influx/efflux.

The determination of unidirectional Na and Cl fluxes in the HeLa and MDCK cell lines under or near physiological conditions proved problematic and it is therefore difficult to determine unequivocally whether these fluxes are inhibited by diuretics.

CHAPTER 5

ROLE OF THE DIURETIC-SENSITIVE K TRANSPORT IN CELL VOLUME REGULATION
IN HELA AND MDCK CELLS AFTER EXPOSURE TO HYPERTONIC MEDIA.

INTRODUCTION.

The diuretic-sensitive K transport present (see chapter 3) in HeLa and MDCK cells may be considered to be a Na K Cl "cotransport" system, since it shares many characteristics with the Na K Cl "cotransport" system(s) of other cells, notably avian erythrocytes (Schmidt and McManus, 1977 a,b,c; Haas et al., 1982), human erythrocytes (Chipperfield 1980; Dunham et al., 1980) and Ehrlich ascites cells (Geck et al., 1980). In epithelial cell lines, Na K Cl "cotransport" has been demonstrated to be involved in Cl reabsorption in the thick ascending limb of the loop of Henle (Greger, Schlatter and Lang, 1983; Greger and Schlatter, 1983 a,b; Greger, Oberleithner, Schlatter, Cassola and Weidtke, 1983; see also review by Greger, 1985) and the marine teleost intestine (Musch, Orellana, Kimberg, Field, Halm, Krasny and Frizzell, 1982). Na K Cl "cotransport" of MDCK cells (Brown and Simmons, 1981) and in a variety of preparations (see review by Petersen and Maruyama (1984)) is involved in Cl secretion. However, the importance of this transport system to the non-epithelial cell types is still unclear.

Maintenance of cell volume is now generally considered to be a balance between the active and passive trans-membrane ion movements with the colloid pressure exerted by the intracellular macromolecules. Animal cells have a finite maximal volume above which lysis occurs since the plasma membrane cannot withstand the high osmotic pressure which would be imposed by the colloid-osmotic swelling. Cells must therefore extrude ions at a rate which is nearly equivalent to the dissipative entry of ions down their chemical gradients, i.e. the

"pump - leak" hypothesis (Tosteson and Hoffman, 1960) and is reviewed by MacKnight and Leaf (1977), Hoffmann (1985) and Cala (1983). However, if other transport systems besides the Na K pump and the passive permeabilities mediate a net transport of osmotically active particles the above hypothesis must be modified to include these ion transport pathways (see Geck, Heinz and Pfeiffer, 1981).

Regulation of cell volume is mediated by controlled changes in the "leak" pathways (ouabain-insensitive) in a variety of cell types, e.g. human lymphocytes (Grinstein, Clarke, Dupre and Rothstein, 1982; Grinstein, Clarke and Rothstein, 1982, 1983; Grinstein, Clarke, Rothstein and Gelfand, 1983; Grinstein, Cohen, Goetz and Rothstein, 1985), avian erythrocytes (Kregenow, 1971 a,b, 1973, 1981; Kregenow, Robbie and Orloff, 1976; Schmidt and MacManus, 1977 a,b,c) and dog erythrocytes (Parker and Castranova, 1984), see also review by Cala (1983). Diuretic-sensitive Na K Cl "cotransport" system(s) have been implicated in cell volume regulation particularly for avian erythrocytes and Ehrlich ascites cells (Geck et al., 1981; Schmidt and McManus, 1977 a,b,c; Hoffmann, 1982, 1985; Hoffmann et al., 1983; Ueberschar and Bakker-Grunwald, 1983). In shrunken cells, the Na K Cl "cotransport" system(s) can mediate net salt uptake, such that an accumulation of permeant ions with their osmotically obliged water results in the re-swelling of the cell (regulatory volume increase, R.V.I.). Haas et al., (1982) have demonstrated that net ion movement via a Na K Cl "cotransport" system is dependent upon the ion gradients, therefore under certain circumstances diuretic-sensitive net salt loss and thus cell shrinkage may occur even in cells exposed to hyperosmotic media (Schmidt and McManus 1977a; Hoffmann, 1982).

The idea that the diuretic-sensitive "cotransport" system(s) are

in volume regulation per se under physiological conditions may not necessarily hold true. Shrunken cells undertake R.V.I. only if the external K concentration is markedly elevated above its physiological concentration (Orskav, 1954; Kregenow, 1977; Schmidt and McManus, 1977a; Hoffmann, 1982), or if the intracellular KCl content is first depleted by pre-treatment in hypotonic media before re-exposure to isosmotic media, resulting in cell shrinkage (Ussing, 1982; Hoffmann et al., 1983). Haas and McManus (1984) have reported a possible functional relationship between the swelling-induced KCl leak (see below) and the Na K Cl "cotransport" of the avian erythrocyte, where the activation of the Na K Cl "cotransport" by nor-adrenaline counteracted the swelling-induced KCl leak. Therefore, the primary function of the Na K and Cl "cotransport" system(s) in non-epithelial cells may be K homeostasis as suggested by Duhm and Goebel (1984).

Exposure of animal cells to hypo-osmotic media produces an initial increase in cell volume, consequently the cells lose KCl with its osmotically obliged water thus reducing their volume to near control level, a phenomenon which may be considered to be a regulatory volume decrease (R.V.D) (Cala, 1985; Hoffmann, 1985). This response to cell swelling has also been reported in HeLa and MDCK cells (Tivey et al., 1985; Simmons, 1984) with R.V.D. being mediated by a specific increase in the passive K membrane permeability. A variety of other cell types exhibit similar responses, (Bakker-Grunwald, 1983; Bui and Wiley, 1981; Hoffmann, 1982; Cala, 1977); as the K conductance is limited by the Cl permeability, cell swelling may induce separate conductive channels for K and Cl (Grinstein et al., 1982, 1983; Cala, 1985; Hoffmann, 1985). The involvement of the diuretic-sensitive transport in R.V.D. cannot be excluded, since sheep erythrocytes (Dunham and Ellory, 1981), Ehrlich ascites cells (Thornhill and Laris,

1984) and the erythrocytes of the oyster toad fish (Lauf, 1982) exhibit a furosemide-sensitive anion dependent KCl transport, stimulated by cell swelling. In sheep erythrocytes however the affinity for furosemide is lower than that expected of the "cotransport" system, suggesting separate molecular entities (Ellory et al., 1982).

Since HeLa and MDCK cells possess a "loop" diuretic-sensitive K transport which may be considered to be Na K Cl "cotransport" (see chapter 3), I have examined this K flux under hyperosmotic (shrunken cells) conditions in order to assess the involvement of this transport system in volume regulation in these cell lines. Data in this chapter have been presented in Aiton, Simmons and Tivey, 1984; Tivey et al., 1985; and Simmons and Tivey, 1985.

RESULTS.

Cell volume: exposure to hyperosmolar media.

Exposure of HeLa and MDCK cells to hyperosmolar media produced significant ($P < 0.001$) decreases in their cellular volume, determined by electronic cell sizing, chapter 2 (table 5.1). The relative cell volume of HeLa and MDCK cells incubated in hyperosmolar media with respect to their volume in isosmotic media, calculated from their cell volume (μm^3) or cell water per plate (μl) are in close agreement with the theoretical relative volume calculated from the ratio of the media tonicities. This verifies the ideal osmotic behaviour of these cell types previously reported by Simmons (1984) and Tivey et al., (1985) for MDCK and HeLa cells respectively.

Effect of hypertonicity upon K (^{86}Rb) fluxes.

The effect on the K fluxes of the HeLa and MDCK cell lines of increasing the external tonicity, by adding 200 mM mannitol, is shown in figures 5.1 a,b and 5.2 a,b. Total K (^{86}Rb) influx is increased in both HeLa and MDCK cells. Subdivision of the K (^{86}Rb) influx into its components using ouabain and "loop"-diuretics (see chapter 3) gives the ouabain-sensitive, diuretic-sensitive and residual flux components. This shows that in control media the stimulation of the total K (^{86}Rb) influx is predominantly the consequence of an increased diuretic-sensitive component of the K (^{86}Rb) influx. There is a small stimulation of the ouabain-sensitive K influx in both HeLa and MDCK cells and a significant decrease in the residual component in the HeLa cell line.

Table 5.1 Effect of hyperosmolar media (510 mOsm/kg, 200 mM
mannitol addition) upon the cell volume of HeLa and
MDCK cells^(a)

Cell type	Medium osmolality (mOsm/kg)	Cell Volume (μm^3)	Cell water per plate (μm)
HeLa	310	3144 \pm 32	3.18 \pm 0.04
	510 (0.61)	1854 \pm 23 ^(b) [0.59]	1.74 \pm 0.02 ^(b) [0.55]
MDCK	300	1751 \pm 144	1.96 \pm 0.13
	484 (0.62)	1187 \pm 32 ^(b) [0.68]	1.36 \pm 0.07 ^(b) [0.69]

(a) Cell water per plate determined by electronic cell sizing was calculated from the total cell number and assuming that 0.16 of the cell volume represents the non-osmotically active fraction of cell volume. Figures in square brackets are the relative cell volumes compared to isosmotic media (300 mOsm/kg). Figures in parenthesis are the ratio of media tonicities. Data are the mean \pm S.D., n = 3, of a single representative experiment.

(b) Significant difference from 300 mOsm/kg media values by Student's t-test; p < 0.001.

Figure 5.1

Effect of exposure of HeLa cells to hyperosmolar media (500 mOsm/kg; 200 mM mannitol addition to a standard Krebs solution) upon the K (^{86}Rb) influx (A) and efflux (B). Experimental data were obtained using cells of the same batch and all cultures were pre-incubated identically for 3 hours to match cells loaded with ^{86}Rb for efflux experiments. (A) Diuretic-sensitive K (^{86}Rb) influx was determined over 5 minutes in the presence of 1 mM ouabain; stippled histograms represent control data. (B) The K (^{86}Rb) efflux was determined in control (●), hyperosmolar (■), control plus 0.1 mM furosemide (▲) and hyperosmolar plus 0.1 mM furosemide (□) media. Using measured values of intracellular K content and the rate constant of K (^{86}Rb) efflux at time 6 minutes following exposure to hypertonicity in the presence or absence of furosemide the calculated values of efflux (in $\text{nmol}/10^6$ cells.min.) were 6.6 and 3.1 for isosmotic and isosmotic plus furosemide respectively, and in hypertonic media 8.3, plus 0.1 mM furosemide 2.4. Data are the mean \pm S.E., n=3.

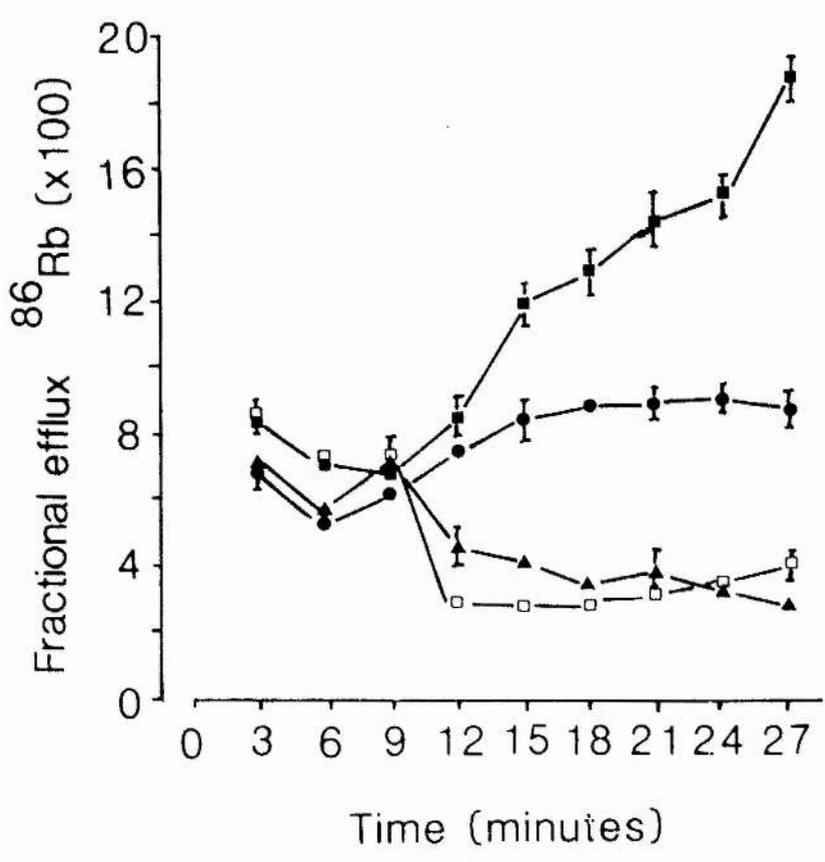
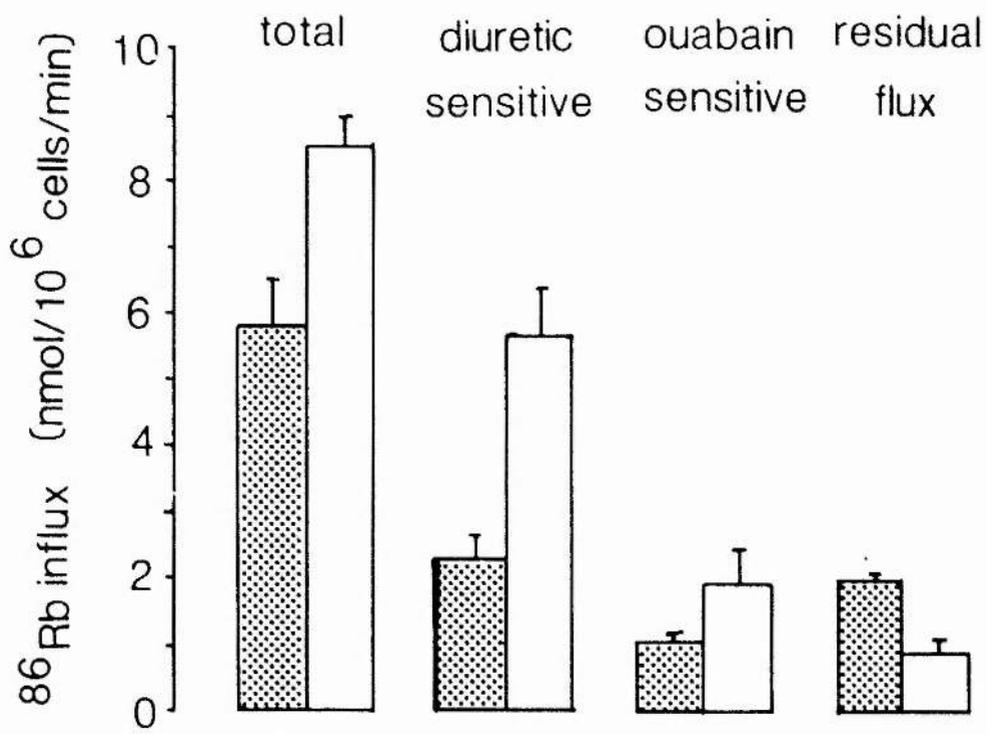
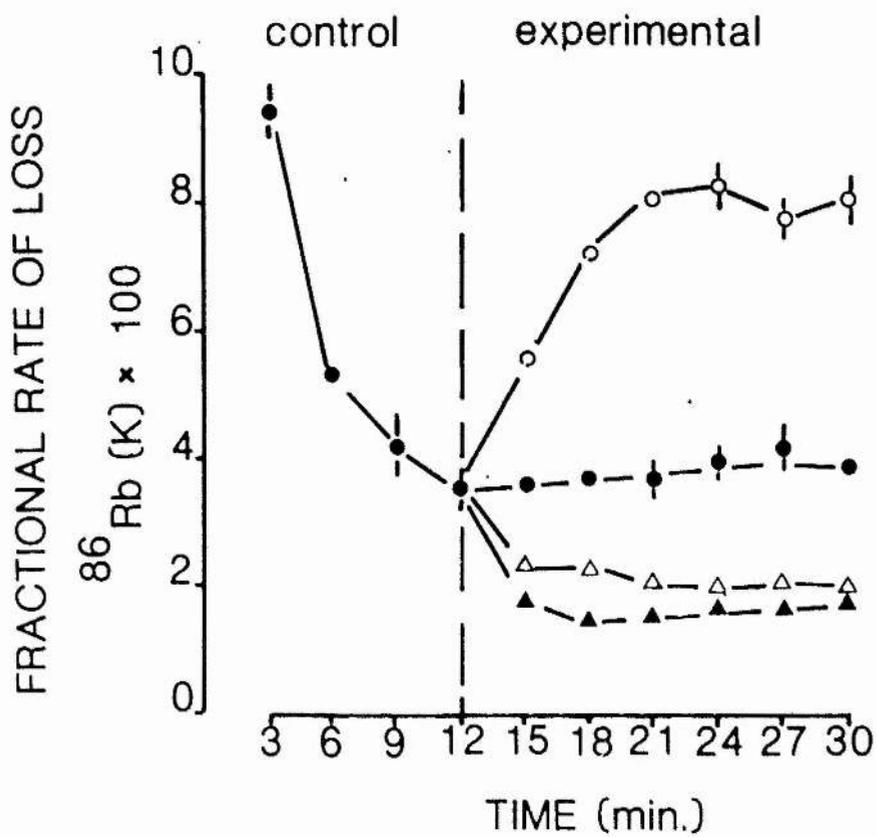
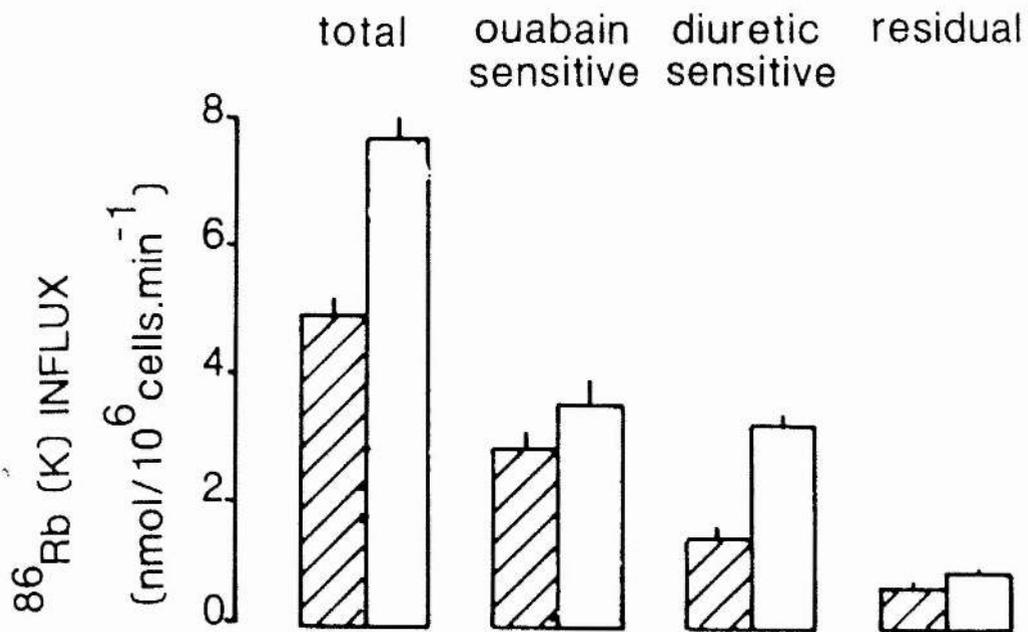


Figure 5.2

The effect of exposure of MDCK cells to hyperosmolar media (500 mOsm/kg; 200 mM mannitol addition to standard Krebs solution) upon the components of K (^{86}Rb) influx (A) and efflux (B) measured in the same cell batch and all cultures were pre-incubated identically for 3 hours to match cells loaded with ^{86}Rb for efflux experiments. (A) K (^{86}Rb) influx determinations were over a 5 minute period; stippled histograms represent control data. The diuretic-sensitive flux component was determined in the presence of 1 mM ouabain. (B) The K (^{86}Rb) efflux was determined in control (●), hyperosmolar (○), control + furosemide (△) and hyperosmotic + furosemide (▲) media. Using measured values for the intracellular K content and the rate constant following 6 minute exposure to hypertonicity, the calculated values of efflux in $\text{nmol}/10^6 \text{ cells min.}$ were 3.7 ± 0.3 (isosmotic) and 7.3 ± 0.2 (hyperosmotic). Data are the mean \pm S.E., n=3.



As discussed in chapter 3, the pharmacological agents ouabain and "loop" diuretics have separate and unique actions, assuming little change in intracellular ionic composition. In shrunken cells, intracellular volume is reduced in proportion to the ratio of the media osmolalities as shown in table 5.1. Since intracellular ion content of HeLa and MDCK cells is not affected by cell shrinkage (see below), it follows that ion concentrations must be increased. Activity of the Na K pump is dependent upon the intracellular Na concentration (see review, Schwartz, Lindenmayer and Allen, 1975; Sachs, 1977), thus, in cells exposed to hyperosmotic media, increased ouabain-sensitive K influx should be observed in comparison with cells incubated under isosmotic conditions, as shown in figures 5.1 and 5.2. This would mediate a net loss of Na(Cl) plus its osmotically obliged water through the Na K pump in the shrunken cell and cause further cell shrinkage. This does not occur however, since HeLa and MDCK cells behave as perfect osmometers, table 5.1 (Simmons, 1984; Tivey et al., 1985) and maintain their intracellular Na content (see below). Any effect of increased Na K pump activity must be balanced by an increased passive Na influx. If an increase in the "passive" Na influx is via a Na K Cl "cotransport" pathway in shrunken cells, then the ouabain-sensitive K influx should be less in "loop" diuretic poisoned cells due to a decrease in intracellular Na concentration.

Since the inhibition of K influx by ouabain and "loop" diuretics was approximately additive (table 5.2), the actions of these agents are independent in shrunken cells which is similar to the situation in control cells (see results chapter 3). For all HeLa experiments (table 5.2), the ouabain-sensitive K influx was not affected by the presence of a "loop" diuretic. Any increase in the passive Na influx

Table 5.2 Additivity of ouabain-sensitive K^+ ($^{86}\text{Rb}^+$) influx determined in the presence (B-OB) or absence (T-O) of 0.1 mM diuretic, and diuretic-sensitive K^+ ($^{86}\text{Rb}^+$) influx determined in the presence (O-OB) or absence (T-B) of 1.0 mM ouabain, in HeLa and MDCK cells to hyperosmolar media (= 500 mosm/kg, mannitol addition)

K^+ ($^{86}\text{Rb}^+$) influx	Cell type	Total no. experiments	$(T-O) = (B-OB)$ (a)		$(T-O) > (B-OB)$ (a)		$(T-O) < (B-OB)$ (a)	
Ouabain- sensitive	HeLa	8	100%	-	-	-	-	
	MDCK	14	64.3%	7.2%		28.5%		
			$(T-B) = (O-OB)$		$(T-B)$	$(O-OB)$ (a)	$(T-B)$	$(O-OB)$ (a)
Bumetanide- sensitive	HeLa	8	100%	-	-	-	-	
	MDCK	14	64.3%	7.2%		28.5%		

(a) Significant difference between the two determinations of ouabain-sensitive and the diuretic-sensitive K^+ ($^{86}\text{Rb}^+$) influx determined by Student's t-test; $p < 0.05-0.01$.

in this cell type must therefore be diuretic-insensitive. In MDCK cells (table 5.2), no significant difference was observed in the ouabain-sensitive K influx determined in the presence or absence of "loop" diuretics in 64% of experiments performed. In 7% of experiments, ouabain-sensitive K influx was significantly greater ($P < 0.05$) in the absence rather than in the presence of "loop" diuretics, while for 28.5% of experiments the reverse is true. This latter represents an outwardly directed Na flux which is diuretic-sensitive. In the majority of MDCK experiments however, the ouabain-sensitive K influx is independent of the presence of "loop" diuretics, suggesting that cells must remain in balance with respect to their Na content via diuretic-insensitive passive pathways.

As the ouabain-sensitive and diuretic-sensitive K influxes are additive in all HeLa and the majority of MDCK experiments, this is evidence that the Na K Cl "cotransport" pathway mediates little or no net flux in shrunken cells.

Exposure of HeLa and MDCK cells to hyperosmotic media produced a progressive stimulation of the K (^{86}Rb) efflux, (figures 5.1b and 5.2b), which reached a plateau (figures 5.2b, 5.3b, 5.5a,b) after 6-9 minutes' exposure to hyperosmotic conditions. It was possible to completely inhibit the stimulation of the K (^{86}Rb) efflux by the inclusion of 0.1mM diuretic into the incubation media. The progressive nature of the stimulation of the diuretic-sensitive "cotransport" contrasts with the inhibitory action of the diuretics (figures 5.1b and 5.2b) and the rapid cell shrinkage of HeLa and MDCK cells on exposure to hypertonic media (Tivey et al., 1985; Simmons, 1984) monitored using a coulter counter.

In view of the fact that the diuretic-sensitive K (^{86}Rb) influx and efflux were determined in cells of the same cell batch and under near identical experimental conditions, a direct comparison of these diuretic-sensitive K (^{86}Rb) fluxes can be made if the efflux is expressed in $\text{nmol}/10^6 \text{ cells} \cdot \text{min}$. This may be accomplished using measured values for intracellular K (expressed in $\text{nmol}/10^6 \text{ cells}$) and the fractional rate of efflux at time six minutes (see legends to figures 5.1 and 5.2). For HeLa cells, the diuretic-sensitive K (^{86}Rb) influx and efflux in isotonic conditions (310 mosmol/kg) were not significantly different, 2.28 ± 0.38 and 3.34 ± 0.34 (mean \pm S.E., $n=3$) respectively, and in hypertonic media (510 mosmol/kg, mannitol addition), K (^{86}Rb) influx, $6.75 \pm 0.19 \text{ nmol}$ (mean \pm S.E., $n=3$), did not differ significantly from K (^{86}Rb) efflux, 6.8 ± 0.16 (mean \pm S.E., $n=3$). Similar findings were seen for MDCK cells, where the diuretic-sensitive K (^{86}Rb) influx and efflux in isotonic media were 1.37 ± 0.16 and 1.14 ± 0.24 (mean \pm S.E., $n=3$) respectively, and in hypertonic media 3.32 ± 0.16 and 4.59 ± 0.11 (mean \pm S.E., $n=3$) respectively. Thus, both cell types can mediate unidirectional K (^{86}Rb) influx and efflux which is diuretic-sensitive, thus substantiating the results of chapter 3. These diuretic-sensitive K (^{86}Rb) fluxes in both control (isosmolar conditions) and shrunken (hyperosmolar conditions) cells were seen to be balanced, thus little or no net transport of ions was mediated by the Na K Cl "cotransport" system(s) of HeLa and MDCK cells under either experimental condition.

Effect of hypertonicity upon intracellular ion contents.

If the diuretic-sensitive K transport was mediating a significant net transfer in shrunken cells, then inhibition of the Na K Cl "cotransport" pathway should alter the intracellular ion content.

Total cation content of HeLa and MDCK cells was determined in control and shrunken cells with or without "loop" diuretic (table 5.3). A 90 minute incubation in the presence of a "loop" diuretic had no significant effect upon the intracellular K and Na content of either HeLa or MDCK cells exposed to isosmotic media, thereby confirming the results presented in chapter 3. The effect of a 90 minute incubation in hyperosmolar media did not alter significantly the intracellular cation content of HeLa and MDCK cells (table 5.3). Moreover, inclusion of the "loop" diuretic in the hyperosmolar incubation had no significant effect on the K and Na content of either cell type. Therefore, the elevated, diuretic-sensitive K (^{86}Rb) influx observed in HeLa and MDCK cells exposed to hyperosmolar media may be considered to be balanced with little or no net flux, similar to the K (^{86}Rb) fluxes in control cells (chapter 3).

Intracellular Cl content (expressed in $\text{nmol}/10^6$ cells) of control and shrunken HeLa cells was determined by coulometry (see footnote table 5.3). Exposure to hyperosmolar media produced no significant effect on the Cl content of HeLa cells, which is similar to the results obtained for cellular K and Na (see above).

Effects of external K upon diuretic-sensitive K efflux.

As discussed in chapter 3, the demonstration of balanced diuretic-sensitive fluxes poses the question: does the diuretic-sensitive ion transport represent an obligatory exchange flux? Haas et al. (1982) demonstrated that Na K "cotransport" in duck erythrocytes did not exhibit a trans-concentration dependence for either Na or K, and that cation fluxes were solely dependent on cis-concentrations of Na and K.

Table 5.3 The effect of hypertonicity and 0.1 mM diuretic (90 minute incubation) upon the intracellular K^+ and Na^+ content of HeLa and MDCK cells (a)

Cell type	0.1 mM diuretic	n	Ion contents (nmol/ 10^6 cells)			
			310 mOsm/kg	K^+	Na^+	510 mOsm/kg
HeLa	-	[12]	276.8 ± 11.2	35.0 ± 2.9	284.0 ± 15	33.9 ± 2.9^{ns}
	+		313.0 ± 23.0^{NS}	38.3 ± 7.0^{NS}	299.0 ± 12.9^{NS}	37.6 ± 8.3^{NS}
	-		279.5 ± 7.9	24.7 ± 2.7	261.8 ± 3.6^{ns}	26.9 ± 1.1^{ns}
MDCK	+	[3]	285.0 ± 5.3^{NS}	25.8 ± 2.1^{NS}	272.0 ± 7.2^{NS}	29.1 ± 2.1^{NS}
	-					

(a) Data are the mean \pm SD of [] determinations in a single representative experiment. Ion contents were tested for significance of difference from 310 mOsm/kg values (lower case lettering) and for cells incubated in diuretic with respect to control cells (upper case lettering) by Student t-test. NS = not significant.

Footnote:

Intracellular $[Cl^-]$ of HeLa cells determined by coulometry in control and shrunken cells were 60.8 ± 13.7 mM and 82.9 ± 7.1 mM respectively. These $[Cl^-]$ are equivalent to 105.7 ± 23.8 and 113.20 ± 9.7 nmol/ 10^6 cells for control and shrunken cells respectively.

For MDCK cells, the effect of varying external K concentrations on the K (^{86}Rb) efflux is shown in figures 5.3 a-c. Reducing external K to 0.04 mM produced a transient increase in the K efflux in isosmotic conditions compared to K (^{86}Rb) efflux from MDCK cells in 5 and 15 mM external K media. This stimulation was mirrored by an increase in the "loop" diuretic-insensitive K (^{86}Rb) efflux however and may result as a secondary effect of the inhibition of the Na K pump. The diuretic-sensitive component of K (^{86}Rb) efflux of MDCK cells in isosmotic media was not affected by varying external K concentrations (figures 5.3 a-c and also results chapter 3). Exposure of MDCK cells to hyperosmolar conditions produced a significant increase in the fractional loss of K (x100), irrespective of the external K concentration. In the three K concentrations the stimulated K efflux was inhibited by the "loop" diuretic and the magnitude of the "loop" diuretic-sensitive component determined in 0.04 mM K media did not differ significantly from that determined in 15 mM K media, 5.33 ± 0.43 (x100) and 7.63 ± 1.79 (x100), mean \pm S.E. n=3, respectively. Thus in MDCK cells the diuretic-sensitive unidirectional K (^{86}Rb) fluxes of stimulated by hypertonicity do not represent an obligatory exchange flux, and are similar to the K flux observed under isosmotic conditions (see chapter 3)*. Qualitatively identical results were observed in HeLa cells and have been reported in Tivey et al. (1985).

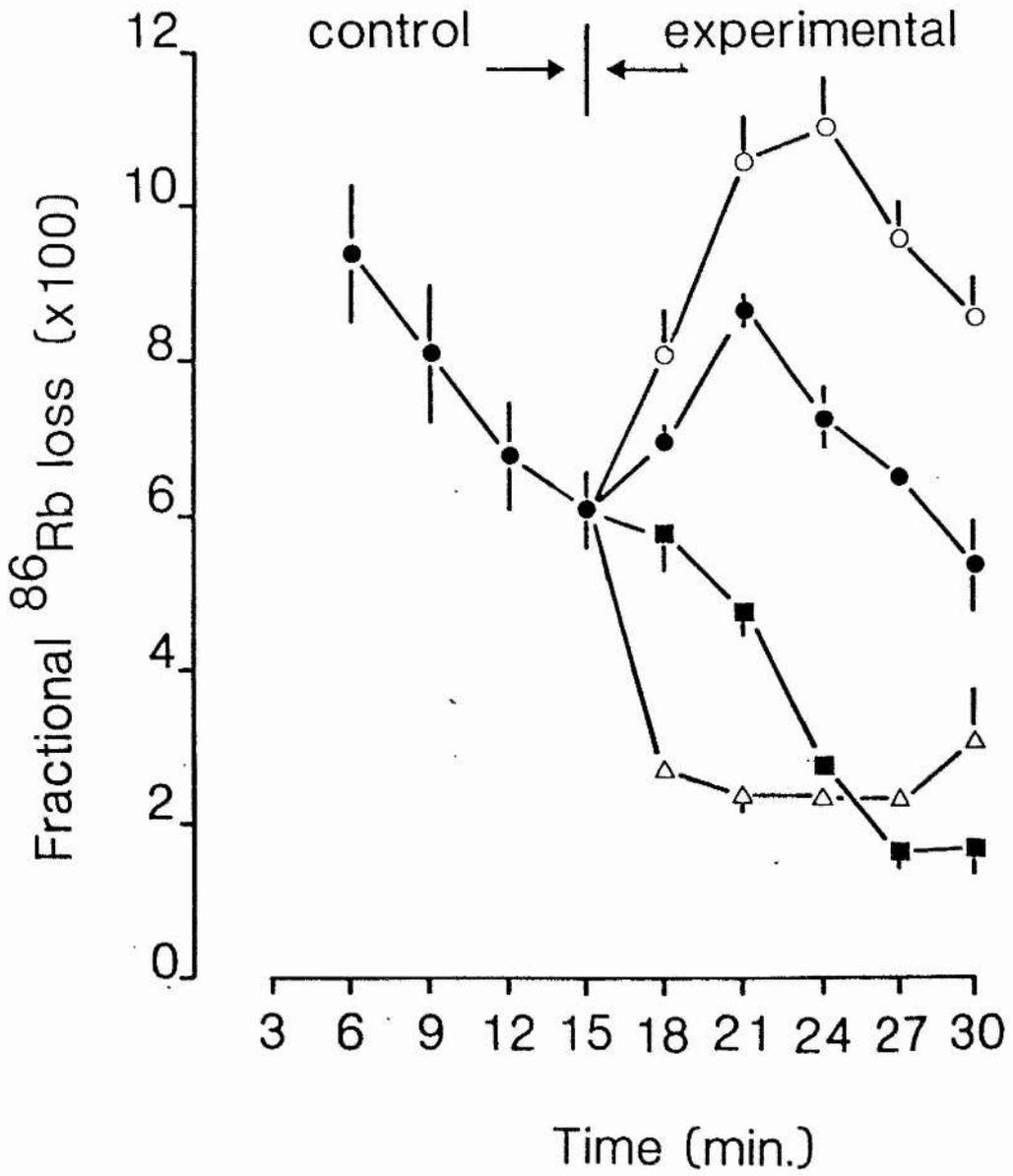
* Note in proof.

Recent reports by Brugnara, et al. (1986) and Canessa, et al. (1986) demonstrate that trans ion concentrations can stimulate and/or inhibit the Na K "cotransport" pathway in human red blood cells. However, the major portion (75%) of the ion fluxes through this transport pathway in this cell type are independent of the trans ion concentrations, thereby indicating the non-obligatory exchange nature of the major portion of these diuretic-sensitive ion fluxes. These reports do not affect the conclusions drawn from data presented in this chapter for the non-obligatory exchange nature of the diuretic-sensitive K fluxes stimulated by cell shrinkage in both HeLa and MDCK cells.

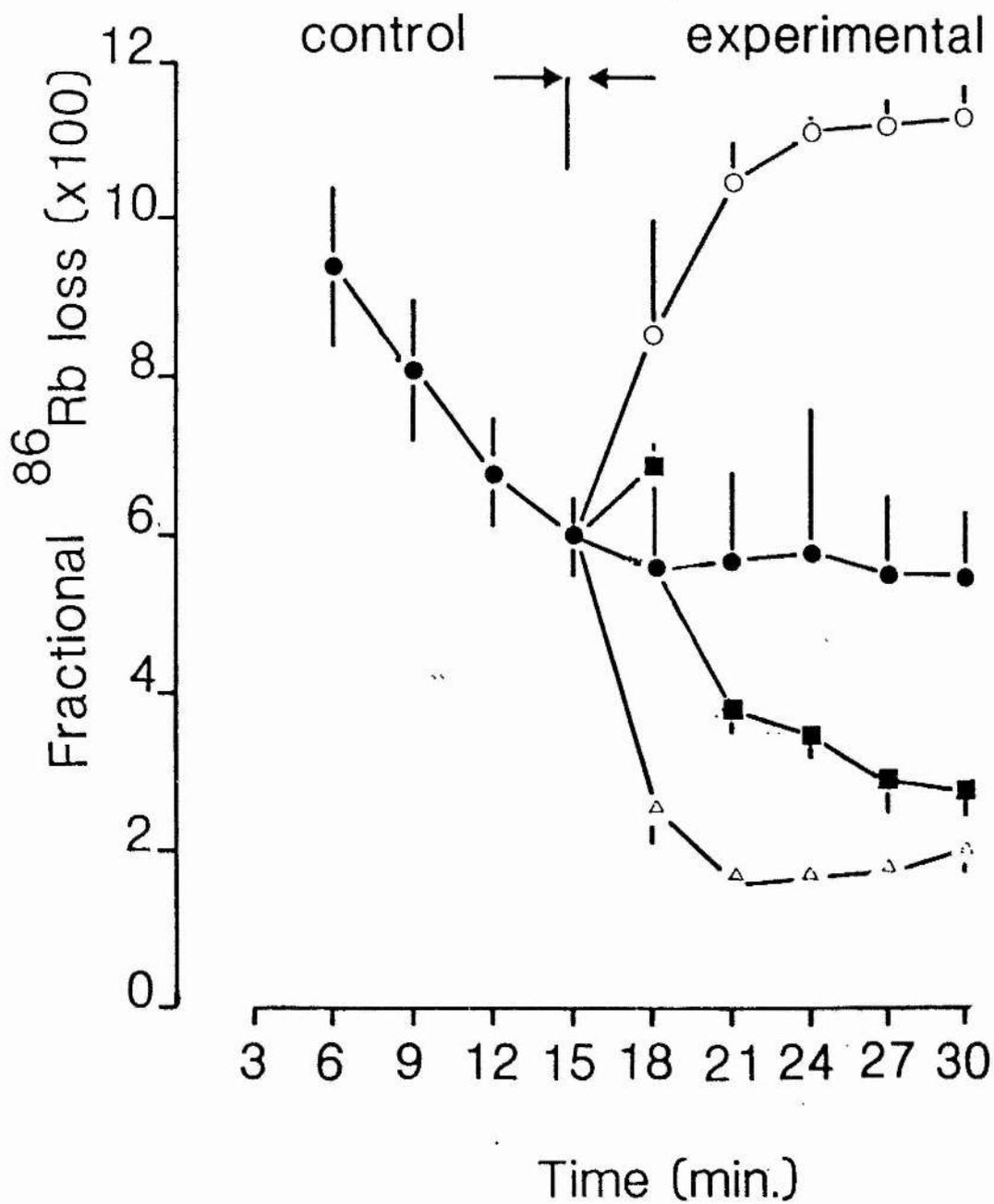
Figure 5.3 a-c

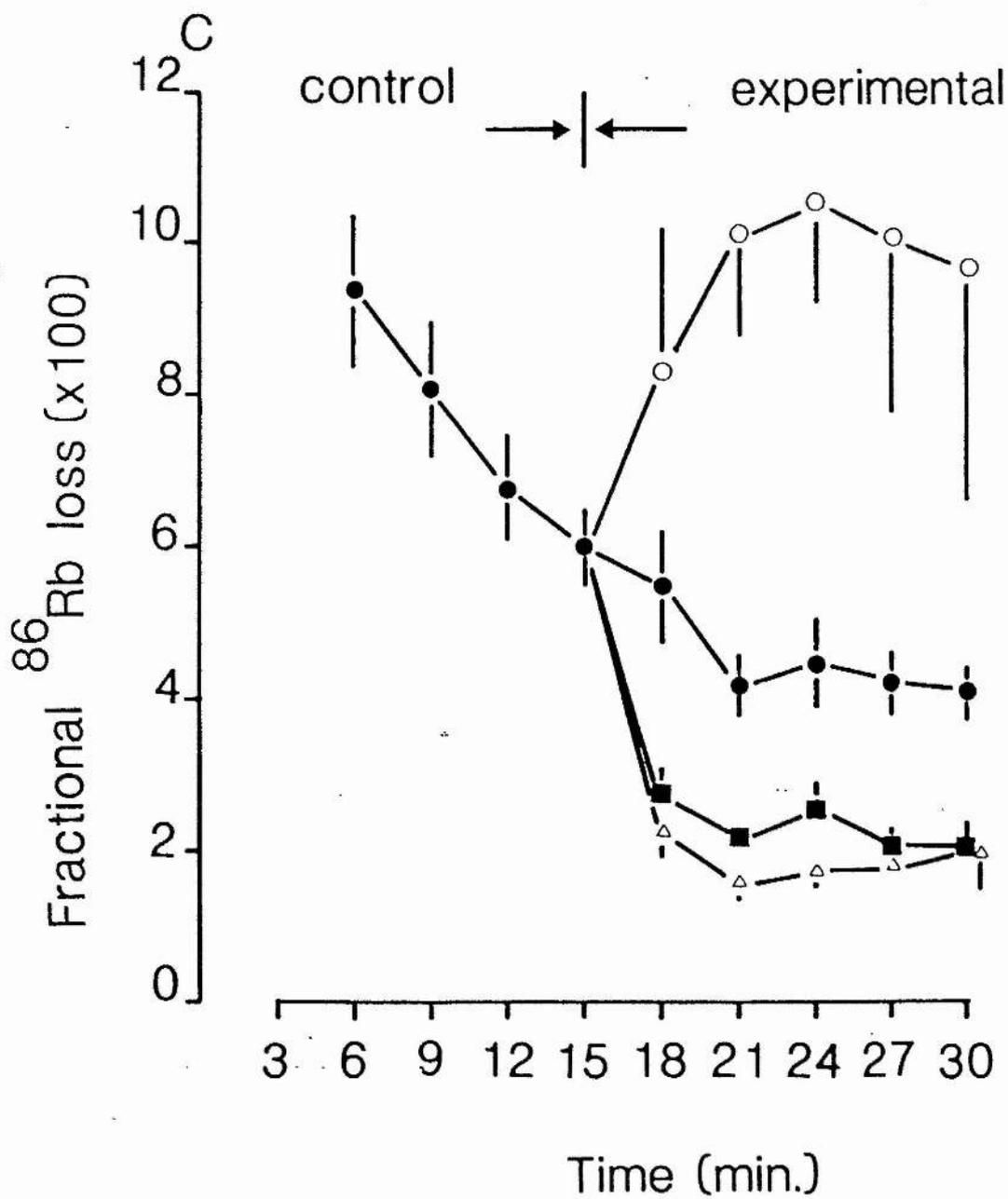
Stimulation of the diuretic-sensitive K (^{86}Rb) efflux by hypertonicity (mannitol addition) in media containing varying K concentrations, (a) 0.04 mM K, (b) 5.4 mM K and (c) 15.4 mM K. K (^{86}Rb) efflux was determined in control (●), control + 0.1 mM diuretic (■), hyperosmotic (○) and hyperosmotic + 0.1 mM diuretic (△). All data are the mean of three separate determination \pm S.D..

A



B





Effect of graded hyperosmolar media on K (^{86}Rb) efflux.

Ueberschar and Bakker-Grunwald (1983) have demonstrated that when turkey erythrocytes are exposed to solutions of increasing hypertonicity, the diuretic-sensitive K (^{86}Rb) efflux increased in a saturable fashion. Figure 5.4 shows data from experiments designed to investigate the K efflux stimulated by hypertonicity and its dependence on the extracellular tonicity in the HeLa cell line. The diuretic-sensitive K (^{86}Rb) efflux increased in a saturable manner from control values, saturation being observed at an external tonicity of 380-400 mosomol/kg. It is also important to note the decrease in the furosemide-insensitive K (^{86}Rb) efflux, as the media tonicity increases, similar to that shown in figure 5.1a.

Reversibility of hypertonic stimulation.

Figures 5.5 a and b show data from experiments designed to investigate the reversibility of the hypertonic stimulation of the K (^{86}Rb) efflux from HeLa and MDCK cells. For HeLa cells (figure 5.5a), it is once again observed that exposure to hyperosmotic media produces a progressive stimulation of the K efflux from the cells, which reaches a plateau after 9 minutes and which has an approximate $T_{1/2}$ of 3 minutes. On reincubation in isotonic media, a complete reversal of the increase in K (^{86}Rb) efflux was observed, with the fractional rate of K (^{86}Rb) efflux approaching that of controls and an approximate $T_{1/2}$ of 3 minutes. A qualitatively identical result was obtained for the MDCK cell line, except that on re-exposure to isotonic media, the fractional rate constant for efflux returned to the control value (figure 5.5b).

Figure 5.4

Effect of increasing media osmolality upon K (^{86}Rb) efflux in the absence (●) or presence of 0.1 mM furosemide (×). Mannitol addition was used to increase media osmolality. Results are expressed as the mean \pm S.D; where not shown, data points lie within the experimental points.

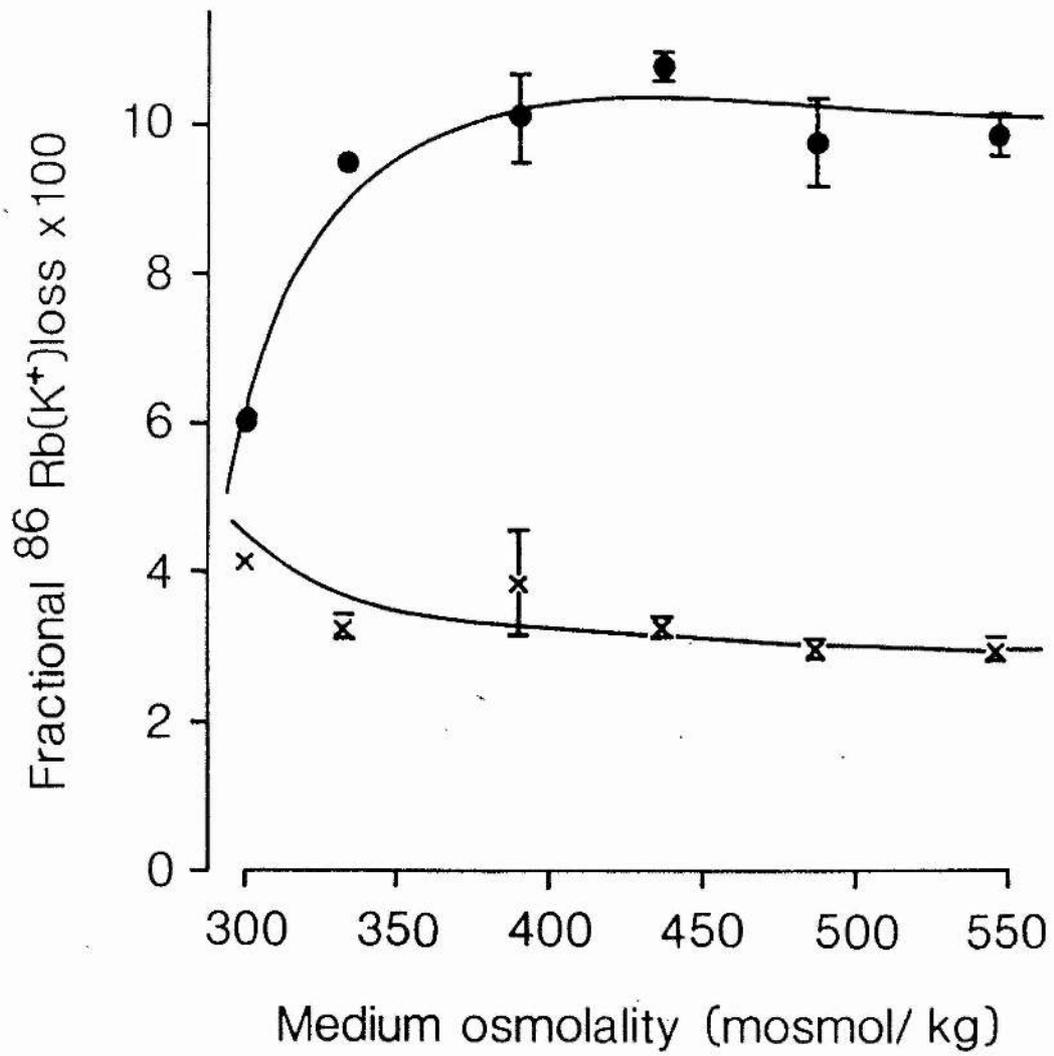
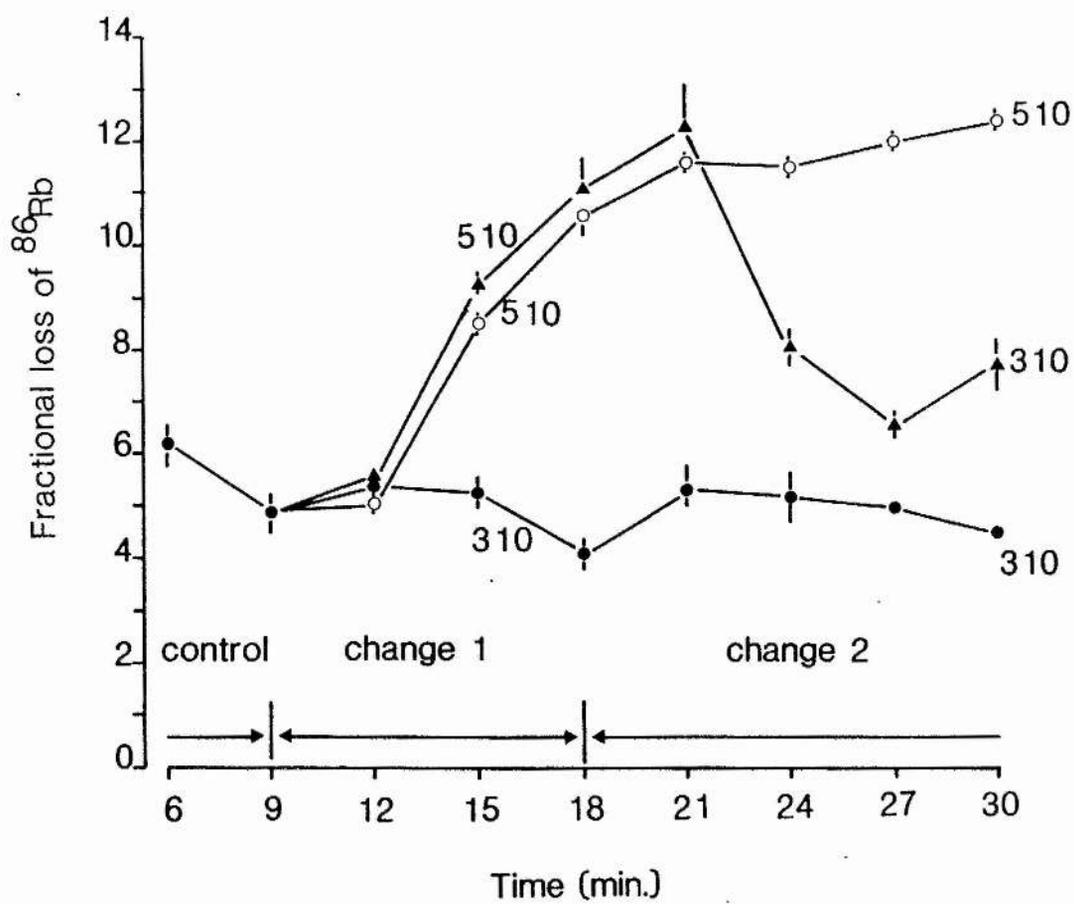


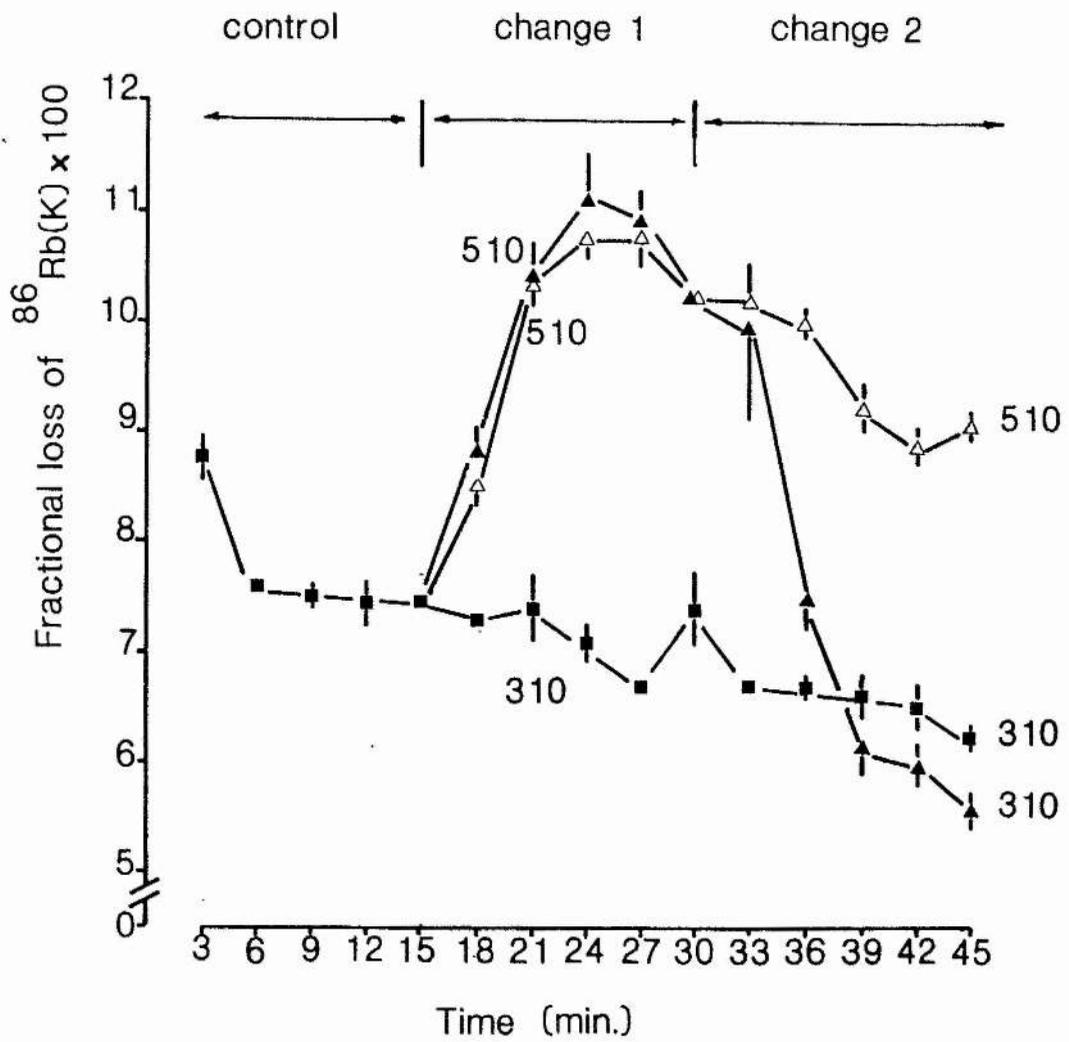
Figure 5.5 a,b

Reversibility of hypertonic stimulation of the K (^{86}Rb) efflux from (a) HeLa and (b) MDCK cells. K (^{86}Rb) efflux was determined in control media (●,■), hyperosmotic media (○,△) and hyperosmotic medium with re-exposure to isosmotic medium (▲). Data are the mean \pm S.E., n=9 (HeLa), n=3 (MDCK).

A



B



Cation and anion dependence of the hypertonicity stimulated diuretic-sensitive K (^{86}Rb) influx.

In chapter 3 and previous reports of Aiton et al., (1981 and 1982) and McRoberts et al. (1982), it has been shown that the "cotransport" flux, measured using K (^{86}Rb) influx, is dependent upon the presence of Na K and Cl in the media, the cation and anion specificity being distinct and limited in nature. Kinetic analysis revealed hyperbolic saturation kinetics for Na and K activations of the diuretic-sensitive K (^{86}Rb) influx, with Cl dependency exhibiting sigmoidal kinetics. In addition, inhibition by "loop" diuretics is of high affinity and characteristically the inhibitory potency of bumetanide exceeds that of furosemide. In order to assign the increased fluxes of K to "cotransport" unambiguously and to ascertain whether hypertonic exposure alters any of the operational parameters, the kinetic parameters of the diuretic-sensitive K (^{86}Rb) influx of HeLa and MDCK cells were determined in cells exposed to hypertonic media.

a) K dependence.

The dependence of the diuretic-sensitive K (^{86}Rb) influx which is present in HeLa and MDCK cells (see chapter 3) on external K was determined under isosmotic and hyperosmotic conditions (figures 5.6 and 5.7). The effect on HeLa and MDCK cells of increasing the K concentration in isosmotic media was comparable with the results presented in chapter 3. Hyperbolic activation curves were produced for both cell types, which saturated near 3 mM K. These data were

Figure 5.6

K activation of the ouabain-insensitive but diuretic (furosemide)-sensitive K (^{86}Rb) influx of HeLa cells in control media (320 mOsm/kg) (\circ), and hypertonic media (520 mOsm/kg, mannitol addition) (\square). The medium Na was 137 mM and KCl was varied in the range 0.5-13 mM (mannitol being used to maintain isosmotic conditions). The curves are the best-fit lines of the data to Michaelis-Menten kinetics, fitted as described in the methods. The kinetic constants V_{max} and K_m were: a) for 320 mOsm/kg data 1.91 ± 0.2 (S.D.) $\text{nmol}/10^6 \text{ cells} \cdot \text{min}.$, and 1.49 ± 0.26 (S.D.) mM respectively; b) for 520 mOsm/kg data, 5.08 ± 0.51 (S.D.) $\text{nmol}/10^6 \text{ cells} \cdot \text{min}.$ and 3.16 ± 0.67 mM (S.D.), respectively.

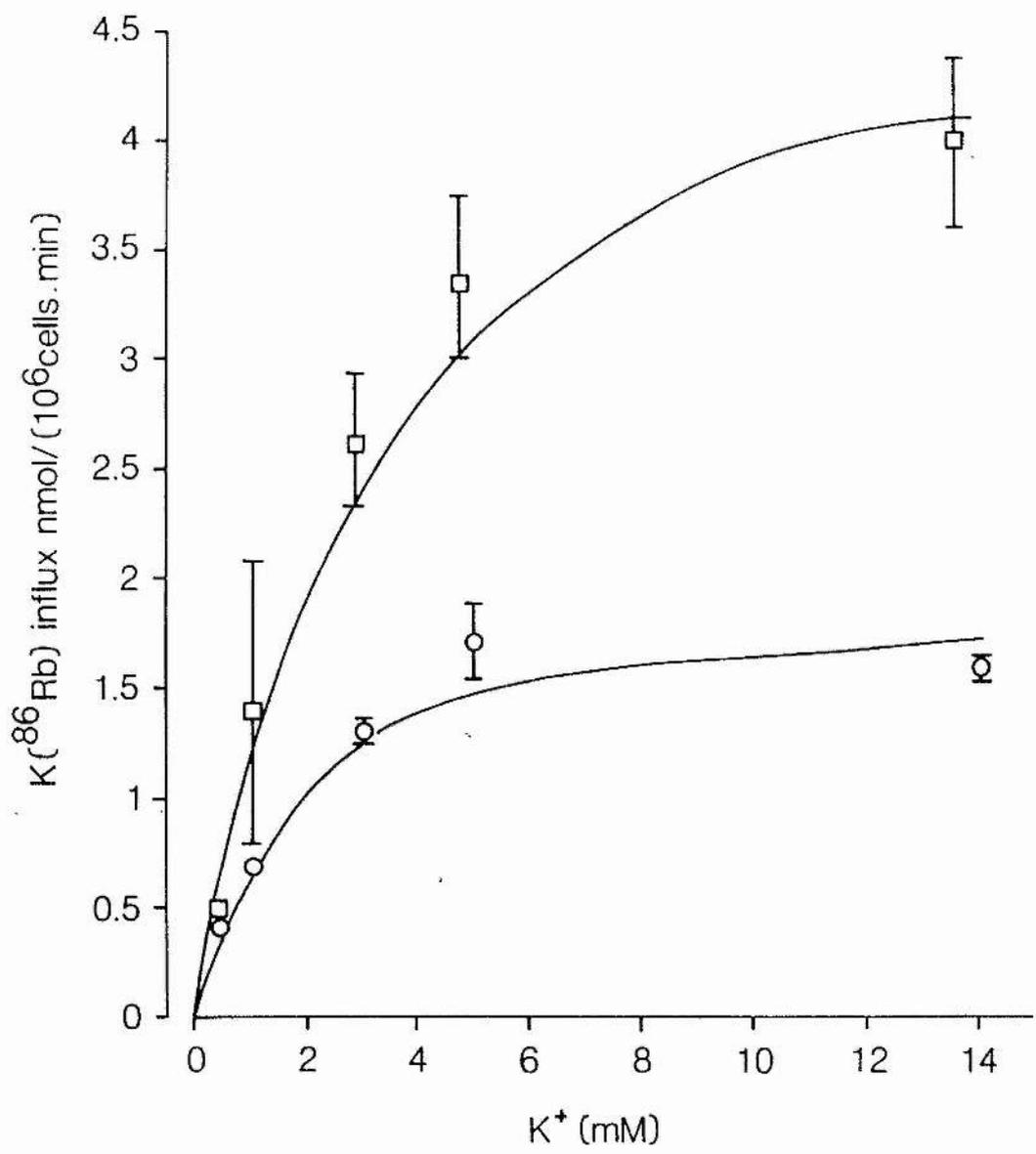
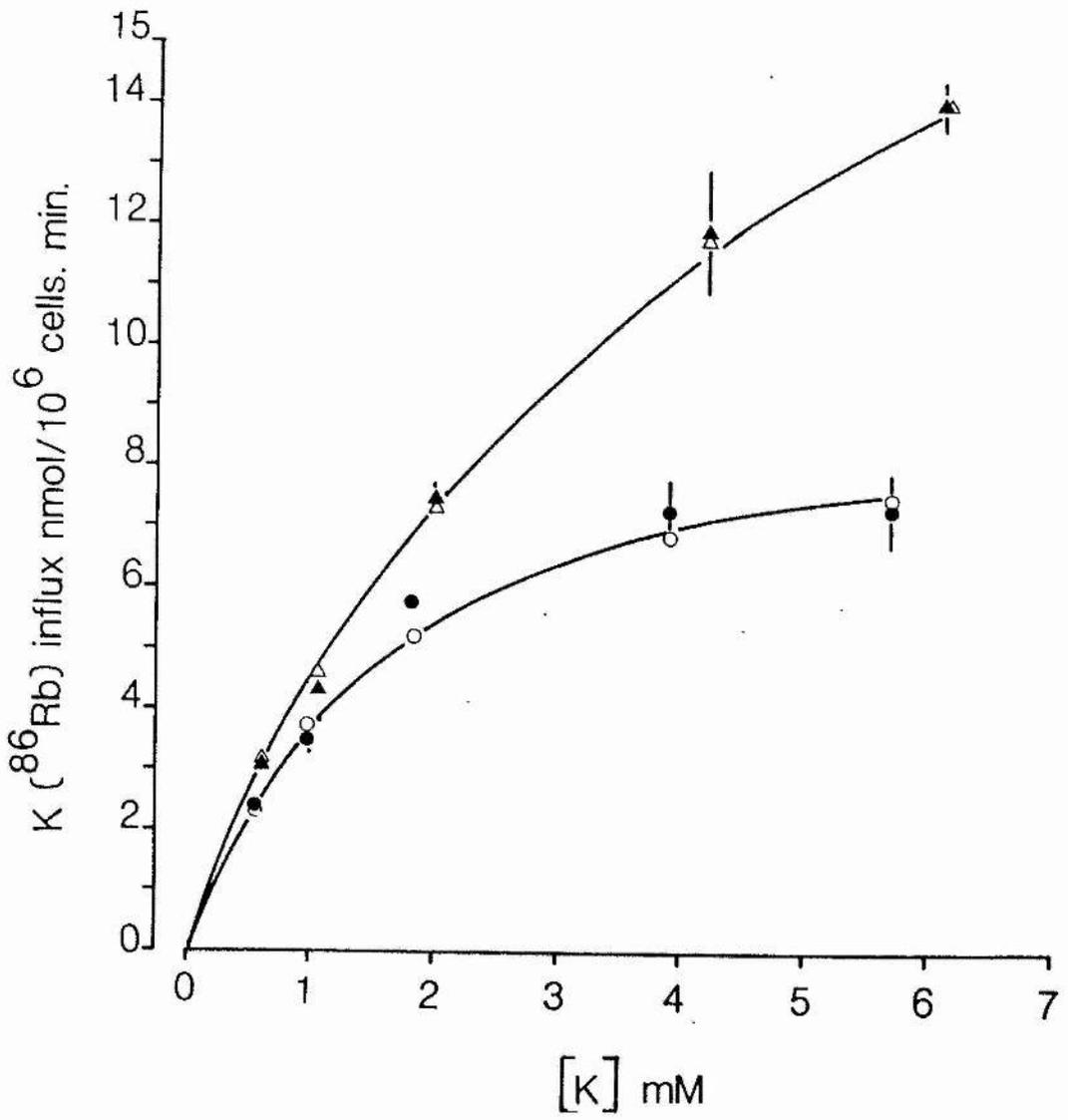


Figure 5.7

K activation of the ouabain-insensitive but diuretic (bumetanide)-sensitive K (^{86}Rb) influx of MDCK cells in control media (310 mOsm/kg) (\bullet), and hypertonic media (510 mOsm/kg, mannitol addition) (\blacktriangle). The media was 137 mM Na and K varied in the range of 0.5-7.0 mM (mannitol being used to maintain isosmotic conditions). The curves are the best-fit lines of the data to Michaelis-Menten kinetics fitted as described in the methods. The kinetic constants V_{max} and K_{M} were a) for 310 mOsm/kg data 9.58 ± 0.61 (S.D.) $\text{nmol}/10^6 \text{ cells.min.}$ and 1.60 ± 0.23 (S.D.) mM respectively; b) for 510 mOsm/kg data 24.71 ± 1.91 (S.D.) $\text{nmol}/10^6 \text{ cells.min.}$ and 4.58 ± 0.59 (S.D.) mM, respectively.



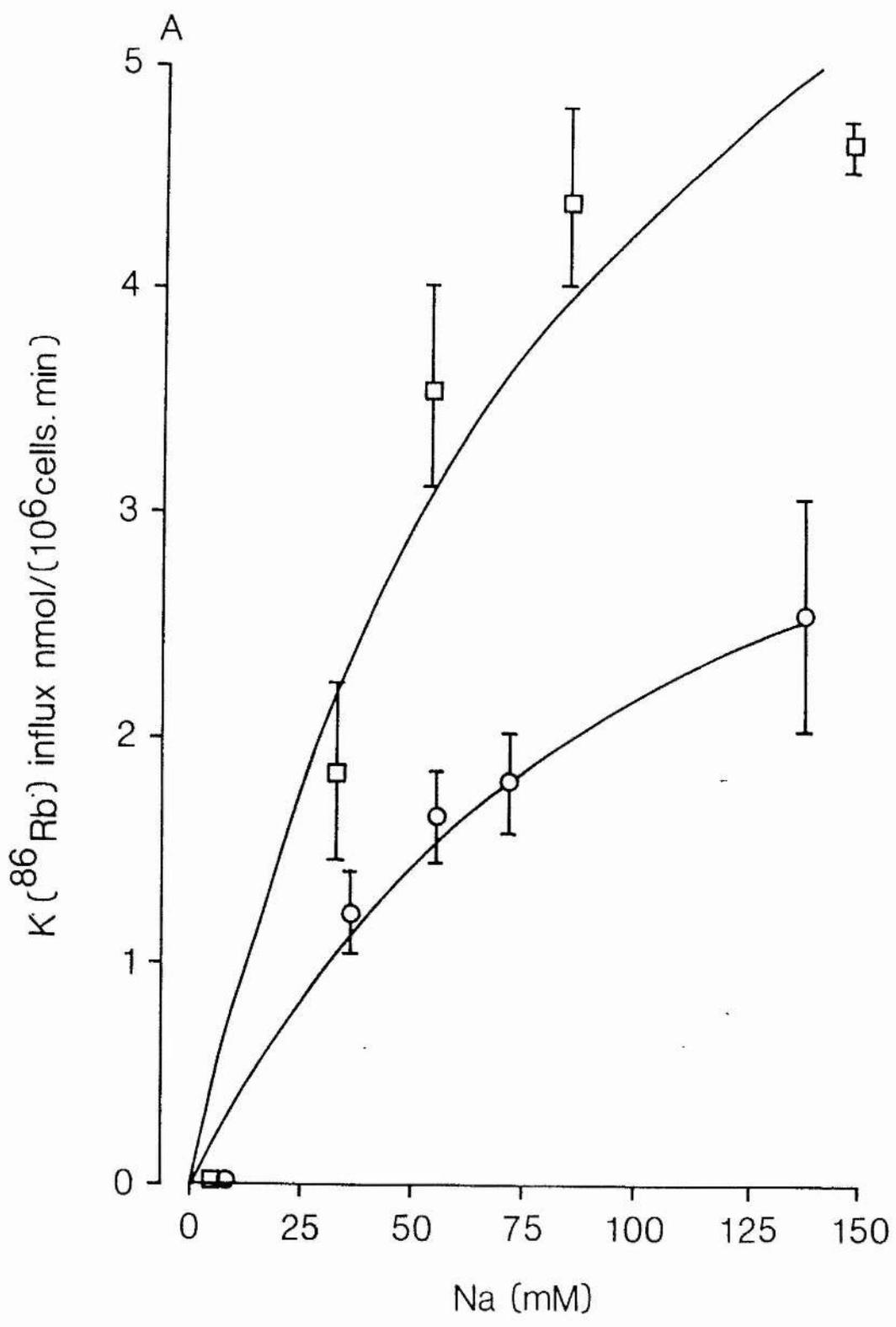
fitted to the Michaelis-Menten equation and gave apparent K_m values (in $\text{mM} \pm \text{S.E.}$) of 1.49 ± 0.26 and 1.60 ± 0.23 and maximal velocity of influx (in $\text{nmol}/10^6 \text{ cells.min} \pm \text{S.E.}$) of 1.91 ± 0.12 and 9.58 ± 0.61 , for HeLa and MDCK cells respectively. In shrunken HeLa and MDCK cells, the activation of diuretic-sensitive K (^{86}Rb) influx again generated hyperbolic curves. Fitting the data to the Michaelis-Menten equation gave apparent K_m (in $\text{mM} \pm \text{S.E.}$) values of 3.16 ± 0.67 and 4.58 ± 0.59 for HeLa and MDCK cells respectively, which are significantly greater than the apparent K_m 's determined in cells exposed to isosmotic conditions ($0.05 < P < 0.01$). The main effect of hypertonicity however is to increase significantly ($0.01 < P < 0.001$) the maximal velocity of the diuretic-sensitive K influx (in $\text{nmol}/10^6 \text{ cells.min.}$) in HeLa and MDCK cells to 5.08 ± 0.51 (S.E.) and 24.91 ± 1.91 (S.E.), respectively.

b) Na dependence.

The diuretic-sensitive K transport of HeLa and MDCK cells as discussed in chapter 3 and by Aiton et al., (1981) is dependent on the presence of media Na. Figures 5.8 a-b and 5.9 show that the activation of the diuretic-sensitive K transport gave hyperbolic kinetics in both HeLa and MDCK cells in isosmotic and hyperosmotic conditions. Kinetic analysis of these data revealed the principal effect of exposure to hyperosmolar media to be a significant ($0.05 < P < 0.001$) increase in the apparent maximal velocity (V_m , in $\text{nmol}/10^6 \text{ cells.min}$) in both HeLa and MDCK cells, irrespective of the Na substitute used (figure legends 5.8 a-b and 5.9). In HeLa cells (figure 5.8 a-b), the diuretic-sensitive pathway's apparent K_m for Na (CholineCl substitute) was not significantly affected by exposure to hypertonicity. However, if the Na substitute N-methyl-D-glucamine is used, a marked decrease

Figure 5.8 a,b

Na activation of the ouabain-insensitive but diuretic (furosemide)-sensitive influx of HeLa cells in (○,●) control media (310 mOsm/kg) or (□,■) hypertonic media (510 mOsm/kg, mannitol addition). Medium K was 5.4 mM, NaCl was varied by isosmotic substitution with a) cholineCl or b) N methyl-D-glucamine titrated to pH 7.0 by HCl. Curves are the best-fit lines of the data to Michaelis-Menten kinetics, fitted as described in the methods. The kinetic constants V_{max} and K_m for choline substitution were: for 310 mOsm/kg data 4.2 ± 0.9 (S.D.) $\text{nmol}/10^6$ cells.min. and 95.5 ± 36.1 (S.D.) mM; for 510 mOsm/kg data, 8.7 ± 2.1 $\text{nmol}/10^6$ cells.min. and 102 ± 42.8 mM respectively. For N methyl-D-glucamine substitution, the kinetic constants V_{max} and K_m were: for 310 mOsm/kg data, $V_{max} = 1.8 \pm 0.2$ $\text{nmol}/10^6$ cells.min. and the $K_m = 15.3 \pm 7.6$ mM, whilst for hypertonic data these constants were 4.1 ± 0.4 $\text{nmol}/10^6$ cells.min. and 57.2 ± 10.8 mM respectively.



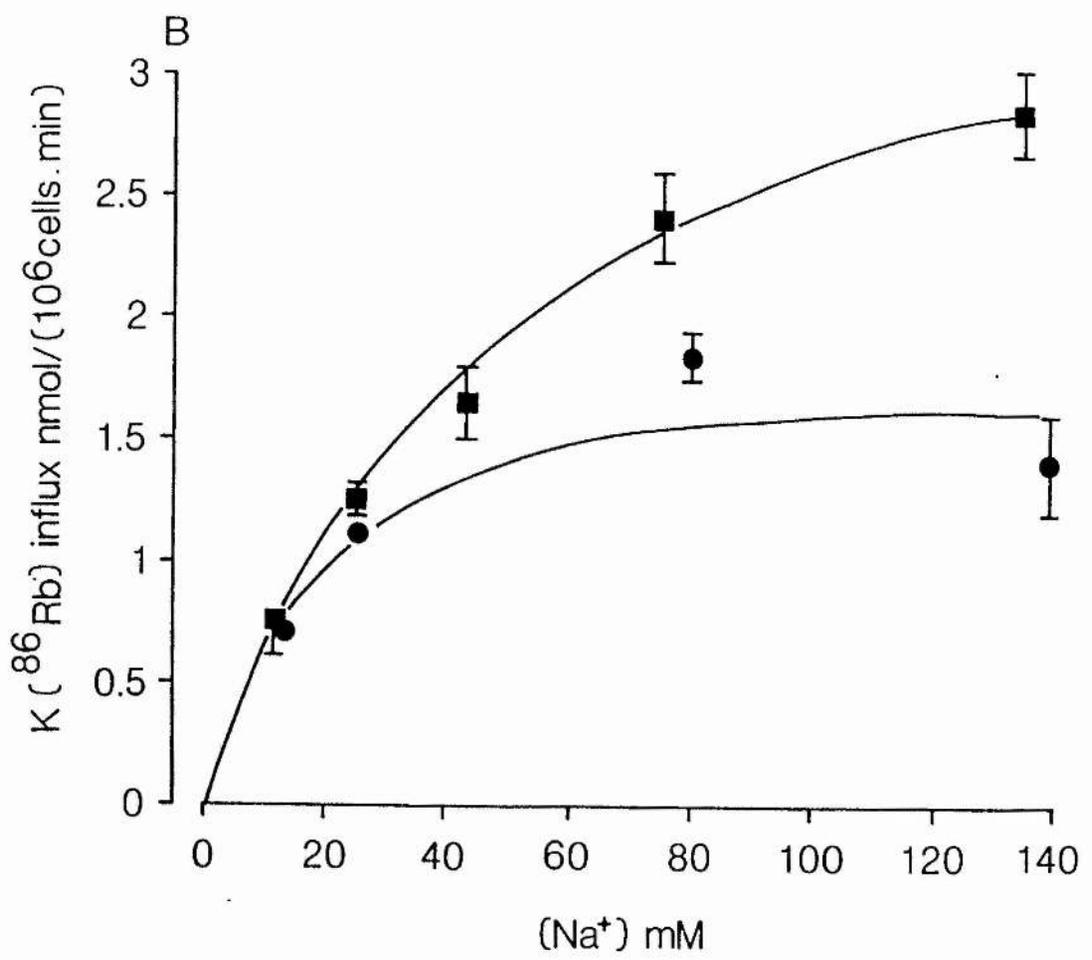
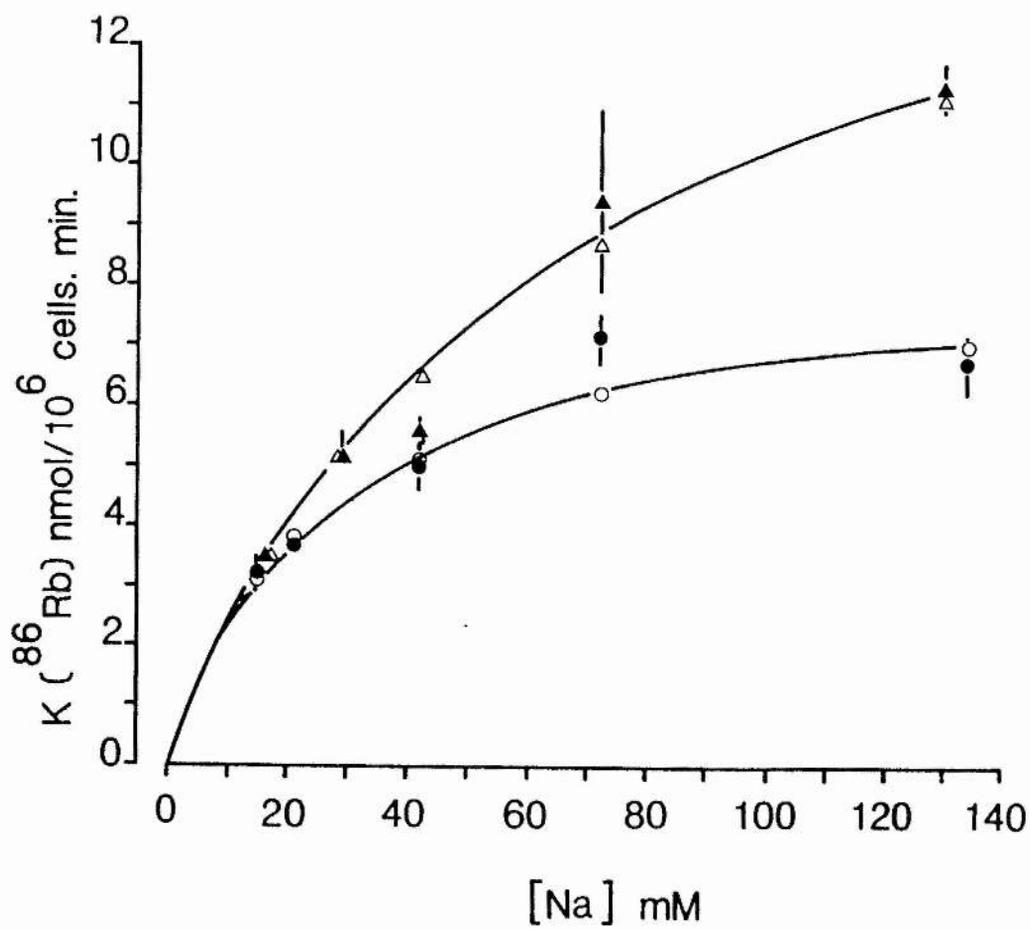


Figure 5.9

Na activation of the ouabain-insensitive but diuretic (bumetanide)-sensitive K (^{86}Rb) influx of MDCK cells in control media (310 mOsm/kg) (\bullet), and hypertonic media (510 mOsm/kg, mannitol addition) (\blacktriangle). Medium K was 5.4 mM, NaCl was varied by isosmotic substitution with N methyl-D-glucamine titrated to pH 7.0 by HCl. Curves are the best-fit lines of the data to Michaelis-Menten kinetics as described in the methods. The kinetic constants V_{max} and K_{m} for 310 mOsm/kg data were $8.37 \pm 0.58 \text{ nmol}/10^6 \text{ cells}\cdot\text{min.}$ and $25.04 \pm 4.59 \text{ mM}$ respectively, and for 510 mOsm/kg data $16.97 \pm 2.46 \text{ nmol}/10^6 \text{ cells}\cdot\text{min.}$ and $68.91 \pm 18.04 \text{ mM}$ respectively.



in the affinity for Na (in mM) was observed, from 15.3 ± 7.6 to 57.2 ± 10.8 ($P < 0.01$) and from 25.04 ± 4.59 to 68.91 ± 18.04 ($P < 0.01$) in HeLa and MDCK cells respectively on exposure to hyperosmolar media, this being similar to the effect on the apparent K_m for K of the diuretic-sensitive pathway in shrunken cells (see above).

The increase in the "residual" component of K influx in CholineCl and N-methyl-D-glucamine media (chapter 3) and the significant reduction of the residual component in hypertonic media (figures 5.1 and 5.4) serve to underline the fact that the residual "passive" K flux does not behave as a simple leak pathway.

c) Cl dependence.

Chipperfield (1985) has demonstrated that the Cl activation curves for (Na + K) "cotransport" in human erythrocytes are dependent upon the Cl replacement used; this is also true for HeLa cells (chapter 3). For this reason - three anion substitutes, nitrate (NO_3^-), isethionate $^-$ and gluconate $^-$ - were used (figures 5.10 a-c and table 5.4). The replacement of Cl in the extracellular media is complicated by changes in the Cl concentration not only in the extracellular media but also in the intracellular compartment, depending upon the relative penetrability of the replacement anion. The secondary effects of changes in the membrane potential and/or intracellular pH may also occur.

Figure 5.10 a,b,c and table 5.4 show the NO_3^- , gluconate $^-$ and isethionate $^-$ replacement for Cl for HeLa cells. The increase in the external Cl concentration (NO_3^- substitution, figure 5.10 a) activated

Figure 5.10 a-c

Cl activation of the ouabain-insensitive but diuretic (furosemide)-sensitive K (^{86}Rb) influx of HeLa cells in control conditions (310 mOsm/kg) (O) or in solutions made hypertonic (510 mOsm/kg) (●) by the addition of mannitol. All Cl salts were replaced by salts of the appropriate anion; a) NO_3^- , b) gluconate $^-$, c) isethionate $^-$. HCl was replaced by HNO_3 . Cl concentrations in all solutions were determined directly by coulometry. In all conditions, a 5 minute pre-incubation was performed prior to the commencement of the isotopic flux measurements. Data are the mean \pm S.D. of at least 3 determinations. Where not shown, error bars lie within the experimental data points. The curves were drawn from the kinetic constants shown in table 5.4.

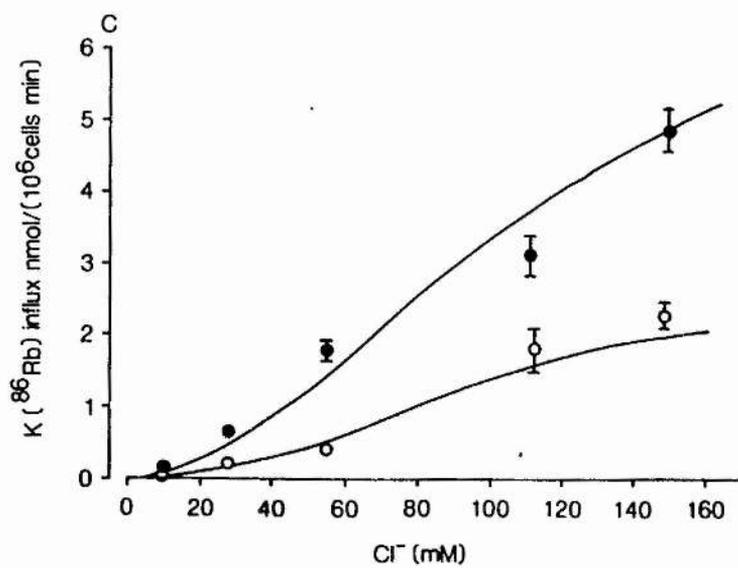
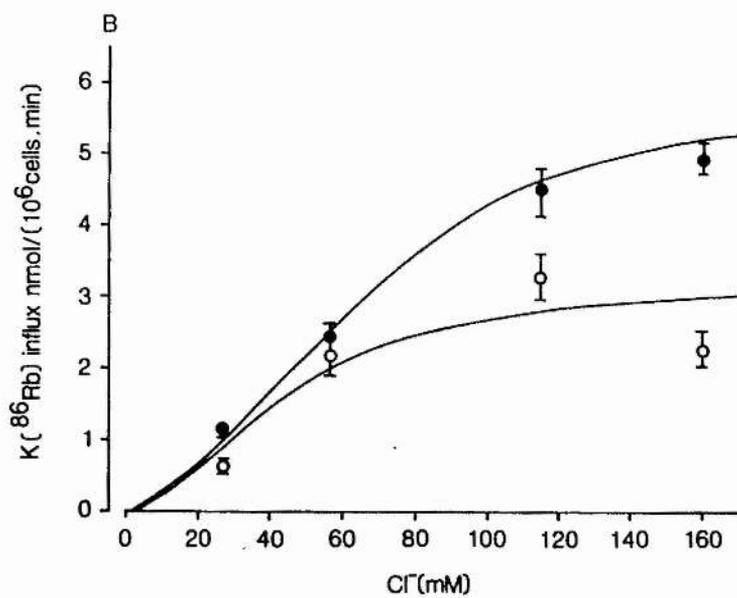
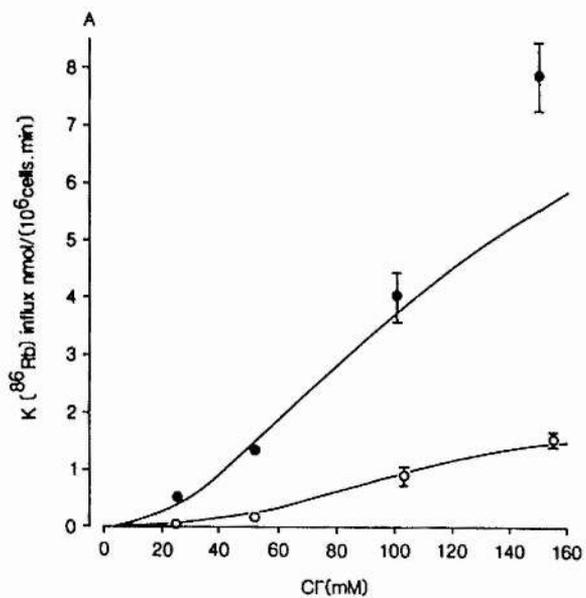


Table 5.4 Kinetic constants for Cl⁻ activation of the ouabain-insensitive but diuretic-sensitive ⁸⁶Rb⁺ influx in control media and hypertonic media, using NO₃⁻, gluconate⁻ or isethionate⁻ as the substituent anions. Experimental data and fitted curves are shown in Figure 5.10 a-c. All data were fitted to the Hill equation with initial estimates of the Hill coefficient (H) between 1.4 and 2.6

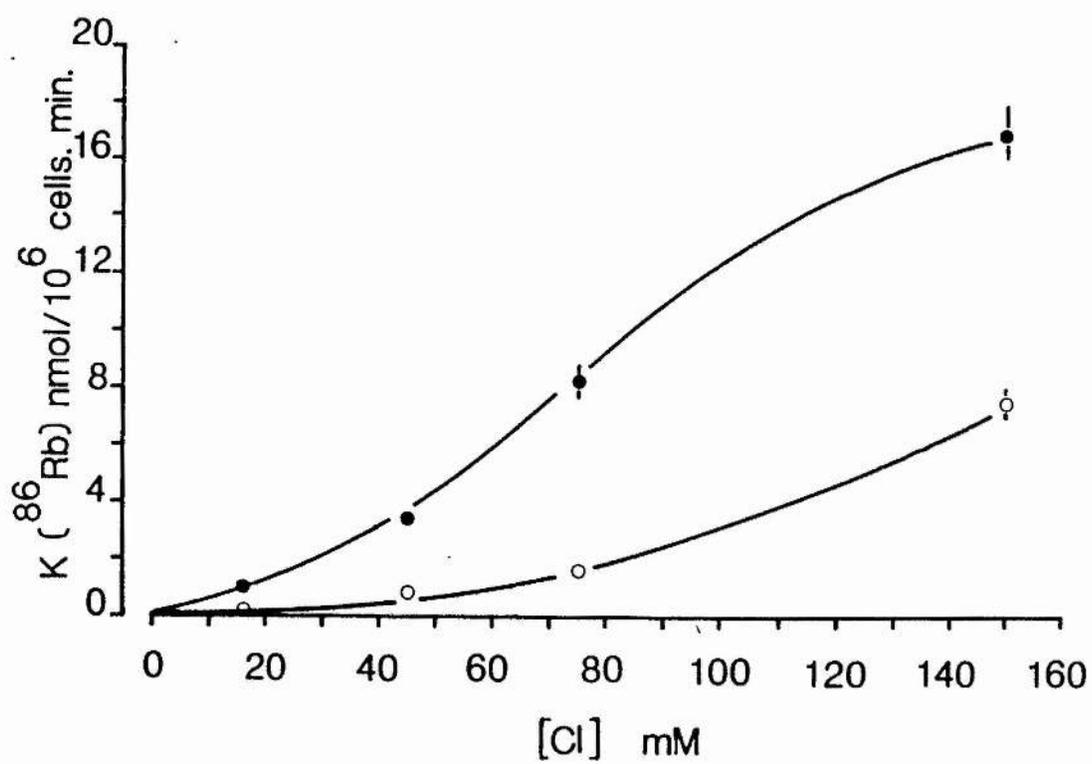
Substituent Anion for Cl ⁻		Medium osmolality (mOsm/kg)	
		310	510
NO ₃ ⁻	V _{max} (nmol/(10 ⁶ cells.min))	2.0	9.9
	K _{1/2} (mM)	111.8	132.0
	H	2.6	1.9
Gluconate ⁻	V _{max} (nmol/(10 ⁶ cells.min))	3.3	6.4
	K _{1/2} (mM)	44.1	70.5
	H	1.9	1.9
Isethionate ⁻	V _{max} (nmol/(10 ⁶ cells.min))	2.9	8.3
	K _{1/2} (mM)	105	123
	H	2.3	1.9

the diuretic-sensitive K (^{86}Rb) influx sigmoidally and the data could be fitted to the Hill equation, producing a Hill coefficient, n , close to 2. In hypertonic media, the diuretic-sensitive K (^{86}Rb) influx at all Cl concentrations was increased, with the main effect being an increase in the maximal velocity of diuretic-sensitive K transport, while little effect was seen on the Cl concentration that ellicates 50% activation (apparent $K_{1/2}$) of the K (^{86}Rb) influx. Similar results were obtained for the gluconate $^-$ (figure 5.10 b, table 5.4) and isethionate $^-$ (figure 5.10 c, table 5.4) replacements for Cl. The sigmoid nature of the Cl activation was more pronounced however for the gluconate $^-$ substitute. The apparent $K_{1/2}$ for Cl activation of the diuretic-sensitive "cotransport" appears to be dependent upon the replacement anion, since the gluconate $^-$ substitution resulted in a markedly smaller estimation of the apparent $K_{1/2}$ than if the Cl substitutes NO_3^- and isethionate $^-$ were used (table 5.4).

The Cl dependency of the diuretic sensitive K (^{86}Rb) influx of MDCK cells was only investigated in one anion substitute, NO_3^- (figure 5.11). In isotonic media, the Cl activation produced a non-saturating upward sloping curve, which became more sigmoidal on exposure to hypertonic media, both of which could be fitted to the Hill equation with Hill coefficients near to 2. The predominant effect of hypertonicity was the increased apparent maximal velocity of K transport via the "cotransport" pathway(s), but no marked effect was seen upon the apparent $A_{1/2}$ concentration of Cl.

Figure 5.11

Cl activation of the ouabain-insensitive but diuretic (bumetanide)-sensitive K (^{86}Rb) influx of MDCK cells in control (310 mOsm/kg) (\circ) or in hypertonic media (510 mOsm/kg, mannitol addition) (\bullet). All Cl salts were replaced by the appropriate NO_3^- salts. Cl concentrations in all solutions were determined by coulometry. In all conditions, a 5 minute pre-incubation was performed prior to commencement of the isotopic flux measurements. The initial estimate to the Hill equation gave kinetic constants V_{max} , $K_{1/2}$ and Hill coefficient (n) for 310 mOsm/kg data of 3.89 nmol/ 10^6 cells.min., 55.2 mM and 1.96 respectively and for 510 mOsm/kg data 14.46 nmol/ 10^6 cells.min., 66.71 mM and 1.96 respectively.



Sensitivity of the hyperosmotically stimulated K transport to "loop" diuretics.

The inhibitory potency of "loop" diuretics upon K (^{86}Rb) influx under isotonic and hypertonic conditions is shown in figures 5.12 a and 5.12 b. For both HeLa and MDCK cells, the increased K (^{86}Rb) influx in hyperosmotic media, was inhibited with high affinity by furosemide and bumetanide respectively. No significant difference in the apparent half maximal inhibitory concentration (apparent K_i) for furosemide in HeLa cells and bumetanide in MDCK cells was observed between hypertonic or isotonic conditions.

The above data for the diuretic inhibition of the hypertonicity-stimulated K transport, taken in conjunction with the cation and anion dependence of this K flux, are strong evidence that this stimulated K flux is mediated by the same pathway as the diuretic-sensitive pathway in control cells (chapter 3).

Ouabain stimulation of diuretic-sensitive K flux in hypertonic media.

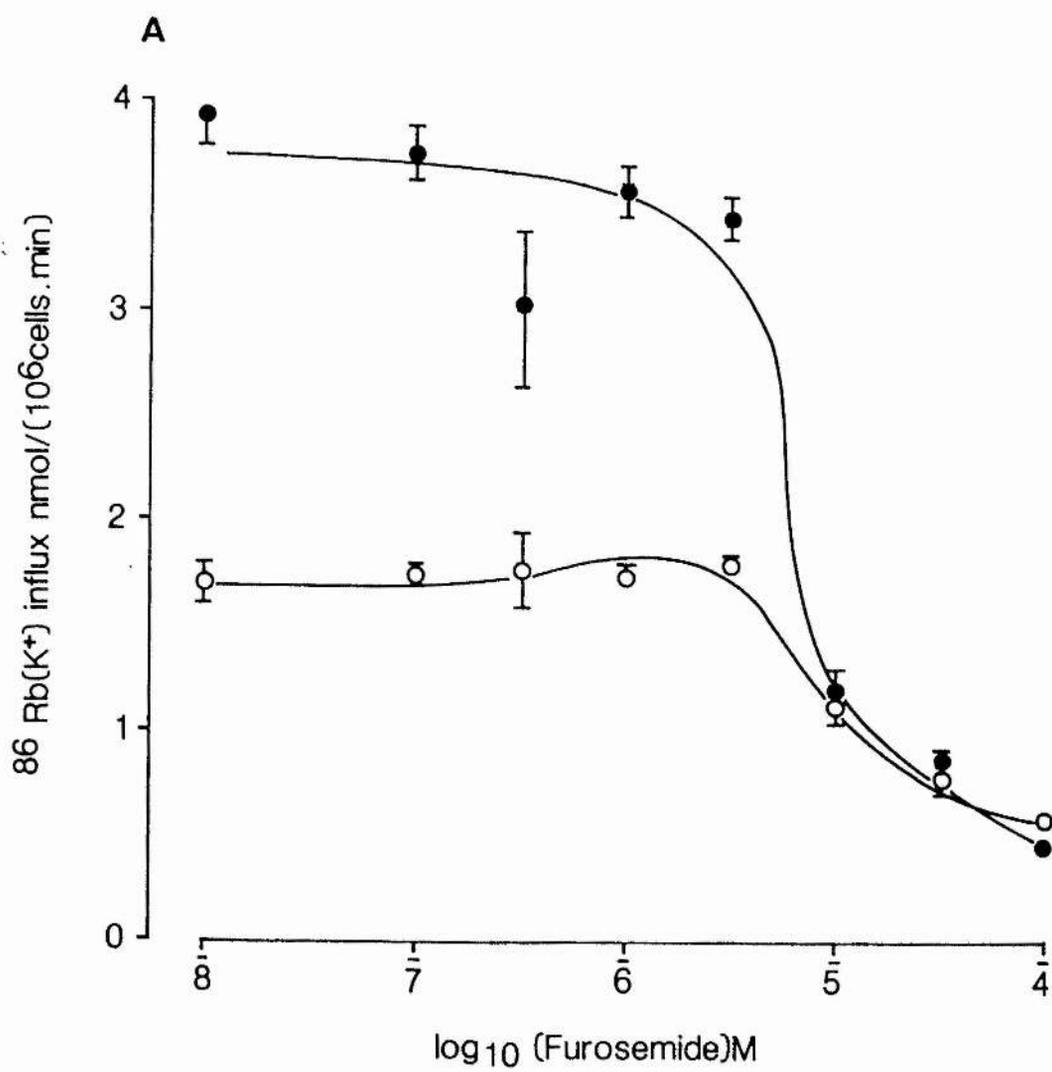
It has already been reported that in HeLa and MDCK cells exposed to ouabain a progressive stimulation of the component of flux which is diuretic-sensitive is observed (Aiton and Simmons, 1984). As discussed in chapter 3 and Aiton and Simmons (1984), incubation with 1 mM ouabain (isotonic) media significantly increased the fractional loss ($\times 100$) of K (^{86}Rb) from both HeLa and MDCK cells. This increase is "loop" diuretic-sensitive (table 5.5). Similarly, exposure to hypertonic media markedly increased the rate of K (^{86}Rb) efflux

Figure 5.12 a,b

Sensitivity of the ouabain-insensitive K (^{86}Rb) influx of HeLa (a) and MDCK (b) cell lines to inhibition by furosemide and bumetanide respectively in control (O) and hyperosmolar (●) conditions. The curves were fitted by eye. From linear regression of data to the Hill equation:

$$\log (V/V_{\max}-V) = n \log S - \log K$$

where V_{\max} is the maximal inhibition by diuretic and S the diuretic concentration, n is the Hill coefficient and K is $S_{0.5}$, control HeLa data gave constants of $n = 0.99 \pm 0.12$ and $K = 5.3 \times 10^{-6}\text{M}$ whilst for hyperosmolar conditions $n = 1.38 \pm 0.15$ and $K = 6.2 \times 10^{-6}\text{M}$. For MDCK control data; $n = 0.87 \pm 0.13$ and $K = 7.4 \times 10^{-7}\text{M}$ and for hyperosmotic data, $n = 1.1 \pm 0.05$ and $K = 1 \times 10^{-7}\text{M}$.



B

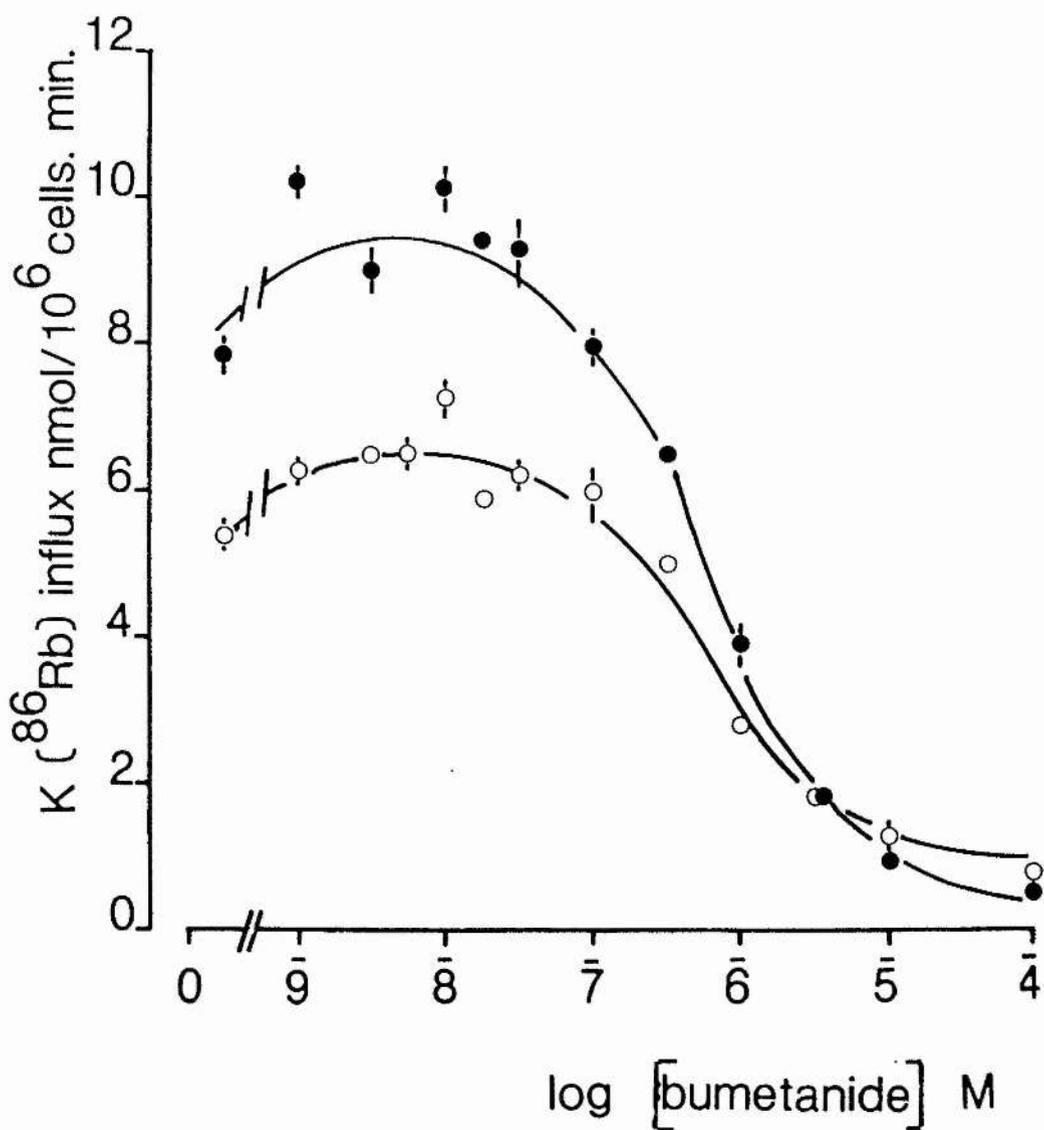


Table 5.5 Effect of ouabain upon the fractional rate constant ($\times 100$) of $K^{86}Rb^+$ efflux at time 30 minutes

From (a) HeLa and (b) MDCK cells. Data are the mean \pm SD, n = 3

Cell type	Media tonicity	(1)	(2)	(3)	(4)	(1-4)	(2-3)	(1-2)
		Control	+ Ouabain	+ Ouabain + Bumetanide	+ Bumetanide	Total - Bumetanide	Ouabain - Ouabain + Bumetanide	Ouabain-stimulated
MDCK	310	3.9 \pm 0.3	8.2 \pm 1.1	2.4 \pm 0.2	2.0 \pm 0.2	1.9 \pm 0.2	5.8 \pm 0.2	4.3 \pm 0.7
	510	8.0 \pm 0.70	15.3 \pm 0.6	2.2 \pm 0.1	1.8 \pm 0.1	6.2 \pm 0.4	13.1 \pm 0.4	7.3 \pm 0.5
HeLa	310	4.2 \pm 0.4	6.2 \pm 0.2	4.1 \pm 0.5	3.7 \pm 0.4	0.5 \pm 0.3	2.1 \pm 0.4	2.0 \pm 0.4
	510	13.4 \pm 1.3	19.3 \pm 0.9	4.2 \pm 0.1	4.4 \pm 0.9	9.0 \pm 0.9	15.1 \pm 0.5	5.9 \pm 0.9

Footnote:

HeLa: Expected stimulation of the diuretic-sensitive K efflux by ouabain and hypertonicity would be 6.2 + 5.8 - 1.9 = 10.1; actual stimulation is 13.1 \pm 0.4.

MDCK: The expected stimulation of the diuretic-sensitive K efflux by ouabain and hypertonicity would be 9.0 + 2.1 - 0.5 = 10.6; actual stimulation is 15.1 \pm 0.5.

compared to that observed in isotonic media and this increase was fully inhibited by bumetanide (see above). Since ouabain incubation is associated with only a small change in cell volume, it is most probable that cell shrinkage and ouabain stimulation of diuretic-sensitive K transport do not result from identical mechanisms, and I therefore tested hypertonic incubation in the presence of ouabain (table 5.5). The inclusion of 1 mM ouabain in the hypertonic media further increased the fractional loss of the K (^{86}Rb) from HeLa and MDCK cells which was again fully inhibited by bumetanide. The effect of ouabain plus hypertonicity was synergistic (see footnote table 5.5) and the stimulatory effect of ouabain on the fractional rate of K (^{86}Rb) efflux increased from 4.3 ± 0.65 to 7.3 ± 0.53 ($P < 0.05$) in MDCK cells and from 1.95 ± 0.36 to 5.9 ± 0.9 ($P < 0.02$) in HeLa cells exposed to hypertonic media. Thus hypertonic and ouabain stimulation of the "loop" diuretic-sensitive K (^{86}Rb) efflux was via separate but interacting pathways. Non-additivity of ouabain stimulated, and hypertonicity stimulated, diuretic-sensitive unidirectional K transport has also been observed in SV 3T3 cells (Bakker-Grunwald, Ogden and Lamb, 1982).

DISCUSSION.

The primary interest of this chapter was the response of HeLa and MDCK cells to cell shrinkage with respect to their diuretic-sensitive K transport. Identification of the K transport pathways affected is based on their pharmacological sensitivity. Furosemide is reported in the literature as exhibiting certain non-specific effects, for example the inhibition of the Cl-HCO_3 exchange in human red cells (Brazy and Gunn, 1976) and the volume-stimulated KCl transport of sheep red cells (Dunham and Ellory, 1981). Therefore the identification of specific membrane transport systems by pharmacological agents is solely dependent on the specific nature of the actions of these agents. The analysis of the Na K and Cl dependencies has also been used to provide extra criteria in the identification of the diuretic-sensitive "cotransport" pathway, similar to the Na K Cl "cotransport" of a variety of cell types (Palfrey and Rao, 1983). Therefore, the diuretic-sensitive K (^{86}Rb) transport may be considered to be a Na K 2Cl "cotransport" system (see also the discussion in chapter 3).

HeLa (Tivey et al., 1985) and MDCK (Simmons, 1984) cells behave as perfect osmometers and they are unable to regulate their cell volume on exposure to hyperosmotic media (table 5.1). Similar behaviour was observed in the Ehrlich ascites cell (Hendil and Hoffmann, 1974), volume regulation, mediated by the "cotransport" pathway, being only apparent when the intracellular KCl is lowered prior to cell shrinkage (Hoffmann et al., 1983).

Shrinkage of HeLa and MDCK cells produced a large stimulation of

the K fluxes predominantly associated with the ouabain-resistant but diuretic-sensitive Na K Cl "cotransport". The stimulation of the K flux was fully reversible on re-exposure to isosmotic media. Similar stimulations of the ouabain-insensitive K transport without volume regulation have been observed in avian erythrocytes (Kregenow, 1977) at physiological values of external K and only when the external K concentration was perturbed from the physiological concentration was volume regulation observed.

Measurement of the total cation content of HeLa and MDCK cells shows no change upon incubation in hypertonic media, thus confirming the lack of volume regulation in these cells. As discussed in chapter 3, and demonstrated by Schmidt and McManus (1977 c) and Haas et al., (1982) for avian erythrocytes the net flux through the Na K Cl "cotransport" system is dependent upon the sum of the chemical potential gradients of the ions involved. Therefore in conditions where no net transport through the Na K Cl "cotransport" pathway is observed, then the sum of the chemical potential gradients should be zero, which holds true for HeLa cells incubated under isosmotic conditions (see discussion chapter 3). However, exposure to hypertonicity produced an increased K transport via the "cotransport" pathway, but did not effect a net uptake of ions and hence volume regulation. Therefore, in shrunken cells, where the osmotic addition is a non-penetrant inert solute (mannitol), the external concentrations of Na K and Cl remain constant compared with the isotonic condition, but the internal concentrations were increased by a factor directly proportional to the ratio of the osmolalities (Tivey et al., 1985; Simmons, 1984; Ueberschar and Bakker-Grunwald, 1983).

Data presented for HeLa and MDCK cells (figures 5.1 and 5.2,

table 5.3) show that the diuretic-sensitive K fluxes via the "cotransport" pathway were in balance in shrunken cells. This contradicts equations 1 and 2 (see discussion chapter 3), because, under these experimental conditions, an outwardly-directed gradient would be predicted, resulting in a loss of K and further shrinkage of the cells, which did not in fact occur. Therefore, under the experimental condition described in this chapter, equations 1 and 2 are not applicable to HeLa and MDCK cells. Assuming that the driving force for the "cotransport" system is the sum of the chemical gradients, as proposed by Haas et al., (1982), then an explanation for this would be that the effective intracellular concentration of the Na K and Cl is less than that predicted from the measured concentrations of these ions in controls multiplied by the ratio of the osmolalities.

Decreased cell volume results in a large stimulation of the unidirectional diuretic sensitive K (^{86}Rb) flux. From the kinetic analysis of the Na K and Cl activations in both HeLa and MDCK cells, the predominant effect was an increase in the maximal velocity of the K (^{86}Rb) transport, which was diuretic-sensitive. The affinity of the "cotransport" for the activating ions Na and K decreased in hyperosmotic conditions. For Na, this decrease in affinity depended upon the Na substitute used, CholineCl (Na replacement) leaving the apparent K_m unaffected by hyperosmotic media. The mechanism which caused the increased K (^{86}Rb) transport via the "cotransport" system has not been addressed in this chapter. The increased apparent V_m and apparent K_m , with the lack of trans-stimulation of external K on the K (^{86}Rb) efflux for the MDCK and HeLa cells (see Tivey et al., 1985), suggests that stimulation may result from an increase in the number of functional units and that the new units may have reduced affinities

for Na and K. The sensitivity to inhibition by diuretic was unchanged by exposure to hyperosmotic media. Thus, if the stimulation was due to an increase in the number of functional units, the sensitivity of the new units to "loop" diuretics does not appear to differ greatly from those in the control cells. This would appear to be unlikely considering the results presented in chapter 8. Alternatively the number of functional transport units may remain constant, and the increased K transport through the Na K Cl "cotransport" pathway being affected by an increased rate of turnover (molecules/site.second).

The stoichiometry of the diuretic-sensitive "cotransport" of the HeLa and MDCK cells in hyperosmotic media may be considered to be Na K 2Cl, this being based upon the assumptions described in the discussion of chapter 3 (see also McRoberts, et al., 1982) and derived from the hyperbolic activation of K influx by K and Na and the sigmoidal dependence of the K influx upon Cl (with the Hill coefficient near 2).

The inability of the HeLa and MDCK cell lines, which possess a "cotransport" system, to undertake volume regulatory increase, thus differ from the avian erythrocyte in that response to cell shrinkage. It should be noted however that the avian erythrocyte only regulates volume when the extracellular K is elevated (Kregenow, 1977; Schmidt and McManus, 1977a). Therefore the "cotransport" system of the MDCK and HeLa cell lines may not be regarded as a volume regulatory system per se, but rather as a system concerned with K homeostasis (see the discussion of Duhm and Goebel, 1984).

As discussed above the diuretic-sensitive K transport of HeLa and MDCK cells is stimulated by cell shrinkage. Under these

experimental conditions intracellular ion concentrations will be increased in proportion to the cell volume (see above). Could these increased ion concentrations regulate the activity of the Na K Cl "cotransport" pathway in the shrunken cell? It is unlikely that intracellular ions, in particular Na, regulate this transport pathway, primarily because the time courses for the stimulation of K transport and cell shrinkage are markedly different, with the former being half maximally activated after 6-9 minutes exposure to hyperosmolar media, whereas the cell shrinkage occurs rapidly as determined by the coulter counter. Secondly, no trans-membrane ion dependency of the diuretic-sensitive K efflux has been reported in this present work.

Thirdly, if intracellular Na does regulate the Na K Cl "cotransport" pathway then the effect of increasing intracellular Na is saturable, since increasing media tonicity above 380 mosmol/kg produced no further stimulation of the diuretic-sensitive K transport despite continued decreases in cell volume. This transport pathway may also be stimulated by incubation in ouabain-containing media (Aiton and Simmons, 1984; see chapter 3), and a similar action of ouabain on the activity of this transport system has been reported in Ehrlich ascites cells and SV 3T3 cells (Bakker-Grunwald, Andrew and Neville, 1980; Bakker-Grunwald, Ogden and Lamb, 1982). The nature of this stimulation is unknown (Bakker-Grunwald, et al., 1982) but is most probably an indirect effect of ouabain, for example changes in intracellular ion concentrations. If this is indeed true, the addition of ouabain to hyperosmolar media should have no further stimulatory effect upon the Na K Cl "cotransport" pathway in the shrunken cell, assuming the response to cell shrinkage to be regulated by intracellular Na, and saturable. However this is not true, since exposure of HeLa and MDCK cells to hyperosmotic media in the presence

of ouabain is synergistic, thus these activations are mediated through separate and interacting pathways. Therefore, it would appear improbable that increases in the intracellular ion concentrations can account for the hyperosmolar stimulation of the Na K Cl "cotransport" system in shrunken HeLa and MDCK cells.

CHAPTER 6

THE ROLE OF Ca AND cAMP IN THE REGULATION OF THE PASSIVE K TRANSPORT.

INTRODUCTION

It is now well established that when avian erythrocytes are exposed to either catecholamines or hyperosmotic media, there is a stimulation of net salt and water uptake (Schmidt and McManus, 1977 a-b; also reviews by Kregenow, 1977; McManus and Schmidt, 1978; Palfrey and Rao, 1983). Schmidt and McManus (1977 a-b) also demonstrated that this salt uptake was mediated via a ouabain-insensitive but "loop" diuretic-sensitive Na plus K "cotransport" pathway. This Na and K "cotransport" in the avian erythrocyte not only requires extracellular Cl (Schmidt and McManus, 1977c) but also shows Cl-driven diuretic-sensitive Na and K transport and so may be considered to be a Na K 2Cl "cotransport" (Bakker-Grunwald, 1981; Haas, et al., 1982).

Catecholamines mediate their stimulatory action through a B adrenergic receptor and the second messenger cAMP (Kregenow, Robbie and Orloff, 1976). The involvement of cAMP has been further substantiated in that external application of 1mM 8-Br-cAMP stimulates the K transport of the avian erythrocyte (Palfrey et al., 1980; Palfrey and Rao, 1983). The catecholamine stimulation of the Na K 2Cl "cotransport" has been demonstrated to be accompanied by the simultaneous phosphorylation of a membrane protein by cAMP and Ca-dependent protein kinases (Alper, Beam and Greengard, 1980; Alper, Palfrey, Deriemer and Greengard, 1980) and in this respect, this action of B-adrenoceptor stimulation via a second messenger and protein phosphorylation is typical of a large number of cAMP-regulated systems. Hypertonic stimulation of the K influx of avian erythrocytes is not accompanied by either an accumulation of intracellular cAMP

(Kregenow, et al., 1976) or a phosphorylation of a membrane protein (Alper et al., 1980 and 1980).

Due to the similarities of the hypertonic- and catecholamine-stimulated Na K 2Cl "cotransport" system of the avian erythrocyte, it has been inferred (Kregenow et al., 1976; Ueberschar and Bakker-Grunwald, 1983) that both activating pathways converge on a common distal system. The lack of cAMP involvement in the hyperosmotic stimulation of the K transport raises the possibility that second messengers other than cAMP may be involved in the regulation of the avian Na K 2Cl "cotransport" system. However, for erythrocytes of human (Garay, 1982) or ferret (Palfrey, 1984) origin, the effect of increasing the cellular cAMP by incubating in 1mM 8-Br-cAMP inhibits the Na K and Na K 2Cl "cotransport" system(s), which is in contrast to avian erythrocytes (see above).

It is now clear that Ca triggers various cellular events; for example, the release of transmitter substances, activation of contractile systems and the regulation of membrane permeability (for references see Flatman, 1982). Garay (1982) has also demonstrated that increased intracellular Ca inhibits the Na K "cotransport" of human red blood cells.

Haas and McManus (1984) have demonstrated that the catecholamine stimulation of Na K 2Cl "cotransport" counter-balances the swelling-induced KCl loss in avian red cells, thereby substantiating the earlier work of Riddick and co-workers (1971), who demonstrated that duck erythrocytes, suspended in an artificial salt solution (K 2.5 mM), required nor-adrenalin to prevent a loss of cell K and water in isosmotic media. Thus, the physiological significance of the

"cotransport" system in the avian red cell may be one of K homeostasis. In mammalian erythrocytes, although they do not possess B adrenergic-stimulated adenylate cyclase activity (Sheppard and Burghardt, 1969; Rasmussen, Lake and Allen, 1975), the action of cAMP on the Na K "cotransport" (see above) may indicate a regulatory role of circulating hormones on the "cotransport" system(s) of non-erythrocyte cells (Garay, 1982).

The diuretic-sensitive K (⁸⁶Rb) transport, Na K Cl "cotransport", of the cultured cell lines MDCK and HeLa (Aiton et al., 1981, 1982; McRoberts et al., 1982; Rindler et al., 1982) may be stimulated by exposure of the cells to hyperosmotic media (see chapter 5; Simmons and Tivey, 1985; Tivey et al., 1985). The mechanism by which this response is mediated is unknown, therefore, in the light of the responses of erythrocytes of avian and mammalian origin, the regulatory role of the cAMP and Ca upon the "cotransport" system has been investigated. Results in this chapter have also been presented in Simmons and Tivey (1985).

RESULTS

A) DIURETIC-SENSITIVE K (⁸⁶Rb) TRANSPORT: REGULATORY ROLE OF Ca.

Manipulation of extracellular and intracellular Ca.

External Ca can be lowered by omitting the Ca salt from the standard Krebs solution and including the Ca-chelating agent, ethyleneglycol-bis-(β -amino-ethyl ether) tetraacetic acid (EGTA (Ca < 1nM)). Manipulation of intracellular Ca can be achieved by the use of the Ca ionophore A23187, which will cause a large uptake of Ca by the cells when the latter are incubated in Ca-containing media. However, if the external media is nominally free of Ca and contains the Ca-chelating agent EGTA (2 mM), then the ionophore, A23187, causes the depletion of intracellular Ca stores (Campbell, 1983). However, it is important to note that the ionophore A23187 will also affect intracellular Mg as well as intracellular Ca (see Flatman, 1982 for references). Since intracellular Ca is low compared to intracellular Mg (Ca < 1.0 μ M, low intracellular Ca maintained by intracellular Ca buffering and the Ca pump (see Flatman, 1982, for references), any effect of A23187 upon the cell is most probably due to changes in the intracellular Ca concentration (see below), rather than in the substantially higher Mg levels.

Effect of increasing intracellular Ca.

(i) Action of Ca ionophore A23187.

Brown (1983) has reported that A23187 increases K permeability of

the MDCK cell line in a dose-dependent fashion. In this present study (table 6.1), inclusion of A23187 (10 μ M) produced a highly significant ($P < 0.001$) increase in the K (^{86}Rb) efflux from this cell line and removal of the extracellular Ca (plus 2mM EGTA) abolished the effect of the ionophore on K efflux (table 6.1). These results are in agreement with previous reports of a Ca-activated K channel that may be activated either by A23187 or physiologically by adrenergic stimulation (Brown and Simmons, 1982). This increase in the K efflux from MDCK cells represents net loss KCl (table 6.2). The KCl is accompanied by its osmotically-obliged water resulting in a significant cell shrinkage ($P < 0.01$). Little or no effect was observed on the intracellular Na content of this cell line.

To observe any effects of increased cellular Ca on the Na K Cl "cotransport" flux, the problem of the increased K permeability and cell shrinkage needs to be overcome. Is it possible to block the Ca activated K conductance effectively ?

(ii) Blockade of Ca-activated K channels.

Ca-dependent K transport in human red cell ghosts and intact human red blood cells is sensitive to inhibition by the carbocyanine dye DiS C₂(5) (Simons, 1979). Garay (1982) used DiS C₂(5) to block the Ca activated K transport when studying the effects of intracellular Ca on the Na K "cotransport" of the human red cell.

Adrenalin has been demonstrated to initiate a Ca-activated K transport (Brown and Simmons, 1982) and this action of adrenalin is substantiated in figure 6.1. This Ca-activated increase of K permeability of the MDCK cell may be inhibited by 10 μ M DiS C₂(5).

Table 6.1 Effect of a 3 minute exposure to the Ca²⁺
ionophore A23187 on the K⁺(⁸⁶Rb⁺) efflux
from MDCK in the presence or absence of
medium Ca²⁺ a

Condition	Fractional rate of K ⁺ (⁸⁶ Rb ⁺) efflux (x 100)
Control	4.2 ± 0.2
+ A23187 (10 μM)	55.0 ± 0.5 ^b
+ A23187 (10 μM) - Ca ²⁺ (+ 2 mM EGTA)	4.1 ± 0.4 ^{ns}

a Data are the mean ± SD of 3 observations of a single representative experiment. Significance of difference from control data tested by Student's t-test; ns = not significant.

b p < 0.001

Table 6.2 Effect of a 15 minute incubation in the presence of A23187 on the cell number, cell volume and Na⁺, K⁺ contents of MDCK^a

Condition	Cell Number	Cell Volume	Ion contents (nmol/10 ⁶ cells) Na ⁺	K ⁺
Control	1.26 ± 0.06	1754 ± 58	125.2 ± 10.8	404.1 ± 7.1
+ A23187 (10 μM)	1.55 ± 0.22 ^{ns}	1681 ± 61 ^{ns}	163.1 ± 36.0 ^{ns}	193.8 ± 41.0 ^d
+ A23187 (20 μM)	1.64 ± 0.19 ^b	1480 ± 86 ^c	170.1 ± 12.3 ^c	172.5 ± 16.4 ^d

a Data are the mean ± SD of 3 observations of a single representative experiment.

Significance of difference from control data tested by Student's t-test; ns = not significant.

b p < 0.05

c p < 0.01

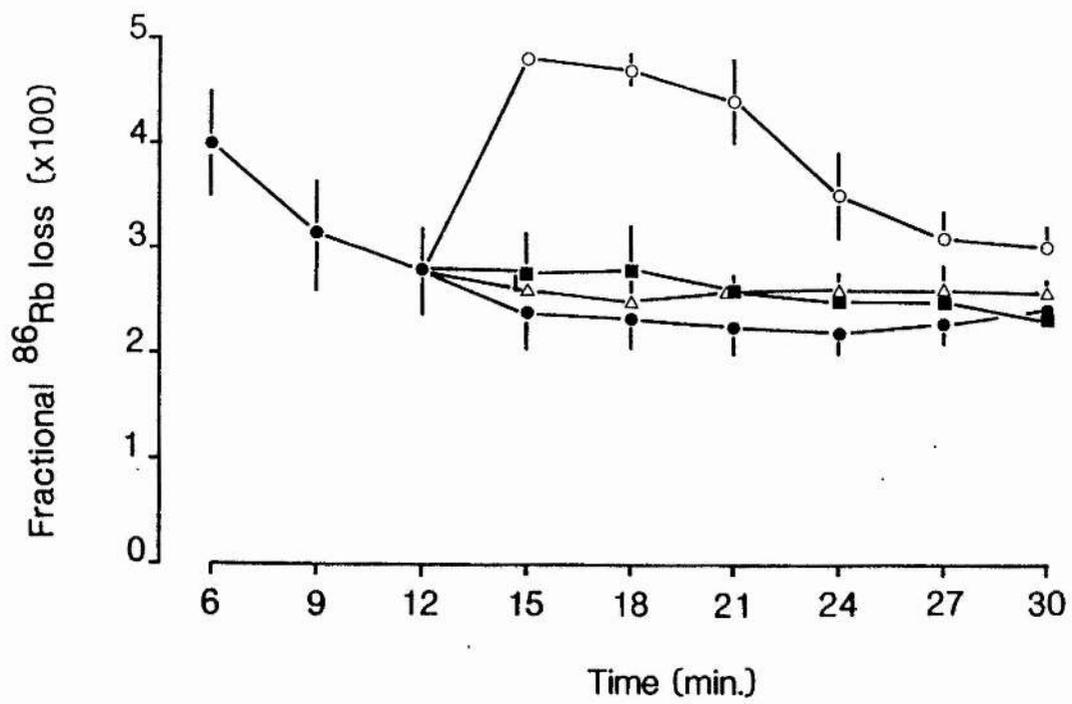
d p < 0.001

Figure 6.1

Inhibition by the carbocyanine dye DiS C₂(5) (10 μ M) of adrenalin (100 μ M) stimulation of Ca²⁺-activated K channels in sub-confluent monolayers of MDCK cells. Cells were incubated under control (■), adrenalin (○), DiS C₂(5) (●) and adrenalin plus DiS C₂(5) (Δ), all conditions pre-incubated in near 0 mM K media + DiS C₂(5). Data are the mean \pm S.D., n=3, of a single representative experiment.

Footnote:

In three experiments performed the apparent half maximal concentration for DiS C₂(5) inhibition of the adrenalin-stimulated Ca-dependent K efflux was $1.8 \pm 0.7 \mu$ M, mean \pm S.D..



However, to effect this inhibition, the DiS C₂(5) must be preincubated in near 0 mM K media prior to the application of 100 μM adrenalin. Simons (1979) demonstrated the action of DiS C₂(5) to be dependent upon the extracellular K concentration. An incubation in near zero K media produced a high affinity and near irreversible inhibition of the Ca-dependent K permeability of the human red cell ghost (Simons, 1979). This inhibition of the adrenalin-initiated K transport of MDCK cells was dose dependent, with an apparent half maximal inhibition (K_i) of 1.8 ± 0.7 μM (mean ± S.D. of three experiments, see footnote to the legend of figure 6.1). That this inhibition by DiS C₂(5) can be considered to be a blockage of the K channel, rather than due to an interaction with the adrenergic receptor is evident from the fact that inhibition of the ATP stimulation of the Ca-activated K efflux through a purine receptor (Brown, 1983) is also sensitive to inhibition by DiS C₂(5) (table 6.3). Therefore, in order to examine the effect of increased cellular Ca upon the "cotransport" flux, an attempt was made to inhibit the A23187-initiated, Ca-activated K efflux from MDCK cells by DiS C₂(5) (figure 6.2). Employing the same pre-incubation, 0 mM K media, protocol as above, DiS C₂(5) did not produce an inhibition of the Ca-activated K efflux in the MDCK cells (figure 6.2). The inclusion of the diuretic furosemide (0.1 mM) in the incubation media produced a significant decrease (P < 0.01) in the fractional rate of efflux at time 20 minutes (figure 6.2), but was without initial effect.

Due to the lack of inhibition by DiS C₂(5) on the A23187-initiated, Ca-activated K channel, the inhibitory action of Ba on the Ca-activated K channel was investigated, since it is known that Ba blocks Ca-activated K conductances in renal and epithelial tissue (Nagel, 1979; Biagi, Kubota, Sohtell and Giebisch, 1981; Matsumura,

Table 6.3 Inhibition of ATP (3 minute exposure) stimulated

K^+ (^{86}Rb) efflux from MDCK cells by $10 \mu\text{M DiS C}_2(5)^a$

Experiment	Control	+ATP	+ATP +DiS $\text{C}_2(5)$
1	6.90 ± 0.53	20.86 ± 0.45	$10.30 \pm 0.69^{(11)}$
2	5.30 ± 0.70	14.80 ± 1.83	$8.66 \pm 0.83^{(1)}$

a Data are mean \pm SD, n = 3.

b Significant decrease of ATP stimulated K^+ ($^{86}\text{Rb}^+$) efflux by DiS $\text{C}_2(5)$ tested by Student's t-test.

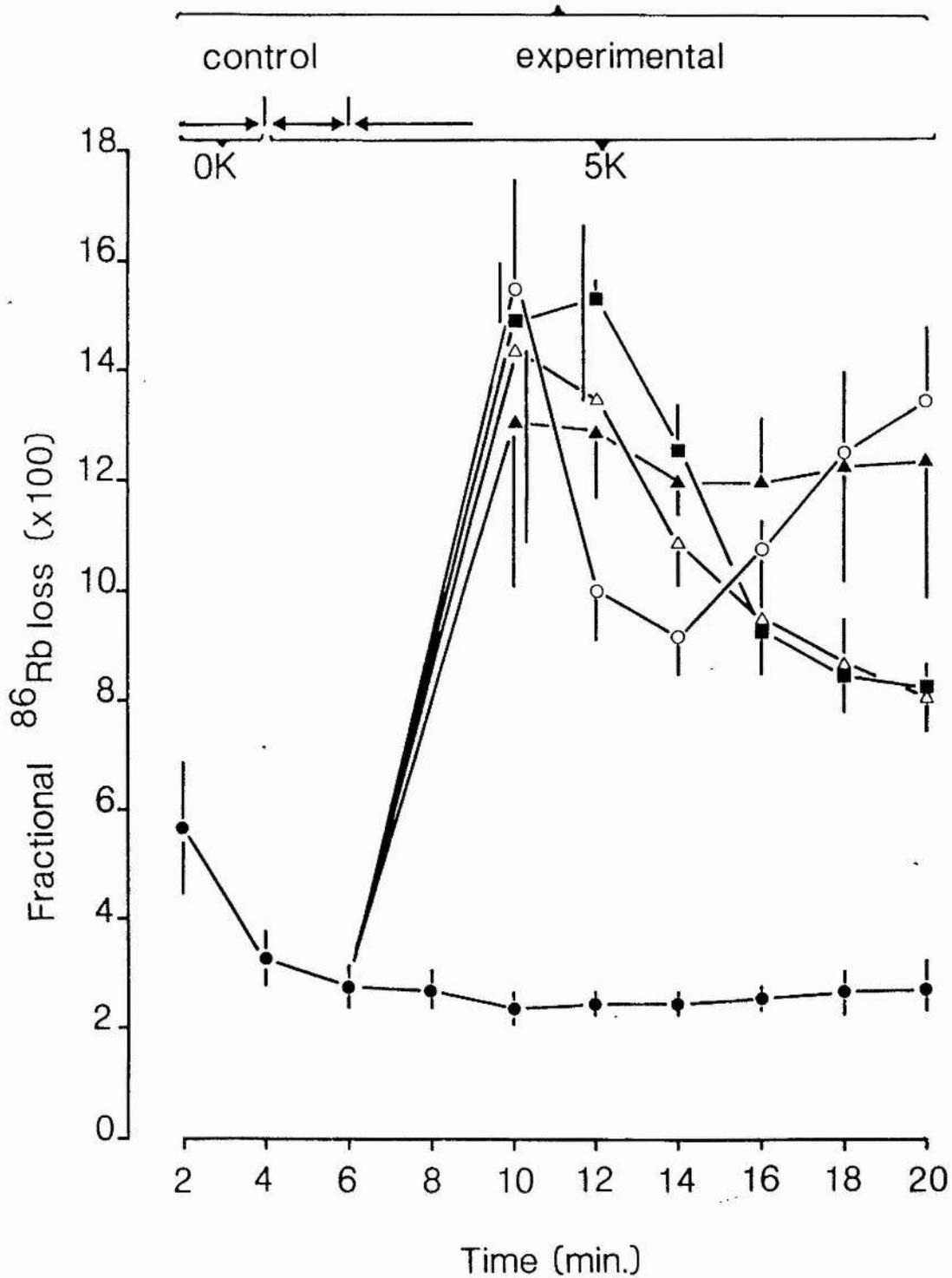
(1) p < 0.01

(11) p < 0.001

Figure 6.2

Stimulation of the Ca-activated K efflux by the Ca ionophore A23187, 10 μ M. Cells were incubated in control (●), A23187 \pm 0.1 mM furosemide (○,△), A23187 and DiS C₂(5) \pm 0.1 mM furosemide (▲,■), all conditions were pre-incubated in near 0 mM K media \pm 10 μ M DiS C₂(5). Data are the mean \pm S.D. of a single representative experiment.

\pm DiSC₂(5)



Cohen, Guggino and Giebisch, 1984). Adrenalin and ATP stimulation of the K efflux was inhibited in a dose-dependent manner by external application of Ba with an apparent half maximal inhibitory concentration (app. K_i) of 3.2 and 2.1 mM Ba respectively (figure 6.3 a-b). However, an initial dose-dependent inhibition of the A23187-stimulation of K efflux (with an app. K_i of 2 mM) was followed on continued exposure to A23187 by a progressive stimulation of the K efflux (figure 6.3 c) to levels seen in controls even in the presence of 4-10 mM Ba.

Taken together, these data suggest the existence of DiS $C_2(5)$ - and Ba- sensitive K channels and DiS $C_2(5)$ - and Ba- insensitive K channels activated by different intracellular Ca concentrations. The differences in the time courses of the activation of Ca- activated K transport by adrenalin, ATP and A23187, and the sensitivity of the A23187-initiated response to furosemide, further support this conclusion (table 6.4). These differences may be due to pharmacological effects of A23187 compared with the physiological increases in intracellular Ca (Flatman, 1982).

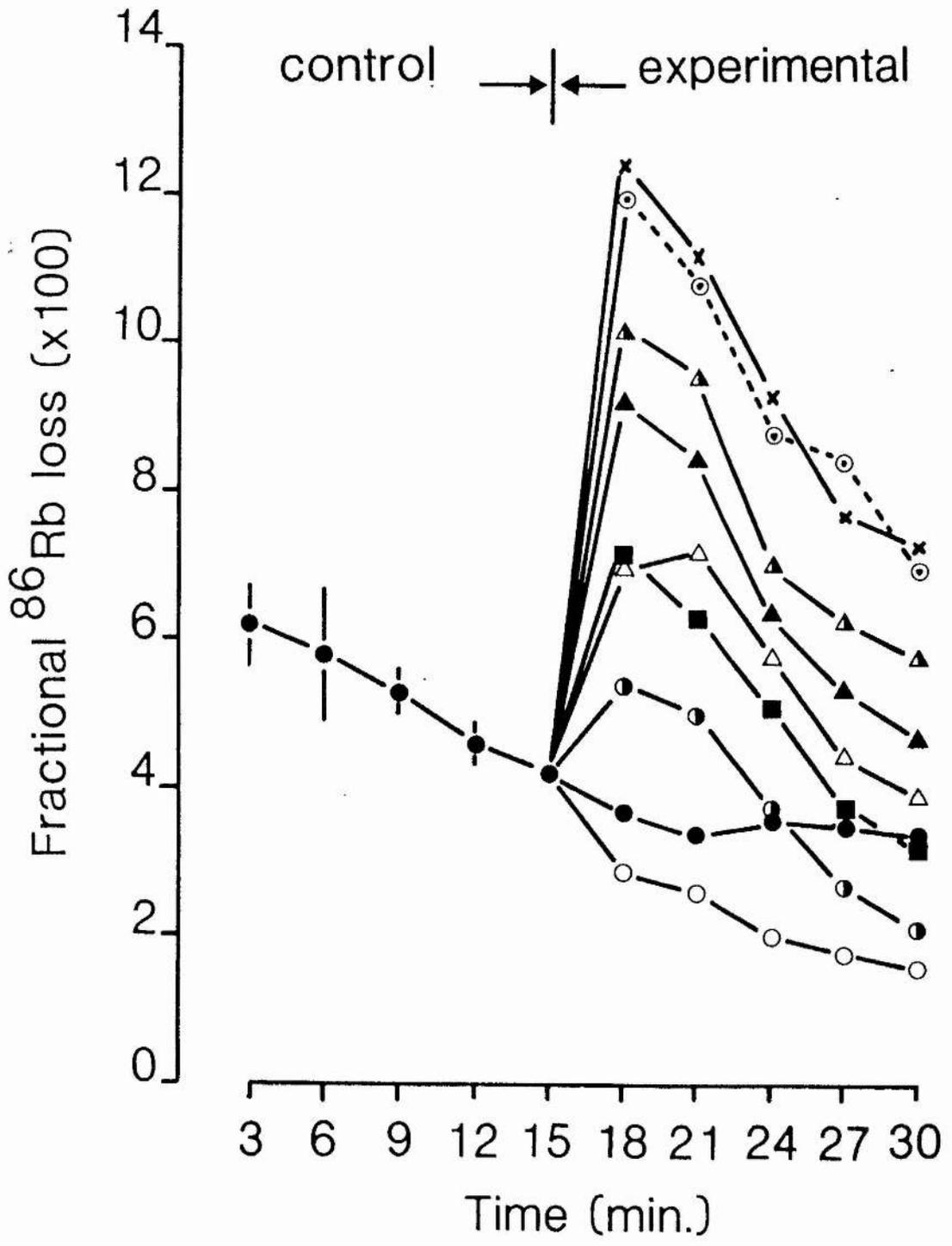
(iii)"Loop" diuretic effects in conditions of elevated intracellular Ca

Does the diuretic-sensitive component of the K efflux observed in figure 6.2 upon A23187-stimulation represent a stimulation by Ca of the Na K Cl "cotransport" pathway? Figure 6.4 shows that 0.1 mM furosemide inhibits a substantial part of the control K efflux, and that the component inhibited by "loop" diuretic (furosemide) in the presence of A23187 is considerably larger. By using the appropriate pharmacological criteria (i.e. that the Na K Cl "cotransport" system is inhibitable by three diuretics, bumetanide, piretanide and

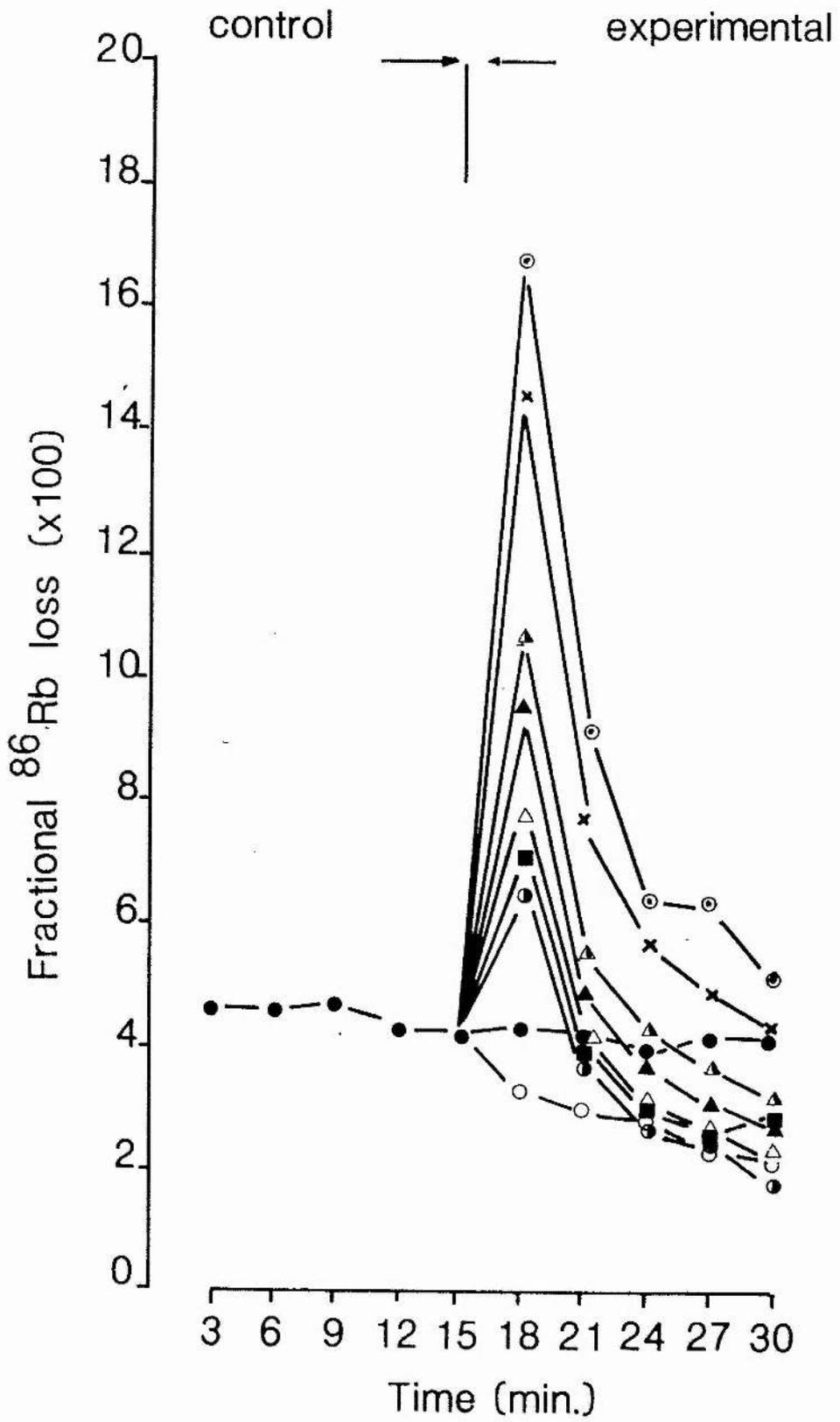
Figure 6.3 a-c.

Ba inhibition of the Ca-activated K efflux stimulated by (a) 100 μ M adrenalin, (b) ATP and (c) 10 μ M A23187. Cells were incubated under control conditions + 10 mM Ba (\bullet , \circ) and in experimental media containing 0, 0.1, 1.0, 2.0, 4.0, 6.0, and 10.0 mM Ba (\times , \odot , \blacktriangle , \triangle , \blacksquare , \square , \ominus). In all media SO_4^- salts were replaced with the appropriate Cl salt in order to prevent the precipitation of BaSO_4 . The apparent half maximal inhibitory concentrations for the Ba inhibition of Ca-activated K efflux, determined from the ratio of fractional efflux rates at 18 minutes/15 minutes were (a) adrenalin, 3.2 mM Ba, (b) ATP 2.1 mM Ba and (c) A23187 2.0 mM Ba. Data are the mean of three observations. Error bars are omitted for clarity but were within 10% of the mean for each datum.

A



B



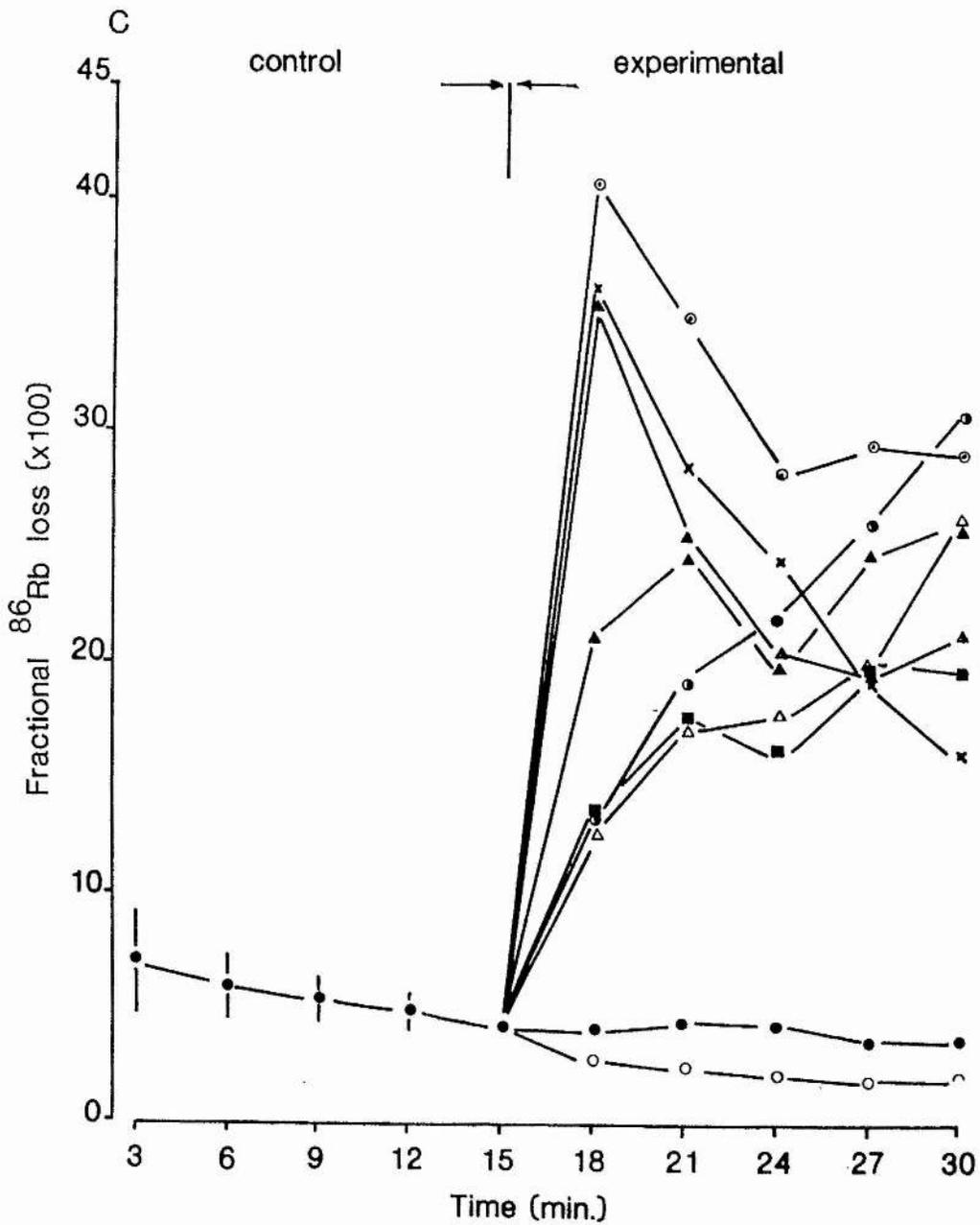
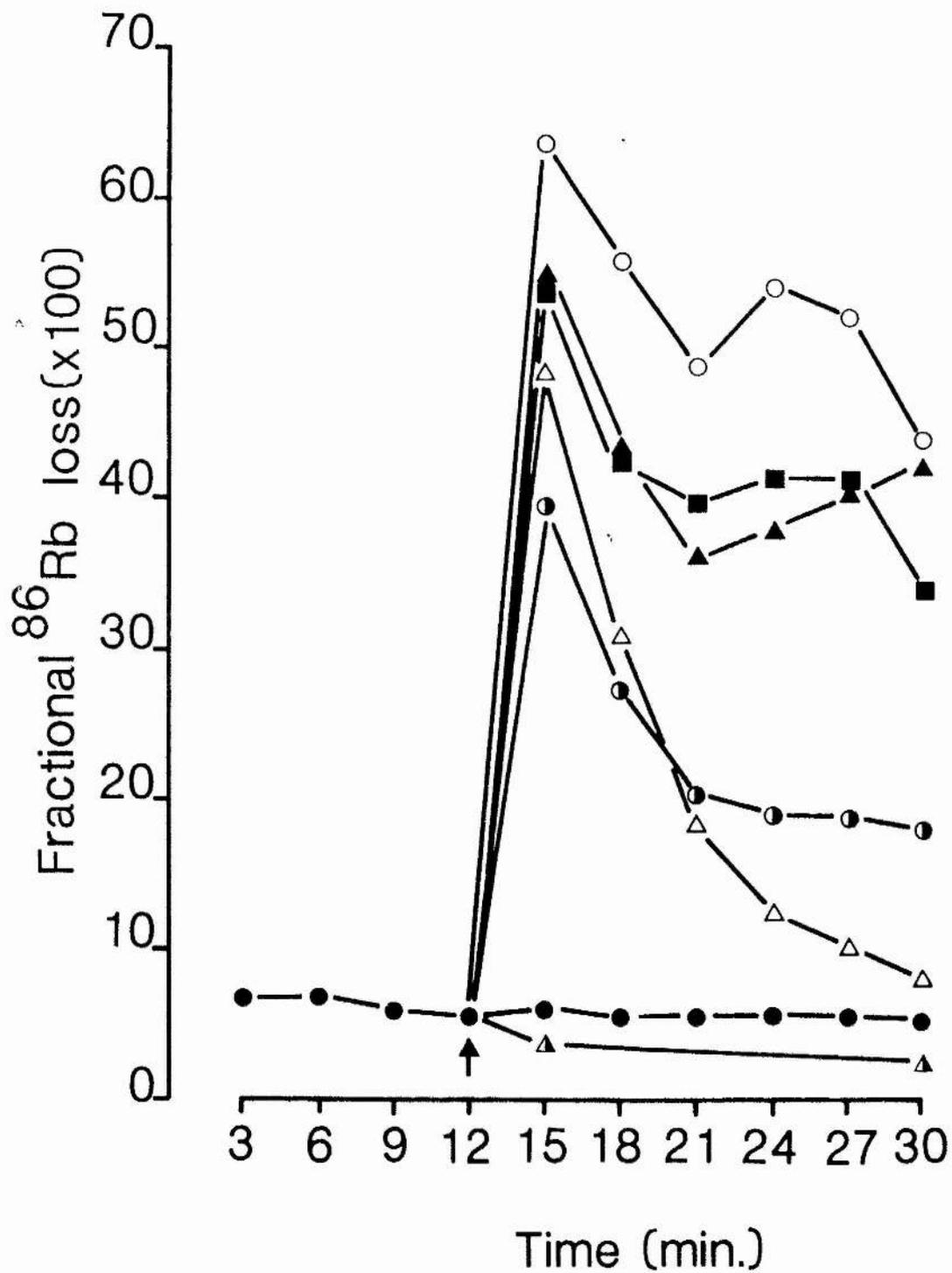


Table 6.4 Summary of data for Ca^{2+} -activated K channels initiated by ATP, adrenalin and A23187, possible existence of two types of channels^a

Initiator	DiS $\text{C}_2(5)$	Ba^{2+}	Diuretic Sensitivity	Time Course
ATP	+	+	-	
Adrenalin	+	+	-	
A23187	-	Transient	+	

a Diuretic sensitivity of ATP and adrenalin-initiated, Ca^{2+} -activated K^+ channels taken from Brown (1983).
A23187 Data, K^+ efflux sensitive only to furosemide.



furosemide, when these are present at 0.1 mM (see chapter 3) but is insensitive to the anion exchange inhibitor DIDS (Palfrey and Rao, 1983)), it is clear that inhibition by furosemide of the A23187-stimulated K efflux cannot be ascribed to the "cotransport" system because the increased efflux was insensitive to 0.1 mM bumetanide and inhibited by 10 μ M DIDS. Complete anion substitution of Cl by nitrate (figure 6.4) results in a maintenance of the A23187-stimulated K efflux; thus the inhibition by furosemide is not due to "cotransport" inhibition but perhaps to a reduction in anion conductance. These data also emphasise that caution must be exercised when ascribing diuretic effects to a single transport system. The high affinity derivative, bumetanide, is likely to show less non-specific actions.

Whether intracellular Ca directly modulates Na K Cl "cotransport" activity is unclear, due to the stimulation of other K transports which obscure the Na K Cl "cotransport" flux. Despite the difficulty in inhibiting the Ca-activated K efflux initiated by A23187, it was thought worthwhile to investigate further the effect of increased cellular Ca on the Na K Cl "cotransport" flux in the presence of Ba, and whether Ca modulates the hyperosmolar stimulation of the Na K Cl "cotransport" reported in chapter 5.

Does the progressive increase in the K efflux in the presence of Ba (4-10 mM), observed in figure 6.3 C, represent a modulation of the Na K Cl "cotransport" flux by Ca under isosmotic conditions?. Inclusion of 10 μ M A23187 had no significant effect upon the bumetanide-sensitive K efflux in isosmotic media, the fractional rate of efflux at time 30 minutes being 5.0 ± 0.4 and 2.6 ± 1.8 (mean \pm S.E., n=3) for control and A23187 conditions respectively (figure

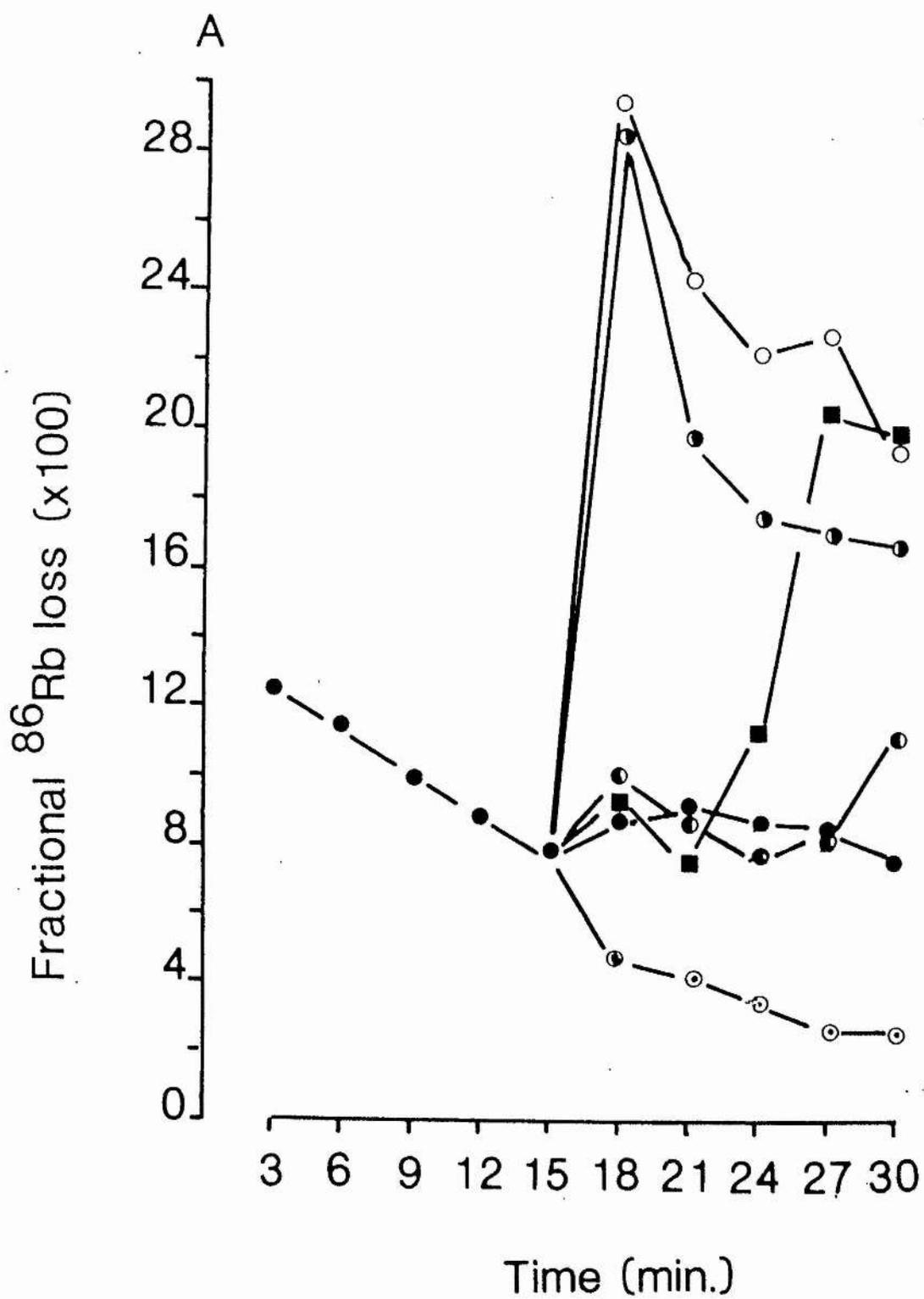
6.5a). This is in contrast to human red blood cells, where increased intracellular Ca significantly inhibited their bumetanide-sensitive Na and K efflux (Garay, 1982). In A23187- and 5 mM Ba-containing media (figure 6.5 a), the progressive increase in the K efflux was seen to be sensitive to 0.1 mM bumetanide. This inhibition at 30 minutes (8.3 ± 0.9 , mean \pm S.E., n=3) was significantly greater ($P < 0.001$) than the bumetanide inhibition of K efflux observed in control conditions (5.0 ± 0.4 , mean \pm S.E., n=3) at this time point.

Under hyperosmotic conditions (figure 6.5), the addition of the ionophore significantly potentiated the bumetanide-sensitive K efflux, with the fractional rate of efflux being increased from 10.6 ± 0.21 under control hyperosmotic conditions to 31.2 ± 4.2 in the presence of A23187 (at 30 minutes, mean \pm S.E., n=3), $P < 0.001$. In A23187- and 5mM Ba-containing media (figure 6.5 b), the progressive stimulation of K efflux in isosmotic conditions (figure 6.5 a) was not observed and the potentiation of the hyperosmolar stimulation of K efflux by A23187 was also abolished. However, prolonged incubation with A23187 and 5 mM Ba produced a progressive increase in the bumetanide-insensitive component (figure 6.5 b) and the bumetanide-sensitive component of the K efflux was markedly reduced ($P < 0.001$) at time 30 minutes, 2.3 ± 1.24 (mean \pm S.E., n=3) compared with control hyperosmolar conditions (10.6 ± 0.21 , mean \pm S.E., n=3).

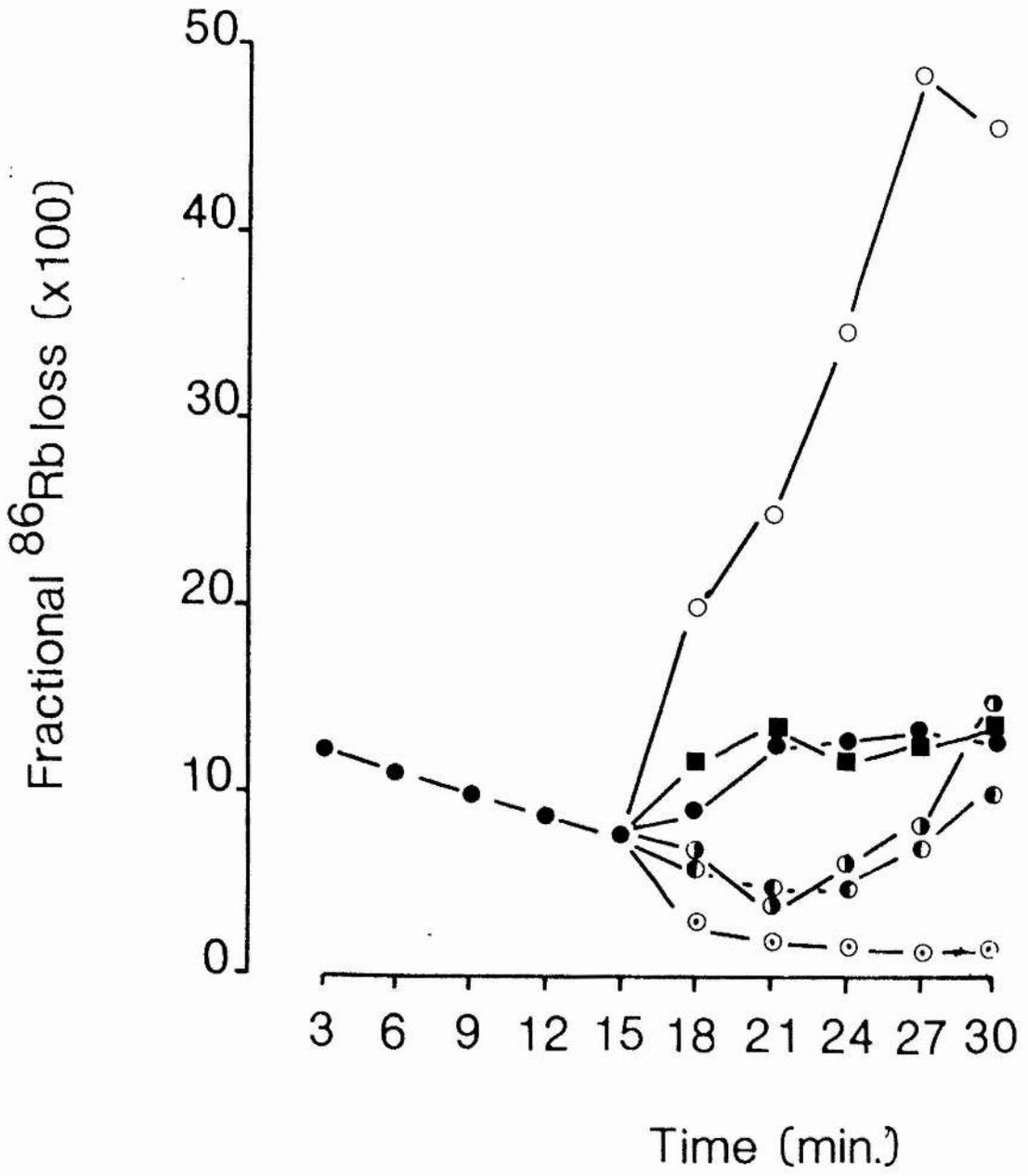
The various effects observed (figure 6.5 a, b) on the bumetanide-sensitive K efflux of MDCK cells in A23187 media in the presence or absence of 5 mM Ba under isosmotic and hyperosmotic conditions are complex and may reflect differences in intracellular ion concentration, pH, possible effects of membrane potential and changes in cell volume, all of which could be affected in this

Figure 6.5 a,b.

Effect of modulating intracellular Ca upon the K (^{86}Rb) efflux from MDCK cells under (a) isosmotic and (b) hyperosmotic (200 mM mannitol addition) conditions. Cells were exposed to control media \pm 0.1 mM bumetanide (\bullet, \odot), 10 μM A23187-containing media \pm 0.1 bumetanide (\circ, \ominus) and 10 μM A23187 plus 5 mM Ba-containing media \pm 0.1 mM bumetanide (\blacksquare, \ominus). Data are the mean of three determinations. Error bars were omitted for clarity but S.D. were within 10% of the mean for each datum.



B



experimental protocol. The effect of increasing intracellular Ca on the bumetanide-inhibited K efflux by means of the ionophore A23187 (+ 5mM Ba) requires further investigation to be carried out into the nature of this efflux. The sensitivity to the pharmacological agents, furosemide, piretanide and DIDS, needs to be determined as well as the anion dependence in order to ascribe this efflux to the Na K Cl "cotransport" pathway unequivocally.

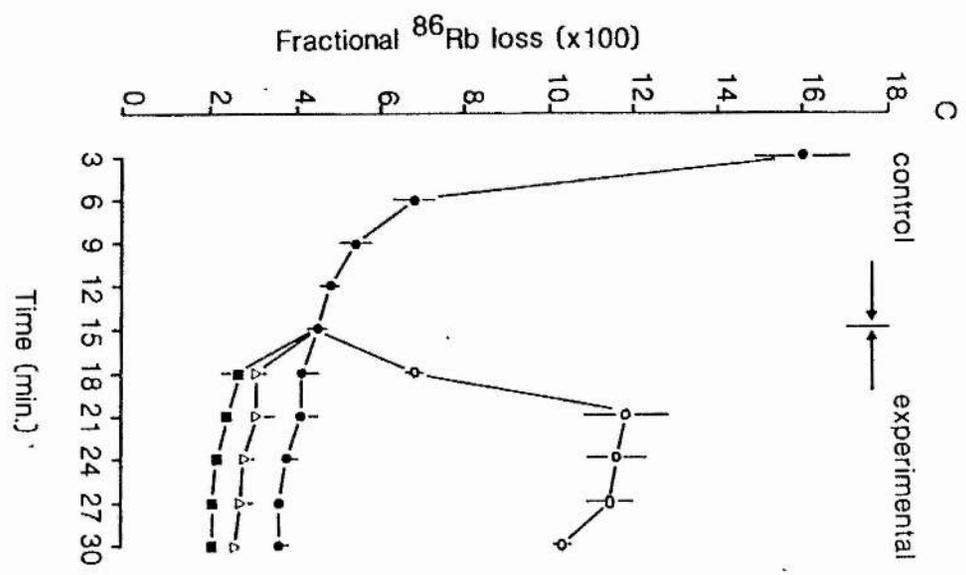
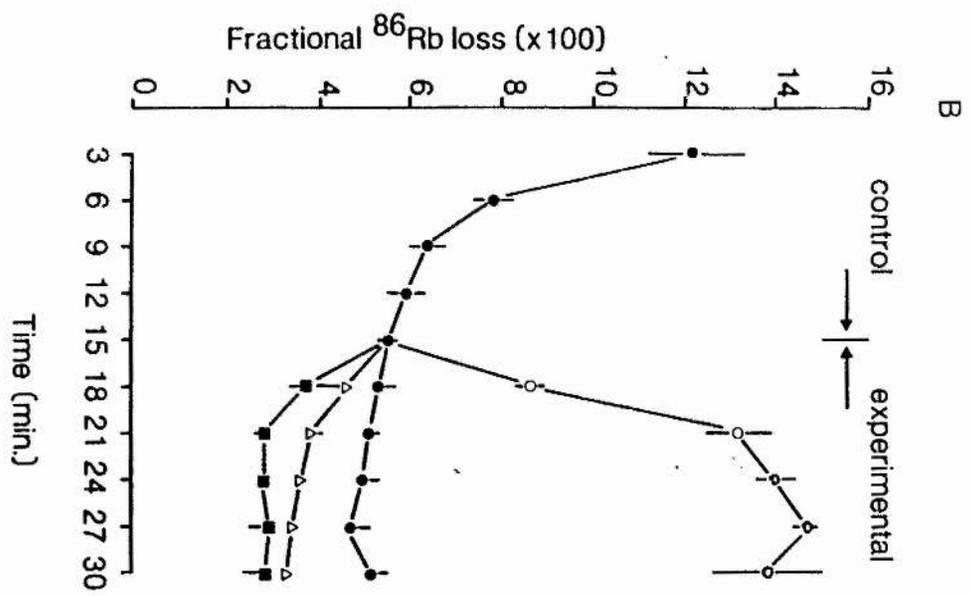
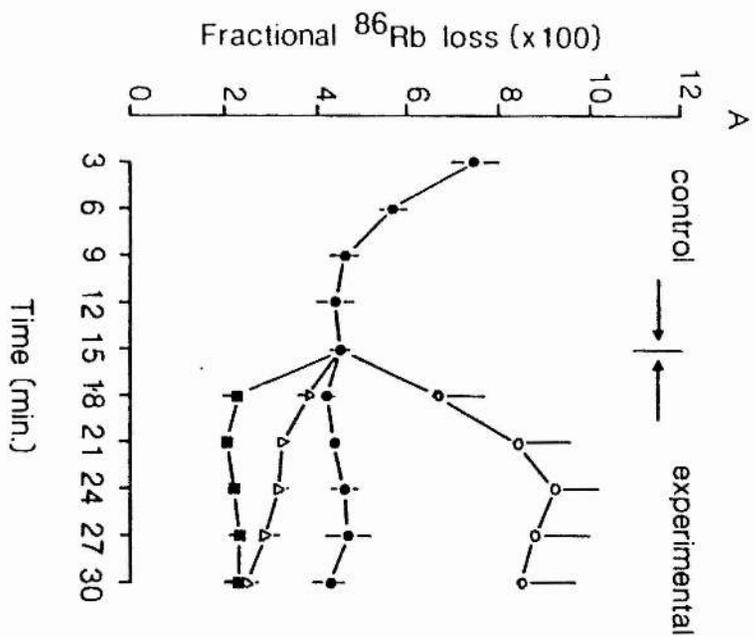
Effect of depleting extracellular and intracellular Ca.

The possible regulatory role of Ca upon Na K Cl "cotransport" and its involvement in the stimulation of Na K Cl "cotransport" in hypertonic media was also approached in Ca depletion experiments. The complication of Ca-activated K channels was obviated in these experiments. MDCK cells were exposed to isosmotic and hyperosmotic media, nominally free of Ca (plus 2mM EGTA), in the presence or absence of A23187 (figure 6.6 A-C). In the absence of external Ca but in the presence of A23187, no sustained, marked increase in the K (^{86}Rb) efflux was noted, thus demonstrating the Ca dependency of this response (see above). Similar to data already presented (figure 5.2), hypertonicity significantly stimulated the K (^{86}Rb) efflux compared with isosmotic conditions - 8.47 ± 0.70 and 4.33 ± 0.20 (at 30 minutes, mean \pm S.E., $n=3$; $P < 0.01$) respectively - and this K (^{86}Rb) efflux was inhibited by 0.1mM bumetanide in Ca-containing media (figure 6.6 A).

In nominally Ca-free Krebs solution (figure 6.6b), the fractional rate of K (^{86}Rb) efflux sensitive to diuretics in isosmotic media did not differ significantly from that observed in Ca-containing media - 1.97 ± 0.26 and 1.80 ± 0.26 (at time (t) 30 minutes, mean \pm

Figure 6.6 a-c.

Stimulation of K (^{86}Rb) efflux from MDCK cells by cell shrinkage (hyperosmolar exposure, 200 mM mannitol addition), in (a) Ca-containing media, (b) nominally Ca-free media and (c) nominally Ca-free media plus 10 μM A23187. Cells were incubated under isosmotic conditions \pm 0.1 mM piretanide (\bullet, Δ) and hyperosmotic conditions \pm 0.1 mM piretanide (\circ, \blacksquare). Data are the mean \pm S.D. n=3, of a single representative experiment.



S.E., n=3) respectively. Exposure to Ca-free media which had been rendered hyperosmotic significantly increased the fractional rate of K (^{86}Rb) efflux compared with isosmotic conditions - 13.80 ± 0.7 and 5.3 ± 0.2 (at time (t) 30 minutes, mean \pm S.E., n=3, $P < 0.001$) respectively. Similar to figure 6.6a, the hypertonic stimulation of the K (^{86}Rb) efflux in the absence of external Ca was inhibited by 0.1mM bumetanide. The magnitude of the stimulated K (^{86}Rb) efflux was markedly greater under nominally Ca-free conditions in comparison with that observed in Ca-containing media - 13.80 ± 0.70 and 8.47 ± 0.70 (at time (t) 30 minutes, mean \pm S.E., n=3, $P < 0.01$) respectively. This potentiation of the effect of cell shrinkage indicated a negative regulatory role for Ca but may also be due to the opening of the "tight" junction of the MDCK monolayers, which thereby increases access to the basolateral surface, since all components of K (^{86}Rb) influx into confluent monolayers of MDCK cells was significantly increased in nominally Ca-free media, whereas this treatment did not increase the K (^{86}Rb) influx in sub-confluent monolayers (table 6.5).

The effect of Ca depletion by incubation in Ca-free media in the presence of A23187, is seen in figure 6.6 c. MDCK cells responded to hyperosmotic conditions in a similar manner to cells under the incubation conditions of figures 6.6a and 6.6b. Under isosmotic conditions, the Ca depletion of the MDCK cells did not affect the diuretic-sensitive component of K (^{86}Rb) efflux significantly in comparison with the efflux observed in Ca-containing media - 0.87 ± 0.14 and 1.80 ± 0.26 (at time (t) 30 minutes, mean \pm S.E., n=3) respectively. Exposure to hyperosmotic media (Ca-free, $10\mu\text{M}$ A23187) markedly increased the rate of K (^{86}Rb) efflux compared with isosmotic conditions - 10.30 ± 0.15 and 3.5 ± 0.12 (at time (t) 30 minutes, mean \pm S.E., n=3, $P < 0.001$) respectively. Once again this stimulation was

Table 6.5 Effect of nominally free Ca^{2+} on K^+ ($^{86}\text{Rb}^+$) influx determined in sub-confluent and confluent monolayers of MDCK cells^a

$[\text{Ca}_0^{++}]$	Cell Number ($\times 10^5$)	Confluency	Total	K^+ ($^{86}\text{Rb}^+$) influx: nmol/ 10^6 cells.min		Residual
				Ouabain sensitive	Bumetanide sensitive	
2.5 mM	1.13 \pm 0.02	Confluent	7.71 \pm 0.14	2.66 \pm 0.17	4.57 \pm 0.09	0.47 \pm 0.06
<1 nM	1.13 \pm 0.02		15.16 \pm 1.29 ^d	7.34 \pm 1.30 ^c	7.04 \pm 0.39 ^e	0.78 \pm 0.03 ^e
2.5 mM	0.31 \pm 0.09	Sub-confluent	27.31 \pm 1.10	11.68 \pm 1.86	14.26 \pm 0.24	1.40 \pm 0.10
<1 nM	0.31 \pm 0.09		26.82 \pm 1.52 ^{ns}	13.71 \pm 1.58 ^{ns}	11.16 \pm 0.41 ^c	1.95 \pm 0.09 ^b

a Data are the mean \pm SE of 3 observations. Test for significant difference from Ca^{2+} media; ns = not significant.

b $p < 0.05$

c $p < 0.02$

d $p < 0.01$

e $p < 0.001$

inhibited by the inclusion of 0.1 mM bumetanide.

Therefore, these data show that the marked stimulation of diuretic-sensitive Na K Cl "cotransport" is not dependent upon external Ca (Ca influx) or upon the release of Ca from internal stores (Ca depletion + A23187). Under isosmotic conditions a diuretic-sensitive component of K efflux of comparable magnitude was observed in the three experimental conditions of figure 6.6 a-c. Thus there is no evidence of Ca-dependent inhibition of Na K Cl "cotransport" as reported by Garay (1982) and decreasing the external and internal Ca does not affect the MDCK cells' ability to increase their diuretic-sensitive K (^{86}Rb) efflux in response to hyperosmotic shrinkage (see also Simmons and Tivey, 1985).

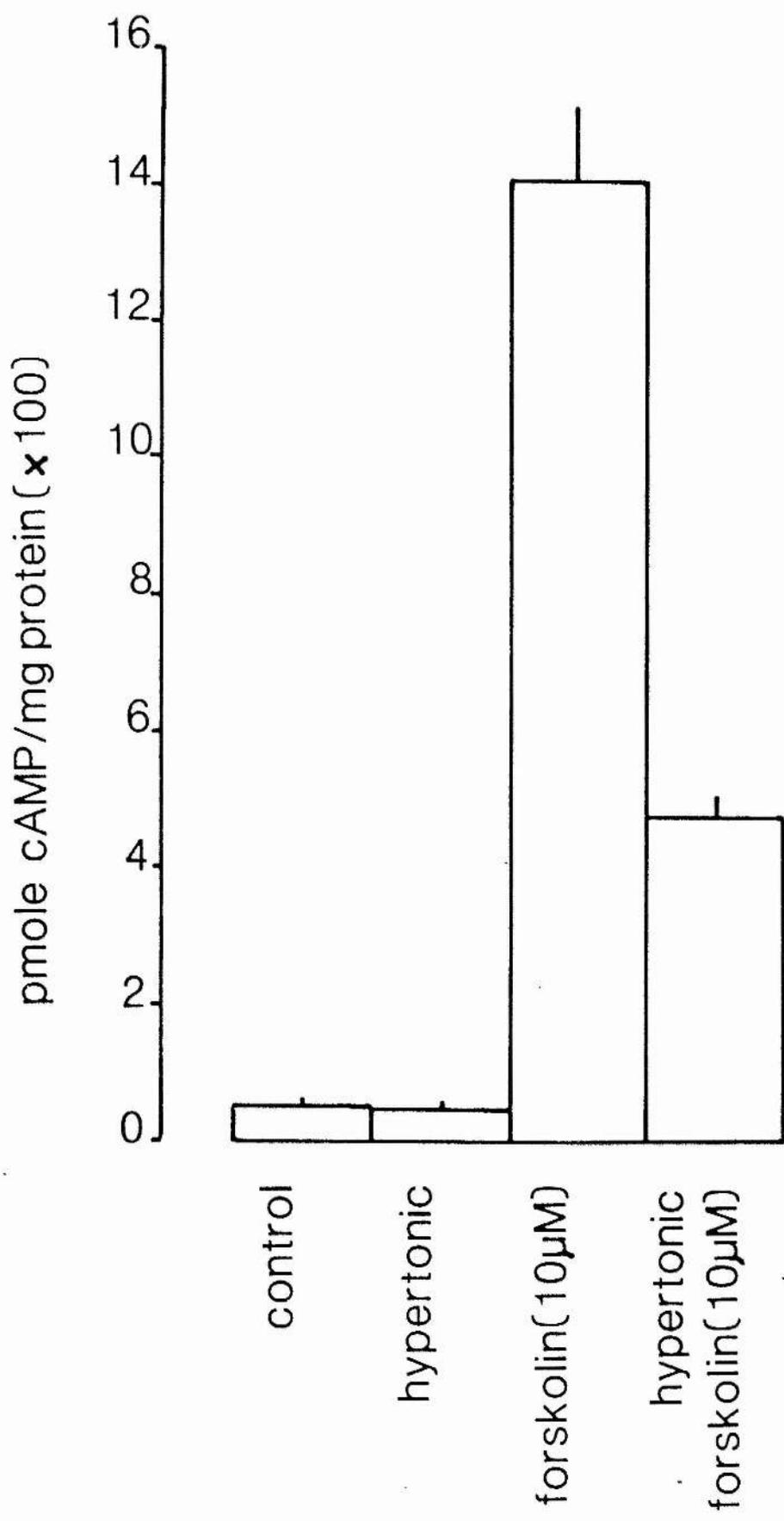
B) DIURETIC-SENSITIVE K TRANSPORT: REGULATORY ROLE OF INTRACELLULAR cAMP.

Effect of hypertonicity and (10 μM) forskolin on intracellular cyclic AMP levels.

The intracellular cyclic AMP content of MDCK cells was determined in cells incubated in isosmotic and hyperosmotic media in the presence or absence of 10 μM forskolin (figure 6.7). Forskolin is a general activator of the adenylate cyclase system at the catalytic subunit (Seaman and Daly, 1983). Inclusion of 10 μM forskolin in the isosmotic incubations produced a highly significant ($P < 0.001$) increase in the intracellular cAMP (pmole/mg protein) levels of the MDCK cells, confirming the previous work of Rugg and Simmons (1984). The stimulation by forskolin of the intracellular cAMP was investigated in shrunken cells and once again there was a highly

Figure 6.7.

Determination of intracellular cAMP in control and shrunken cells (hypertonicity, 200 mM mannitol addition) in the presence or absence of 10 μ M forskolin, after a 5 minute incubation. Data are the mean \pm S.D. of 5 determinations.



significant ($P < 0.001$) stimulation compared with both isosmotic and hyperosmotic controls. However, intracellular cAMP produced by means of the action of forskolin (pmole/mg protein) was markedly less than that produced by cells incubated in isotonic media ($P < 0.001$). This may be explained by either the decrease in cellular volume of cells exposed to hyperosmotic media which gives similar concentrations of the cAMP in both control and shrunken cells, or the increase in the rate of efflux of cAMP from the cells. The salient point is that the use of forskolin to increase the intracellular cAMP is appropriate for cells in isosmotic and hyperosmotic media. Hyperosmotic shock alone did not significantly affect the intracellular cAMP levels of the MDCK cells, a feature which has also been reported for duck erythrocytes (Kregenow, Robbie and Orloff, 1976).

Effect of cAMP upon K (^{86}Rb) flux.

A decrease in the cellular volume by hypertonic shock (510 mosmol/kg, mannitol addition) produced a stimulation of the K (^{86}Rb) efflux (figure 6.8A-B) from MDCK cells, which was inhibited by the inclusion of 0.1mM bumetanide in the incubation media, as previously discussed (chapter 5).

The presence of forskolin (10 μM) in isosmotic conditions did not markedly affect the fractional rate of K (^{86}Rb) efflux over a 15 minute incubation period in comparison with control conditions - 3.53 ± 0.07 and 3.07 ± 0.09 (mean \pm S.E. ($\times 100$), $n=3$) respectively (figure 6.8A). The effect of forskolin upon K (^{86}Rb) influx in isosmotic media in the MDCK cell line was investigated (figure 6.9), with no significant effects upon the total, ouabain-sensitive, diuretic-sensitive and residual components of the K influx by cAMP

Figure 6.8 a,b

Effect of 10 μ M forskolin upon the K (86 Rb) efflux from MDCK cells under (a) isosmotic or (b) hyperosmotic (200 mM mannitol addition) conditions. Cells were incubated in control media \pm 0.1 mM bumetanide (O, \blacksquare) and 10 μ M forskolin-containing media \pm 0.1 mM bumetanide (\bullet , \blacktriangle). Data are the mean \pm S.D., n=3, of a single representative experiment.

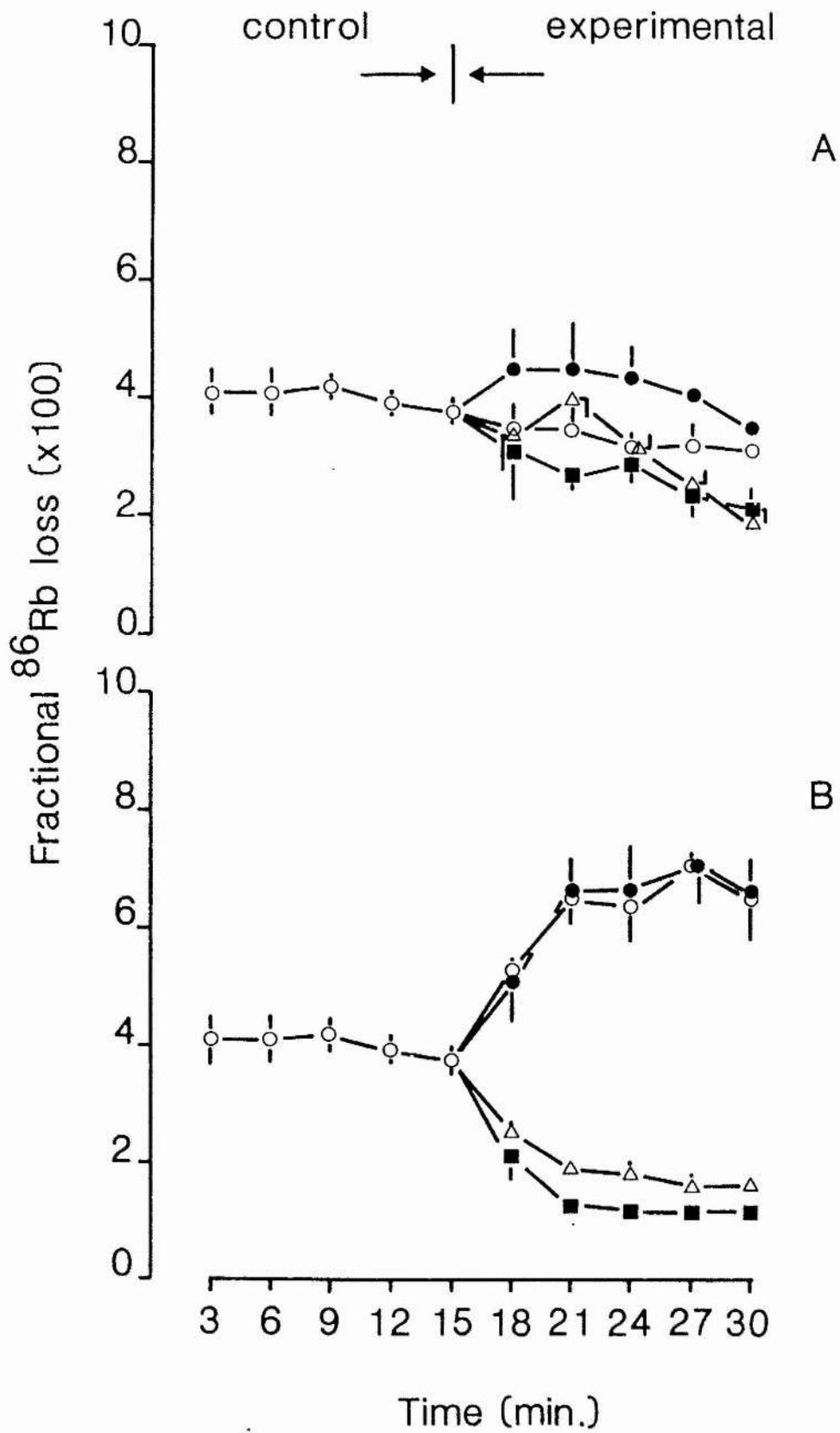
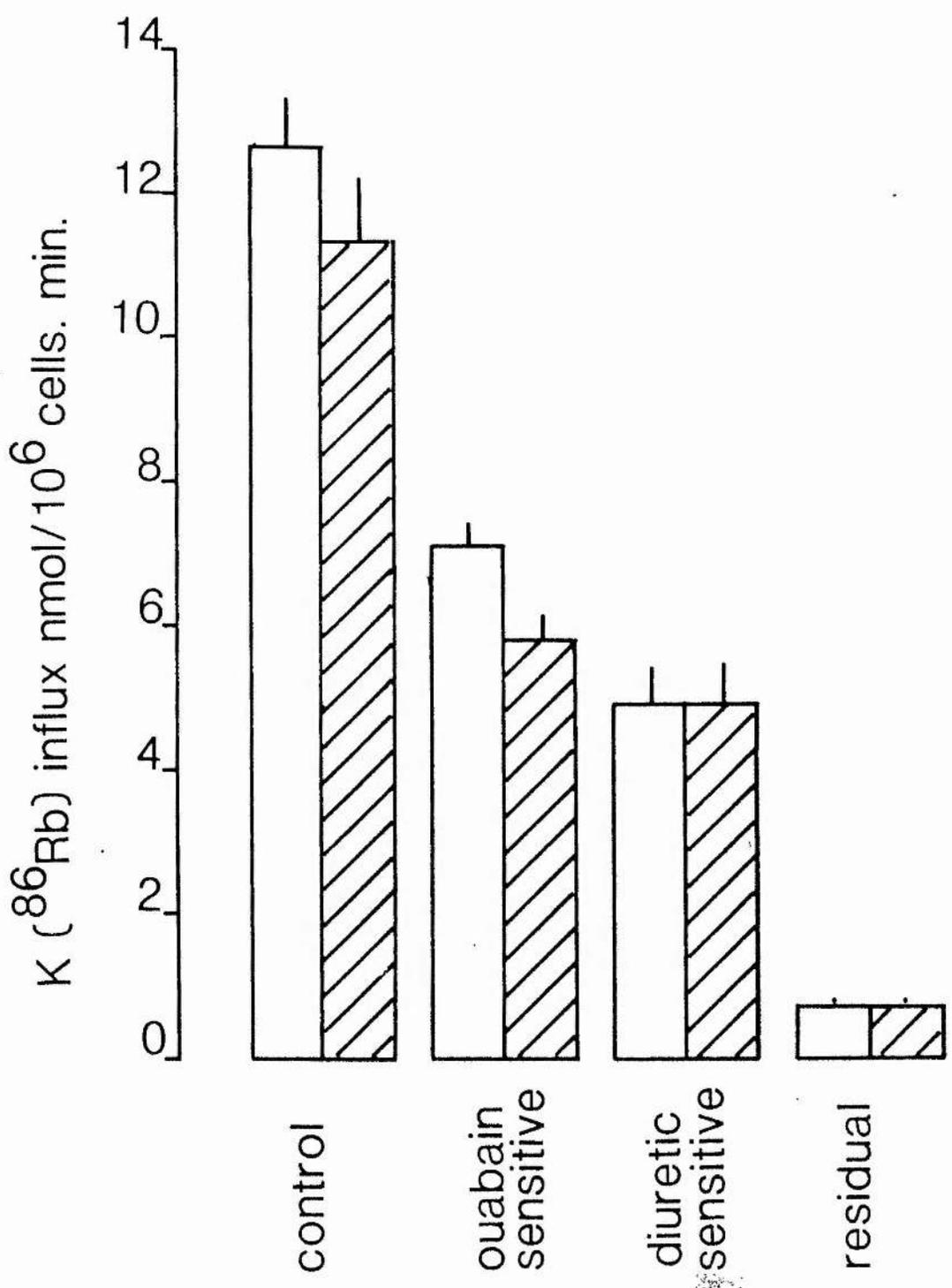


Figure 6.9.

Effect of 10 μ M forskolin (Stippled bars) upon the K (^{86}Rb) influx of MDCK cell determined over a 5 minute incubation as described in the methods. K (^{86}Rb) influx was characterised into ouabain-sensitive, diuretic-sensitive and residual components. Data are the mean \pm S.D. of 3 determinations.



being observed. These results are dissimilar to those obtained in erythrocytes of avian origin, in which the diuretic-sensitive K transport can be stimulated by increasing the intracellular cAMP via adrenoceptor activation of the adenylate cyclase system (Kregenow et al., 1976; Schmidt and McManus, 1977b; Palfrey et al., 1980; Ueberschar and Bakker-Grunwald, 1985), and those obtained in erythrocytes of human and ferret origin where increased cAMP causes a decrease in the Na K "cotransport" (Garay, 1982; Palfrey, 1984). The stimulation of the bumetanide-sensitive K (^{86}Rb) efflux by hypertonicity (figure 6.8b) was not modified by forskolin. After a 15 minute incubation period the bumetanide-sensitive K (^{86}Rb) effluxes for control and forskolin-treated cells were 5.27 ± 0.42 and 4.90 ± 0.36 (mean \pm S.E. ($\times 100$), $n=3$) respectively.

An increase in the intracellular cAMP may also be achieved by the inhibition of phosphodiesterase with 1 mM isobutylmethylxanthine (IBMX) (Rugg and Simmons, 1984). Incubation of MDCK cells for 15 minutes with 1mM IBMX had no significant effect upon total or diuretic-sensitive K (^{86}Rb) efflux in isosmotic conditions (table 6.6), thus confirming previous work of Brown and Simmons (1982).

Since forskolin is a general activator of adenylate cyclase, the action of forskolin on K (^{86}Rb) flux in the HeLa cell line in isosmotic media was also investigated (table 6.7). The total K (^{86}Rb) influx ($\text{nmol}/10^6$ cells min.) was not significantly affected by 10 μM forskolin - 5.34 ± 0.35 and 5.56 ± 0.28 (mean \pm S.E., $n=3$). The ouabain-sensitive, diuretic-sensitive and residual K (^{86}Rb) influx components were similarly unaffected by the application of forskolin to HeLa cells. Efflux of K (^{86}Rb) was also seen to be unaffected by forskolin after a 15 minute incubation period, with the fractional

Table 6.6 Effect of a 15 minute exposure to (100 μ M) IBMX on the fractional rate (x 100) of K^+ ($^{86}Rb^+$) efflux from MDCK cells under isosmotic conditions^a

Condition	Fractional rate of K^+ ($^{86}Rb^+$) efflux (x 100)	
	Control	+ IBMX
Total (1)	5.3 \pm 0.3	4.80 \pm 0.7 ^{ns}
+ Furosemide (2) (0.1 mM)	3.8 \pm 0.2	3.4 \pm 0.1 ^{ns}
Furosemide- sensitive (1-2)	1.5 \pm 0.2	1.4 \pm 0.4 ^{ns}

a Data are the mean \pm SD, n = 3. Significance of difference from control values tested by Student's t-test; ns = not significant

Table 6.7 Effect of a 15 minute exposure to 10 μ M forskolin on K^+ ($^{86}\text{Rb}^+$) influx and efflux determined in HeLa cells^a

Influx	nmol/ 10^6 cells.min	
	Control	+ 10 μ M forskolin
Total	5.34 \pm 0.61	5.56 \pm 0.49 ^{ns}
Ouabain-sensitive	3.36 \pm 0.40	3.91 \pm 0.32 ^{ns}
Diuretic-sensitive	1.65 \pm 0.19	1.30 \pm 0.21 ^{ns}
Residual	0.33 \pm 0.06	0.37 \pm 0.26 ^{ns}

Efflux	Fractional rate of efflux (x 100)	
	Control	+ 10 μ M forskolin
Total	3.40 \pm 0.29	2.80 \pm 0.35 ^{ns}
+ Diuretic	2.33 \pm 0.36	2.40 \pm 0.17 ^{ns}

a Data expressed as the mean \pm SD of 3 observations. Significant differences from control values tested by Student's t-test; ns = not significant.

rate of K efflux from control cells being 3.4 ± 0.10 and from cells plus forskolin 2.80 ± 0.12 (mean \pm S.E., n=3).

In the cultured cell lines MDCK and HeLa, an increase in intracellular cAMP does not stimulate a diuretic-sensitive K transport under isosmotic conditions, or modify the response of the MDCK cell to hyperosmotic media.

DISCUSSION.

Garay (1982) has demonstrated that an increase in intracellular Ca inhibits the Na K "cotransport" system of human erythrocytes. Intracellular Ca in these studies was manipulated by the Ca ionophore A23187 in 1 mM Ca media. However, this protocol mediates a Ca-dependent increase in K permeability (Gardos, 1958), which was circumvented by the use of the carbocyanine dye DiS C₂(5) (Simons, 1979).

Since the application of A23187 in Ca-containing media gives rise to a large increase in the K permeability in MDCK cells, with consequent loss of cell K, gain of Na and cell shrinkage, experiments were performed to see if DiS C₂(5) was an effective inhibitor of Ca-mediated increases in K permeability. Although DiS C₂(5) blocked the ATP- and adrenalin-initiated increases in K permeability, it had no effect on that stimulated by A23187. Thus another general K channel blocker, Ba, was tested, though giving blockade of ATP- and adrenalin-initiated increases in K permeability only a transient decrease of the A23187-initiated K permeability was observed. Despite the ambiguity associated with the use of DiS C₂ and Ba, it was still felt worthwhile to see whether the fraction of K efflux inhibited by "loop" diuretics was altered when intracellular Ca was modulated.

In the presence of A23187 alone in Ca-containing media the "loop" diuretic furosemide gave a significant decrease in the K efflux. This inhibition of K efflux is not due to inhibition of the "loop" diuretic-sensitive Na K Cl "cotransporter", since this inhibition by diuretic cannot be extended to bumetanide, and the

sensitivity to furosemide may be a non-specific effect of this diuretic (Brazy and Gunn, 1976). This, coupled with the flux being maintained by the Cl substitute NO_3^- and inhibited by the anion exchange inhibitor DIDS, is strong evidence for the Ca-activated K efflux elicited by A23187 being limited by the Cl permeability via the Cl/HCO_3^- anion exchanger (see review by Cala, 1983).

The response of the MDCK cells to A23187 and adrenalin may be due to activation of different Ca-activated K channels. The differential sensitivity to Ba and DiS C₂(5) and the different time courses support this. The A23187-stimulated increase in the membrane's permeability to K by the raised intracellular Ca is further set apart from the adrenalin response in its inhibition by the "loop" diuretic furosemide, since the adrenalin response was insensitive to furosemide (Brown and Simmons, 1982). To ascertain this, direct measures of the single channel conductance would be required (Petersen and Maruyama, 1984).

The effect of raised intracellular Ca on the diuretic-sensitive "cotransport" system of MDCK cells is dependent upon the inhibition of the Ca-activated K efflux. Where A23187 was applied without Ba, striking effects on K transport in hyperosmotic media were seen, both in the hypertonicity-stimulated, diuretic-sensitive K efflux and on the diuretic-insensitive component. The potentiation of the hyperosmotic activation of bumetanide-sensitive transport may be due to the action of Ca on the Na K 2Cl "cotransport" system. In the presence of Ba, the increase in the bumetanide-sensitive K efflux was observed in the isosmotic condition, this being in contrast to the report by Garay (1982). Whether these contrasting responses are a result of differing intracellular Ca concentrations is not known, and

ideally the Ca concentration should be measured by using the Quin 2 method (Crutch and Taylor, 1983). The nature of the bumetanide-sensitive K efflux also requires further characterisation in order to ascertain whether Ca has a regulatory role on the Na K 2Cl "cotransport" system of the MDCK cell. Removal of the external Ca or the depletion of the intracellular Ca did not stimulate the Na K 2Cl "cotransport" system in isosmotic conditions, nor was the response of MDCK cells to hypertonicity modified by the nominally Ca-free conditions (see also Simmons and Tivey, 1985).

Therefore, if Ca plays a regulatory role in the "cotransport" system of the MDCK cell, it is via increases in cytosolic Ca and not decreases in the Ca concentration. The mechanism of regulatory response is not known and was not addressed in the present work, but could conceivably be via a Ca-dependent protein kinase with the subsequent phosphorylation of a membrane protein (Alper et al., 1980 and 1980).

Similar to avian erythrocytes (Kregenow et al., 1976), exposure of MDCK cells to hyperosmotic media and the subsequent activation of the diuretic-sensitive "cotransport" system were not accompanied by an increase in the intracellular cAMP level, other than the increase due to the reduced cellular volume. This activation of the "cotransport" system by a decrease in the cell volume may be fitted to the model proposed by Geck and co-workers (1981) that the activity of the "cotransport" system is determined by the discrepancy of the real cell to reference volume in the cellular "memory". From the present data it would appear that the mechanism which recognises the change in cell volume is not cAMP. The hyperosmotic stimulation of the Na K 2Cl "cotransport" of MDCK cells was not modified by increases in the

intracellular cAMP brought about by the application of $10\mu\text{M}$ forskolin (see also Simmons and Tivey, 1985).

Elevating the intracellular cAMP by the external application of forskolin, IBMX (as in the present work) or adrenalin (Brown and Simmons, 1982) did not stimulate the diuretic-sensitive "cotransport" of MDCK cells under isosmotic conditions, contrasting with the catecholamine stimulation of Na K 2Cl "cotransport" in avian red blood cells (Schmidt and McManus, 1977 b-c; Haas et al., 1982; Ueberschar and Bakker-Grunwald, 1983). The catecholamine response is via increases in intracellular cAMP (Kregenow et al., 1976), this dependence being confirmed by the stimulation of "cotransport" by the external application of 8-Br-cAMP (Palfrey et al., 1980). However, in the erythrocytes of man and ferret, the action of cAMP is one of inhibition and not stimulation (Garay, 1982; Palfrey, 1984). Although avian, human and ferret erythrocytes possess (an) analogous diuretic-sensitive "cotransport" system(s) to MDCK cells, the response to increases in cellular cAMP is dependent upon the species from which the cell originates.

In the MDCK cell line, cAMP neither regulates the activity of the Na K 2Cl "cotransport" system in contrast to the erythrocytes of avian, human or ferret origin, nor does it modify the hyperosmotic activation. The role of Ca is more complex, with the removal of extracellular Ca or depletion of intracellular Ca having no effect upon the activity of "cotransport" under either isosmotic or hyperosmotic conditions. Increasing cellular Ca produced a complex response, with Ba-free media modifying the bumetanide-sensitive K efflux under hyperosmotic conditions, whereas in media containing Ba and under isosmotic conditions an activation of the

bumetanide-sensitive K efflux was observed. These differences may be the result of differing intracellular ion composition, intracellular pH, and changes in the cell volume. However, the bumetanide-sensitive K transport which is increased by raised Ca concentrations has not been unequivocally demonstrated to represent the Na K 2Cl "cotransport" system of the MDCK cell line.

CHAPTER 7

MEMBRANE POTENTIAL AND INTRACELLULAR pH DETERMINATIONS IN ISOSMOTIC
AND HYPEROSMOTIC CONDITIONS: EFFECT OF ANION SUBSTITUTION AND
DIURETICS.

INTRODUCTION.

MDCK and HeLa cells possess a "loop" diuretic-sensitive Na K 2Cl "cotransport" system (McRoberts et al., 1982; Rindler et al., 1982; Aiton et al., 1981, 1982). In general, this transport system is thought to be electroneutral, so that transport per se should not be affected by the membrane potential. However, intracellular ion activities are dependent upon the sum of the electrochemical gradients and the passive ion conductances present. Thus secondary effects upon the Na K 2Cl "cotransport" system are possible. Characterisation of this "cotransport" system in control (isosmotic) and shrunken (hyperosmotic) cells revealed marked differences in the anion activation of the diuretic-sensitive K influx depending upon the anion substitute used (chapters 3 and 5). Similar observations have been reported for other cell types, namely erythrocytes of human and ferret origin (Chipperfield, 1984; Palfrey, 1984). These variations between differing anion substitutes (penetrant versus non-penetrant) may be due to secondary effects of membrane potential and/or intracellular pH. The pH dependency of the "cotransport" has been determined in human red blood cells (Garay, Adragna, Canessa and Tosteson, 1981) and was shown to be bell-shaped with maximal activity at pH 7.0 and inhibition occurring above pH 8.3 and below pH 6.0.

In order to assess whether changes in the membrane potential and/or intracellular pH are important in anion activation and diuretic inhibition experiments, both parameters must be assessed. The size of the MDCK cells means that puncture by microelectrodes requires ultra-fine tips and therefore these measurements are not readily made. In this present work, the membrane potential probe (^{14}C -labelled

methyl triphenyl phosphonium iodide (TPMP)) and the pH probe (^{14}C -labelled 5,5-dimethyl oxazolidine-2,4-dione (DMO)) have been used. Data on the verification of the use of both the membrane potential probe, TPMP⁺, and pH probe, DMO, are also presented.

RESULTS.

A) VERIFICATION OF ^{14}C -METHYL TRIPHENYL PHOSPHONIUM IODIDE (TPMP^+) AS A MEMBRANE POTENTIAL (E_m) PROBE:

TPMP^+ accumulation.

The accumulation of TPMP^+ in MDCK cells in the presence or absence of $2\ \mu\text{M}$ tetraphenylboron (TPB^-), as shown in figure 7.1, was maximal at 30 minutes and remained constant for a further 90 minutes. Thus, in all subsequent studies with TPMP^+ (+ TPB^-), an incubation period of 30 minutes was employed. The maximal level of TPMP^+ accumulation was markedly increased by the inclusion of $2\ \mu\text{M}$ TPB^- in the incubation media. TPB^- is usually included as a counter ion to accelerate the rate of membrane penetration but should not affect the final accumulation ratio of TPMP^+ (Deutsch et al., 1979).

A qualitatively similar increase in the TPMP^+ accumulation ratio by TPB^- has been reported for human erythrocytes (Cheng et al., 1980), in which the difference was attributed to a change in membrane potential rather than to a specific ionophoretic effect of TPB^- . If TPMP^+ is to be used as a membrane potential probe, the increased TPMP^+ ratio affected by TPB^- should not be due to an increase in the passive K permeability, since the membrane potential is determined by the distribution and permeability of ions across the cell membrane (Hope, 1971). Therefore, the effect of TPMP^+ plus $2\ \mu\text{M}$ TPB^- upon ^{86}Rb fluxes and intracellular ion content of MDCK cells was investigated (figure 7.2 a-b and table 7.1). No significant effect of TPMP^+ + $2\ \mu\text{M}$ TPB^- upon the total, ouabain-sensitive, diuretic-sensitive and residual

Figure 7.1.

Time dependence of TPMP⁺ uptake (○) and in the presence of (●) 2 uM TPB⁻ in the MDCK cell line. The binding of TPMP⁺ to plates void of cells (▲) was minimal and independent of time. Data are the mean ± S.D., n=3, of a single representative experiment.

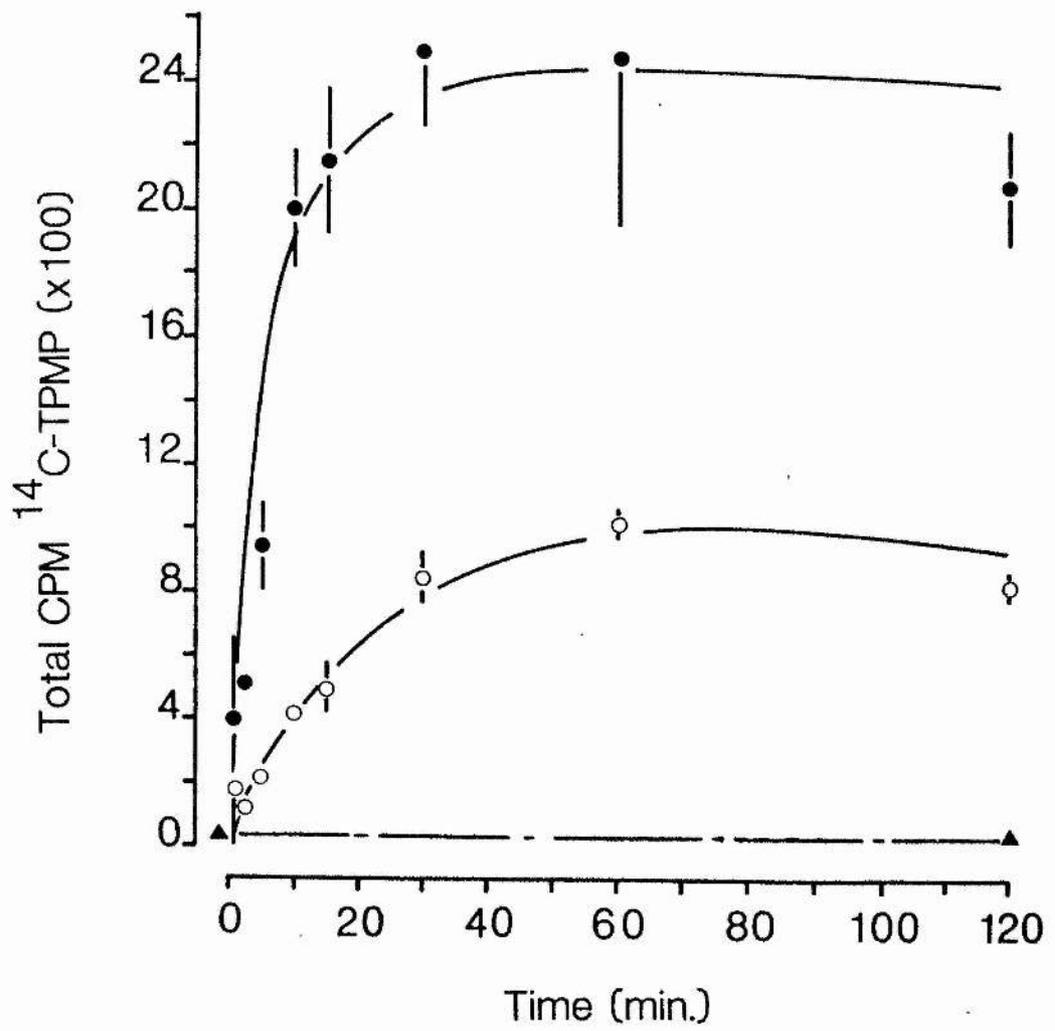
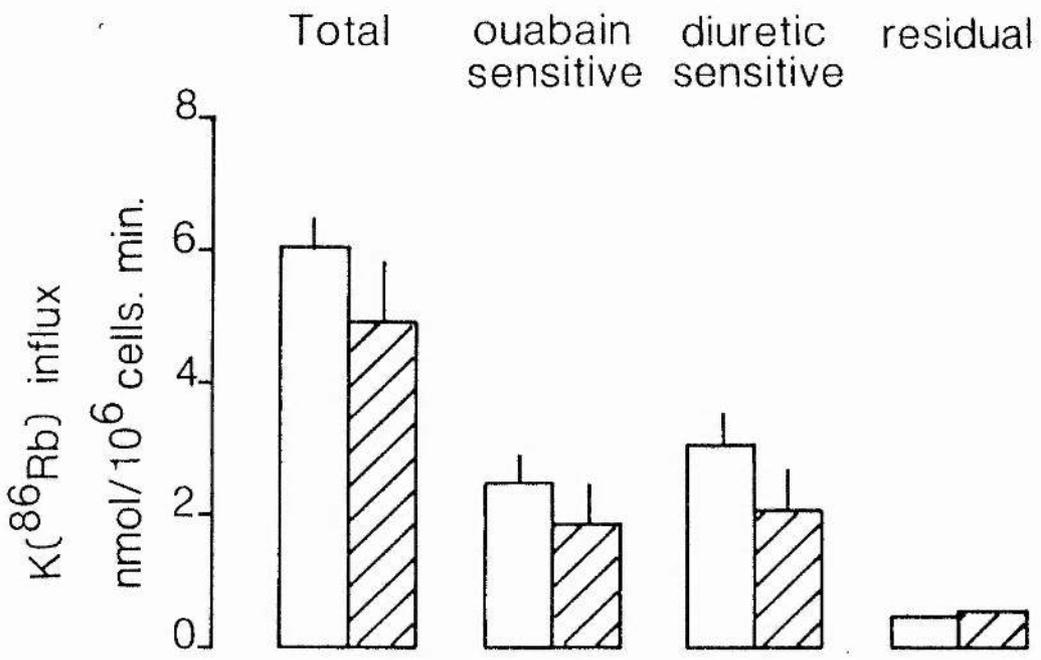


Figure 7.2 a, b.

The effect of TPMP⁺ and TPB⁻ upon the K (⁸⁶Rb) fluxes of MDCK cells. (a) Influxes were performed over a 5 minute incubation and separated into the ouabain-sensitive, diuretic-sensitive and residual components, non-stippled histograms represent control data. (b) K (⁸⁶Rb) efflux was determined in (●) control media and (○) plus TPMP⁺/TPB⁻. Data are the mean ± S.D. of three observations.

a



b

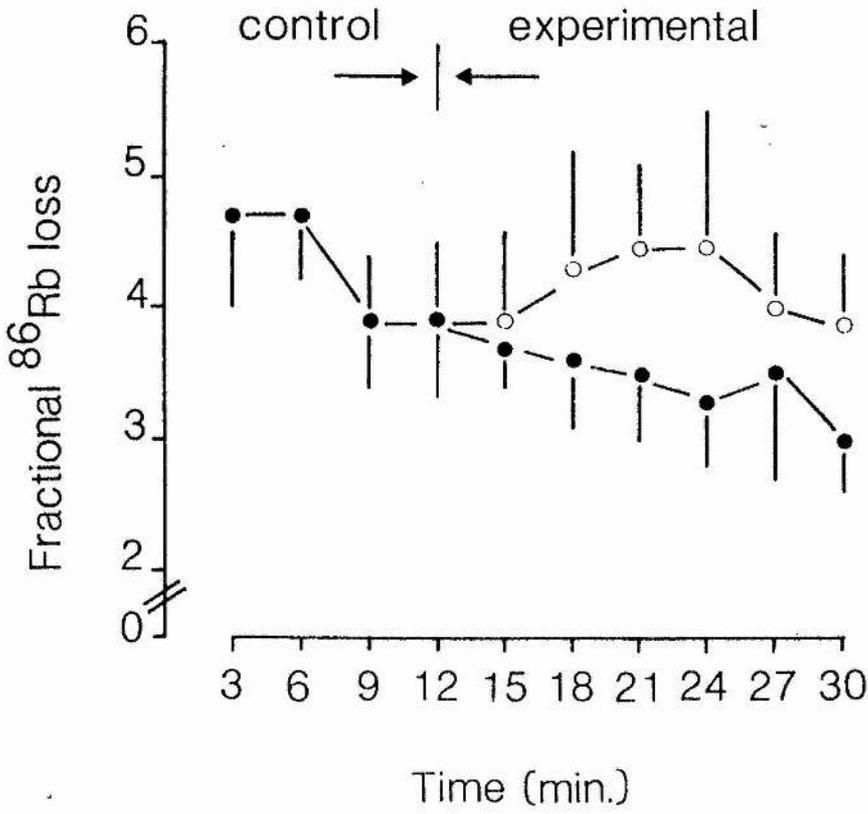


Table 7.1 Effect of TPMP⁺/TPB⁻ upon the cellular cation content (nmol/10⁶ cells) after 10 and 60 minutes incubations^a

Time	nmol/10 ⁶ cells	
	Na _i ⁺	K _i ⁺
Control	10.0 ± 3.8	249.0 ± 4.5
10 min		
TPMP ⁺ /TPB ⁻	8.2 ± 2.7 ^{ns}	245.0 ± 4.1 ^{ns}
Control	30.1 ± 16.6	235.0 ± 9.6
60 min		
TPMP ⁺ /TPB ⁻	13.7 ± 2.7 ^{ns}	264.0 ± 5.0 ^b

a Data are the mean ± SD of 3 observations. Significance of difference from control values tested by Student's t-test: ns = not significant.

b p < 0.01

components of the ^{86}Rb influx were observed (figure 7.2 a). and similarly, the ^{86}Rb efflux was unaffected by exposure to TPMP^+ plus $2\ \mu\text{M}$ TPB^- (figure 7.2 b). Over a 10 minute incubation, $\text{TPMP}^+ + 2\ \mu\text{M}$ TPB^- had no significant effect upon the cation content of MDCK cells, although, after a prolonged incubation (1 hour) a small but significant increase in intracellular K was observed (table 7.1). Thus, TPMP^+ plus TPB^- had no significant effect upon passive K permeability in MDCK cells.

Where additional intracellular membranous compartments exist in the MDCK cell, the significantly greater accumulation of TPMP^+ plus $2\ \mu\text{M}$ TPB^- over TPMP^+ as shown in table 7.2 (49.8 ± 0.6 and 9.47 ± 0.49 (mean \pm S.D., $n=3$, $P < 0.001$) respectively), may be due to either an additional accumulation by cytosolic organelles, i.e. mitochondria, which have a large membrane potential (Scott and Nicholls, 1980), or a reduction in the cytosolic activity coefficient of TPMP^+ by complexation with TPB^- , similar to the dibenzylidimethylammonium uptake by yeast cells (Hoeberichts and Borst-Pauwels, 1975). No evidence for or against the latter occurring in MDCK cells is presently available.

As described by Felber and Brand (1982 a), exposure of lymphocytes to valinomycin not only affects the plasma membrane K permeability, but also leads to a depolarisation of mitochondrial membrane potential, by setting their potential to the K diffusion potential between the cytosol and mitochondrial matrix. Inclusion of $10\ \mu\text{M}$ valinomycin in the incubation media should markedly decrease the accumulation of TPMP^+ in the presence of TPB^- if mitochondrial accumulation of TPMP^+ is present in MDCK cells. Whereas, a single action of valinomycin upon the plasma membrane should hyperpolarise

Table 7.2 The effect of 10 μ M valinomycin and elevated external K^+ upon the accumulation ratio of TPMP⁺ (intracellular [TPMP_i⁺]/extracellular [TPMP_o⁺]) and the depolarisation of membrane potential in 140 mM K^+ for MDCK cells in the presence or absence of TPB⁻ ^a

K^+ _o mM	Valinomycin	TPMP ⁺ accumulation ratio	
		TPMP ⁺	TPMP ⁺ /TPB ⁻
5.4	-	9.47 ± 0.49	49.8 ± 0.6
	+	9.97 ± 0.57	6.43 ± 0.48 ^b
140	-	4.39 ± 0.35 ^c	9.20 ± 0.16 ^c
	+	2.78 ± 0.22 ^{b,c}	1.93 ± 0.19 ^{b,c}
Change in membrane potential mV			
5.4-140	-	19.5 ± 4.7	44.5 ± 1.6 ^e
5.4-140	+	33.8 ± 3.0 ^d	32.2 ± 4.3

- a Data are the mean ± SD of 3 observations of a single representative experiment. Change in membrane potential calculated as described in text. Significance of difference tested by Student's t-test.
- b Significant effect of valinomycin; 0.01 < p < 0.001
- c Significant effect of 140 mM K; p < 0.001
- d Significant effect of valinomycin; 0.02 < p < 0.01
- e Significant effect of TPB⁻; p < 0.001

Footnote

The change in membrane potential (5.4-140 mM K^+ media) determined by TPMP⁺/TPB⁻ protocol was 51.8 ± 7.4 mV, mean ± SE of 5 experiments.

the membrane potential and result in increased TPMP⁺ accumulation, accumulation of TPMP⁺ in the presence of TPB⁻ was reduced from 49.8 ± 0.6 to 6.4 ± 0.5 (mean ± S.D., n=3, P < 0.001) in the presence of valinomycin, thereby indicating a mitochondrial accumulation of the TPMP⁺ in MDCK cells (table 7.2). Valinomycin was also effective at the plasma membrane, since the rate of ⁸⁶Rb accumulation was accelerated in the presence of 10 μM valinomycin (figure 7.3). The above is consistent with the hypothesis that the additional TPMP⁺ accumulation observed in the presence of TPB⁻ is due to a mitochondrial accumulation.

If the K⁺ permeability (pK) is greater than the Na⁺ permeability (pNa), then isosmotic replacement of NaCl by KCl should cause a depolarisation and therefore reduce the TPMP⁺ accumulation ratio. The accumulation of TPMP⁺, under the four experimental protocol conditions given in table 7.2, was decreased significantly upon exposure to 140 mM K⁺ media, thus indicating a depolarisation of the plasma membrane. Absolute values for the change in potential elicited by the elevated external K concentration may be derived from the Nernst equation:

$$E_m = -61.4 \cdot \log \text{TPMPi} / \text{TPMPo}$$

where TPMPi and TPMPo are the intracellular and extracellular concentrations respectively. The change in E_m will be given by:

$$E_{m_c} - E_{m_e} = (-61.4 \cdot \log(\text{TPMPi}/\text{TPMPo}))_c - (-61.4 \cdot (\log \text{TPMPi}/\text{TPMPo}))_e$$

where E_{m_c} and E_{m_e} are the potential differences for control and experimental conditions respectively. Therefore:

Figure 7.3

Time dependent ^{86}Rb accumulation by MDCK cells in the presence (O) or absence (●) of 10 μM valinomycin. Data are the mean \pm S.D. of three observations.

$$E_m_c - E_m_e = -61.4 \cdot \log \left(\frac{(TPMPi/TPMPo)_c}{(TPMPi/TPMPo)_e} \right)$$

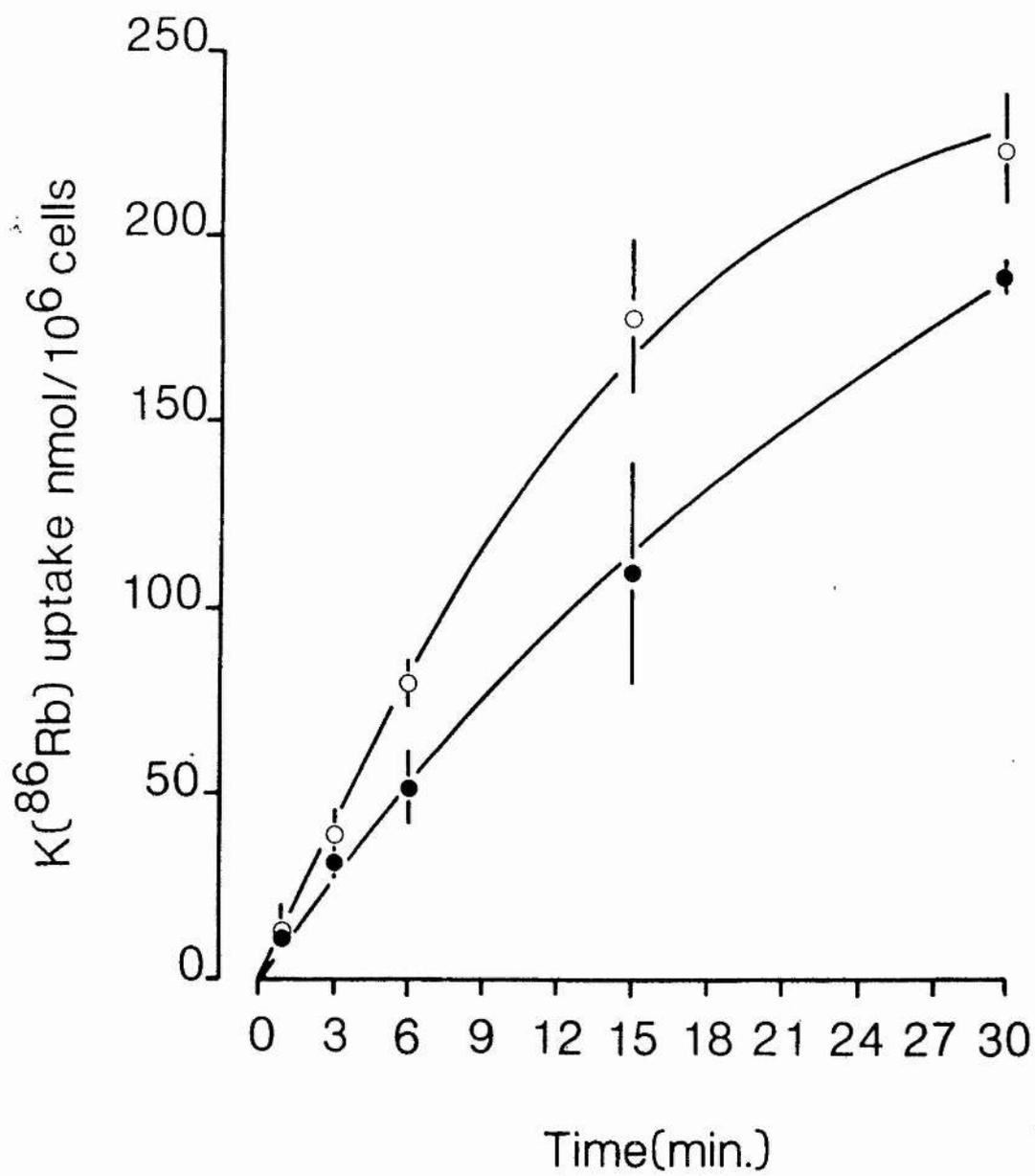
Assuming that extracellular TPMP⁺ remains constant,

$$E_m_c - E_m_e = -61.4 \cdot \log \left(\frac{(TPMPi)_c}{(TPMPi)_e} \right)$$

This latter equation has been used to calculate the potential differences given in table 7.2.

Under experimental conditions where no concentration gradients are present for Na and K, any accumulation is due to intracellular compartments, and the change in potential difference observed between 5.4 and 140 mM K media should therefore approximate the resting membrane potential. Clearly, from table 7.2, the change in potential measured by TPMP⁺ accumulation is dependent upon the protocol used. However, under conditions where the effects of mitochondrial accumulation are either saturated or abolished, i.e. TPMP⁺ + TPB⁻ or TPMP⁺ + TPB⁻ in the presence of valinomycin (see above), the potential measured by these protocols are in good agreement (44.5 ± 1.6 and 32.2 ± 4.3 / 33.8 ± 2.9 mV respectively). In five experiments where TPMP⁺ accumulation was measured in the presence of TPB⁻ but without valinomycin, a mean depolarisation by 140 mM K media of 51.8 ± 7.4 mV (S.E.) was observed (footnote table 7.2). This value is in good agreement with plasma membrane potentials of MDCK cells determined by microelectrode studies (-40.5 ± 15.0 mV (Stefani and Cereijido, 1983) and -20.8 mV (Paulmichl, Gstraunthaler and Lang, 1985)).

Although the absolute determination of change in potential difference is dependent upon the protocol used for determining the TPMP⁺ accumulation, variations in membrane potential, by K



depolarisation are reported in the presence or absence of TPB^- . Since the accumulation of TPMP^+ into mitochondria is variable in the absence of TPB^- (Scott and Nicholls, 1980), and higher accumulation ratios favour more accurate determination of radioactive counts within the cell, it was felt that measurements of changes in membrane potential should be made in the presence of TPB^- . Under these conditions mitochondrial accumulation of TPMP^+ is maximal (Felber and Brand, 1982 b).

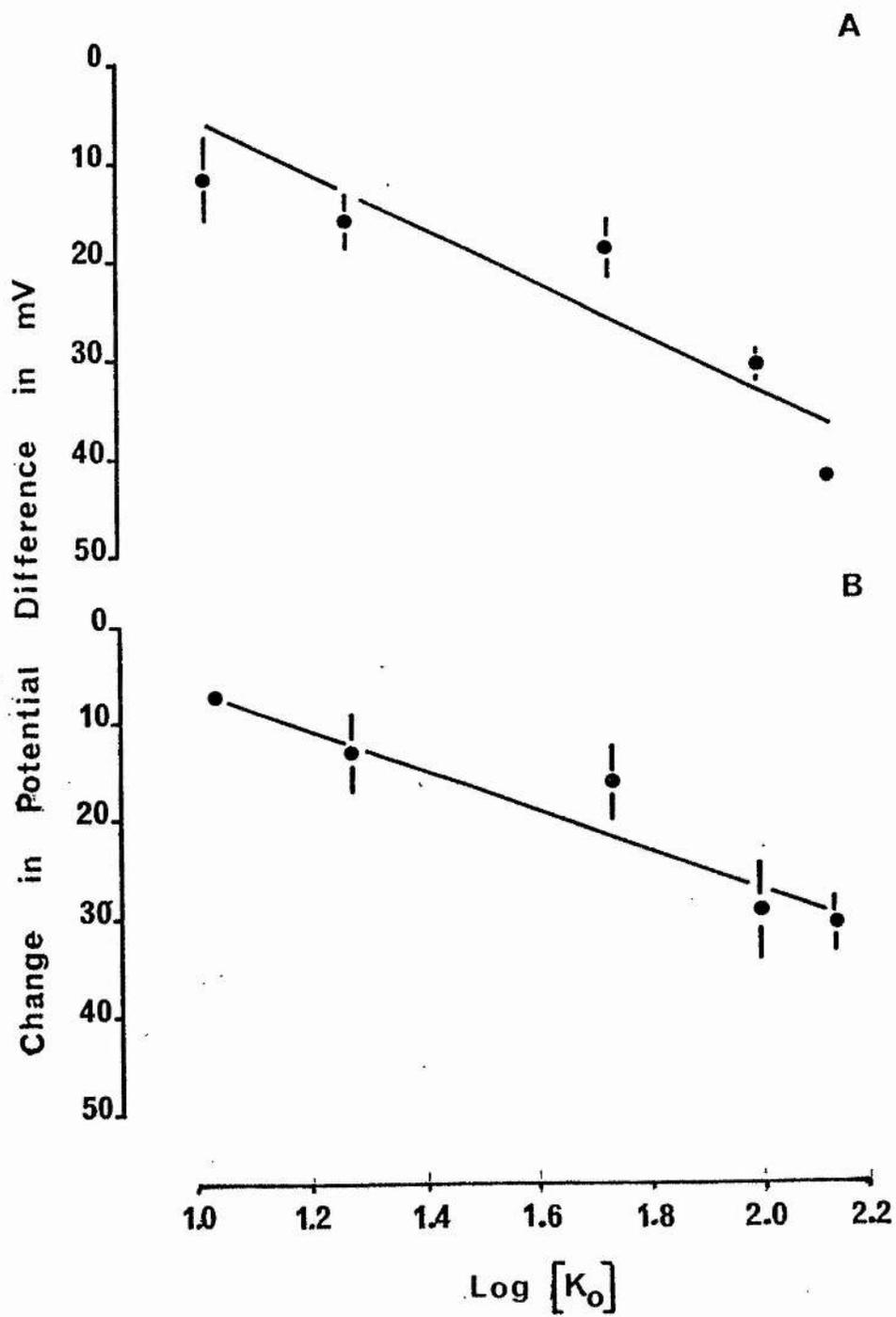
The dependence of TPMP^+ accumulation (membrane potential) upon extracellular K was further investigated using protocols of $\text{TPMP}^+/\text{TPB}^-$ in the absence or presence of 10 μM valinomycin (figure 7.4 a, b). Similar to data presented above, an increase in external K produced a significant depolarisation of the plasma membrane potential. Figure 7.4 a shows the isosmotic replacement of NaCl by KCl, the potential change induced is clearly not Nernstian with respect to K (slope of 29.0 ± 7.0 (S.E.)/decade change in extracellular K concentration). Thus MDCK cells do not represent a K electrode, with membrane potential set at the K diffusion potential, and these cells are likely to follow a Goldman relationship involving K, Na and probably Cl.

$$E_m = -61.41 \log \frac{pK \cdot K_i + pNa \cdot Na_i + pCl \cdot Cl_o}{pK \cdot K_o + pNa \cdot Na_o + pCl \cdot Cl_i}$$

where pK , pNa and pCl are the permeabilities of the three ions. The slope of the K depolarisation in figure 7.4a is in good agreement with that derived from data obtained by microelectrode studies (Paulmichl et al., 1985), which yielded a slope of 34 mV/decade. Similarly, Ehrlich ascites cells have a K dependence of membrane potential of 21

Figure 7.4 a, b.

Effect of external K concentration on the accumulation TPMP⁺ (+ TPB⁻) in the presence or absence of 10 uM valinomycin in MDCK cells. NaCl was isosmotically replaced by KCl and cells were washed (x5) in the appropriate K media prior to the determination of TPMP⁺ uptake over 30 minutes. (a) TPMP⁺/TPB⁻; potential changed 29.1 ± 7.8 mV/decade change in K concentration ($r = 0.91$, $P < 0.034$), (b) TPMP⁺/TPB⁻ in the presence of 10 uM valinomycin; potential changed 21.1 ± 3.5 mV/decade change in K concentration ($r = 0.961$, $P < 0.009$). Change in membrane potential calculated as described in text, using 5.4 mM K as the reference point (TPMP_{control}). Data are the mean \pm S.D., n=6, of a single representative experiment.



mV/decade (Hoffman, et al., 1979). Inclusion of 10 μM valinomycin (figure 7.4 b) had no effect upon the slope of the K depolarisation; if valinomycin had greatly increased pK over pNa, then the plasma membrane potential would approach the K diffusional potential. Therefore, either the increase in the pK is insufficient to greatly affect the membrane potential, or the concurrent depolarisation of mitochondrial membrane potential (see above) may have secondary effects upon the cellular ATP causing a decrease in the Na K pump and hence the dissipation of the K gradient, as reported for lymphocytes (Felber and Brand, 1982 a; Negendank and Shaller, 1982), or changes in intracellular pH affecting ion permeability particularly Na (Rindler and Saier, 1981) resulting in the pNa/pK ratio remaining constant.

B) TPMP ACCUMULATION UNDER VARIOUS EXPERIMENTAL CONDITIONS.

Does replacement of external Cl by SO_4^- , gluconate $^-$ or NO_3^- affect the TPMP $^+$ accumulation/ membrane potential in MDCK cells?

Table 7.3 shows data for TPMP $^+$ accumulation in MDCK cells under isosmotic conditions in the presence of differing external anions. Replacing extracellular Cl by SO_4^- produced no significant effect upon the TPMP $^+$ accumulation ratio. However, a decrease in TPMP $^+$ uptake was observed in NO_3^- media and to a greater extent in gluconate $^-$ media and this represented depolarisations of 4.8 ± 1.5 mV and 25.1 ± 2.0 mV respectively. These depolarisations are the result of lower membrane permeability of the replacement anion. Relative permeabilities for the anions in isosmotic media would be $\text{Cl} = \text{SO}_4^- < \text{NO}_3^- \ll \text{gluconate}^-$.

TPMP $^+$ accumulation in Cl and NO_3^- media was unaffected by cell

Table 7.3 Effect of anion substitution upon the TPMP⁺ (+TPB⁻) accumulation ratio determined in isosmotic media containing 5.4 mM K⁺ in MDCK cells. Change in membrane potential determined using the accumulation in Cl⁻ media as the reference point^a

Anion	TPMP ⁺ accumulation ratio	Change in membrane potential mV
Cl ⁻	75.5 ± 4.3 (6)	4.8 ± 1.5
NO ₃ ⁻	65.1 ± 3.7 (6) ^b	
Cl ⁻	66.8 ± 5.3 (6)	25.1 ± 2.0
Gluconate ⁻	27.7 ± 2.1 (6) ^c	
Cl ⁻	56.8 ± 1.3 (3)	-0.42 ± 0.4
SO ₄ ²⁻	56.8 ± 0.8 (3) ^{ns}	

a Data are the mean ± SD, number of observations in brackets (). Positive values for change in membrane potential indicate a depolarisation. Significant differences from TPMP⁺ accumulation ratio determined in Cl⁻ media tested by Student's t-test: ns = not significant.

b p < 0.01

c p < 0.001

shrinkage (table 7.4) indicating that there was no significant change in membrane potential, despite the increases in intracellular ion concentration in proportion to the ratio of media tonicities (see chapter 5). In cells containing (in mM) 174 K, 13.5 Na and 60 Cl and using a pNa/pK ratio of 0.125 (calculated from the K dependence) and a pCl/pK ratio of 0.375 reported by Hoffman et al., (1979) for the Ehrlich ascites cells, the calculated membrane potential would be -43.9 mV, and in shrunken cells with the commensurate increase in ion concentrations, the calculated membrane potential would be -47.1 mV. Both these values are within the range determined experimentally by TPMP⁺ accumulation under isosmotic conditions (see above). In gluconate⁻ media under isosmotic conditions a depolarisation of the membrane potential was observed (see above). Exposure of MDCK cells to hyperosmolar gluconate⁻ media produced a highly significant hyperpolarisation of -27.4 ± 0.5 mV compared with gluconate controls. This hyperpolarisation was equivalent to the depolarisation observed in control cells when media Cl was substituted by gluconate⁻. The effect of cell shrinkage must affect the anion permeability of the MDCK cells. Two possibilities arise either resulting in the same effect: first, cell shrinkage increases gluconate⁻ permeability to that of Cl and NO₃⁻; or secondly, the Cl permeability is reduced in shrunken cells. Thus in shrunken cells, the relative anion permeabilities would be Cl = NO₃⁻ = gluconate⁻.

Effect of "loop" diuretics on the membrane potential in control or shrunken MDCK cells.

No significant effect on the TPMP⁺ accumulation ratio was observed when 0.1 mM furosemide was included in the incubation media, which is similar to the tetraphenylphosphonium accumulation in Ehrlich

Table 7.4 Effect of hypertonicity upon the TPMP⁺ (+ TPB⁻) accumulation ratio determined in Cl⁻, NO₃⁻ and gluconate⁻ media of MDCK cells. Change in membrane potential calculated using the TPMP⁺ accumulation ratio in isosmotic media of appropriate anion as the reference point^a

Anion	Media tonicity	TPMP ⁺ accumulation ratio	Change in membrane potential mV
Cl ⁻ (mean of 3 experiments)	Isosmotic	76.0 ± 5.76	0
	Hyperosmotic	68.0 ± 8.66	3.7 ± 1.8
NO ₃ ⁻	Isosmotic	65.1 ± 1.5 (6)	0
	Hyperosmotic	62.3 ± 4.4 (6) ^{ns}	1.2 ± 0.6
Gluconate ⁻	Isosmotic	27.7 ± 0.8 (6)	0
	Hyperosmotic	77.5 ± 1.5 (6) ^b	-27.4 ± 0.5

a Data are the mean ± SE, number of observations given in brackets (). Positive values for change in membrane potential indicate a depolarisation. Significant differences compared with corresponding isosmotic values tested by Student's t-test: ns = not significant.

b p < 0.001

ascites cells and mouse J774.2 cells (Geck et al., 1980; Bourrit, Atlan, Fromer, Melmed and Lichtstein, 1985) (table 7.5). Thus, the inhibition of the diuretic-sensitive Na K 2Cl "cotransport" has no effect upon the membrane potential, which indicates that this transport system mediates no net transfer under these experimental conditions (see chapters 3 and 5) and that the system is electroneutral. However, to use TPMP⁺ accumulation data as the sole criteria for ascribing electroneutrality is erroneous, because the known electroneutral Na K 2Cl "cotransport" of TALH cells, when inhibited by "loop"-diuretic, would effect a membrane depolarisation. This is due to the "cotransport" pathway mediating net ion transfer and thereby generating ion gradients across the plasma membrane (Greger, Oberleithner, Schlatter, Cassola and Weidtke, 1983; Greger, Schlatter and Lang, 1983).

C) DETERMINATION OF INTRACELLULAR pH: 5,5-dimethyl (2-¹⁴C) oxazolidine-2,4-dione (DMO) distribution.

The use of DMO as an indirect measure of the H⁺ gradient has been employed in the human erythrocyte (Bromberg, Theodore, Robin and Jensen, 1965). However, caution should be exercised in accepting the DMO distribution as a true measure of intracellular pH (see the discussion of Funder and Wieth, 1966). Any discrepancies between the intracellular pH determined by DMO or other pH probes (e.g. Trimethylacetic acid and trimethylamine (Deutsch et al., 1979)) and the pH measured directly in lysed cells (Funder and Wieth, 1966) may be due to binding of the probe(s) to cellular membranes or proteins (Deutsch et al., 1979). For lymphocytes, the intracellular pH calculated from the DMO distribution is a linear function of the external pH, thus the pH probe DMO has the potential to detect changes

Table 7.5 The effect of inhibiting the Na K Cl 'cotransport' system of MDCK cells upon the TPMP⁺ (+ TPB⁻) accumulation ratio, and change in membrane potential, in isosmotic and hyperosmotic media. Change in membrane potential calculated using diuretic free media as the reference point^a

Media Tonicity	Diuretic 0.1 mM	TPMP ⁺ (+ TPB ⁻) accumulation ratio	Change in membrane potential (mV)
Isosmotic	-	86.7 ± 5.3	0.7 ± 2.3
	+	84.6 ± 7.1 ^{ns}	
Hyperosmotic	-	81.8 ± 7.1	0.6 ± 1.0
	+	80.0 ± 2.8 ^{ns}	

a Data are the mean ± SD of 6 determinations. Positive values for change in membrane potential indicate a depolarisation. Significant effect of diuretic (0.1 mM) tested by Student's t-test: ns = not significant

in the intracellular pH if not the absolute pH value (Deutsch et al., 1979).

Rindler and Saier (1981) have used DMO distributions to estimate the intracellular pH (pH_i) of MDCK cells. They addressed the possibility of an accumulation of DMO by mitochondria which are known to generate a pH gradient of their own (Mitchell, 1979). The inclusion of the mitochondrial uncoupler antimycin in the incubation media reduced DMO uptake at all external pH (pH_o) tested by 30%, however this reduction was not of qualitative importance to the results. Figure 7.5 confirms the previous reports that DMO uptake is sensitive to pH_o , the DMO uptake being greater in low pH media, and the calculated pH_i (see methods) varied as a linear function of pH_o in both control and shrunken cells, similar to lymphocytes (Deutsch et al., 1979).

Upon exposing MDCK cells to hyperosmolar media the pH_i varied linearly with increasing pH_o . However, a progressive intracellular alkalinisation was observed when pH_o increased from 6.0 to 8.3 compared to isosmotic conditions. These increased pH_i s were highly significant ($P < 0.001$) at pH_o s of 7.4 and 8.3, (figure 7.5 and table 7.6) compared to control cells. This data suggests the activation of Na:H exchange in shrunken cells producing an intracellular alkalinisation concurrent to increased Na uptake since increased intracellular H has been demonstrated to activate a Na:H antiport in this cell line (Rindler and Saier, 1981). It has been discussed previously that an increased Na uptake by furosemide-insensitive pathways should occur in shrunken cells (chapter 5). The ionic mechanisms of pH_i regulation are dependent upon the cell type. Snail neurones are reported to have

Figure 7.5.

Intracellular pH determined by (^{14}C) DMO distribution calculated as described in the methods; dependence on the extracellular pH in (●) isosmotic and (○) hyperosmotic media. Data are the mean \pm S.D. of 3 determinations.

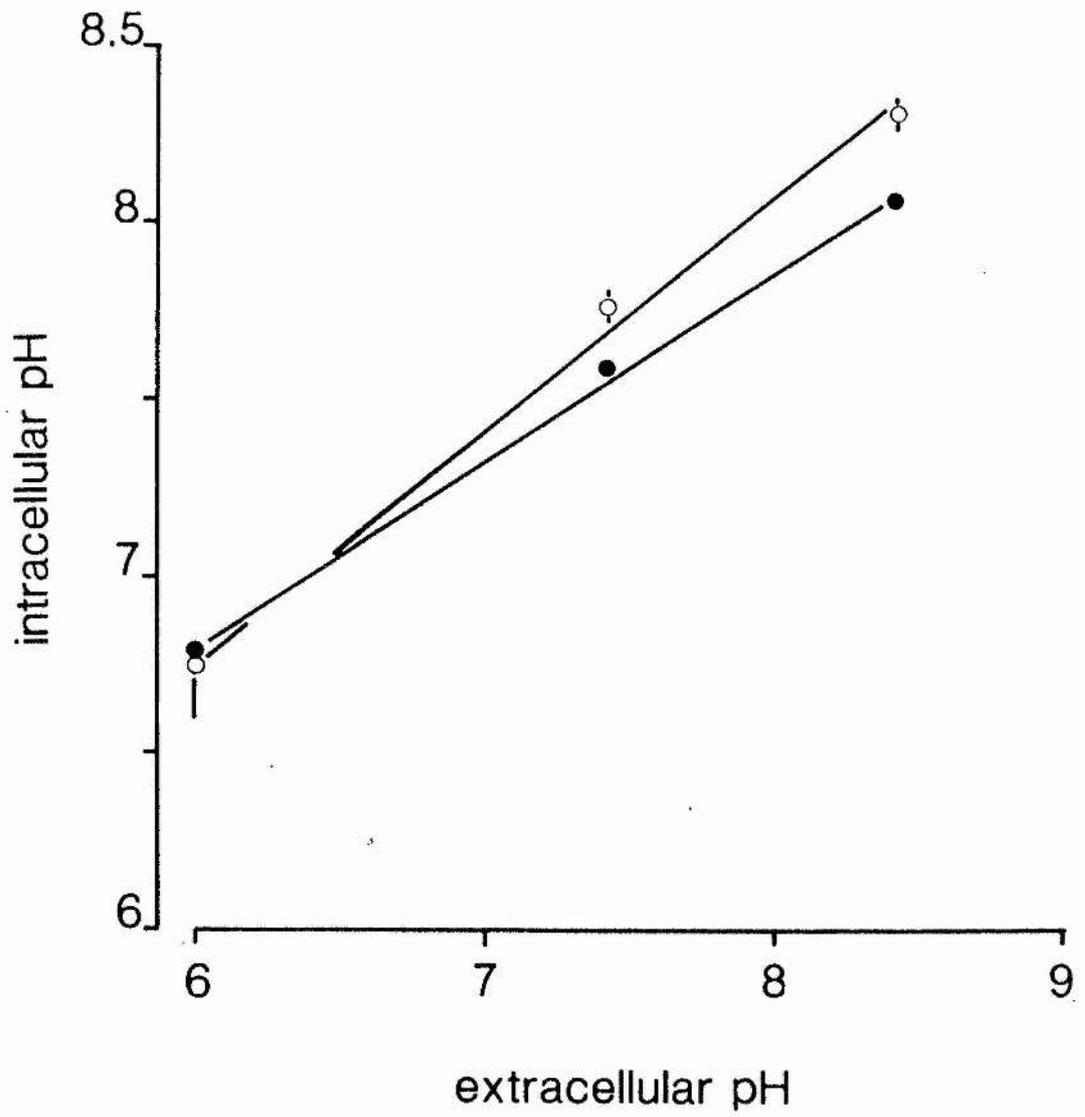


Table 7.6 Measurement of intracellular pH, using DMO distribution.
The effect of anion substitutions and hypertonicity upon
intracellular pH^a

Anion	Intracellular pH	
	Isosmotic	Hyperosmotic
Cl ⁻	7.59 ± 0.11 (9)	7.84 ± 0.18 (9) ^e
NO ₃ ⁻	7.55 ± 0.03 (6) ^b	7.92 ± 0.03 (6) ^{b,e}
Gluconate ⁻	8.10 ± 0.03 (6) ^c	8.12 ± 0.05 (6) ^{c,d}

a Data are the mean ± SD, number of observations given in brackets ().

Significant differences compared with Cl⁻ values

b Not significant

c p < 0.001

Significant differences compared with isosmotic condition in equivalent anions

d Not significant

e p < 0.001

closely linked Na:H and Cl:HCO₃, with the Na electrochemical gradient driving these ion movements (Thomas, 1977). Removal of external HCO₃ did not completely abolish the pH_i regulation, however, the residual regulation presumably being maintained by the dissolved CO₂ which is in equilibrium with the atmosphere and/or produced from cellular metabolism. Other cell types depend predominantly on a Na:H antiport, for example lymphocytes (Grinstein et al., 1985) and mouse skeletal muscle (Aickin and Thomas, 1977), although in the latter a small but separate involvement of an ATP driven Cl:HCO₃ was observed. A pH regulatory role of the Na:H antiport in Amphiuma red blood cells is redundant, due to the robust characteristics of these cells Cl:HCO₃ exchange (for references see Cala, 1983).

For the human erythrocyte, the distribution of Cl and H is a Donnan distribution (for references see Funder and Wieth, 1966). This concept was supported by the Cl and H distribution after the addition of a non-penetrant anion to the extracellular media. In these conditions the plasma membrane potential is inverted and there is an alkalinisation of the cytosol (Funder and Wieth, 1966). If the distribution of Cl and H ions in MDCK cells behaves like that in red blood cells, variation of the external Cl may be expected to affect pH_i. Thus, the DMO uptake by MDCK cells (pH 7.4 at 37° C) in Krebs solution with the major anion being either NO₃⁻ or gluconate⁻, was studied in isosmotic and hyperosmotic media (table 7.6). No significant effect upon the intracellular pH was observed in isosmotic media when Cl was substituted with the penetrant anion NO₃⁻, reflecting the equivalent permeability of Cl and NO₃⁻ (see above). However, a highly significant (P < 0.001) alkalinisation of the cytosol occurs in isosmotic gluconate⁻ media. A modifier site has been proposed for the

Na:H antiport of lymphocytes, with the pH sensitivity of this site being increased in shrunken cells (Grinstein et al., 1985). Speculatively, the depolarisation observed in gluconate⁻ media (see above) may give rise to a similar shift on the pH sensitivity of the proposed modifier site thus activating the Na:H antiport. Alternatively, under these conditions the Cl_o/Cl_i ratio will decrease, thus if the distribution of small ions is similar to human red cells, then a commensurate shift in the H_i/H_o must occur. Since pH_o is constant, a decrease in the intracellular concentration of H must result, giving rise to an alkalisation of the cytosol.

Exposure to hypertonicity produced a significant alkalisation of the cytosol in Cl and NO_3^- media over their respective controls, whereas cell shrinkage in gluconate⁻ media was without effect upon the pH_i compared to gluconate⁻ controls. For Cl and NO_3^- , this may represent a Na:H antiport stimulation, with NO_3^- behaving in a similar manner to Cl due to their similar membrane permeabilities as indicated by the membrane potential (see above). In shrunken cells the relative permeabilities of Cl, NO_3^- and gluconate⁻ were equivalent as adjudged from membrane potential determinations. Thus in shrunken cells in gluconate⁻ media, a possible activation of a Na:H antiport similar to that seen in Cl or NO_3^- media may be occurring.

Substitution of extracellular Cl by non-penetrant or penetrant anions produced markedly different secondary effects upon membrane potential and pH_i in isosmotic media, but no difference in shrunken cells.

DISCUSSION.

Although the determination of membrane potential and intracellular pH is technically easy by using membrane penetrant cations (TPMP⁺) and weak acids (DMO), caution must be exercised in applying these methods. The equations used to determine the membrane potential and pH_i should use intra- and extracellular activities; these are only approximated by concentrations. There are two caveats against accurate determination of intracellular concentration of TPMP⁺ and DMO; first, if there is a significant binding of the probes to intracellular proteins and secondly, the possible accumulation of these probes by intracellular organelles, particularly mitochondria. Both these problems will result in an over-estimation of the intracellular probes' concentrations and thereby give erroneous estimations of membrane potential and pH_i . For membrane potential, the problem of mitochondrial accumulation may be obviated by ensuring that accumulation in these organelles is saturated. Binding of the probe to intracellular protein can be eliminated by determining the TPMP⁺ uptake with membrane potential set at zero. On combining these two experimental conditions, changes in the membrane potential if not the absolute value for the membrane potential are indicated by TPMP⁺ uptake. Similarly, DMO distributions are affected by a mitochondrial component but elimination of this uptake did not qualitatively affect experimental results (Rindler and Saier, 1981), thus in this study DMO was used to monitor changes in rather than absolute intracellular pH.

The MDCK cell line has a well characterised Na K 2Cl "cotransport" system, as reported by Rindler et al., (1982), McRoberts et al., (1982) and Aiton et al., (1982), and this may be stimulated by

cell shrinkage (see chapter 5). Inhibition of the "cotransport" system by 0.1 mM furosemide, in control or shrunken cells, had no effect upon the membrane potential, as with previous results reported for Ehrlich ascites cells (Geck et al., 1980) and mouse J774.2 cells (Bourrit et al., 1985). It is erroneous to use TPMP accumulation and hence membrane potential, as the sole criterion for ascribing electroneutrality to a transport pathway, since in cells that possess a known electroneutral diuretic-sensitive "cotransport" pathway, inhibition of the pathway by diuretic significantly affects the membrane potential (Greger, et al., 1983 a and b). Therefore the lack of effect upon the membrane potential by inhibition of the Na K Cl "cotransport" pathway indicates that this transport pathway does not mediate a net movement of ions under these experimental conditions, and confirms data presented in chapters 3 and 5.

Shrinkage of the cells will increase the intracellular ion concentration in proportion to the ratio of the media osmolalities (Ueberschar and Bakker-Grunwald, 1983). Thus, for the MDCK cells, whose total cation content is unaltered by hypertonicity (chapter 5), and assuming the membrane potential to be dominated by Na and K, a hyperpolarisation of 13 mV (using ion concentrations and pNa/pK values discussed in the results section of this chapter) would be expected. However, this was not the case and the TPMP accumulations under isosmotic and hyperosmotic conditions were equivalent, indicating that the membrane potential was unaltered. To account for this, assuming that the membrane potential is dominated by the Na K gradients in a similar manner to that of rat white adipocytes (Cheng et al., 1980), the pNa/pK ratio must double in shrunken compared with control MDCK cells. However, if a component for Cl is introduced in the

determination of the membrane potential (using the Cl concentration and the pCl/pK ratio given in results of this chapter), the expected hyperpolarisation in shrunken cells would only be 2.8 mV. Since the Na K Cl "cotransport" system of the MDCK cells does not mediate net movement and is most probably electroneutral, secondary effects of membrane potential, if present are unlikely to be important.

The Cl activation of the diuretic-sensitive "cotransport" system(s) of human erythrocytes, ferret erythrocytes, HeLa and MDCK cells (Chipperfield, 1984; Palfrey, 1984; and chapter 3 and 5) is dependent upon the anion substitute used. Using a penetrant (NO_3^-) which changes the Cl concentration in both the intracellular and extracellular compartments, as compared with a non-penetrant (gluconate $^-$) anion, where only the external concentration is affected, secondary effects upon the membrane potential and/or pH might occur.

In nitrate-replaced media, no effects on the membrane potential or the pH were observed in cells incubated under isosmotic conditions. In shrunken cells, both in Cl and NO_3^- media, a significant intracellular alkalinisation occurred. In gluconate $^-$ media, significant depolarisation of the membrane potential and alkalinisation of the cytosol occurred in isosmotic conditions, but, on shrinkage, the membrane potential was equivalent to that observed in Cl and NO_3^- media, due to the relative permeability of gluconate $^-$ being similar to that of Cl and NO_3^- . The effect of gluconate $^-$ under isosmotic conditions is similar to previous work on human erythrocytes (Chipperfield and Shennan, 1984). Under these conditions, a significant rise in the passive Na and K movement of human red blood cells was observed (Cotterrell and Whittam, 1971; Chipperfield and Shennan, 1983; and Shennan, 1984). Piretanide-sensitive K efflux was

affected by pH_o , and in conditions of constant pH_i but varying membrane potential no effect of extracellular pH was observed and these changes in the passive Na and K fluxes of erythrocytes were attributed to a change in pH_i rather than in membrane potential (Chipperfield and Shennan, 1984).

In the present work there is no evidence of the activation of the ouabain- and diuretic-insensitive component of the K influx in gluconate-replaced media (chapter 3). If a secondary effect of gluconate⁻ media on the activity of Na K Cl "cotransport" is present it is most likely to be pH_i rather than membrane potential as this transport system does mediate a net movement of ions under these experimental conditions. The pH_i dependency of the Na K "cotransport" of human erythrocytes has been investigated (Garay et al., 1981), with the elevation of the pH_i from 6.0 to 7.1 increasing the maximal activity of the Na K "cotransport" but this activity declined when pH_i approached 8.3. Thus, the intracellular pH may be important in regulating the activity of the Na K Cl "cotransport" of MDCK and HeLa cells.

Under isosmotic conditions does the greater sigmoidicity of the Cl activation of Na K Cl "cotransport", using gluconate⁻ as compared with NO_3^- for the replacement of media Cl (chapter 3 and 5), represent secondary effects of pH_i ? One might expect the shape of the Cl activations in hyperosmolar media, where the intracellular pH and membrane potential of the cells in both NO_3^- and gluconate⁻ media are similar, to be similar. The degree of sigmoidicity of the Cl activation in shrunken cells is similar in both NO_3^- and gluconate⁻ Cl substitutions (chapter 5). The question arises whether the Na K Cl "cotransport" system of HeLa and MDCK cells have similar pH profiles

to the human erythrocyte? Further studies are required for elucidation of this question.

CHAPTER 8

[³H] BUMETANIDE UPTAKE IN MDCK CELLS: EFFECT OF CELL SHRINKAGE BY
HYPEROSMOLAR MEDIA.

INTRODUCTION.

Na K Cl "cotransport" in the MDCK cell line has been shown in this present work to be stimulated by hyperosmolar media i.e. cell shrinkage (see chapter 5). This activation was similar to the response to hypertonicity seen in avian erythrocytes (Schmidt and McManus, 1977 a, c; Kregenow et al., 1976; Ueberschar and Bakker-Grunwald, 1983).

Na K Cl "cotransport" system(s) share many common features, in particular their relative sensitivity to the "loop" diuretics bumetanide, piretanide and furosemide (Palfrey and Rao, 1983). Bumetanide has a relatively high specificity for the "cotransport" system, compared with the "loop" diuretic furosemide with little inhibition by bumetanide of other transport pathways i.e. the band 3 anion exchanger of red blood cells (Lant, 1984; Brazy and Gunn, 1976). Therefore, radiolabelled bumetanide should be a useful tool in the investigation of the nature and characterisation of the Na K Cl "cotransport" system (see Ruzg, Simmons and Tivey, 1985 a, b).

The presence of a Na K Cl "cotransport" system which is inhibited by furosemide has already been demonstrated in the thick ascending limb of the loop of Henle (TALH) of mammalian kidney (Greger, 1981; Greger and Schlatter 1981; Greger, Schlatter and Lang, 1983). Therefore, in membrane preparation of the outer renal medulla, a high density of the Na K Cl "cotransport" system should be found. Indeed, binding of [³H] bumetanide, benzetanide to these membrane preparations has been demonstrated, with a saturable component being displaced by concentrations of unlabelled "loop" diuretics (Forbush

and Palfrey, 1983; Jorgensen, et al., 1984) which are known to inhibit the Na K Cl "cotransport". The concentration of [^3H] bumetanide required for 50% of maximal binding in the membrane preparation of renal medulla was 0.3 μM (Forbush and Palfrey, 1983), which is similar to the 50% inhibitory concentration of K transport by the Na K Cl "cotransport" system in a variety of cell types (Palfrey and Rao, 1983). Similarly, the Na K Cl "cotransport" of the dogfish rectal gland has been demonstrated to bind the ^{14}C labelled "loop" diuretic N-methylfurosemide, with a saturable portion being displaceable by unlabelled diuretics (Hannafin, Kinne-Saffran, Friedman and Kinne, 1983). However, membrane isolated from renal medulla will be derived from a heterogenous cell population and it is important to be aware of the existence of secretory processes resulting in the clearance of diuretic (Olsen, 1977) and the secreted tubular proteins known to bind diuretics (Greven, Kolling, Bronewski-Schwarzer, Junker, Neffgen and Nilius, 1984).

The uptake of [^3H] bumetanide by the MDCK cell line has been investigated in this laboratory by Simmons and co-workers (1985 a, b). Total [^3H] bumetanide uptake by the MDCK cell could be separated into two components; a) a non-saturable linear uptake and b) a component displaying sigmoidal kinetics with half maximal uptake at 0.33 μM , which was comparable with the half-maximal inhibition by bumetanide (0.26 μM) of the K (^{86}Rb) flux through the Na K Cl "cotransport" system. This saturable component of [^3H] bumetanide uptake was seen to be displaceable by unlabelled "loop" diuretics. The magnitude of the saturable component of [^3H] bumetanide uptake was correlated to the magnitude of the K (^{86}Rb) flux in the MDCK cells. Cellular localisation of the [^3H] bumetanide indicates that the uptake sensitive to displacement by piretanide was largely bound to membranous

material.

The uptake of [3 H]bumetanide which is sensitive to displacement by 0.1mM piretanide may represent binding to the Na K Cl "cotransport" entity. Since this transport pathway can be stimulated by hyperosmolar media (cell shrinkage), the primary objective of the data presented in this chapter was to investigate the effect of cell shrinkage (hyperosmolar media) on the [3 H] bumetanide uptake in MDCK cells.

RESULTS.

A) [^3H] bumetanide uptake by the MDCK cell (isotonic media):

The biological activity of the [^3H] bumetanide was determined directly by assessing the inhibition of the K (^{86}Rb) influx (table 8.1). In the presence or absence of 1mM ouabain, the K (^{86}Rb) influx of MDCK cells inhibited by 8 μM [^3H] bumetanide was not significantly different from that inhibited by 8 μM unlabelled bumetanide. Thus the addition of [^3H] to the bumetanide had no effect on the biological activity of bumetanide.

In this laboratory, Simmons and co-workers (1985 a,b) have demonstrated the uptake of the [^3H] bumetanide to be time-dependent, and the piretanide-displaceable uptake to be complete after 15 minutes. The cellular uptake of the [^3H] bumetanide was seen to be dependent upon the external concentration of bumetanide (figure 8.1). Similar to previous reports (Rugg et al., 1985 a,b), the total [^3H] bumetanide uptake is non-linear with respect to the external [^3H] bumetanide concentration. This uptake may be resolved into two components, with the inclusion of 0.1mM piretanide resulting in a linear relationship ($r = 0.994$, $P < 0.001$, slope 5.41 ± 0.12 pmol/ μM and intercept -0.138 ± 0.06) and comprises the major portion of the [^3H] bumetanide uptake and is analogous to the observed binding to membranes prepared from dog kidney (Forbush and Palfrey 1983).

To determine the saturable component, the uptake in the presence of maximal inhibitory concentration of piretanide was subtracted from the total uptake and this demonstrated sigmoidal kinetics. A Scatchard

Table 8.1 Inhibition of K(⁸⁶Rb) influx by [³H]-Bumetanide (Batch RO10-6338-706) and bumetanide in MDCK cells. Data are mean \pm SD of 3 observations

Conditions	⁸⁶ Rb influx nmol/10 ⁶ cell.min	Bumetanide- sensitive
A Total no addition	13.45 \pm 0.51	
B 10 ⁻³ M ouabain	8.12 \pm 0.37	
C 8 x 10 ⁻⁶ M Bumetanide	6.54 \pm 0.27 0.1 < p < 0.05	(A-C) 6.91 \pm 0.57 ns
D 8 x 10 ⁻⁶ M [³ H]-Bumetanide	5.07 \pm 0.89	(A-D) 8.38 \pm 1.03
E 10 ⁻³ M ouabain + 8 x 10 ⁻⁶ M Bumetanide	1.83 \pm 0.09	(B-E) 6.29 \pm 0.38
F 10 ⁻³ M ouabain + 8 x 10 ⁻⁶ M [³ H]-Bumetanide	2.62 \pm 0.47 ^a	(B-F) 5.50 \pm 0.59 ns
G 10 ⁻⁴ M bumetanide	5.96 \pm 0.28	(A-G) 7.49 \pm 0.58
H 10 ⁻³ M + 10 ⁻⁴ M bumetanide	1.30 \pm 0.16	(B-H) 6.02 \pm 0.40

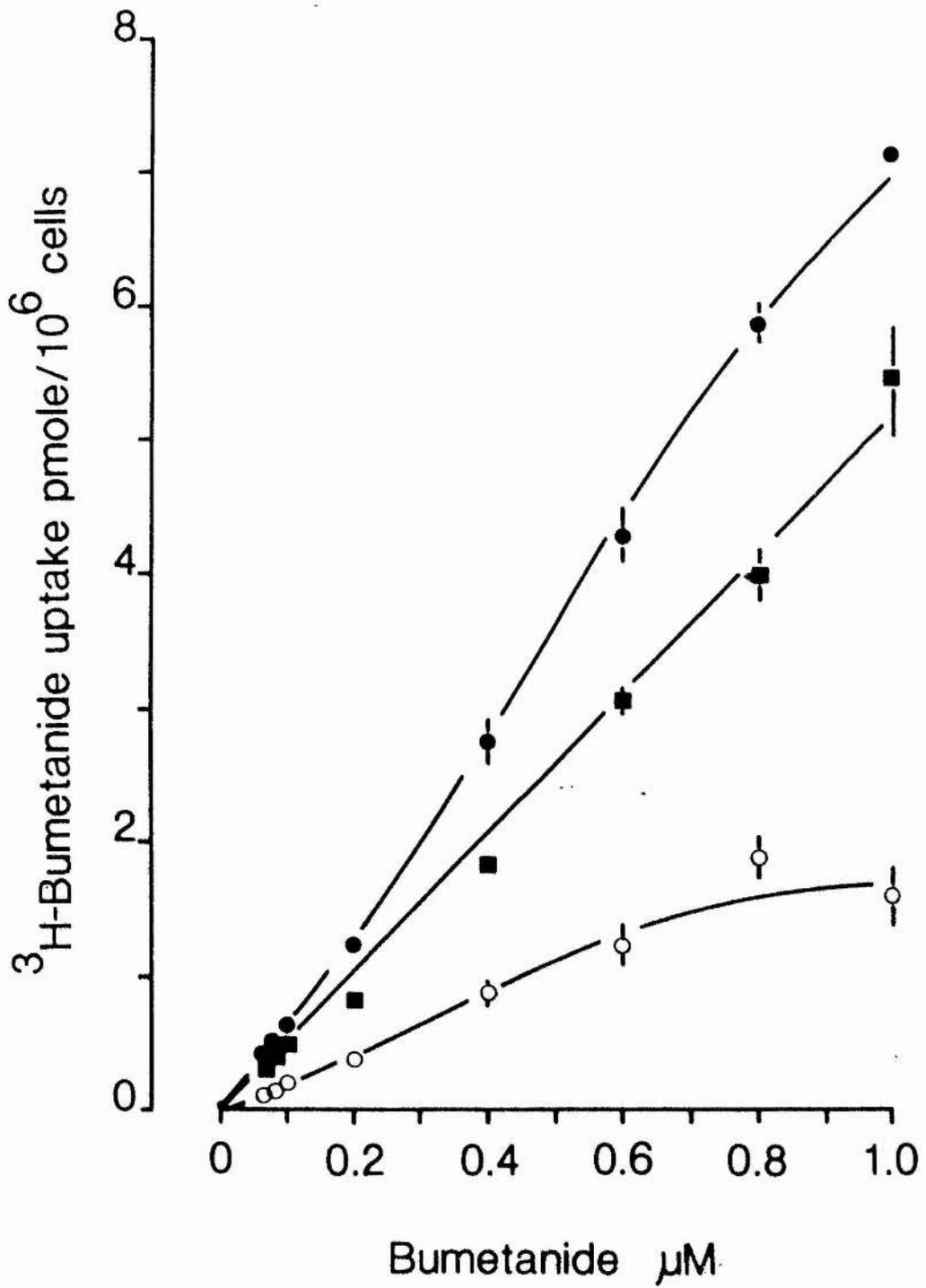
Significantly different from unlabelled bumetanide

ns = not significantly different

(a) = p < 0.05

Figure 8.1

Variation of [^3H]bumetanide uptake in MDCK cells with extracellular [^3H]bumetanide concentration in the range 0-1.0 μM . Total cellular uptake (\bullet), and the cellular uptake in the presence of a competing ligand (piretanide) (\blacksquare). Linear regression analysis of the line for [^3H]bumetanide uptake in the presence of piretanide gave $r=0.994$, $P < 0.001$, $n=24$, intercept of -0.138 ± 0.06 (S.E.) and slope of 5.41 ± 0.11 (S.E.) pmole/ μM . The piretanide-sensitive uptake (\circ) is determined by subtraction, total- piretanide. Data are the mean \pm S.D., $n=3$, of a single representative experiment.



plot of the data presented in figure 8.1 is shown in figure 8.2a. The non-linear nature of this plot infers a Hill coefficient (H_n) greater than one, which is similar to the binding of furosemide to the Tamm-Horsfall protein (Greven et al., 1984). Subsequent Hill analysis, assuming the maximal uptake to be that observed in $1 \mu\text{M}$ bumetanide, is given in figure 8.2b. Linear regression of these data gave a slope (Hill coefficient) of 1.67 ± 0.08 (\pm S.E.) and a calculated half-maximal uptake at a $[^3\text{H}]$ bumetanide concentration of $0.27 \mu\text{M}$, this value being similar to the half-maximal inhibition of the K (^{86}Rb) influx by bumetanide $0.55 \pm 0.26 \mu\text{M}$, mean \pm S.D., $n=4$ (see chapter 3). Table 8.2 shows summarized data of 4 experiments, where the half maximal saturation was observed at $0.15 \pm 0.04 \mu\text{M}$ and maximal uptake was between 0.52 and $1.50 \text{ pmol}/10^6$ cells and a Hill coefficient near 2. The mean piretanide-sensitive uptake of $0.89 \text{ pmol}/10^6$ cells corresponds to a cellular density of 5.35×10^5 sites/cell and is in good agreement with the cellular density of the Na K ATPase (Lamb, Ogden and Simmons, 1981) and the previously reported value for $[^3\text{H}]$ bumetanide uptake (Rugg et al., 1985 a,b) in the MDCK cell line. These data represent the binding of the bumetanide to the "cotransport" system of this cell line.

B) Effect of hyperosmotic media on $[^3\text{H}]$ bumetanide uptake:

The ability to label the Na K Cl "cotransport" system of the MDCK cell with radio-labelled "loop" diuretics should facilitate the study of the regulation of this system. The Na K Cl "cotransport" system is stimulated in MDCK cells exposed to hyperosmolar media (chapter 5 of this present work). Therefore, if this stimulation is due to an increased number of Na K Cl "cotransport" units in the membrane, then exposure to hyperosmotic media should increase the

Figure 8.2 a,b

Analysis of the piretanide-displaceable [^3H] bumetanide uptake (a) Scatchard plot (b) data fitted to the Hill equation assuming B_{max} to be the uptake in $1 \mu\text{M}$ [^3H] bumetanide. Linear regression gave slope (H_n) of 1.67 ± 0.08 (S.E.), regression coefficient = 0.98, $P < 0.001$ and a calculated $A_{1/2}$ of $0.27 \mu\text{M}$.

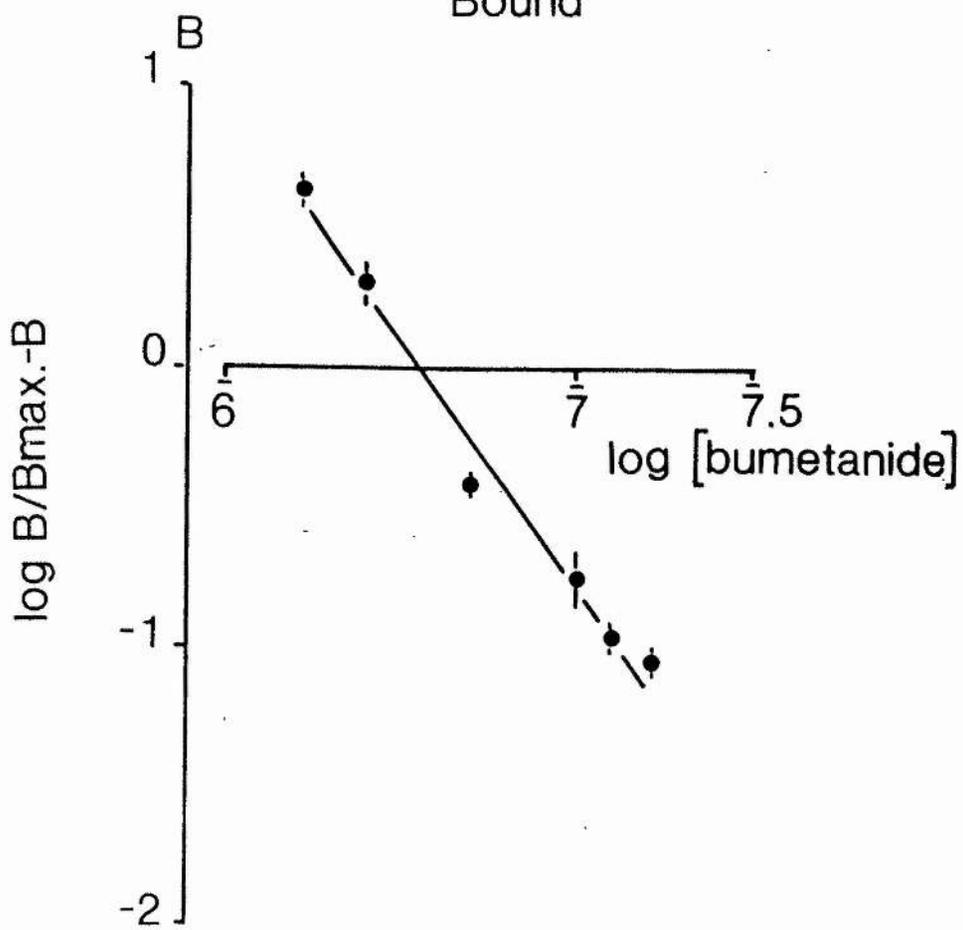
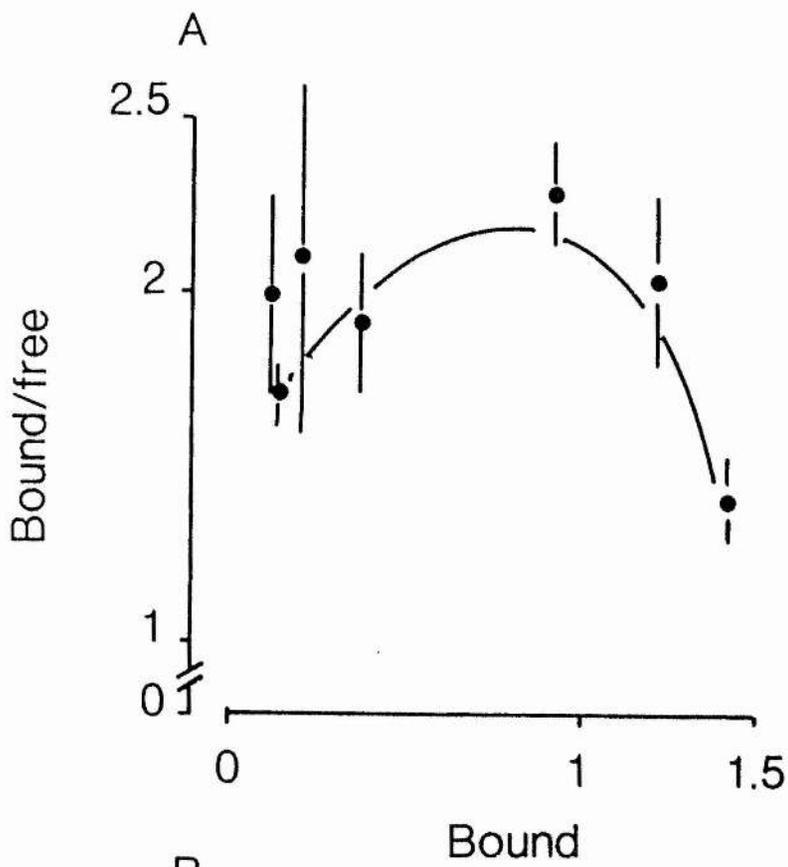


Table 8.2 Initial estimate of Bmax, $A_{1/2}$ and the Hill coefficient (H), linear regression to the Hill equation (Kinfit program, see Methods). Calculations for 4 experiments

Exp	Bmax	$A_{1/2}$	H
1	0.54	0.15	1.9
2	0.98	0.12	1.9
3	1.50	0.21	1.9
4	0.52	0.12	1.96
	0.89 ± 0.46	0.15 ± 0.04	1.9

amount of piretanide-sensitive [^3H] bumetanide uptake by the cells. Table 8.3 shows the piretanide-sensitive [^3H] bumetanide uptake (external [^3H] bumetanide = 0.4 μM) for the MDCK cell line exposed to isosmotic and hyperosmotic media. In 6 experiments increased media tonicity resulted in an increase in the uptake of piretanide-sensitive [^3H] bumetanide, and this increase is highly significant in 50% of the experiments performed, with P values between 0.05 and 0.001. The non-significance of the increase observed for the remaining experiments may be due to the error inherent in determining the relatively small diuretic-sensitive component of the [^3H] bumetanide uptake by subtraction of the large "non-specific" component from the total uptake. Therefore, if the experimental error is large in these components, then the error of the piretanide-sensitive component will mask any small increases in this component caused by exposure to hyperosmotic media. An alternative explanation may be that small differences in the media tonicity of the isosmotic condition may cause increases or decreases in the [^3H] bumetanide uptake since this uptake is extremely sensitive to media tonicity (see below (figure 8.3)).

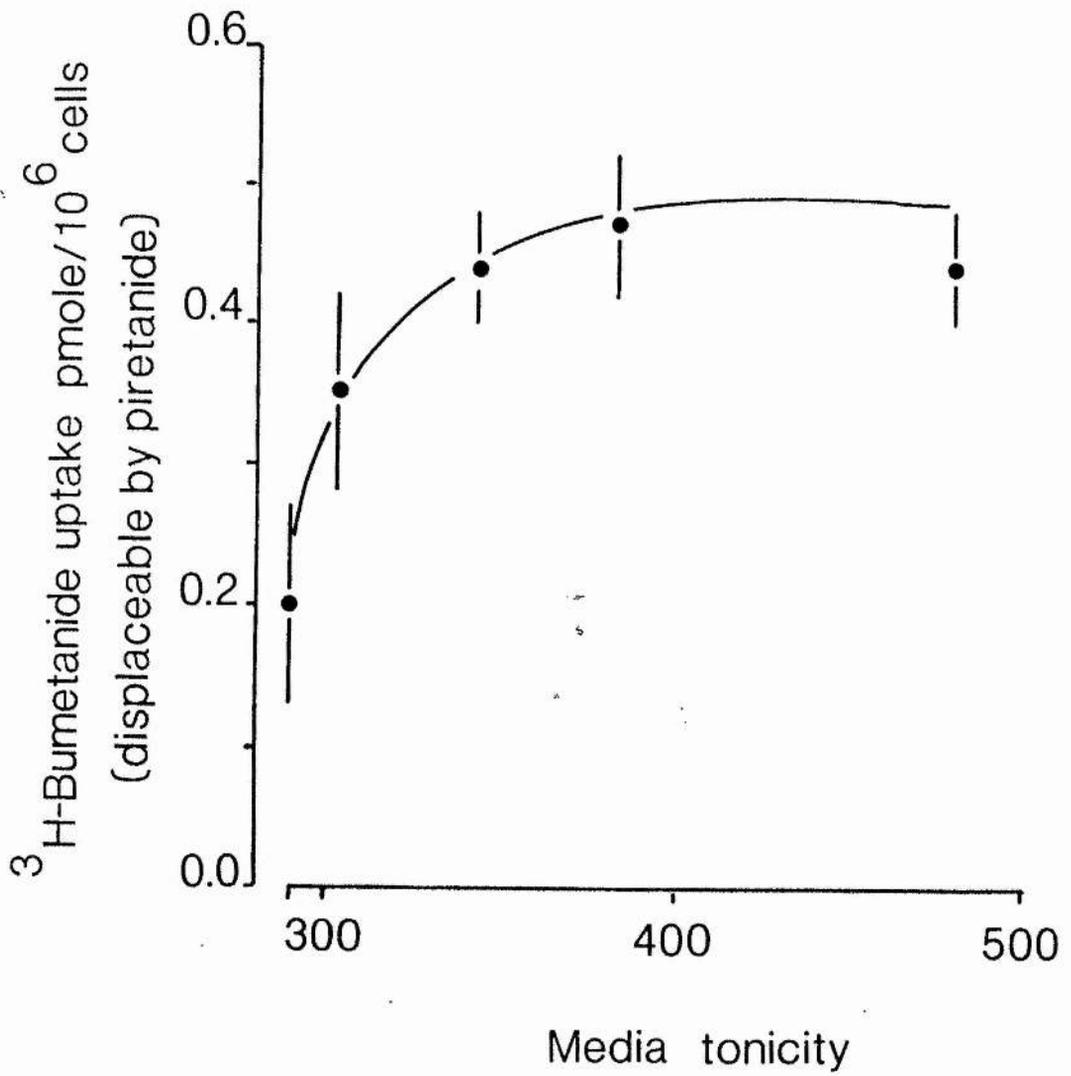
Stimulation of the piretanide-sensitive [^3H] bumetanide uptake by increased media tonicity (mannitol addition) is saturable (figure 8.3). In 290 and 480 mosmol/kg media the uptake of piretanide-sensitive [^3H] bumetanide was 0.20 ± 0.07 and 0.44 ± 0.04 pmol/ 10^6 cells (mean \pm S.D., $n=6$, $P < 0.001$) respectively. This stimulation is complete at 340 to 380 mosmol/kg media and is similar to the effect of increased media tonicity on the diuretic-sensitive K (^{86}Rb) efflux from HeLa cells (Chapter 5 of this present work) and avian erythrocytes (Ueberschar and Bakker-Grunwald, 1983). It appears from these data that the [^3H] bumetanide uptake responds to cell shrinkage in a similar manner to the K fluxes via the Na K Cl

Table 8.3 Effect of hypertonicity upon the piretanide displaceable [³H] Bumetanide uptake. [³H] Bumetanide outside was at 4×10^{-7} M. Data are the mean \pm SD of 6 individual experiments

Exp	Isotonic	Hypertonic	Hypertonic-isotonic	Significance
1	0.17 \pm 0.13	0.34 \pm 0.12	0.17	ns
2	0.29 \pm 0.08	0.48 \pm 0.07	0.19	p < 0.05
3	2.01 \pm 0.02	3.65 \pm 0.50	1.64	p < 0.001
4	0.52 \pm 0.07	0.60 \pm 0.05	0.08	ns
5	0.20 \pm 0.07	0.44 \pm 0.04	0.22	p < 0.001
6	0.65 \pm 0.16	0.72 \pm 0.14	0.07	ns

Figure 8.3

Dependence of the piretanide displaceable component of [^3H] bumetanide (0.4 μM) uptake upon the media osmolality. Media osmolality varied by mannitol addition. Data are the mean \pm S.D. of 6 observations.



"cotransport" pathway.

In order to establish whether an increase in the piretanide-sensitive [^3H] bumetanide uptake (increased number of "cotransport" units) represents the mechanism by which the increased activity of the K (^{86}Rb) transport via the Na K Cl "cotransport" is mediated, it is necessary to determine the K flux and the [^3H] bumetanide uptake in the same cell batch and under near identical experimental conditions. Under isosmotic conditions, the number of [^3H] bumetanide sites per cell and the turnover of ^{86}Rb by the "cotransport" (footnote table 8.4) are in good agreement with previously reported values in the MDCK cell line (Rugg et al., 1985 a,b). Table 8.4 shows data from two experiments where the [^3H] bumetanide uptake and K (^{86}Rb) influx were determined in the same experiment under isosmotic and hyperosmotic conditions. In both experiments the diuretic-sensitive K (^{86}Rb) influx was significantly increased ($P < 0.001$), thus confirming the data presented in chapter 5. Similarly, the [^3H] bumetanide uptake which was piretanide-sensitive was also increased in hyperosmolar media, but this increase was only significant in experiment 2. If the stimulation of the K (^{86}Rb) influx takes place via an increased number of "cotransport" units, it could be assumed that the turnover rate (molecules/site.second) would remain constant. Turnover rate for K (^{86}Rb) transport through the Na K Cl "cotransport" of the MDCK cells exposed to isosmotic and hyperosmotic media differed significantly ($P < 0.01$). However, the direction is not consistent, since an increase of 115 molecules/site.second and a decrease of 398 molecules/site.second were observed in the experiments given in table 8.4.

In the MDCK cell line, cell shrinkage by exposure to

hyperosmolar media has significant effects upon both the diuretic-sensitive K (^{86}Rb) influx and the piretanide-sensitive [^3H] bumetanide uptake. An increase in the number of Na K Cl "cotransport" units per cell is not adequate as a possible mechanism for the increased K flux observed in shrunken cells, suggesting that a control mechanism regulating the rate of turnover of the Na K Cl "cotransport" must be present in the MDCK cells.

Table 8.4 Turnover number, molecules/site.second, of $^{86}\text{Rb}^+$ by the $\text{Na}^+ \text{K}^+ \text{Cl}^-$ 'cotransport' pathway.

Response of MDCK cells, K^{86}Rb^+ influx and ^3H -Bumetanide uptake to hyperosmolar media were determined in the same cell batch. Data are the mean \pm SD of [n] values

Exp	Media tonicity	$\text{nmol}/10^6$ cells.min K^{86}Rb influx	$\text{pmol}/10^6$ cells ^3H Bumetanide uptake, piretanide sensitive	No of site/cell	^{86}Rb molecules/site/sec turnover number
1	Isosmotic	12.57 ± 0.75 (3)	0.52 ± 0.07 (3)	3.13×10^5	400 ± 24
	Hyperosmotic	18.75 ± 0.75 (3) ^c	0.60 ± 0.05 (3) ^{ns}	3.60×10^5	515 ± 24 ^b
2	Isosmotic	12.60 ± 0.30 (3)	0.20 ± 0.04 (6)	1.20×10^5	1050 ± 27
	Hyperosmotic	17.20 ± 0.40 (3) ^c	0.44 ± 0.04 (6) ^c	2.65×10^5	652 ± 15 ^c

a significance of difference from isosmotic values; ns = not significant

b $p < 0.01$

c $p < 0.001$

Footnote:

In isosmotic conditions the number of site/cell were in the range of $1.2\text{--}8.4 \times 10^5$, with a mean value of 4.17 ± 2.64 (SD) for 5 experiments. The turnover number for ^{86}Rb under the same condition were 120-1054 molecules/site/sec with a mean value of 440 ± 358 (SD) for 5 experiments.

DISCUSSION.

The primary purpose of this chapter was to identify a component of [^3H]bumetanide uptake which may be associated with the Na K Cl "cotransport" system of MDCK cells under isosmotic and hyperosmotic conditions. The presence of Na K Cl "cotransport" in the MDCK cells has been reported previously (McRoberts et al., 1982) and is confirmed in this present work (chapter 3 and 5). In this cell type, the Na K Cl "cotransport" exhibits a marked stimulation after exposure to hypertonic media (chapter 5) which is similar to the situation found in avian erythrocytes (Ueberschar and Bakker-Grunwald, 1983).

Simmons and co-workers (1985 a,b) have demonstrated a component of [^3H]bumetanide uptake which is displaceable by high concentrations of competing "loop" diuretics, and the data suggest possible binding to the Na K Cl "cotransport" system. These data are supported in this present work, since a component of [^3H]bumetanide was displaceable by piretanide. The concentration of bumetanide giving half maximal [^3H] bumetanide uptake and half maximal inhibition of the K (^{86}Rb) influx corresponds closely to the values reported in Rugg et al., (1985 a,b). Analysis of the saturable component of the [^3H]bumetanide uptake gave sigmoidal kinetics with Hill coefficients near 2. In many respects the kinetics of [^3H] bumetanide uptake are similar to the binding of furosemide to the tubular secreted proteins (Greven et al., 1984) and of "loop" diuretics to membranes of renal origin (Forbush and Palfrey, 1983; Jorgensen et al., 1984) and dogfish rectal gland (Hannafin et al., 1983). Such kinetics may be expected in the light of the stoichiometry of 2Cl (McRoberts, et al., 1982) and that the inhibition of the Na K Cl "cotransport" is at the Cl site (Haas and McManus, 1983). However, this is not substantiated by the bumetanide

inhibition of the K (^{86}Rb) influx, since Hill analysis yielded Hill coefficients near unity (Rugg et al., 1985 b). The reason for this discrepancy is at present unclear.

To investigate the hypothesis that the stimulation of the K (^{86}Rb) fluxes through the Na K Cl "cotransport" pathway by cell shrinkage is due to an increased number of "cotransport" units, the piretanide-sensitive [^3H] bumetanide uptake has been studied in MDCK cells exposed to hyperosmotic media. Hypertonicity significantly increased the [^3H] bumetanide uptake which was displaceable by the "loop" diuretic piretanide. The dependence of this increase on the tonicity was very similar to that observed for the K (^{86}Rb) fluxes of HeLa cells (see chapter 5) and the avian erythrocyte (Ueberschar and Bakker-Grunwald, 1983). Therefore the number of the Na K Cl "cotransport" units per cell is dependent upon the tonicity of the media and hence the cell volume.

The mechanism by which this is achieved is unknown, but intuitively one can consider it to be an insertion or exposure of pre-formed Na K Cl "cotransport" units rather than de novo synthesis of new protein, principally because the time course is rapid. How cell shrinkage is translated into increases in the number of Na K Cl "cotransport" units is also unknown. Two possible but speculative explanations may be proposed. First, the response is mediated through a second messenger, although it is unlikely to be cAMP since hypertonicity does not increase cAMP in MDCK cells or avian erythrocytes (see chapter 6; Kregenow et al., 1976), whilst GTP and its metabolites are increased by hypertonicity (see chapter 9). Second, the cell shrinkage causes physical changes in the structure of

the cell membrane resulting in either exposure or incorporation of new [^3H] bumetanide binding sites in the membrane; the cytoskeleton may be involved since a polypeptide photolabelled with [^3H] bumetanide can only be extracted in high ionic strength solutions from membrane prepared from outer renal medulla (Jorgensen et al., 1984).

Assuming that [^3H] bumetanide is associated with the membrane, does the increase in the [^3H] bumetanide uptake and hence the increase in the number of Na K Cl "cotransport" units in hyperosmotic media represent the mechanism which mediates the increase in the diuretic-sensitive K (^{86}Rb) fluxes in shrunken cells?. In experiments where both the [^3H] bumetanide uptake and the K (^{86}Rb) flux are measured in the same cell batch and are seen to be increased by hypertonicity, the percentage increase of the [^3H] bumetanide uptake does not correspond to that of the K (^{86}Rb) flux. The turnover for K transport by the Na K Cl "cotransport" pathway reveals either a downward or an upward regulation of the K turnover in shrunken cells compared with controls. The cell must therefore recognise the number of Na K Cl "cotransport" units and adjust the turnover rate giving the required K flux observed in the shrunken cells, but the means by which this regulation is achieved by the cell is at present unknown.

Exposure of the MDCK cells to hyperosmotic media increases both the diuretic-sensitive K (^{86}Rb) influx and the pifretanide-displaceable [^3H] bumetanide uptake. The present data suggest however that the increase in the diuretic-sensitive K transport is a regulation of the turnover of Na K Cl "cotransport" for K transport rather than a simple increase in the number of functional Na K Cl "cotransport" units.

CHAPTER 9

DIURETIC-SENSITIVE K (^{86}Rb) INFLUX: EFFECT OF METABOLIC INHIBITION.

INTRODUCTION.

Beauge and Adragna (1971) originally reported the ouabain-insensitive, Na-dependent ^{86}Rb influx of the human erythrocyte, now considered to be the Na K "cotransport" (Wiley and Cooper, 1974), to be sensitive to metabolic inhibition by iodoacetic acid. These results in the human erythrocyte have recently been confirmed (Adragna, Perkins and Lauf, 1985), using the reversible inhibitor of cellular metabolism, the limited-metabolisable sugar 2 deoxy-D-glucose. Adragna and co-workers demonstrated that the reduction of cellular ATP to 4% of control values inhibited the net cation efflux via the Na K "cotransport" system, and that, on replenishment of cellular ATP by incubation in the metabolisable substrate glucose, this inhibition was reversed. The above apparently contradicts the work of Lauf, Perkins and Adragna (1984), who reported the relative insensitivity of the Na K "cotransport" of the the human erythrocyte to metabolic inhibition. However, these differences in the response of the human erythrocyte to metabolic inhibition may be attributed to the presence or absence of Ca in the extracellular media, with metabolic dependence being observed in Ca-free conditions (Adragna et al., 1985). The apparent dependence of the Na K Cl "cotransport" system on the metabolic status of the cell has also been demonstrated in a number of other cell types, e.g. Ehrlich ascites cells (Geck, Heinz, Pietrzyk and Pfeiffer, 1978), MDCK cells (Rindler et al., 1982) and avian erythrocytes (Palfrey, 1983; Ueberschar and Bakker-Grunwald, 1985)

It should be noted that metabolic inhibition or starvation of

cells will not only affect the absolute level of intracellular ATP, but will also undoubtedly produce changes in either the adenylate energy charge ($ATP + 0.5ADP/ATP + ADP + AMP$) and/or the phosphorylation potential ($ATP/ADP \times P_i$) similar to HA-1 CHO fibroblasts (Calderwood, Bump, Stevenson, Van Kersen and Hahn, 1985), or in other cell metabolites e.g. GTP, GDP. The most convincing evidence for the involvement of ATP has been obtained for the squid axon (Russell, 1983), where the cytoplasmic ATP was reduced by dialysis with a subsequent reduction of the K and Cl furosemide-sensitive influx, but on return to a dialysis fluid containing ATP, furosemide-sensitive transport was restored.

Is ATP required as an energy source for Na K Cl "cotransport" system(s)? The ouabain-insensitive Na-dependent K influx inhibited by ethacrynic acid which was observed in the human erythrocyte by Lubowitz and Whittam (1969) showed no difference in lactate production associated with the operation of this flux. The nature of the K influx determined by Lubowitz and Whittam (1969) has many features in common with the Na K "cotransport" reported by Wiley and Cooper (1974). Thus, it may be inferred that the operation of the Na K "cotransport" of the human erythrocyte has no energy requirement.

More substantial evidence is available for Ehrlich ascites cells (Geck et al., 1980). Inhibition of the Na K 2Cl "cotransport" of Ehrlich ascites cells by the "loop" diuretic furosemide has no effect on the turnover of ATP (Geck et al., 1980). Therefore it seems unlikely that the hydrolysis of ATP is required as an energy source for the Na K Cl "cotransport" system(s). Since there appears to be no requirement of ATP as an energy supply for the operation of the Na K Cl "cotransport" system, this implies that the Na K Cl "cotransport"

pathway is regulated by cellular ATP and/or general cell metabolism.

In chapter 3 of this present work, I have demonstrated the presence of a Na K Cl "cotransport" system in both the HeLa and MDCK cell line, which is stimulated by cell shrinkage (chapter 5). In the light of the above evidence for the metabolic dependence of the Na K Cl "cotransport", particularly for MDCK cells (Rindler, et al., 1982), the primary purpose of this chapter was to investigate a possible regulation of the Na K Cl "cotransport" by cell metabolism under isosmotic and hyperosmotic conditions in both HeLa and MDCK cells.

RESULTS

A) INHIBITION OF METABOLISM BY 2DEOXY-D-GLUCOSE: EFFECT ON CELL NUCLEOTIDE LEVELS.

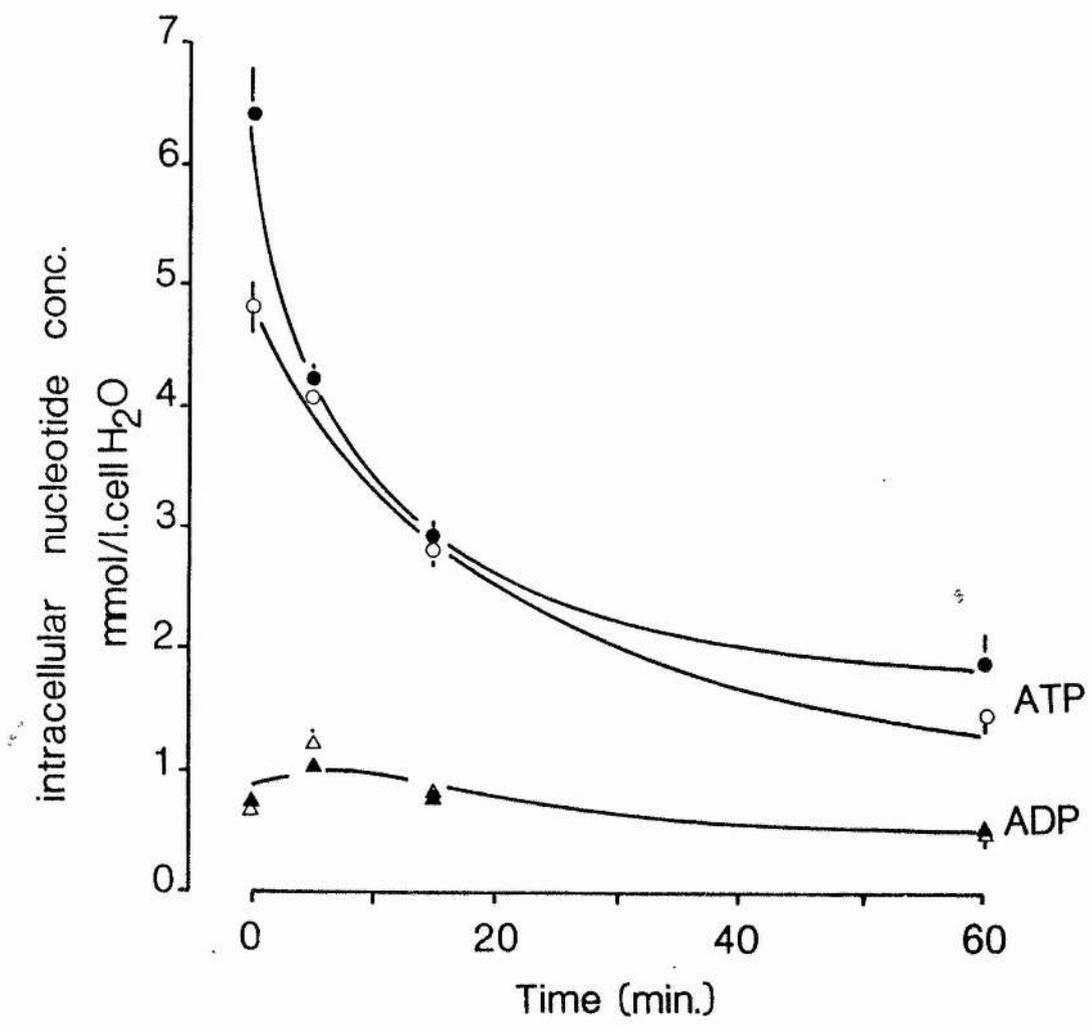
Determination in the HeLa cell line.

ATP and ADP concentrations were determined in HeLa cells exposed to a standard, isosmotic or hyperosmotic (mannitol addition) Krebs solution, where the glucose had been replaced in an equimolar fashion by the limited-metabolisable sugar 2deoxy-D-glucose (figure 9.1). The cellular ADP concentrations demonstrated a significant decrease in both isosmotic (0.72 ± 0.08 to 0.42 ± 0.04 mean \pm S.D., $n=3$, $P<0.01$) and hyperosmotic (0.74 ± 0.01 to 0.48 ± 0.11 , mean \pm S.D., $n=3$, $P < 0.02$) conditions after a 60 minute incubation period in 2 deoxy-D-glucose media, compared with control values (time zero minutes).

Time-dependent decreases in the ATP concentrations of the HeLa cells in both isosmotic and hyperosmotic media in the presence of 2 deoxy-D-glucose media were observed (figure 9.1). After a 1 hour incubation period the ATP concentrations in HeLa cells exposed to either isosmotic or hyperosmotic conditions were 30% of their respective control values. Unfortunately, no data on the AMP or inorganic phosphate concentration in these experimental conditions are available, therefore no analysis of the adenylate energy charge or the phosphorylation potential can be made. However, the response of the ATP and ADP concentrations are similar to HA-1 CHO fibroblasts under starvation (Calderwood et al., 1985). In these cells the adenylate energy charge over a three hour starvation was essentially unaltered

Figure 9.1

Time dependence of metabolic inhibition in HeLa cells. Intracellular ATP and ADP concentrations (in mM) determined by Hplc (see methods). Cells were incubated under isosmotic (○, ATP; ▲, ADP) or hyperosmotic (●, ATP; △, ADP) conditions in the presence of the limited-metabolisable sugar 2 deoxy-d-glucose. Results are the mean \pm S.D. of 3 observations.



due to the failure of AMP and ADP to accumulate, whereas the phosphorylation potential declined with similar kinetics to the ATP decrease (Calderwood et al., 1985).

In the same cell batch, the GTP and GDP were determined (figure 9.2). The GTP concentration (expressed in peak area/ normalised to per litre of cell water) showed a highly significant, time-dependent decrease in both isosmotic (8.40 ± 0.62 to 2.00 ± 0.20 , mean \pm S.D., $n=3$, $P < 0.001$) and hyperosmotic (11.60 ± 0.60 to 4.70 ± 0.84 , mean \pm S.D., $n=3$, $P < 0.001$) media. At all time points, the concentration of GTP was significantly greater in cells exposed to hyperosmolar media. The cellular concentration of GDP was not significantly affected by the exposure to isosmotic, 2 deoxy-D-glucose-containing media. However, shrinkage of the cells produced a significant (2.82 ± 0.46 to 4.6 ± 0.06 , mean \pm S.D., $n=3$, $P < 0.01$) accumulation of the GDP after a 60 minute incubation period in the presence of 2 deoxy-D-glucose.

Determination in the MDCK cell line:

ATP and ADP concentrations were also determined in the MDCK cell line (table 9.1). The initial response to cell shrinkage was an increase in cellular concentrations of ATP and ADP in proportion to the ratio of the media osmolalities. Prolonged exposure to the hyperosmotic conditions has no significant effect upon the ATP and ADP concentrations of the MDCK cells. Substitution of the media glucose with the non-metabolisable sugar, 2 deoxy-D-glucose, reduced the cellular ATP concentration to 20% of control cells. However, no significant difference between control or metabolically depleted cells was observed with respect to their ADP content.

Figure 9.2

Time dependence of metabolic inhibition in HeLa cells. Intracellular GTP and GDP expressed in peak area ($\times 10^6$)/ 1 cell water were determined by Hplc (see methods). Cells were incubated under isosmotic (\circ ,GTP; Δ ,GDP) or hyperosmotic (\bullet ,GTP; \blacktriangle ,GDP) conditions in the presence of the limited-metabolisable sugar 2 deoxy-d-glucose. Data are the mean \pm S.D. of 3 observations.

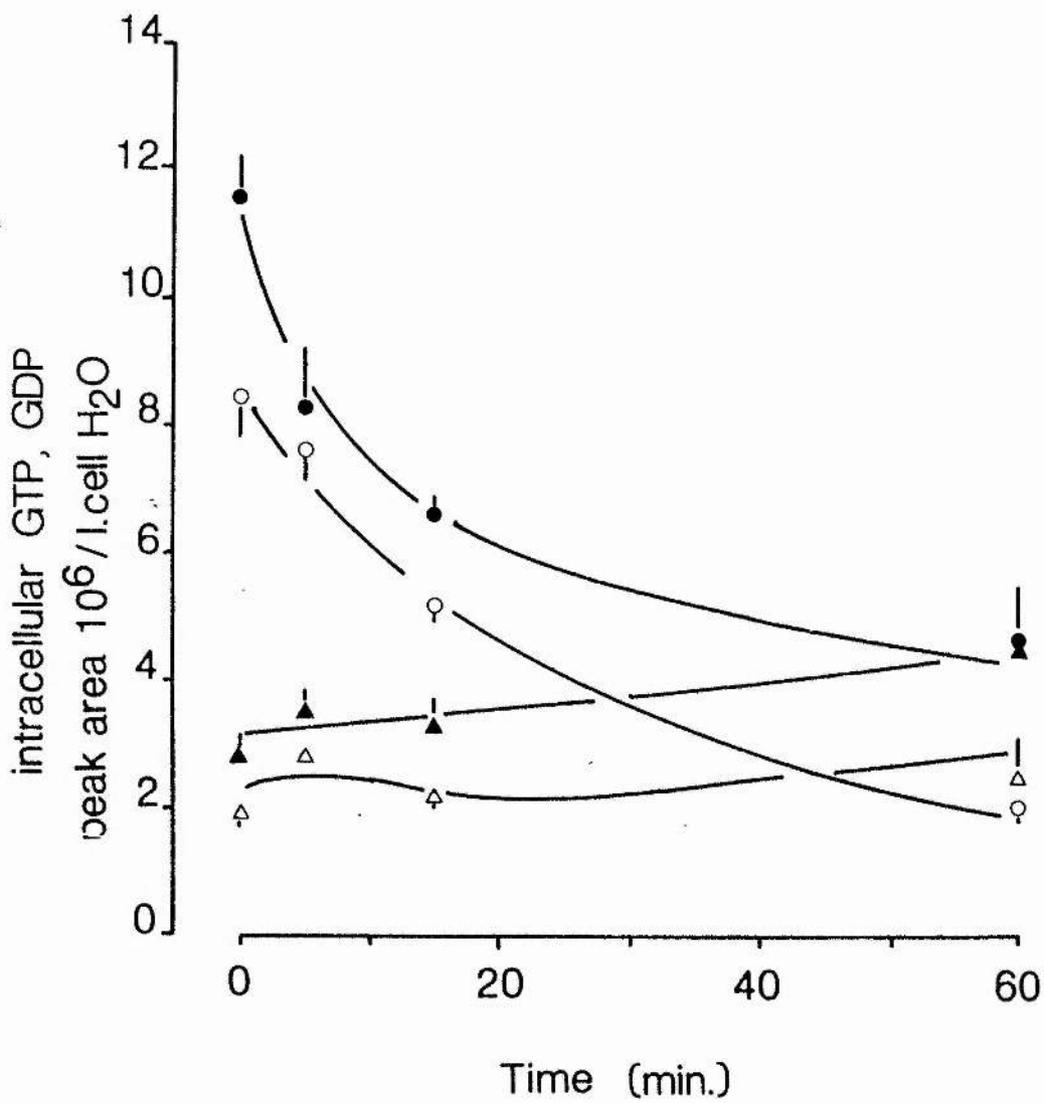


Table 9.1 Intracellular ATP and ADP concentrations of the MDCK cell line determined by HPLC (see Methods). MDCK cells were exposed to isosmotic and hyperosmotic media in the presence of glucose or 2-deoxy-D-glucose for 60 minutes ^(a)

Incubation time min.	Sugar	Media tonicity mosmol/kg	mmol/l. cell water	
			ATP	ADP
zero	glucose	310	6.79 ± 0.46	0.57 ± 0.06
	glucose	510	11.06 ± 0.61	0.81 ± 0.07
60	glucose	310	6.50 ± 0.13 ^{ns}	0.55 ± 0.07 ^{ns}
	glucose	510	10.80 ± 0.20 ^{ns}	0.71 ± 0.06 ^{ns}
60	2-deoxy-D-glucose	310	1.38 ± 0.13 ^b	0.49 ± 0.07 ^{ns}
	2-deoxy-D-glucose	510	2.07 ± 0.14 ^b	0.78 ± 0.11 ^{ns}

(a) Data are the mean ± SD (n=3) of a single representative experiment. Significance of difference from time zero values tested by Student's t-test. ns = not significant.

(b) $p < 0.001$

GTP and GDP levels were unaltered by prolonged incubation in an isotonic glucose containing Krebs solution (table 9.2). However, a 60 minute incubation period in hypertonic media produced significant increases in both these nucleotides. Metabolic inhibition of the MDCK cells by exposure to 2 deoxy-D-glucose for 60 minutes in both isosmotic and hyperosmotic media significantly decreased the intracellular GTP levels to 25 % of the GTP levels of cells incubated in glucose-containing media. GDP levels in shrunken cells were elevated compared with the GTP levels observed in cells incubated under isosmotic conditions, but metabolic depletion greatly enhances the accumulation of the GDP from 8.05 ± 1.24 to 28.7 ± 3.9 ($\times 10^6$)/ 1 cell water.

EFFECT OF METABOLIC INHIBITION UPON THE K INFLUX OF HeLa AND MDCK CELLS.

Determination in the HeLa cell line.

A representative experiment of the effects of metabolic depletion upon the K (^{86}Rb) influx of HeLa cells is shown in table 9.3. The primary effect on the K (^{86}Rb) influx (under isosmotic conditions) of decreasing the metabolic status of the cell was a highly significant reduction in the total K (^{86}Rb) influx. Subdivision into the ouabain-sensitive, diuretic-sensitive and residual fluxes revealed significant reductions in all three components of the K (^{86}Rb) influx (expressed in $\text{nmol}/10^6$ cells.min). These results substantiate the previous reports of apparent metabolic dependence of the Na K Cl "cotransport" system(s) in a variety of cell types,

Table 9.2 Intracellular GTP and GDP concentrations of MDCK cells determined by HPLC (see Methods). Cells were exposed to isosmotic and hyperosmotic conditions in the presence of glucose or 2-deoxy-D-glucose for 60 minutes ^(a)

Incubation time min.	Sugar	Media tonicity mosmol/kg	peak area ($\times 10^6$)/1.cell water	
			GTP	GDP
zero	glucose	310	23.1 \pm 1.6	1.1 \pm 0.2
	glucose	510	37.0 \pm 2.5	1.7 \pm 1.4
60	glucose	310	21.4 \pm 1.7 ^{ns}	2.3 \pm 0.9 ^{ns}
	glucose	510	58.6 \pm 1.0 ^d	8.0 \pm 1.2 ^b
60	2-deoxy-D-glucose	310	5.4 \pm 0.8 ^d	2.4 \pm 0.5 ^c
	2-dexoy-D-glucose	510	13.6 \pm 1.6 ^d	28.7 \pm 3.9 ^d

(a) Data are the mean \pm SD (n=3) of a single representative experiment. Significance of difference from time zero values tested by Student's t-test. ns = not significant.

(b) p < 0.01

(c) p < 0.02

(d) p < 0.001

Table 9.3 K^+ (^{86}Rb) influx in HeLa cells under isosmotic and hyperosmotic (mannitol addition) conditions subdivided into ouabain-sensitive; diuretic-sensitive and residual components, in control or metabolically depleted cells (2-deoxy-D-glucose) (a)

Incubation time min.	Media tonicity mosmol/kg	Extracellular sugar	ATP _i % of control	K^+ (^{86}Rb) influx nmol/10 ⁶ cells.min			Residual
				Total	Ouabain sensitive	Diuretic sensitive	
15	310	glucose	100%	6.90 ± 0.29	4.52 ± 0.43	1.29 ± 0.39	1.09 ± 0.11
		2-deoxy-D-glucose	59%	3.24 ± 0.11 ^e	2.26 ± 0.57 ^e	0.25 ± 0.06 ^c	0.73 ± 0.09 ^c
15	510	glucose	100%	11.81 ± 0.69	3.36 ± 0.01	8.13 ± 0.31	0.33 ± 0.05
		2-deoxy-D-glucose	44%	9.58 ± 0.58 ^c	2.13 ± 0.40 ^d	7.15 ± 0.22 ^c	0.30 ± 0.17 ^{ns}
60	310	glucose	100%	7.64 ± 0.19	3.67 ± 0.21	1.70 ± 0.13	2.27 ± 0.06
		2-deoxy-D-glucose	30%	4.40 ± 0.13 ^e	2.10 ± 0.15 ^d	0.05 ± 0.31 ^d	2.25 ± 0.32 ^{ns}
60	510	glucose	100%	7.53 ± 1.78	3.47 ± 0.16	3.27 ± 0.29	0.39 ± 0.19
		2-deoxy-D-glucose	30%	3.53 ± 0.20 ^b	1.18 ± 0.19 ^b	1.66 ± 0.13 ^e	0.69 ± 0.09 ^{ns}

(a) Data are the mean ± SD (n=3) of a single representative experiment. Significance of difference from respective glucose values tested by Student's t-test. ns = not significant.

(b) p < 0.05

(c) p < 0.02

(d) p < 0.01

(e) p < 0.001

Footnote:

Incubation in the presence of 2-deoxy-D-glucose to 60 minutes (isosmotic conditions) significantly increased the cell volume in all experiments performed. Increase in the representative experiment: 2434 ± 53 to 2699 ± 105 μ³ (mean ± SD, n=6, p < 0.01)

notably MDCK, squid axon, avian and human erythrocytes (Rindler et al., 1982; Russell, 1983; Palfrey, 1983; Adragna, Perkins and Lauf, 1985).

Exposure to hyperosmotic (glucose) media increases the K (^{86}Rb) influx significantly (6.9 ± 0.29 to 11.81 ± 0.69 , mean \pm S.D., $n=3$, $P < 0.001$) which is due to an increased Na K Cl "cotransport", thereby confirming the results presented in chapter 5. The stimulation of the total K (^{86}Rb) influx by cell shrinkage was not abolished by metabolic depletion of the cells (3.24 ± 0.11 to 9.58 ± 0.58 , mean \pm S.D., $n=3$, $P < 0.001$), and this is due solely to an increased K (^{86}Rb) influx via the Na K Cl "cotransport" system. The magnitude of the stimulation of the K (^{86}Rb) influx through the Na K Cl "cotransport" system over the isosmotic conditions in metabolically replete or depleted cells did not differ significantly (6.84 ± 0.28 and 6.9 ± 0.13 , mean \pm S.D., $n=3$, respectively). This is in contrast to the avian erythrocyte where the reduction of cellular ATP ($> 5\%$ of controls) reduced the hypertonicity-stimulated Na K Cl "cotransport" to 28 % of controls (Palfrey, 1983).

To establish whether the discrepancy between the avian erythrocyte and the HeLa cells under hyperosmotic conditions was due to the difference in the cellular ATP concentrations, the effect of a prolonged incubation (60 minutes) in 2 deoxy-D-glucose was investigated (table 9.3). The cellular ATP concentration under these incubation conditions was 30% of the control cells (figure 9.1). Similar to the 15 minute incubation period in isosmotic media, the reduction of the intracellular ATP concentration was accompanied by a significant decrease in the total K (^{86}Rb) influx. This was attributable to a significant decrease in the ouabain-sensitive

component and an almost complete abolition of the Na K Cl "cotransport" pathway for K (^{86}Rb) influx. Exposure to hyperosmotic conditions (cell shrinkage) had no effect upon the total K (^{86}Rb) influx in either control or metabolically depleted cells, because of the markedly reduced residual component of K (^{86}Rb) influx in shrunken as compared with control cells. However, in order to counteract the decrease in the residual component, there were highly significant increases in the K (^{86}Rb) influx through the Na K Cl "cotransport" pathway. Again the hyperosmotic stimulation of the Na K Cl "cotransport" pathway in either the metabolically replete or depleted cells over isosmotic conditions were equivalent (1.57 ± 0.18 and 1.61 ± 0.19 , mean \pm S.D., respectively).

In experiments where HeLa cells had been incubated in the presence of the limited-metabolisable sugar 2 deoxy-D-glucose (isosmotic conditions), significant increases in the cellular volume (determined by electronic cell sizing, see methods), expressed in μm^3 , were observed (see footnote table 9.3). Thus the effect of metabolic depletion on the Na K Cl "cotransport" system of the HeLa cell line may be a secondary effect due to the increased cell volume. As has been shown (chapter 5), the activity of the Na K Cl "cotransport" is dependent upon the cell volume (see also Ueberschar and Bakker-Grunwald, 1983).

Determination in the MDCK cell line.

Similar to the HeLa cell line, the dependence of the K (^{86}Rb) influx of the MDCK cells upon the metabolic status of the cell was investigated and a representative experiment is shown in table 9.4. Under isosmotic conditions the cellular ATP concentration may be

Table 9.4 K^+ ($^{86}Rb^+$) influx in MDCK cells under isosmotic and hyperosmotic (mannitol addition) conditions subdivided into ouabain-sensitive, diuretic-sensitive and residual components in control or metabolically depleted cells
 ([2-deoxy-D-glucose]^a)

Incubation time min.	Media tonicity mosmol/kg	Extracellular sugar	ATP _i % of control	Total	K^+ ($^{86}Rb^+$) influx nmol/10 ⁶ cells·min		
					Ouabain sensitive	Diuretic sensitive	Residual
15	310	glucose	100%	9.60 ± 0.50	3.64 ± 0.34	5.31 ± 0.48	0.49 ± 0.05
		2-deoxy-D-glucose	39%	2.80 ± 0.19 ^d	1.42 ± 0.21 ^d	1.07 ± 0.18 ^d	0.31 ± 0.06 ^b
15	510	glucose	100%	14.08 ± 0.37	2.21 ± 0.56	11.60 ± 0.39	0.23 ± 0.39
		2-deoxy-D-glucose	33%	14.98 ± 1.06 ^{ns}	2.50 ± 0.68 ^{ns}	12.16 ± 0.13 ^{ns}	0.27 ± 0.02 ^{ns}
60	310	glucose	100%	10.99 ± 0.65	4.40 ± 0.46	6.16 ± 0.27	0.43 ± 0.52
		2-deoxy-D-glucose	21%	4.58 ± 0.17 ^d	2.73 ± 0.13 ^c	1.40 ± 0.09 ^d	0.45 ± 0.06 ^{ns}
60	510	glucose	100%	11.51 ± 0.82	3.10 ± 0.60	8.26 ± 0.36	0.15 ± 0.05
		2-deoxy-D-glucose	19%	10.38 ± 0.89 ^{ns}	0.87 ± 0.09 ^c	8.92 ± 0.29 ^{ns}	0.59 ± 0.09 ^c

(a) Data are the mean ± SD (n=3) of a single representative experiment. Significance of difference from respective glucose values tested by Student's t-test. ns = not significant.

(b) $p < 0.02$

(c) $p < 0.01$

(d) $p < 0.001$

Footnote:

Incubation in the presence of 2-deoxy-D-glucose for 60 minutes significantly increased the cell volume in all experiments performed. Increase in this experiment: 2189 ± 121 to 2831 ± 172 μ^3 (mean ± SD, n=6, $p < 0.001$)

reduced to 38% and 20% of control values after 15 or 60 minute incubations respectively in 2 deoxy-D-glucose media (tables 9.1 and 9.4). Under these experimental conditions the total K (^{86}Rb) influx was significantly decreased and this is associated with reductions in both the ouabain-sensitive and diuretic-sensitive components of the K (^{86}Rb) influx. The effect upon the diuretic-sensitive component confirms the previous report of Rindler and co-workers (1982), who demonstrated that metabolic inhibition of the MDCK cell by iodoacetic acid plus oligomycin or antimycin (ATP < 3% of controls) markedly inhibited the Na K Cl "cotransport" system of the MDCK cell line.

Exposure to hyperosmotic media produced significant increases in the total K (^{86}Rb) influx (15 minute incubation) in both metabolically replete and depleted cells (table 9.4). These increases are entirely associated with the diuretic-sensitive K (^{86}Rb) influx (Na K Cl "cotransport"). However, the hyperosmotic stimulation of the K (^{86}Rb) influx over the control value is markedly greater in the ATP depleted cells compared with control cells (510 - 310 influx values yield 11.09 ± 0.13 and 6.29 ± 0.36 nmol/ 10^6 cells.min., respectively, mean \pm S.D., n=3, P < 0.001). A similar effect on the diuretic-sensitive K (^{86}Rb) influx after a 60 minute incubation period was also observed.

Incubation in 2 deoxy-D-glucose for 60 minute (isosmotic media) produced marked cell swelling (see footnote table 9.4). Thus, the argument applied to the HeLa cell line, that the effect of the metabolic perturbation of the cell on the Na K Cl "cotransport" system may be a secondary one as a result of cell volume changes, may also be applied to the MDCK cell line. The possibility of regulatory roles of other nucleotides (i.e GTP, GDP) cannot be discarded at present.

DISCUSSION

The primary object of the experiments presented in this chapter was to investigate the effect of metabolic depletion of the HeLa and MDCK cells with respect to their K (^{86}Rb) influx, via the Na K Cl "cotransport" pathway. Perturbation of metabolism was achieved by the non-metabolisable sugar 2 deoxy-D-glucose which, after phosphorylation to 2 deoxy-D-glucose phosphate, cannot be further processed through the glycolytic pathway, resulting in a decline of cellular ATP levels (Lauf, 1983; Adragna et al., 1985). In the established cell lines used the relative concentrations of glycolytic enzymes present indicate that glucose is a prime energy source via glycolysis (Gstraunthaler, Pfaller and Kotanko, 1985). For both the HeLa and MDCK cell lines, the effect upon the intracellular ATP of incubation in 2 deoxy-D-glucose media was assessed by Hplc of cell extracts.

The inhibition of the cell metabolism not only affected the ATP concentration but also reduced the ATP/ADP ratio and the GTP/GDP ratio in both cell lines. The complete nucleotide profiles of the cells, illustrated in figure 2.2 of the materials and methods, were affected, producing notably large increases in the peaks whose retention times were 5 - 8 minutes. Therefore, in assessing the effect of metabolic depletion upon the K (^{86}Rb) influxes, caution should be exercised in directly attributing any response of the K (^{86}Rb) influx solely to changes in the cellular ATP concentrations.

In all experimental conditions, incubation in 2 deoxy-D-glucose-containing media significantly reduced the activity of the Na K Pump, which is similar to the response of the Na K Pump to

metabolic inhibition in avian erythrocytes (Palfrey, 1983). It is unlikely that this is due to the reduced ATP concentration, since the ATP is still at saturating concentration for the Na K ATPase (see Jorgensen, 1982) even after a 60 minute incubation in 2 deoxy-D-glucose media.

The response of the cell types Ehrlich ascites cells, human erythrocytes, avian erythrocytes, squid axon and MDCK cells (Geck et al., 1978; Adragna et al., 1985; Palfrey, 1983; Russell, 1983; Rindler et al., 1982) to metabolic inhibition demonstrate consistent effects upon the Na K Cl "cotransport" system(s) of these cells. The present work on the effect of the inhibition of cell metabolism on the K (⁸⁶Rb) influx of the HeLa and MDCK cells maintained in isosmotic conditions corroborates reports in the literature for the other cell types and MDCK cells. However, the radical degree of metabolic inhibition required to affect the Na K Cl "cotransport" system(s) in the previous reports (cellular ATP less than 5% of controls) was not necessary in the present work, as an ATP concentration of 40-60 % of control value reduced the K (⁸⁶Rb) influx through the Na K Cl "cotransport" pathway to 20% of the controls. This discrepancy is particularly evident in MDCK cells, since protocols which produce a similar reduction in ATP to this present work (antimycin (mitochondrial inhibitor), ATP - 50% of control, Rindler et al., 1982) had no effect on the Na K Cl "cotransport". The reason for this is most probably due to secondary effects on cell volume (see below), but it may also be due to differences in mitochondrial and glycolytic inhibition of metabolism. No comparison of the nucleotide profile can be made as all previous studies assessed only cellular ATP as a measure of the cell metabolic status by assaying for ATP by the Luciferin luciferase technique.

MDCK and HeLa cells exposed to isosmotic media containing 2 deoxy-D-glucose underwent a significant cell swelling (see Results), possibly due to the reduced activity of the Na K Pump and/or a significant accumulation of the limited-metabolisable 2 deoxy-D-glucose phosphate, which would be similar to the human erythrocyte (Adragna et al., 1985). The activity of the Na K Cl "cotransport" system for both the HeLa and MDCK cells is sensitive to relatively small changes in cell volume (see chapter 5), with cell shrinkage producing a marked stimulation of the Na K Cl "cotransport" system, while exposure of the HeLa cell line to hyposmotic media (210 mOsm/kg) significantly decreased the activity of this transport pathway (Tivey et al., 1985). A possible mechanism for the action of the metabolic inhibition of the HeLa and MDCK cells on their Na K Cl "cotransport" systems may be a secondary event related to cell volume rather than a direct regulatory role of the ATP or its metabolites on the Na K Cl "cotransport" pathway.

If increased volume accounts for the decrease in the activity of the Na K Cl "cotransport" system(s) of metabolically depleted HeLa and MDCK cells, then shrinkage of the cells by exposure to hyperosmolar media should reactivate the Na K Cl "cotransport" in metabolically depleted cells. This is the case, with the K (^{86}Rb) influx of the MDCK cells (controls or metabolically depleted) via the Na K Cl "cotransport" pathway being equivalent in hypertonic media (see results). In HeLa cells the stimulation of the Na K Cl "cotransport" by cell shrinkage over the isosmotic level of K (^{86}Rb) influx in either the metabolically replete or depleted cells were equivalent. However, as the stimulation of the diuretic-sensitive K (^{86}Rb) influx by cell shrinkage in ATP-depleted cells was not to the level of K

(⁸⁶Rb) influx in shrunken metabolically replete cells, a portion of the Na K Cl "cotransport" flux was not reactivated by cell shrinkage. This may mean that a portion of this flux is dependent upon the metabolic status of the cell, but the reason for this is unclear. The primary conclusion is that the hyperosmolar stimulation of the Na K Cl "cotransport" pathway is apparently independent of the metabolic status of the cell within the limits of ATP achieved and this is similar to the effect of metabolic depletion upon the diuretic-sensitive K transport of avian erythrocytes (Palfrey, 1983; Ueberschar and Bakker-Grunwald, 1985).

A speculative second explanation of the above may be that GTP and/or its metabolites has a regulatory role, since, under all experimental conditions, the levels of GTP were elevated by hypertonic shrinkage of the cells. Thus, as suggested by Kregenow et al., (1976), the intracellular cGMP may act as a regulator of the Na K Cl "cotransport" in shrunken cells. Further work is required however to substantiate this hypothesis. The mechanism by which the stimulation by hypertonicity of the Na K Cl "cotransport" pathway of the HeLa and MDCK cell lines occurs is independent of the cellular ATP concentration of the cell and thus remains unclear.

CHAPTER 10

CONCLUDING REMARKS

The primary finding of this thesis is that the diuretic-sensitive K transport of the established cell lines, HeLa and MDCK (chapter 3), may be stimulated by cell shrinkage, initiated by media rendered hyperosmotic by the addition of 200 mM mannitol (chapter 5). This was principally due to an increase in the maximal velocity of this K transport. In both control and shrunken (HeLa and MDCK) cells, the diuretic-sensitive K transport may be considered to be mediated by a Na K 2Cl "cotransport", based on kinetic analysis of ion activation studies and the pharmacological sensitivity to "loop" diuretics of this K transport, as outlined by Palfrey and Rao (1983). Under the experimental conditions of this present work, direct evidence for inhibition of unidirectional Na and Cl fluxes by "loop" diuretics was technically impossible (chapter 4). However, the striking similarities between the diuretic-sensitive K transports of HeLa and MDCK cells, with the latter being known to possess a Na K 2Cl "cotransport" system (McRoberts et al., 1982), are strong evidence to indicate a coupled trans-membrane transport of the three ions Na K and Cl, rather than a Na- and Cl-dependent, diuretic-sensitive K transport in the HeLa cell line.

This stimulation of Na K Cl "cotransport" in shrunken HeLa and MDCK cells represents an activation of this transport system similar to that observed in other cell types in particular avian erythrocytes (Schmidt and McManus 1977 a,b,c; Ueberschar and Bakker-Grunwald, 1983) and Ehrlich ascites cells (Geck et al., 1981; Hoffmann 1982, 1985; Hoffmann et al., 1983) undergoing volume regulation. In control and shrunken HeLa and MDCK cells, the Na K Cl "cotransport", although mediating a substantial unidirectional K flux, did not contribute to a

net trans-membrane movement of ions, thereby indicating balance between influx and efflux via this transport system. The driving force of the Na K Cl "cotransport" system(s) is considered to be the sum of the chemical gradients of the ions involved (Schmidt and McManus, 1977c; Haas et al., 1982); thus in control and shrunken cells, the sum of the chemical gradients should be zero since no net transport of ions has occurred. This is true for control cells (see discussion chapter 3), but in shrunken cells, since both HeLa and MDCK cells behave as perfect osmometers (chapter 5; Tivey et al., 1985; Simmons, 1984) and cellular ion content remains constant, then the intracellular concentrations should increase in proportion to the media tonicity. Thus the equations derived by Schmidt and McManus (1977c) and Haas et al., (1982) will clearly not yield a value of zero for the sum of chemical gradients in shrunken cells. In truth, the gradients are such that a large net efflux should be observed, since no change in intracellular ion content occurs, the predicated outward transport is clearly not tenable. Thus the equations cited in chapter 3 either do not hold true in shrunken cells or a significant component of the intracellular ions is not osmotically active.

Therefore, in HeLa and MDCK cell lines a decrease in cell volume stimulated the Na K Cl "cotransport", but the increased activity of this transport system did not alter cell volume. Cell shrinkage was effected by media rendered hyperosmolar by 200 mM mannitol addition, and absence of cell volume regulation in this experimental protocol is similar to results obtained in Ehrlich ascites cells when shrunken by sucrose addition to the media (Hendil and Hoffmann, 1974). Thus, decreases in HeLa and MDCK cellular volume per se do not initiate the phenomenon of volume regulation. A decreased intracellular Cl appears to be necessary after cell shrinkage for a volume increase to occur

in the frog skin epithelia (Ussing, 1982) and Ehrlich ascites cells (Hoffmann, 1985). Therefore, the Na K Cl "cotransport" of HeLa and MDCK cells may be a system concerned with K homeostasis rather than volume regulation (see discussion Duhm and Goebel, 1984).

The profound effect of cell shrinkage upon Na K Cl "cotransport" gives rise to two important questions. First, how do the HeLa and MDCK cells recognise the decrease in cell volume and thus regulate the Na K Cl "cotransport"? Secondly, how does the Na K Cl "cotransport" achieve the increased K transport?

With regard to the latter question, since the increase in the diuretic-sensitive K transport is principally due to an increased maximal velocity, then this elevated K transport could be achieved by an increase in either the number of Na K Cl "cotransport" entities or the turnover (molecules/site.second) of existing Na K Cl "cotransport" entities. To investigate the above, a marker specific to the Na K Cl "cotransport" is required. As bumetanide is a relatively high affinity inhibitor of Na K Cl "cotransport" (chapter 3 and 5) and exhibits little or no inhibition of other transport systems (Lant, 1984), a radiolabelled form could provide a useful tool for investigating the Na K Cl "cotransport" in cultured cell lines. This has been demonstrated by work performed in this laboratory (Rugg et al., 1985) and is confirmed in chapter 8. Radiolabelled "loop" diuretics have also been used successfully to identify and characterise the presence of Na K Cl "cotransport" in a variety of tissues (Forbush and Palfrey, 1983; Jorgensen, 1984; Hannafin et al., 1983).

Exposure to media of varying osmolalities produced progressive but saturable increases in the diuretic-displaceable [^3H] bumetanide

uptake, and this was similar to the dependence of diuretic-sensitive K transport on the media tonicity (chapter 5). In order to determine whether these two phenomena are correlated, both the uptake of [³H] bumetanide and the diuretic-sensitive K transport in isosmotic and hyperosmotic media should be measured in the same cell batch. The turnover rate of molecules/site.second should remain constant if the increased K flux is due to a greater number of functional Na K Cl "cotransport" entities in the membrane. However, turnover of K by the Na K Cl "cotransport" pathway was either decreased or increased in different experiments. At present, data indicate that the increase in K transport associated with the Na K Cl "cotransport" pathway is effected by cells recognising the number of Na K Cl "cotransport" entities in the membrane and regulating the turnover rate. How the cells interpret the decrease of cell volume into an increased K transport will be the subject of the remaining discussion.

Clearly cell shrinkage will produce marked changes in the intracellular environment; for example, the intracellular ion concentration will increase. Could such changes be responsible for the stimulation of the diuretic-sensitive K transport? Changes in the intracellular ion concentrations would occur due to cell shrinkage, which is rapid compared to the stimulation of the K transport, half time being 6-9 minutes (chapter 5). If simple ion concentration changes produced the increased K transport, one might expect similar time courses for both events. Other parameters which are dependent upon the cytosolic ionic composition are membrane potential and pH. Cell shrinkage had no effect upon the membrane potential of MDCK cells therefore it would appear unlikely to be a regulator of the Na K Cl "cotransport" system.

Intracellular pH was shown to change with cellular volume (chapter 7), with a significant alkalinisation of the cytosol occurring in shrunken cells. If no regulatory mechanism for pH is present, then one would expect an acidification of the cytosol; thus the alkalinisation probably represents an activation of a Na:H antiport which is present in the MDCK cell (Rindler and Saier, 1981) producing an intracellular alkalinisation with a concurrent increased Na uptake. The activation of such an antiport may be a direct response to the increased H concentration which would occur upon the initial shrinkage of the cell or an effect of the second messenger diacylglycerol (Berridge, 1984) assuming that this messenger is elevated by cell shrinkage (see later). For pH to be a regulator of Na K Cl "cotransport" in both the HeLa and MDCK cell lines, then a pH dependency of this transport system in both cell lines must be established. The Na K Cl "cotransport" systems of erythrocytes of human and avian origin (Garay, et al., 1981; Ueberschar and Bakker-Grunwald, 1983) do exhibit a pH dependency. However, the profile for each cell line is different, with human red cells having a bell shaped profile with a maximal activity at pH 7.1, whereas the avian red cells cAMP-stimulated "cotransport" activity increased progressively from pH 6.0-8.0. Clearly if pH is to modulate the activity of the Na K Cl "cotransport" in shrunken cells the absolute intracellular pH, degree of cytoplasmic alkalinisation in shrunken cells and the pH profile of this transport system are required, since alkalinisation of the cytoplasm could result in either the activation or inhibition of this transport system depending upon the pH sensitivity.

The metabolic status of a variety of cell types, e.g. MDCK, squid axon and Ehrlich ascites cells, affects the activity of the Na K

Cl "cotransport" pathway (see Palfrey and Rao, 1983; chapter 9). The requirement of ATP as an energy source for this transport pathway is unlikely (Lubowitz and Whittam, 1969; Geck, et al., 1980), thus any effect of metabolic inhibition implies either a regulatory role for ATP and/or general cell metabolism. In this present work, metabolic inhibition of both HeLa and MDCK cells, achieved using the limited metabolisable sugar 2 deoxy-D-glucose, significantly reduced the activity of the Na K Cl "cotransport" pathway. The activity of this transport pathway is sensitive to relatively small changes in cell volume (chapter 5) and exposure of HeLa cells to hyposmotic media resulted in a significant inhibition of the "cotransport" pathway (Tivey, et al., 1985). Thus inhibition of the Na K Cl "cotransport" pathway in metabolically depleted cells may be considered a secondary event of cell swelling since metabolic inhibition caused a significant increase in cellular volume, with the re-shrinking of metabolically depleted cells reactivating the Na K Cl "cotransport" pathway (chapter 9).

A possible regulation of the Na K Cl "cotransport" pathway may be mediated by other cytosolic nucleotides. In the avian red cell Na K Cl "cotransport" may be activated by cAMP (Kregenow, et al., 1976; Palfrey and Greengard, 1981; Ueberschar and Bakker-Grunwald, 1983), but in the avian red cell this cAMP activation is via an independent pathway to the increased Na K Cl "cotransport" mediated by cell shrinkage (Ueberschar and Bakker-Grunwald, 1983). Further evidence for this dichotomy is the lack of effect upon the cellular cAMP content of the avian red cell when exposed to hyperosmolar media (Kregenow, et al., 1976), which has been corroborated in chapter 6. A role for protein phosphorylation has been proposed in the cAMP-activated pathway (Alper et al, 1980 a,b; Palfrey and Greengard, 1981) but the

mechanism by which cell shrinkage activates the Na K Cl "cotransport" is not known (Palfrey and Rao, 1983). It is noteworthy that cAMP activation of the Na K Cl "cotransport" is not present in either MDCK or HeLa cells nor does increased intracellular cAMP affect these cell lines' response to cell shrinkage (chapter 6).

Cyclic GMP was suggested by Kregenow and co-workers (1976) as the possible intracellular messenger in the activation of the Na K Cl "cotransport" pathway in shrunken cells. Although intuitively cGMP should behave as a second messenger, the precise function of this nucleotide is still ill-defined. However, roles for this nucleotide have been reported, e.g. in the retina, in particular the rod cells' cGMP maintains Na channels open (see Berridge, 1985) and in the intestine of flounder and rabbit ileum, cGMP has an inhibitory action on the Na K Cl "cotransport" (see Palfrey and Rao, 1983). In the present work no direct evidence for cGMP involvement in regulating the Na K Cl "cotransport" of the MDCK and HeLa cell lines has been presented. However, the nucleotides GTP and GDP were consistently elevated in shrunken cells which were both metabolically replete and depleted (chapter 9), therefore it seems likely that cGMP is also elevated under these conditions and the action of cGMP requires elucidation.

Does the raised intracellular GTP in the shrunken cell affect a G regulatory protein in the membrane thereby directly or indirectly effecting an increase, in the Na K Cl "cotransport" system? If so, then it is unlikely to be via the adenylate cyclase system since both HeLa and MDCK cells were unaffected by an increased cellular cAMP (see above). However, G proteins are important in the activation of the Inositol lipid pathway of second messengers (Berridge, 1984, 1985).

With G proteins activating a PIP_2 phosphodiesterase (PDE) which cleaves PIP_2 into the second messengers inositol triphosphate (IP_3) and diacylglycerol (DG). As there is a requirement for GTP in the initial step of this reaction, then an increase in the cellular concentration of this nucleotide may effect a response from the inositol lipid pathway.

Could IP_3 and DG trigger an increase in the activity of the Na K Cl "cotransport" in shrunken cells? The latter's (DG) cellular action is through C-kinase with the subsequent phosphorylation of proteins to their active form e.g. the Na :H antiport (see Berridge, 1984). Thus DG has the potential for activating membrane protein and may be involved in regulating the Na K Cl "cotransport" system. Clearly two questions require investigation: first, does cell shrinkage elevate cellular DG and secondly, would the application of phorbol esters, which act directly upon the C-kinase, affect the activity of the Na K Cl "cotransport" system? The second of the two second messengers derived from PIP_2 is IP_3 , whose action is mediated by modulating the intracellular Ca concentration, with increased intracellular Ca activating the Ca /CaM Kinase which effects a phosphorylation of protein, thus producing the cellular response. The action of this pathway is mimicked by the ionophore A23187 (Berridge, 1984 and 1985). In MDCK cells, increasing intracellular Ca by means of the ionophore A23187 in the presence of the K channel blocker Ba produced a progressive stimulation of K efflux which was bumetanide-sensitive; whether this increased flux is mediated by the Na K Cl "cotransport" requires verification but the results do imply a possible role for cellular Ca as a regulator of the Na K Cl "cotransport" (chapter 6). Thus in future work a detailed investigation of the metabolism of the inositol lipid pathway and intracellular Ca mobilisation in shrunken

cells is required, both of which are now technically possible by HPLC and single cell fluorimetry (using the probe flura II) respectively.

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