MEMBRANE TRANSPORT ALTERATIONS

IN

DISEASE STATES

Thesis submitted for the degree of

Doctor of Philosophy

by

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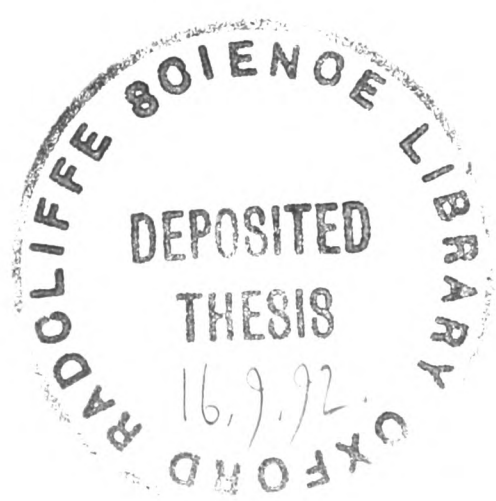

ABSTRACT

MEMBRANE TRANSPORT ALTERATIONS IN DISEASE STATES

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The present thesis investigates cell membrane abnormalities in selected diseases. Membrane transport systems are studied in chronic renal failure (CRF), endstage diabetic nephropathy, renal transplantation and atherosclerotic vascular disease. The role of membrane cholesterol concentration in modulating transport activity is also explored.

Erythrocyte choline uptake is elevated in CRF and here it is shown that transporter activity returns to normal following a successful kidney transplant. Choline uptake recovers in parallel to the plasma creatinine changes. Plasma factors are suggested to play a role in transport activity in uraemia. The activity of the leucocyte sodium-hydrogen antiporter (Na/H) is shown to be normal in CRF with or without end-stage diabetic nephropathy. Erythrocyte lactate transport was investigated at 37°C for the first time. Kinetic parameters of transport were characterized. Lactate uptake in erythrocytes is mediated mainly via the monocarboxylate carrier. No lactate transport alterations are present in uraemic patients when compared with normal subjects.

The possible influence of membrane cholesterol on the activity of transporters in disease was approached in two ways. First, cell cholesterol content was modulated *in vitro* employing liposomes. With this approach, lymphoblast Na/H antiporter activity is inhibited by cholesterol-enrichment while activation is seen with cholesterol-depletion. Erythrocyte choline uptake was insensitive to cholesterol modulation. It is suggested that membrane cholesterol content is unlikely to contribute to the membrane transport abnormalities in the uraemic syndrome. In erythrocytes liposome depletion of cholesterol reduced Na/Li countertransport activity. *In vivo* treatment with the HMG CoA reductase inhibitor simvastatin, which reduces plasma cholesterol levels, was employed to look at Na/Li countertransport activity in patients with atherosclerotic vascular disease. Although simvastatin reduces mean cholesterol by about 30%, there was no change in erythrocyte mean cholesterol content. Erythrocyte Na/Li countertransport was unaffected by simvastatin treatment. Accordingly an *in vivo* relationship between membrane cholesterol and Na/Li countertransport activity could not be tested in this study.

The present results emphasize the selective nature of alterations in membrane composition and function in various disease states.

TO ANA

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CHAPTER 1

GENERAL INTRODUCTION

The research presented in this thesis concerns membrane transport in cells from human subjects with a range of clinical diseases. The cell membrane is essential to the cell and has numerous functions. Alterations in its composition or function can be involved in pathological states. I do not intend to review all membrane transport systems in all diseases- perhaps a impossible task. Rather the objective here is to study selected examples of transport systems and diseases and to use as models for possible interactions between membrane function and clinical disorders.

The study of membrane transport in human disease is important for several reasons other than just providing a better understanding of disease pathophysiology. Membrane transport abnormalities can be the cause of disease, as in the case in cystic fibrosis, where an abnormally low trans-epithelial chloride transport is present [Higgins & Hyde 1991, Riordan et al 1989]. In patients with essential hypertension an abnormally elevated rate of erythrocyte sodium-lithium countertransport (Na/Li countertransport) is manifest and has been postulated to be a disease marker, being valuable for diagnostic and research purposes [Canessa et al 1980]. The presence of transport abnormalities may be a secondary or adaptive response in disease as shown in malaria-infected erythrocytes, where some transport systems show abnormal function and certain cryptic transporters are expressed [Kirk et al 1991, 1992]. Finally membrane transport can be the target for therapeutic action of drugs as in the inhibition of the sodium-potassium ATPase (Na/K ATPase) by the cardiac glycosides used for treatment of cardiac failure and supraventricular arrhythmias [Sweadner & Goldin 1980], or the inhibition of Na/K/Cl cotransport by loop diuretics

[Hendry & Ellory 1988].

The connection between membrane transport and disease has been sought previously. Hypertension is an example of a clinical disorder in which much research on the properties and function of cell membranes has been carried out [Garay et al 1982, Canessa et al 1980, Blaustein 1977, Mongeau et al 1990, Postnov & Orlov 1985]. In this thesis attention will be directed principally towards renal disease, which is my main interest.

The models chosen in the present study were varied, and are listed below. First, erythrocyte choline transport in patients with chronic renal failure (CRF) and following kidney transplantation was analyzed. Erythrocytes were also used to look at the transport of lactate under physiological conditions and in patients with CRF. Experiments on the sodium-hydrogen antiporter (Na/H antiporter) activity and intracellular pH (pHi) in uraemic patients with or without diabetic nephropathy were performed using the white cell as a model system. A novel approach was used to look at the relationship between membrane cholesterol levels and transfer of selected substrates across the cell membrane via the Na/Li, Na/H and choline transporters. Initially, liposomes were employed for the *in vitro* modulation of cell membrane cholesterol content, and membrane transport activity was examined. The models used were the lymphoblast Na/H antiporter, the erythrocyte Na/Li countertransport and the erythrocyte choline carrier. In order to study influences of cholesterol modulation *in vivo* on transport activity, patients suffering from atherosclerotic vascular disease and treated with the HMG CoA reductase inhibitor, simvastatin, were examined. Simvastatin treatment results in reduction of blood cholesterol. The activity of the Na/Li countertransporter was measured in erythrocytes obtained before and following treatment.

For each transporter and cell type, different methods for cell separation and ion transport measurements were required. The various techniques chosen will be introduced and discussed later.

The introduction to this thesis will have two parts. First an introduction to membrane transport and then a description of the association between disease and membrane transport.

The first part of the introduction will start by looking at membrane structure and how it affects transport. This is particularly relevant because some studies involved interference with that structure by modifying cell cholesterol levels.

1.1. MEMBRANE COMPOSITION AND MEMBRANE TRANSPORT

The mechanisms responsible for altered membrane transport in disease are varied. Both endogenous and exogenous factors may play a role, and a combination of different influences is a common feature. Genetic determinants create a predisposition on which environmental factors may act. Factors may act upon the transport protein directly, or on protein synthesis and/or protein regulators which can induce changes that modulate its copy number, activity or function.

In this work special consideration was given to the membrane lipid fraction, and in particular to the membrane cholesterol. The rationale behind this was the known interaction between the major components of the cell membrane. Cholesterol is one of the major components of cell membrane and a molecule very much involved in the concept of "health and disease". It is particularly important when considered in relation to disorders involving increased risk of vascular disease, such as the ones examined here.

In the next section basic concepts of cell membrane structure, composition and function will be reviewed.

1.1.1. MEMBRANE CELL STRUCTURE

All human cells are surrounded by an external membrane (plasma membrane) providing a reactive interface and barrier between external medium and cytoplasm. Although different tissues perform distinct functions, the general structure and function of cell membranes is common. The cell membrane has a wide range of functions including the formation of physical boundaries, maintenance of cell composition, selective transport of substrates or waste products, transduction of chemical signals and the provision of an ideal environment for the appropriate intracellular functions [Evans & Graham 1989].

Singer and Nicolson in the 70's [Singer and Nicolson 1972] described the fluid mosaic model for the membrane structure, and stressed its dynamic aspect. A diagram based on their work is reproduced as figure 1.1.a. The relative proportions of the membrane components do not necessarily reflect reality, since the proportion of protein is usually higher than as shown in the figure [Yeagle 1987, 1989] and varies with cell type [Stein 1990]. The figure shows the basic structure of the cell membrane as a lipid bilayer in which proteins are embedded, together with smaller amounts of associated carbohydrates. Lipids provide the membrane's main structure due to lipid hydrophobic effects and amphipathic characteristics (hydrophobic and hydrophillic groups within the same molecule) [Yeagle 1989]. A hydrophobic environment exists in the middle of the bilayer populated by hydrocarbon chains while the lipid polar heads face the aqueous phase. The relative amounts of lipid also vary with the type of membrane, and table 1.1 illustrates the composition of human erythrocyte. In human cells phospholipids are the predominant component, however the molar ratio of phospholipid:cholesterol is close to 1:1.

TABLE 1.1
ERYTHROCYTE MEMBRANE COMPOSITION

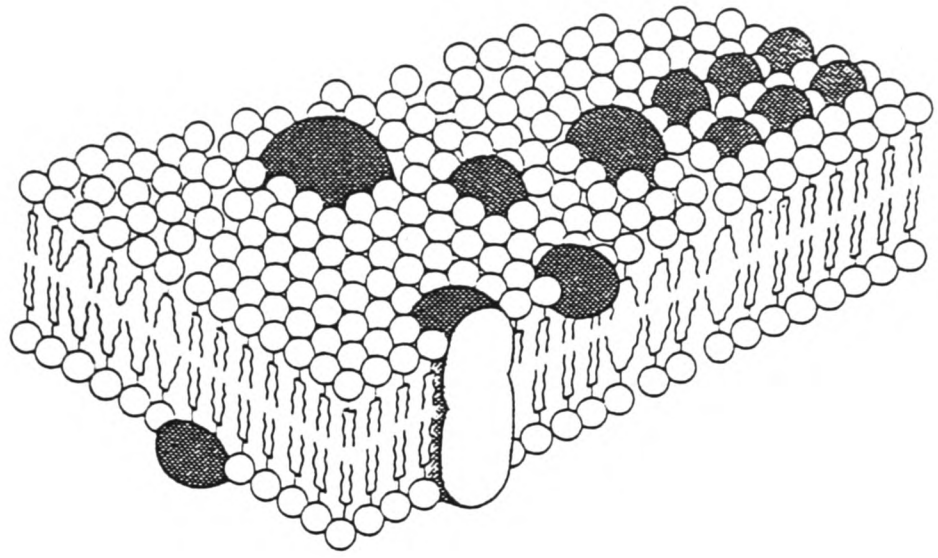
LIPID/PROTEIN RATIO	0.75	
	(weight)[a]	(mole %)[b]
MAJOR LIPIDS		
Phosphatidylcholine	25 %	17%
Phosphatidylethanolamine	22 %	16%
Phosphatidylserine	10 %	6%
Sphingomyelin	18 %	17%
Cholesterol	25 %	45%

a. Gennis 1989; b. Yeagle 1987

The distribution of molecules in the membrane is asymmetrical. Membrane protein asymmetry is a consequence of the way in which proteins are originally inserted into the bilayer [Gennis 1989]. The mechanisms leading to membrane lipid asymmetry is not completely clear. It is possible that physical forces and cytoskeletal interactions may be involved. "Flip-flop" motion of lipids (molecule rotation of 180 degrees about an axis in the plane of the membrane) is helped by the presence of ATP-requiring enzymes "flipases" or "translocases" [Gennis 1989] contributing to the asymmetry. The human erythrocyte membrane lipid composition is an example where phosphatidylcholine and sphingomyelin are the main phospholipids at the external interface and phosphatidylethanolamine and phosphatidylserine at the inner side (figure 1.2) [Verkleij et al 1985]. Lipids, and proteins, can also rotate about their axes and diffuse laterally [Stubbs 1983, Carruthers & Melchior 1988, Yeagle 1989, Spector & Yorek 1985]. This dynamic equilibrium is important and results in domains maintaining diverse functions and regulating biological function.

Cholesterol is a major lipid component of the cell membrane. An interesting feature of cholesterol is that it is essential for normal mammalian cell growth and function, and yet, as stated by Yeagle, often plays a lethal role in the development of vascular disease [Yeagle

A



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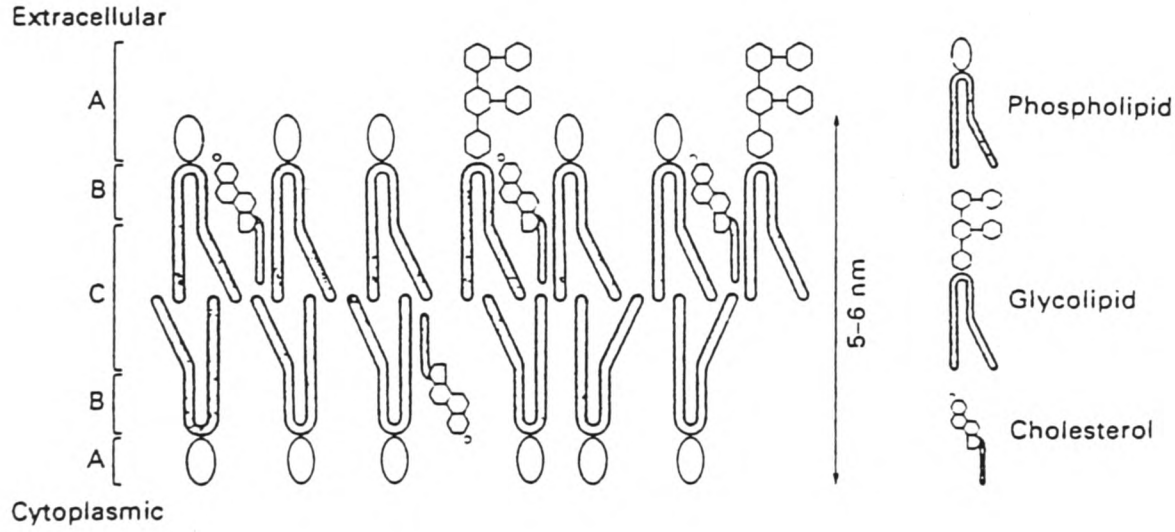


FIGURE 1.1: Cell membrane structure. (a) Model of the cell membrane as suggested by Singer-Nicholson, with a phospholipid bilayer in which proteins are embedded [reproduced from Jain 1988, Copyright © 1988 John Wiley & Sons, Inc. Reprinted by kind permission of John Wiley & Sons, Inc]. (b) Schematic representation of the membrane lipid bilayer with the presence of cholesterol, glycolipid and phospholipid. A refers to polar regions, B non-polar region-fluidity modulated by cholesterol and acyl chain unsaturation, C fluid, non polar region. [reproduced from Evans & Graham 1989 by kind permission of Oxford University Press].

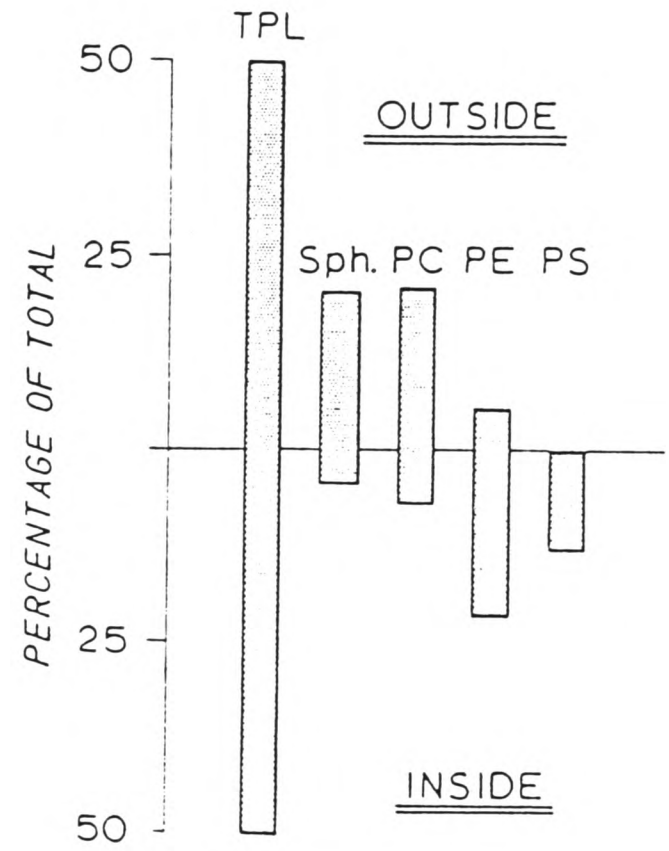


FIGURE 1.2: Distribution of erythrocyte membrane phospholipids is asymmetric [reproduced from Verkleij et al 1985 by kind permission of Elsevier Science Publishers].

1987]. It belongs to a group of lipids that cannot form bilayers by themselves upon hydration, but can interdigitate into bilayers to modify bilayer structure [Carruthers & Melchior 1988]. It consists of a planar, fused-ring nucleus, a polar hydroxyl group, and a hydrocarbon tail. Cholesterol increases ordering of the lipid bilayer due to the effect of its rigid sterol structure on the lipid components of the membrane [Yeagle 1985, 1989]. This effect on membrane fluidity is one of the possible mechanisms by which cholesterol affects membrane permeability [Cooper 1977, Shinitzky et al 1980, Gier et al 1980]. Cholesterol may also alter the behaviour of membrane transport proteins by direct protein-cholesterol interactions [Spector & Yorek 1985].

The ability of cholesterol to move from one membrane to another was used in this thesis to study its participation in regulating membrane function [Bruckdorfer et al 1968a,b, 1969, Dawidowicz 1987, Shinitzky & Inbar 1974]. Cholesterol can exchange between membranes, or net cholesterol flux can occur when there are concentration gradients of cholesterol between the cells and vesicles. In plasma, lipoproteins participate in this cholesterol transfer [Norum et al 1983, Miller 1984, Mahlberg & Rothblet 1992]. Some evidence suggests that this exchange occurs via the aqueous phase, and it is possible that cholesterol is distributed according to thermodynamic equilibrium. Alternatively, it can be maintained by a flux of cholesterol through some sorting process from the site of synthesis to the plasma membrane [Yeagle 1987]. Cholesterol is also asymmetrically distributed in the different cell membranes [Kier et al 1986]. In erythrocytes, cholesterol is nearly symmetrically distributed between the two faces of the lipid bilayer. Rapid transbilayer movement of cholesterol occurs and may assist in the necessary shape changes of erythrocytes in the circulation [Yeagle 1987, Lange et al 1980, 1981].

Membrane lipids are in a fluid state at physiological temperatures where the degree of fluidity depends on chemical composition [Mato 1991]. Passage through this fluid barrier is possible for hydrophobic substances. Charged or hydrophilic groups such as the major

cellular metabolites will seldom cross the lipid barrier of the cell directly. Rather this transport function will be provided by specific membrane proteins. The lipid composition is regulated by the cell, but will also vary with cell age, cell cycle and a variety of stimuli or changes in the environment and physiological state [Stubbs & Smith 1984].

Membrane proteins embedded in the lipid bilayer can be intrinsic or extrinsic. Intrinsic proteins are bound firmly to the membrane and linked by van der Vaals forces to the lipid hydrocarbon chains. Proteins that span the membrane include those that are involved in transmembrane transport of substrates. Lipids and proteins exist in intimate association in the membrane and one might expect cell membrane lipid to play a role in modulating membrane protein activity. Membrane-bound enzymes usually require a lipid bilayer to maintain full activity, and the bilayer provides a medium where the hydrophobic regions of proteins can be anchored [Clandinin et al 1991].

The membrane is organized in such a way that domains exist where lipid-protein interactions are highly specific and organized to reflect specific cellular functions [Yeagle 1989, Clandinin et al 1991]. Specific interactions between lipid and protein led to the concept of a lipid annulus that provides a specific microenvironment around hydrophobic regions of functional membrane proteins, enabling lipid to influence particular membrane proteins independently [Clandinin et al 1985, 1991, Yeagle 1989]. Interactions between those two elements may be achieved by several mechanisms including: associations with membrane cytoskeleton, conformational interactions, changes in acyl chain length and saturation, fluidity and molecular charge [Spector & Yorek 1985, Yeagle 1989, Clandinin et al 1991].

Carbohydrates are attached to the proteins or lipids. The cells also have a cytoskeleton that stabilizes the plasma membrane and maintains cell shape and some cell types may have intercellular junctions. With the rare exception of erythrocytes, human cells usually have

internal membranes, but these are not going to be considered in this thesis.

Endogenous and environmental factors interact together to maintain and regulate membrane composition. Any alterations in the precise balance may contribute to pathological conditions. It was the intention to look at alterations in transport of molecules through the membrane in a range of diseases and to examine the possible role of modulation of lipid composition in affecting certain transporters. The routes by which molecules can cross membranes will now be reviewed.

1.1.2. MEMBRANE TRANSPORT MECHANISMS

There are several good reviews on this topic [Stein 1986, 1989, 1990; Christensen 1975; Jain 1988; Bonting & De Pont 1981; Ellory & Young 1982; Ellory & Lew 1977; Ellory et al 1988a; Gennis 1989, Sachs & Fleischer 1989, Klingenberg 1989, Aronson 1990, Clark 1988, Höfer & Hoggett 1981].

The lipid bilayer provides an effective barrier to most small solutes. Some permeants can move across the cell membrane down an electrochemical gradient "unaided" by membrane components. This *simple diffusion* will depend on substrate factors such as size, charge, hydrophobicity, and on the lipid bilayer characteristics. Mediated transport of substances is mainly protein-mediated and can operate by various mechanisms, such as *pores and channels, facilitated diffusion and primary or secondary active transport.*

Pore and channels consist of proteins that form "tunnels" through the membrane in which binding sites for solutes are accessible from either side at the same time. Pores have usually a poor selectivity and discriminate solutes primarily on the basis of their size. The term channel is usually reserved to describe ion channels which can be highly selective. These proteins

have different mechanisms by which they can be in an open or closed state, allowing, or not, the traffic of solutes; such gating mechanisms are controlled by voltage, ligands, pressure, phosphorylation and other stimuli.

Active transport is defined as transport of substrate against its electrochemical gradient, and requires energy for this uphill transfer, which usually results in accumulation of substrate on one side of the membrane. Primary active transporters are mainly ion pumps, such as the Na-K ATPase, where translocation is coupled directly with an energy-yielding reaction. Chemical gradients are generated by the ion pumps. Secondary active transporters use electrochemical gradients as the driving force for the translocation of their substrates. They operate as countertransporters (or antiporters) or as cotransporters (or symports). In the present thesis Na/H and Na/Li are examples of countertransporters in which Li or H are exchanged for Na present on the other side of the membrane. An example of cotransport is seen when lactate fluxes via the monocarboxylate transporter are analyzed. Lactate is transferred together with a proton, and the energy is provided by a pH gradient (see table 1.2).

The term "carrier" refers to transporters other than primary active transporters, channels or pores. Carrier- mediated transport is relevant to all the chapters of this thesis. Simple carriers represent facilitated diffusion systems, which facilitate solute flux down its chemical or electrochemical gradient. One of the best studied is the glucose transporter in erythrocytes (GLUT1) and other cells [Gould 1992, Holman 1992, Lienhard et al 1992]. The glucose transporter exemplifies a family of proteins which are structurally and functionally related, performing specific roles at cellular level. There are 5 subtypes of glucose transporters identified to date. All transport glucose, but each has discrete characteristics suited to different tissue needs. Alterations in this carrier-mediated transport are believed to play a role in disease states in where insulin resistance is present, such as hypertension, obesity and renal failure. In chapter 3 another

previously identified carrier will be studied in renal failure patients: the choline transporter. A simple carrier can be represented by the diagram in figure 1.3. A carrier moves its substrate from one side of the membrane (cis) to the other compartment (trans). It is able to facilitate flow of substances by changing orientation and conformation during transport.

In the figure, C represents the carrier, and it can be present in two conformational states depending on whether the substrate binding site is facing the intra- or extracellular face of the membrane. The carrier in its external position (C_1) will bind the substrate (S) forming the carrier-substrate complex (CS_1). The carrier will then be reoriented towards the inside of the membrane (CS_2) unloading the carrier (C_2) and liberating the substrate on the trans side (S). The arrows represent the different steps to achieve transport: b refers to the translocation of the loaded carrier, d the reorientation (translocation) of the unloaded carrier, a and c represent the breakdown or formation of the carrier-substrate complex.

One of the characteristics of carrier-mediated transport is trans-acceleration. If transport rate is measured in the absence of substrate on the trans side (e.g. intracellularly) the transporter will cycle as follows $C_1 + S \rightarrow CS_1 \rightarrow CS_2 \rightarrow C_2 + S \rightarrow C_1$, so after transporting and delivering the substrate the carrier travels empty. Trans-acceleration occurs when substrates are present at both sides of the membrane and the transfer of the substrate-carrier complex ($C_1 + S \leftrightarrow CS_1 \leftrightarrow CS_2 \leftrightarrow C_2 + S$) operates at higher flux rates. The substrate-carrier complex translocates in the membrane faster than the empty carrier.

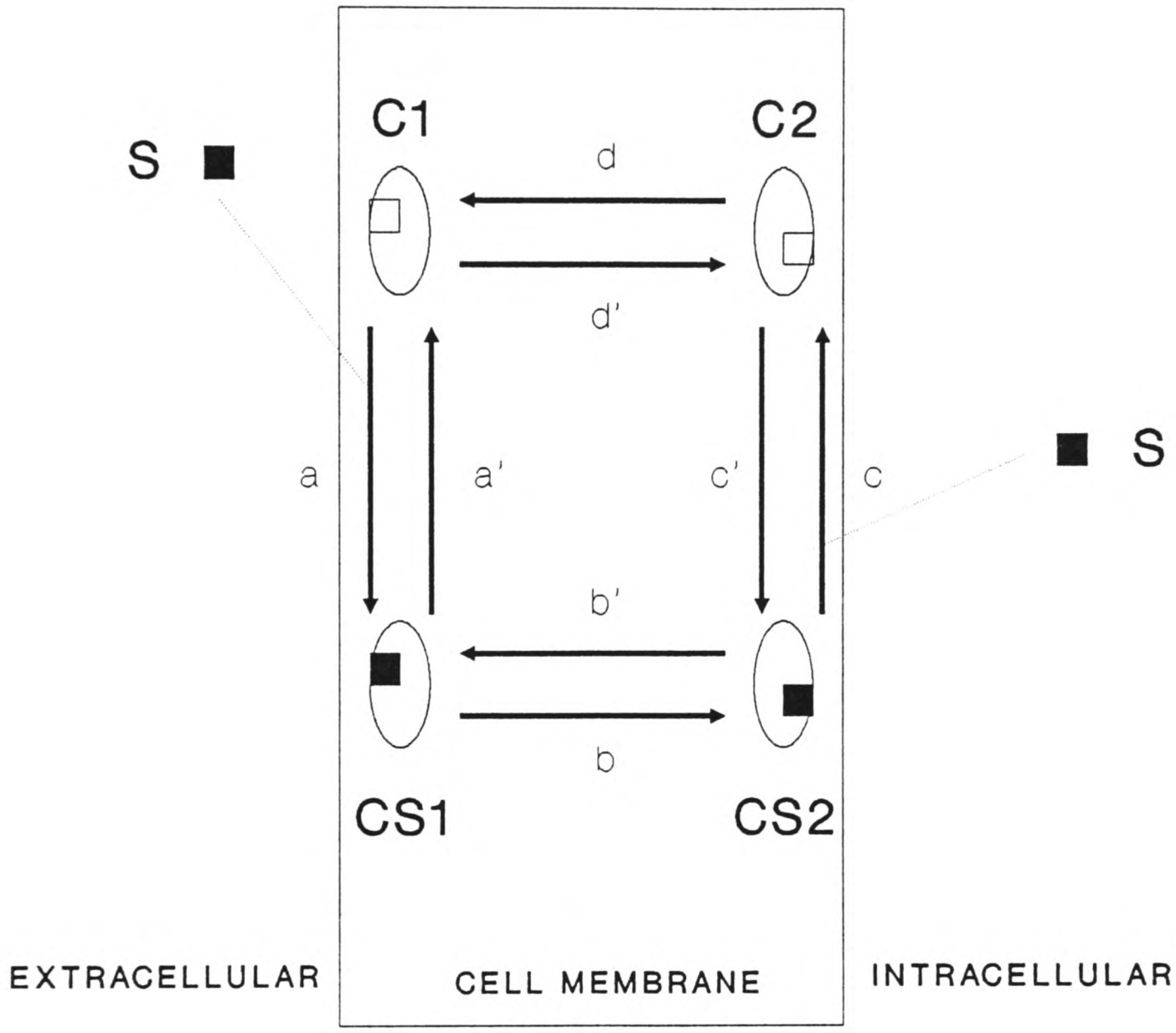


FIGURE 1.3: Schematic representation of the carrier model. S represents substrate; C1 and C2 correspond to the empty carrier; CS1 and CS2 are different conformational states of the carrier-substrate complex while a, a', b, b', c, c', d and d' represent the rate constants for the various steps involved in transport of the substrate. See text for discussion. Adapted from Jain 1988.

The various transporters studied in the present thesis are classified in table 1.2.

TABLE 1.2.
MEMBRANE TRANSPORT SYSTEMS STUDIED - MECHANISMS

CHOLINE	SIMPLE CARRIER
Na/Li COUNTERTRANSPORTER	SECONDARY ACTIVE TRANSPORTER
Na/H ANTIPORTER	SECONDARY ACTIVE TRANSPORTER
MONOCARBOXYLATE CARRIER	SECONDARY ACTIVE TRANSPORTER

Next, the basis for the kinetic parameters used to analyze transport data in this thesis will be presented. Transport function is characterized by showing enzyme kinetic, electrogenicity, specificity, and regulation [Sacks & Fleischer 1989]. Kinetic analysis under defined experimental conditions allows some understanding of the transporter and the steps taken to transfer the substance through the membrane. Various procedures can be used such as measurement under equilibrium exchange, zero trans, infinite trans and infinite cis conditions [Stein 1989]. The use of inhibitors and varying cell substrate conditions can provide information on the type of transporter and its regulation [Stein 1986, 1989, 1990, and reviews above].

1.1.3. KINETICS OF MEMBRANE TRANSPORT

Analysis of data in this thesis will be based on functional measurements of membrane transport activity, and objective parameters should be used for quantifying and comparing fluxes under different conditions. Transporter activity is measured by examining the transfer of the substrate in question from one side of the membrane to the other compartment, as a function of variables such as substrate concentration and time. A complete review is beyond the scope of this thesis [For broader reviews see: Lieb 1982, Stein 1986-1989-1990, Aronson 1990,

Cornish-Bowden & Wharton 1988]. Some of the principles necessary to interpret the parameters used in the present studies will be briefly mentioned below.

Consider the theoretical fluxes seen in figure 1.4.a, plotting flux versus substrate concentration. In this particular example we can see that with increasing concentrations the flux increases up to a saturating level. The resulting curve can be fitted to the Michaelis-Menten formula:

$$v = \frac{S \cdot V_{max}}{(K_m + S)} \qquad (1.1)$$

in which v is flux, S is substrate concentration and V_{max} and K_m are the maximal capacity of transport and the concentration of substrate necessary to obtain half of V_{max} respectively.

This is a typical example of a transporter that exhibits saturation kinetics fitted by the Michaelis-Menten equation used in enzyme kinetics. The uptake increases until all the transporters available are occupied by the substrate, and when the transporter rate is fully occupied the transport is at its maximum (V_{max}).

The concentration of substrate necessary to produce half of the maximum capacity of transport corresponds to the K_m. This parameters defines the affinity of the transporter for its substrate, a higher K_m representing a lower affinity. Usually transporters that are highly specific tend to have a low K_m.

When analyzing the transmembrane transport of a particular substrate more than one transport system can be involved. For example, as shown in figure 1.4.b, both simple diffusion and a carrier-mediated transport can be involved. In simple diffusion flux will increase linearly with substrate concentration, and the rate of transport will depend on the characteristics of the membrane and permeability to the substance.

In figure 1.4.b the transport is the summation of two components, where the

saturable component can be resolved by the subtraction of the diffusion component from the total flux. Diffusion is represented by the straight line shown in the inset. Flux will the represent the sum of the two forms.

$$v = \frac{S \cdot V_{max}}{(K_m + S)} + k_D S \qquad (1.2)$$

in which k_D represents the slope of the linear component.

Experimentally determination of the time course should precede any kinetic observations. This allows the introduction of another important concept: the initial rate of transport. If a substrate is moving from one compartment to the other of a cell membrane, the concentration in both intra- and extracellular compartments will be changing. Under appropriate conditions the initial flux represents the unidirectional flux of substrate, corresponding to a linear relationship between flux with time as seen in figure 1.4.c. Using initial rates of transport the back flux can be disregarded.

As seen above we can differentiate diffusion from mediated transport. In diffusion the rate of transport increases linearly with the concentration of the solute, whereas mediated transport exhibits saturation kinetics. Mediated transport permits a faster movement of substances important for the cell above the rate at which they would cross an lipid bilayer. It should be stressed that usually true simple diffusion of ions is minimal; where the linear component (leak) of transport represents a mediated flux that would only start to show saturation at much higher substrate concentrations.

Identification of the various transport pathways and mechanisms is greatly facilitated by the use of specific inhibitors. Analysis of Na/K pump activity is possible by employing the specific inhibitor ouabain. However, specific inhibitors are not always available. The lack of inhibitors for erythrocyte aminoacid transporters makes the identification of such pathways very difficult.

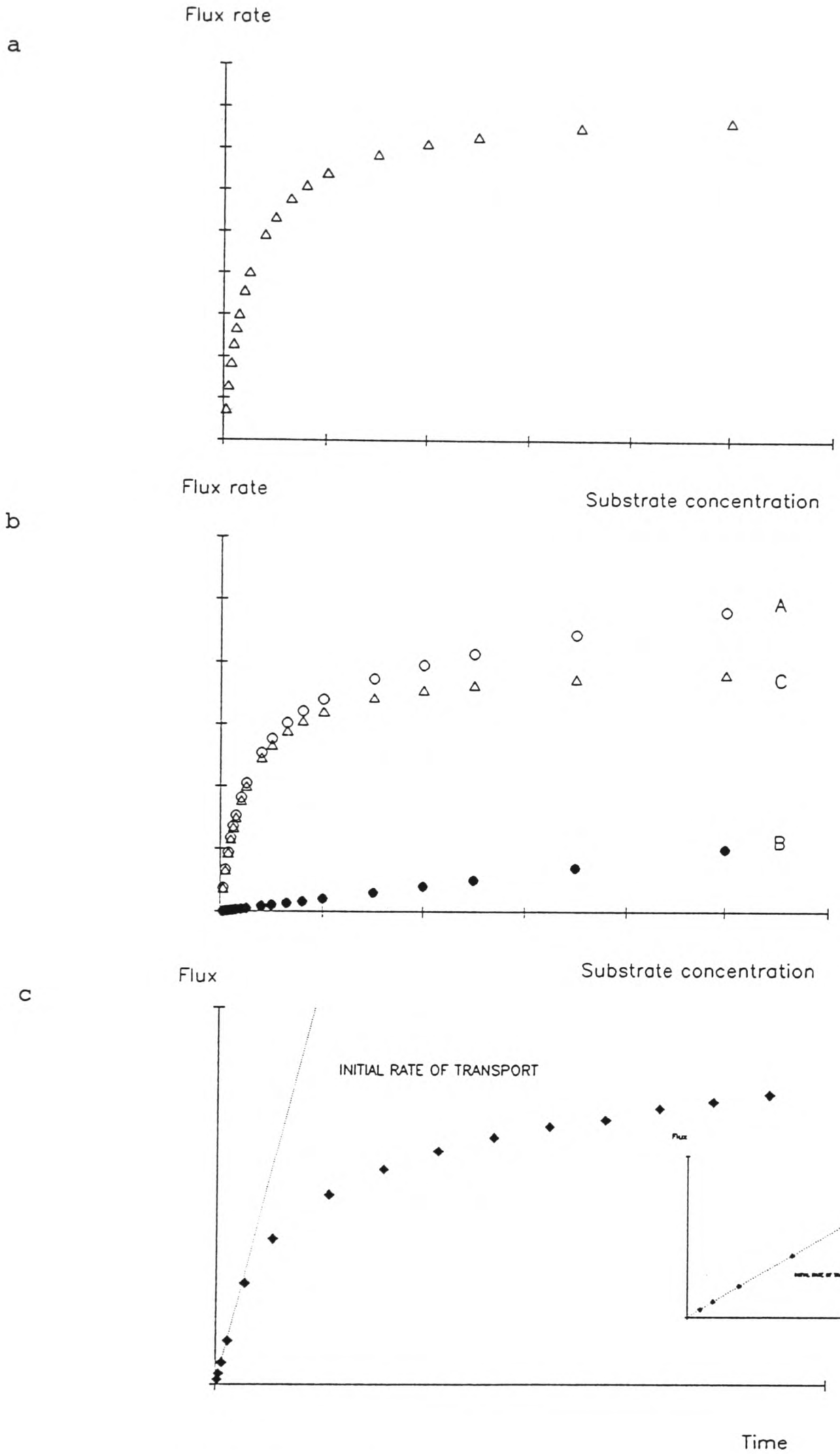


FIGURE 1.4: (a) Concentration dependence of substrate transport rate for a system with typical Michaelis-Menten kinetics. (b) Concentration-dependence of the flux of a substrate transported by more than one system. A is the total flux; B corresponds to flux via "diffusion", which can be evaluated by measuring transport in the presence of a specific inhibitor; C corresponds to the inhibitor-sensitive transport. For a saturable system: C = A-B. (c) Time course. The inset shows the initial rate of transport.

Kinetic parameters obtained under different defined conditions allow the identification and discrimination of discrete transport pathways. Two important kinetic concepts are equilibrium exchange and zero-trans fluxes. In equilibrium exchange (EE) conditions the substrate is present on both sides of the membrane at equal concentrations, i.e. equilibrium. Using EE and zero-trans modes provides information on the mechanism of transport. The discrimination between a carrier and a pore is often possible by this criterion. The presence of substrate on the opposite (trans) side of the membrane can accelerate the rates of transport, trans-acceleration, in the case of a carrier, but never in the case of a channel.

1.1.4. BLOOD CELLS AS MODELS

The cells used in the present thesis were blood cells (erythrocytes and leucocytes). Blood cells are very easily and safely obtained from humans, and represent excellent models of membrane transport systems. Data from work on both erythrocytes and leucocytes have shown that several different membrane transport pathways are altered in disease. There are clearly differences between blood cells and cells from other tissues. However, alterations such as impaired function of Na-K ATPase in patients with CRF have been described initially in erythrocytes and subsequently very similar findings shown for other cells such as leucocytes, muscle, neurones system cells and others [Welt et al 1964, Edmondson et al 1975, Cotton et al 1979, Fraser et al 1985a]. Care should be taken when making generalizations but, as a model, erythrocytes and leucocytes have proved their value.

Some of the present studies (Na/H antiporter activity and membrane cholesterol changes) were performed on lymphoblasts (Epstein-Barr (EB) Virus modified lymphocytes). The volume of blood necessary to perform these in vitro studies on isolated peripheral blood cells

would be very large and cultured lymphoblasts were chosen as an alternative to look at the effects of changing membrane cholesterol on the antiporter activity.

The studies performed in this thesis had the approval of the Oxford Research Ethical Committee and consent was obtained from the patients.

1.2. CLINICAL DISEASE AND MEMBRANE TRANSPORT

In the second part of this introduction some background information on the chosen models of diseases and transporter will be presented. Table 1.3. illustrates the relationship between transporters and disease by showing a few examples of diseases in which the transporters studied in the present thesis have been postulated to play a role in the pathophysiology.

TABLE 1.3

EXAMPLES OF ABNORMAL MEMBRANE TRANSPORT IN DISEASE

TRANSPORTER	INHIBITORS	DISEASES
CHOLINE	N-ETHYLMALEIMIDE[1], IMIDAZOLE[2], QUININE[3].	ALZHEIMER'S[4] DEPRESSION[5] MALARIA[6]
Na/Li	PHLORETIN[7], N-ETHYLMALEIMIDE[8], INHIBITIN[9].	HYPERTENSION[10], HYPERLIPIDAEMIA[11], DIABETES[12], IgA NEPHROPATHY[13].
Na/H	AMILORIDE AND ANALOGUES[14].	HYPERTENSION[15], DIABETES[16].
LACTATE	CINNAMIC ACID[17], MERCURIALS[18], STILBENES[19].	PHENYLKETONURIA, and MAPLE-SYRUP DISEASE[20], MALARIA[21].

REF:1. KRUPKA & DËVES 1988, 2. DËVES & KRUPKA 1987, 3. TALBOT ET AL 1992, 4. MILLER ET AL 1986, 5. UNEY ET AL 1985, 6. KIRK ET AL 1991, 7. PANDEY ET AL 1978, 8. DUHM ET AL 1976, 9. MORGAN ET AL 1989, 10. CANESSA ET AL 1980, 11. HUNT ET AL 1986, 12. KROLEWSKI ET AL 1988, 13. BOERO ET AL 1991 , 14. BENOS 1988, 15. NG & DUDLEY 1989, 16. NG ET AL 1990b, 17. HALESTRAP 1976, 18. DEUTICKE 1982, 19. DEUTICKE ET AL 1982, 20. DENTON & HALESTRAP 1979, 21. KANNANI ET AL 1991.

The principal theme of this thesis is chronic renal failure (CRF) and is discussed next.

1.2.1. CHRONIC RENAL FAILURE AND MEMBRANE TRANSPORT

Renal failure is a result of the inability of the kidneys to perform their function. The causes of renal impairment are multiple and can result in acute (and potentially reversible), or chronic kidney damage depending on the nature of the insult. Impaired function of the kidneys can result in the syndrome known as uraemia. The term uraemia was used initially by Piorry in 1847 to describe an illness caused by "contamination of the blood with urine" [May et al 1991]. The inability of the kidneys to perform their function leads to manifestations that involve multiple organs and systems, as illustrated in table 1.4.

TABLE 1.4
MANIFESTATIONS OF URAEMIA

NEUROLOGIC	**GASTROINTESTINAL**
Drowsiness	Anorexia
Impaired memory	Nausea and Vomiting
Asterixis and myoclonus	Pancreatitis
Confusion and disorientation	Mucosal inflammation
Peripheral neuropathy	Ascites
Restless legs	
Cramps	**DERMATOLOGIC**
Coma	Pruritus
CARDIOVASCULAR	**HAEMATOLOGIC**
Accelerated atherosclerosis	Anaemia
Cardiomyopathy	Platelet dysfunction
Pericarditis	Immunodepression
Hypertension	**ENDOCRINOLOGIC**
PULMONARY	Hyperparathyroidism
Pulmonary oedema	Insulin resistance
Pneumonitis	Hyperlipidaemia
Pleuritis	Altered thyroid function

OTHERS

These are widespread changes, which suggest that either several factors acting independently participate in the development of the uraemic state or that a single factor disturbs cellular metabolism by affecting functions basic to different cell types [May et al 1991]. With symptoms that resemble intoxication, it has been suggested that the syndrome involves accumulation of uraemic toxins [May et al 1991, Ringoir et al 1987]. Dialysis treatment, with removal of accumulated substances, improves several of the uraemic manifestations and is a strong argument in favour of the toxins theory [Maher 1989].

The broad range of membrane functions, the varied manifestations of uraemia and the reported altered intracellular electrolyte composition in patients with CRF suggest that cell membrane changes may play a role in the pathophysiology of uraemia [Mitch & Wilcox 1982, Frommer & Kurtzman 1984]. Metabolites accumulated from protein metabolism, bacterial flora in the gut, or other substances could affect numerous biological processes [Bergström 1989] and have to be distributed within the body compartments, certainly with the participation of cellular membranes.

Membrane transport abnormalities have indeed been demonstrated in renal failure. Welt and co-workers pioneered the field when they described alterations in the erythrocyte Na/K ATPase in a group of patients with renal failure [Welt et al 1964, 1967, Cole et al 1968]. The abnormal erythrocyte, "The Sick Cell" [Welt et al 1967], was suffering from a reduced activity of the Na/K ATPase with an inhibitory effect of about 50% on ouabain-sensitive potassium and sodium fluxes and had a raised intracellular sodium content [Welt et al 1964, 1967]. These initial findings were soon confirmed by others [Cole 1973, Kramer et al 1976, Villamil et al 1968, Francavilla et al 1972]. Interestingly the abnormalities were not restricted to erythrocytes, but also described in other cell types and tissues from both patients and animal models [Patrick et al 1972, Patrick & Jones 1974, Edmondson et al 1975, Minkoff et al 1972, Cunningham et al 1971, Cotton

et al 1979, Penpargkul et al 1976, Fiehn et al 1976].

Welt's group also observed that the abnormalities were reversible, and also found a 16 % reduction of the ouabain-sensitive ATPase activity by incubation of normal cells with uraemic plasma [Welt et al 1967, Cole et al 1968]. Haemodialysis treatment improved the Na-K ATPase function [Welt et al 1967]. Treatment by kidney transplantation was shown later to improve Na pump function [Zannad et al 1982], and the low muscle membrane potential [Cunningham et al 1971], secondary to inhibition of Na-K ATPase, improved with adequate haemodialysis [Cotton et al 1979]. This initial work clearly pointed to the presence of a plasma-borne factor.

The Na-K pump is been the most extensively studied transporter in uraemia. Welt's patients who had pump inhibition, also had a high intracellular Na, and those with higher intracellular sodium seemed to be sicker [Welt et al 1964, 1967, Cole et al 1968]. The inhibition of the Na-K pump seems to be a consistent cellular event in renal failure, despite some apparently controversial results. The discrepancies could be explained by the heterogenicity of the patients, cell age, cell type, methodology and intracellular Na content [Reviewed by Kaji & Kahn 1987 & May et al 1991]. It is also clear that degree of uraemia and dialysis regimen affect the activity of the pump. The defect may be in part due to a reduced pump number [Cheng et al 1984] or reduction in pump turnover [Izumo et al 1984]. When studying Na-K pump activity in plasma it was shown that both dialysed and non-dialysed uraemic patients had reduced Na-K pump function with diverse effects on pump turnover and/or functional pump number depending on the dialysis treatment [Ellory et al 1988, Fervenza et al 1989]. Once again the reversibility of the changes by dialysis and renal transplantation were shown, again suggesting a possible "plasma factor" [Ellory et al 1988b, Fervenza et al 1989a].

The search for such a "circulating factor" or factors has been going on for more

than 25 years. Which uraemic toxin is responsible? Bricker's tradeoff theory suggested that a natriuretic factor could account for the changes in sodium excretion, during the progression of renal failure [Bricker et al 1970 & 1972]. It has been suggested that "middle molecules" (molecules that behave on dialysis as though their molecular weights were in the range of 300-5000 daltons) could be responsible [Schoots et al 1986, Babb et al 1972, 1973, Bergström 1989]. More recently there has been increasing evidence for an endogenous digitalis-like factor [Graves & Williams 1987, Graves et al 1983, Lancet editorial 1991] affecting the Na-K ATPase function in renal failure [Kramer et al 1985]. This postulated factor has cross-reactivity with antibodies to cardiac glycosides [Kramer et al 1985, Kelly et al 1986a], but at least part of the inhibition is due to nonesterified fatty acids (NEFA) and lysophospholipids [Kelly et al 1986b, 1986a]. Renal failure patients with high NEFA content in the erythrocyte membrane did improve Na pump activity after haemodialysis [Kelly et al 1989], and the change in the pump activity correlated with reduction on the membrane NEFA content [Kelly et al 1989]. However, recent evidence against this hypothesis has been presented [Dwight et al 1992].

In summary, the Na-K ATPase activity in patients with uraemia is inhibited, due to either decreased pump turnover and/or pump number. The reversibility of the inhibition with dialysis or transplantation, and the effect of uraemic plasma on normal cells strongly suggests the presence of a circulating inhibitor, perhaps an digitalis-like factor.

So far it may seem that the uraemic syndrome is restricted to only one membrane transport system. However, this is not the case, and membrane transport function in uraemia proved to be an extremely interesting field of investigation. Table 1.5 is an extensive summary of several membrane transporters that have been studied in uraemia.

TABLE 1.5
MEMBRANE TRANSPORT IN URAEMIA

TRANSPORTER CELL/MODEL	FUNCTION	OBSERVATIONS	REFERENCES
Na/K ATPase			
RBC/Human	Inhibited	.	Welt et al 1964.
	Inhibited	HD	Welt et al 1967.
	Inhibited	S+	Cole et al 1968.
	Inhibited	S-	Villamil et al 1968.
	Inhibited	.	Smith & Welt 1970.
	Inhibited	.	Cole 1973.
	Inhibited	S+	Kramer et al 1976.
	Inhibited	TX	Zannad et al 1982.
	Inhibited		Walter & Becht 1983.
	Inhibited	S+, HD	Izumo et al 1984.
	Inhibited	S-	Cheng et al 1984.
	Inhibited	TX	Diez et al 1986.
	Inhibited	TX↑	Kelly et al 1986a.
	Inhibited	.	Cheng et al 1982.
	Inhibited	High early	Swaminathan et al 1982. Swaminathan 1984.
	Inhibited	TX, S+	Fervenza et al 1989a.
	Inhibited	.	Cumberbatch & Morgan 1981.
	Inhibited	.	Brod et al 1984.
	Inhibited	S+	Boero et al 1988.
	.....	TX↑	Cole & Maletz 1975.
	.....	HD	Krzesinski & Rorive 1983.
	.....	TX↑, S+	Cole et al 1978.
	.....	HD	Zannad et al 1985.
	.....	HD	Quarello et al 1985.
	.....	HD- NEFA	Kelly et al 1989a.
	.....	HD-	Woods et al 1983.
	Normal		Corry et al 1986.
	Normal		Corry et al 1987.
	Normal	.	Main et al 1991..
	Activated	.	Jessop & Eales 1977.
RBC/Rats	Inhibited	.	Fiehn 1978.
Leucocyte/Human	Inhibited	Sug	Patrick et al 1972.
	Inhibited	Sug	Patrick & Jones 1974.
	Inhibited	HD	Edmondson et al 1975.
	Inhibited	S+ Diet.	Aparicio et al 1991.
Smooth Muscle/Rat	Inhibited	S+	Simon 1989.
Brain/Rat	Inhibited	.	Fraser et al 1985a.
	Inhibited	.	Minkoff et al 1972.
Adypocyte/Rat	Inhibited	S-	Druml et al 1988.
Muscle/Rat	Inhibited	S+	Druml et al 1988.
	Inhibited	.	Kelly et al 1990.
Muscle/Human	.....	MP HD	Cotton et al 1979.
	.....	MP	Cunningham et al 1971.
	.....	MP HD	Bilbrey et al 1973.
	.....	MP HD, Diet	Cotton et al 1985.
Myocardium/Rat	Inhib/Norm	ARF:I, CRF:N	Penpargkul et al 1976.
	Inhibited	.	Fiehn et al 1976.
	Inhibited	.	Huot et al 1983.
	Normal	.	Kelly et al 1990.
	Normal	.	Druml et al 1990.
	Inhibited	.	Fiehn 1978.
Na,K,Cl Cotransport			
RBC/Human	Inhibited	.	Corry et al 1986.
	Inhibited	.	Corry et al 1987.
	Inhib/Normal	CAPD:N, HD:I HD-	Trevisan et al 1986.
	Inhibited	TX	Diez et al 1986.
	.....	HD	Kelly et al 1985.
	.....	HD	Kelly et al 1989.
	.....	HD	Quarello et al 1985.
	Normal	HD-	Desanto et al 1987.
Adypocyte/Rat	Inhibited	.	Druml et al 1988.
Muscle/Rat	Inhibited	.	Druml et al 1988.
Na-Li Countertransport			
RBC/Human	Normal	.	Corry et al 1986.
	Normal	.	Diez et al 1986.

	Normal	HD-	Desanto et al 1987.
	.....	HD-	Smith et al 1984.
	.....	HD-	Beuckelmann & Erdmann 1985.
	.....	HD-	Kelly et al 1989.
	.....	HD-	Quarello et al 1985.
	Lower?	HD, S+	Woods et al 1983a.
	Activated	HD	Trevisan et al 1986.
	Normal	.	Rutherford et al 1992.
Na-H antiporter			
Leucocyte/Human	Normal	.	Present study.
Anion exchanger			
RBC/Human	Inhibited	.	Maduell et al 1990
Na and/or K passive permeability (leak)			
RBC/Human	Normal	.	Welt et al 1964.
	Normal	.	Corry et al 1987.
	Normal	.	Quarello et al 1985.
	Normal	.	Izumo et al 1984.
	Normal	.	Cheng et al 1984.
	Normal	.	Corry et al 1986.
	Normal	.	Fervenza et al 1989a.
	Reduced	TX	Diez et al 1986.
Smooth Muscle/Rat	Normal	.	Simon 1989.
Adypocyte/Rat	Normal	.	Druml et al 1988.
Skeletal Muscle/Rat			
	Normal	.	Druml et al 1988.
Aminoacid			
y+			
RBC/Human	Activated	.	Ellory et al 1988c.
	Activated	.	Fervenza et al 1989b.
Gly			
RBC/Human	Inhibited	.	Ellory et al 1988c.
	Inhibited	.	Fervenza et al 1990.
ASC			
RBC/Human	Normal	.	Ellory et al 1988.
	Inhibited	.	Fervenza et al 1990.
Skeletal Muscle/Rat			
	Normal	.	Maroni et al 1986b.
L			
RBC/Human	Normal	.	Fervenza et al 1990.
Skeletal Muscle/Rat			
	Normal	.	Maroni et al 1986b.
T			
RBC/Human	Normal	.	Fervenza et al 1990.
A system			
Adypocyte/Rat	Inhibited	S-	Druml et al 1988.
Skeletal Muscle/Rat			
	Inhibited	Insul N	Maroni et al 1986a.
Aminoisobutyric acid transport			
Skeletal muscle/Rat			
	.....	Insul -	Arnold & Holliday 1979.
Ca transport			
RBC/Human	Inhibited		Nieman et al 1983.
Heart/Rat	Inhib/Norm	ARF:I, CRF:N	Penpargkul et al 1976.
Brain/Rat	Activated		Fraser et al 1985b.
SM/Rabbits	Inhibited		Matthews et al 1977.
Glucose transport			
Leucocyte/Human	Inhibited	HD	Haag-Weber et al 1991.
	Inhibited	.	Briggs et al 1983.
Diaphragm/Rat	.....	S+	Balestri et al 1972.
Adypocyte/Rat	Inhibited	S+	Maloff et al 1983.
	Inhibited	.	May et al 1985.
Nucleoside (Uridine)			
RBC/Human	Normal	.	Fervenza et al 1991a.
Lactate			
RBC/Human	Normal	.	Present study.
Choline			
RBC/Human	Activated	.	Fervenza et al 1991a.
	Activated	.	Bibby et al 1991.
	Activated	.	Talbot et al 1992.
	Activated	TX	Present study.
Water transport			
Brain/Rat	Normal	.	Verkman & Fraser 1986.
Urea transport			
Brain/Rat	Normal	.	Verkman & Fraser 1986.

MEMBRANE TRANSPORT IN URAEMIA/ EPITHELIA

TRANSPORTER CELL MODEL	FUNCTION	OBSERVATIONS	REFERENCES
Na/K ATPase			
Kidney/Rabbits	Activated	.	Salehmoghaddam et al 1985.
Kidney/Rat	Activated	.	Fanestil 1968.
	Activated	.	Schon et al 1974.
	Activ/Norm	.	Mujais & Kurtzman 1986.
	Activated	.	Jacobson et al 1980
	Normal	.	Salihagic et al 1988.
Rectal mucosa/Human			
	Normal	.	Hene et al 1985.
Na/H antiporter			
Kidney/Dog	Activated	Sug	Hruska et al 1982.
	Activated	.	Cohn et al 1982a.
	Activated	.	Cohn et al 1982b.
Kidney/Rabbit	Activated	.	Nord et al 1985.
Kidney/Rat	Activated	.	Harris et al 1984.
	Activated	.	Salihagic et al 1988.
	Activated	.	Preisig & Alpern 1991.
Na-Phophate cotransport			
Kidney/Dog	Inhibition	.	Hruska et al 1982.
Na-Glucose Cotransport			
Kidney/Dog	Inhibited	.	Hruska et al 1982.
Kidney/Rat	Normal	.	Harris et al 1984.
Na-Proline Cotransport			
Kidney/Dog	Inhibited	.	Hruska et al 1982.
Na-alanine cotransport			
Kidney/Rat	Normal	.	Harris et al 1984.
Na/3HCO$_3$			
Kidney/Rat	Activated	.	Preisig & Alpern 1991.
Na or H conductive pathways			
Kidney/Dog	Activated	.	Cohn et al 1982b.
Kidney/Rat	Normal	.	Harris et al 1984.
Kidney/Rabbit	Normal	.	Nord et al 1985.
Choline			
Kidney/Mice	Normal	.	Katz et al 1976.
Kidney/Rats	Activated	.	Toback 1984.

ABBREVIATIONS: HD: Alteration in the transport function is improved or reversible with haemodialysis; HD-: No effect on transport function by haemodialysis; S+: Plasma or serum effect on transport function; S-: No plasma or serum effect; TX: Alteration in the transport function is improved or reversible with kidney transplant; TX↑: Higher transporter activity following renal transplant; High early: Elevated activity in early stages of uraemia; NEFA: Correlation of transport activity with red cell nonesterified fatty acids; Sug: Study only suggests the changes in transport; Diet: Improvement of transporter function with dietary treatment; MP: Low membrane potential; ARF: Acute renal failure; CRF: Chronic renal failure; N: Normal; I: Inhibited; Insul:N- Normal transport activity with insulin; Insulin:-=Reduced sensitivity to insulin.

The table has been divided in two parts. The second part refers to epithelial cells (mainly kidney cells), in which specific adaptative changes occur in renal failure. Renal hypertrophy is a feature of some stages of progressive renal disease or some models of renal failure [Fine et al 1992]. There is increased biosynthesis of lipids and proteins and the activity of transporter proteins in kidney cells may actually reflect an effect of the renal hypertrophy, rather than renal failure. As shown in the table, effects such as Na-K ATPase activation in epithelial cells seems to be present, while inhibition seems to be the rule in other tissues. Further discussion will concentrate only on nonepithelial tissues.

There are two striking features in the Table. First, the specificity of the transport changes: inhibition of transport in uraemia is not universal. In fact the changes seem to be specific for each particular transport system and cell type. Activation, inhibition and normal rates of transport can all be found. For some system's the affinity for the substrate, measured as K_m, is altered without modification on the maximal capacity of transport, V_{max}. In other cases V_{max} is the only parameter affected.

The aetiology of the membrane abnormalities in uraemia is unresolved. As discussed above, there is strong evidence for a digitalis-like plasma factor. Circulating factors have been suggested, with some controversy, for other transporters such as Na,K,Cl cotransport and Na-Li countertransport [Kelly et al 1985, 1987, Quarello et al 1985, Corry et al 1986, 1987, Woods et al 1983]. Several other studies suggest abnormal transport of aminoacids or glucose in different organs [Clark & Mitch 1983, May et al 1985, Garber 1978, 1978, Tizianello et al 1987, Deferrari et al 1987]. Some investigations have been performed in whole organs with disparate techniques, and it is difficult to know whether the changes reflect abnormal membrane transport, or rather they reflect abnormal cell metabolism [Morgan & Morgan 1964, May et al 1985, Kahn 1978, Garber 1978, 1978]. Incubation of cells or tissues with uraemic serum has provided further evidence for

the presence of a plasma-borne factor inducing abnormal cell function, which can affect glucose uptake in this syndrome [Balestri et al 1972, Morgan & Morgan 1964, Dzurik 1986, Maloff et al 1983, McCaleb et al 1984]. The influence of insulin on cellular transport/metabolism of different substrates has also been studied in CRF. It is suggested that uraemia is associated with an insulin-resistant state, where there is defective glucose or aminoacid transport in peripheral tissues (muscle, fat). However splanchnic substrate uptake is varied, but for glucose hepatic uptake is usually normal even in CRF [Mondon et al 1978, May et al 1985, Arnold & Holliday 1979, Lacy 1970, Frölich et al 1977, 1974, Kahn 1983].

The plethora of the clinical manifestations and cellular transport changes in uraemia reflects their widespread nature. Cell membranes are obvious candidates for the etiology of the changes: physical properties could be at least partially responsible. Reduced erythrocyte membrane fluidity, increased osmotic fragility and abnormal membrane composition all have been reported in uraemia [Ota et al 1980, Giardini et al 1984, Komidori et al 1985] .

The reversibility of the membrane transport alterations is another fascinating aspect. If a circulating uraemic factor is present it has to react or associate with the membrane in order to exert its effect. Removal of the offending uraemic environment should tend to reverse the abnormalities.

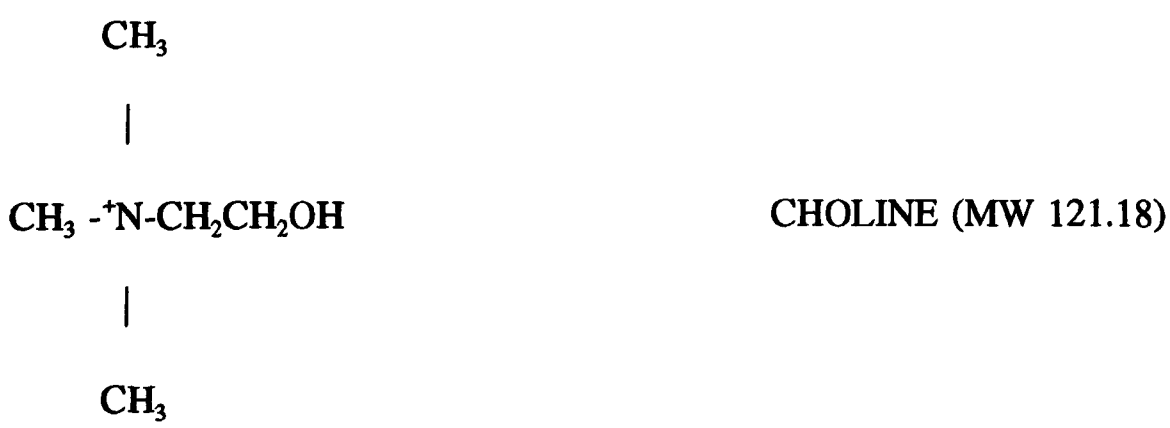
The next few sections will introduce the specific transport systems that were examined in patients with chronic renal failure in the present thesis.

1.2.1.1. CHOLINE TRANSPORT

Of all the transport systems looked at in uraemic patients, the one that shows the most dramatic alterations is the erythrocyte choline carrier [Fervenza et al 1991b]. A consistent

2 to 3 fold difference in the maximum rate of erythrocyte choline uptake in uraemic patients was found when compared with normal controls [Fervenza et al 1991b]. The choline uptake in patients with CRF was higher than in controls, with no overlap between the two groups. Changes of such magnitude are not common in clinical situations where an overlap is frequently present. Fervenza et al [1991b] also described choline transport in a group of renal transplant patients with kidney grafts of different ages and function. A wide scatter of choline uptake activity was seen. However, choline uptake rates exhibited a significant correlation, when plotted against creatinine clearance. The significance of the alteration is not clear. It is possible that it is secondary to circulating plasma factors. Alternatively it could be due to membrane changes induced during erythropoiesis or due to modifications during cell aging. The changes are consistent and reproducible [Fervenza et al 1991b].

Choline is a quaternary ammonium ion required to make membrane phospholipid, as a precursor for the biosynthesis of the neurotransmitter acetylcholine and as an important source of labile methyl groups [Zeisel et al 1991]. There is an endogenous pathway for the de novo biosynthesis of choline, but choline is an essential nutrient for humans, present in the food primarily as phosphatidylcholine [Zeisel et al 1991].



CHOLINE (MW 121.18)

Choline cannot cross cell membranes at a significant rate by simple diffusion, nor is it a usual substrate for ion-specific channels [Stein 1990], although it enters Cl-channels in

malaria infected cells [Kirk et al 1992]. Askari [1966] found that choline could be transported into human erythrocytes. The transporter does not have an obvious physiological role in mature red blood cells, and it is probably vestigial considering that choline is neither incorporated into phospholipid nor converted into a metabolite in the erythrocyte [Askari 1966, Martin 1968]. This makes erythrocytes a very useful model for studying the choline transporter [Martin 1977].

The erythrocyte choline transporter has been studied extensively and its kinetics have been characterized. Two distinct mechanisms of choline uptake in red cell have been identified, simple diffusion and a facilitated mechanism which becomes saturated at low extracellular concentrations of the ion [Askari 1966]. At extracellular choline concentrations of 100 mM or higher, the flux through the saturable component is a negligible function of the total cation flux [Askari 1966]. At micromolar concentration of extracelullar choline (physiological plasma concentrations) the erythrocyte choline uptake is larger than that expected via simple diffusion and follows Michaelis Menten kinetics. The half-maximal infiux is around a choline concentration of 20 to 30 μM [Martin 1968, 1972, 1977; Askari 1966], near the physiological mean plasma concentration of 8-20 μM [Bligh 1952, Zeisel et al 1991, Greenwald et al 1985, Toback 1984]. The choline carrier can perform choline-choline exchange or unidirectional choline fluxes. The kinetics of the carrier are somewhat complex [Martin 1968, Edwards 1973, Deves & Krupka 1979a, b, c, 1981a, b, 1983,1986, Krupka & Deves 1980a, b].

The alterations in choline transport in renal failure, the mechanisms of which remain unclear, deserve more investigation. The present study was undertaken to expand the understanding of the molecular and clinical basis of this disturbance. It involved patients undergoing kidney transplantation, which is a valuable opportunity to study membrane transport changes after acute changes in renal function. The study was designed in a longitudinal fashion allowing the same patient to be followed for several months.

The rates of choline transport were also measured in a group of CRF patients who had never been submitted to dialysis treatment. The association of transport with renal function will be shown.

Erythrocyte life span is reduced in CRF, so a larger fraction of the cell population will be represented by younger cells [Desforges 1970, Eschbach 1989a]. To look at the effects of cell aging in the rate of choline transport, experiments were performed in young and old red cell fractions separated from normal blood.

1.2.1.2. SODIUM HYDROGEN ANTIPORTER AND INTRACELLULAR pH

Free hydrogen ions exist at very low concentration in physiological media, and H^+ activity (concentration for practical purposes) is represented by its negative logarithm, or pH. Maintenance of a stable pH is essential for cell and tissue function. Abnormal composition of body fluids, as mentioned above, is one of the striking features of uraemia. The kidneys are important regulators of the body pH: acid is excreted and bicarbonate is reabsorbed. Deterioration of renal function with glomerular filtration rates below 20-30 ml/min will result in acidosis. Metabolic acidosis, or uraemic acidosis, induces several metabolic derangements and may eventually explain some of the clinical manifestations of the uraemic syndrome [Warnock 1988, Madias & Kraut 1989]. It is surprising that pHi and its regulation in uraemia has received little attention. Bearing those facts in mind we studied pHi and the activity of an important acid extruding membrane transport system, the Na/H antiporter, in leucocytes from CRF patients.

Several vital cell functions are dependent on intracellular pH and its values are kept reasonably stable by regulatory mechanisms. If H^+ and HCO^-_3 were passively distributed across the plasma membrane, according to the membrane potential most cells would have a more

acid pHi. This tendency to acidosis is counteracted by acid extruding mechanisms [Hoffmann & Simonsen 1989, Boron 1989b]. Cells deal with acid or base loads by the action of cytoplasmic buffers that will minimize the changes in pH and then finally the acid-base disturbances will be corrected by transport mechanisms. In the steady-state pH is determined by the rate of addition or removal of H^+ or OH^- equivalents [Boron 1989b], by metabolism and acid-base transport via the cell membrane. It seems logical to expect disturbances in intracellular acid-base homeostasis and regulation in patients with renal failure.

Regulation of pHi is necessary to counteract the tendency of cells to became acidotic. Acid extrusion mechanisms involved in pH regulation are frequently driven by a concentration gradient for another ion (secondary active transport). An illustration of the main mechanisms regulating intracellular pH can be seen in figure 1.5.

The main transport systems are the Na/H antiporter, a acid extruding transporter, and the HCO^-_3 dependent systems (Na-dependent and independent Cl^-/HCO^-_3 exchangers) [Bock & Marsh 1988, Grinstein 1987, Ross & Boron 1981, Busa & Nuccitalli 1984, Saleh & Batlle 1991, Boron 1985, 1987, 1989b, Aalkaer 1990, Kahn et al 1990, 1991, Swallow et al 1990, Hoffmann & Simonsen 1989]. The Na-dependent Cl^-/HCO^-_3 exchanger is mainly involved in acid-extrusion, while the Na-independent Cl^-/HCO^-_3 exchanger is involved in HCO^-_3-extrusion [Simchowitz & Davis 1990, Kikeri et al 1990, Reinertsen et al 1988, Thomas 1989, Ganz et al 1989]. The monocarboxylate carrier involved in lactate transport and pH regulation will be discussed later. The monocarboxylate carrier has been suggested as one of the main acid-extruding systems in neutrophils [Simchowitz et al 1991].

The work described in chapters 3 and 4 concerns the activity of the Na/H antiporter. The Na/H antiporter in mammalian cells was initially demonstrated in 1976 using rat small intestine and kidney proximal tubule cell brush-border membranes [Murer et al 1976]. The

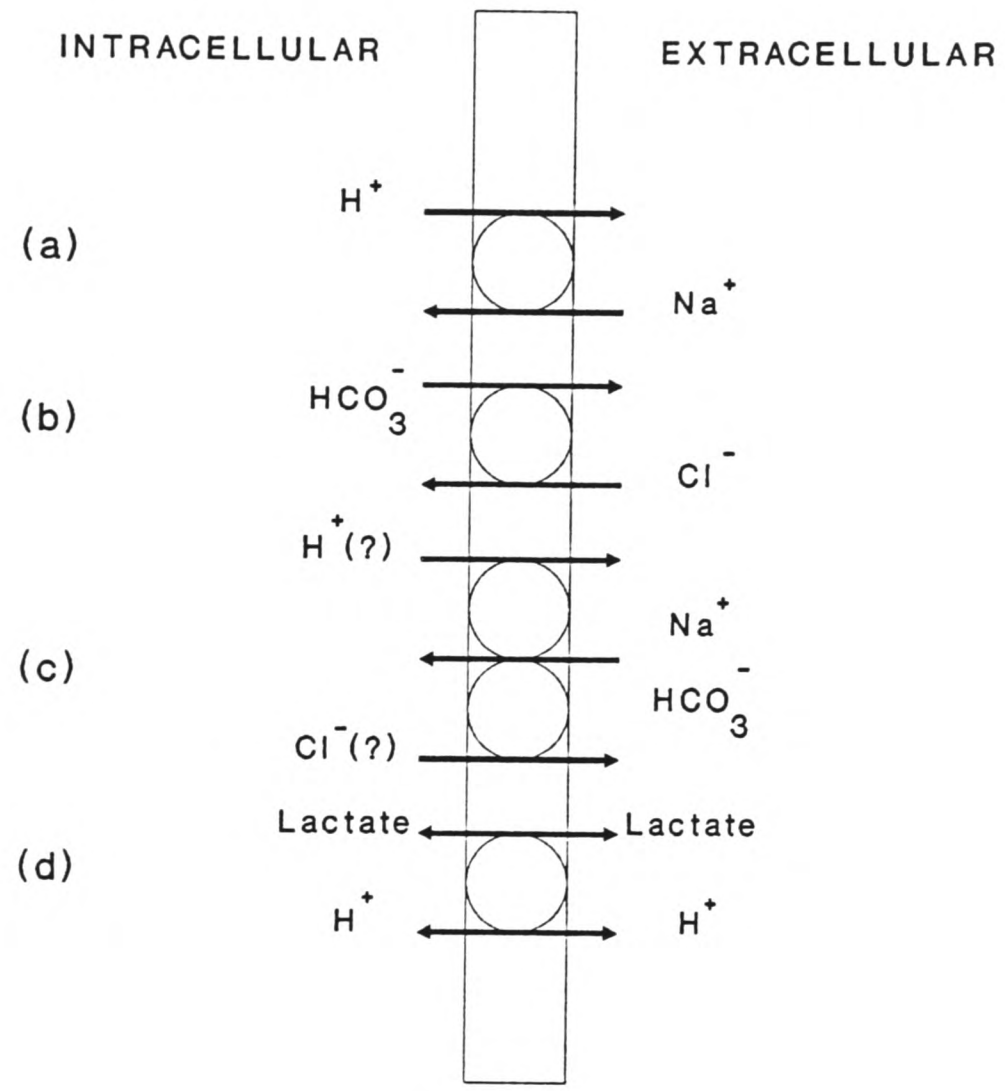


FIGURE 1.5: Principal membrane transport systems involved in pHi regulation in human cells. (a) Na/H antiporter, (b and c). Na-dependent and -independent anion-bicarbonate exchanges, (d). Monocarboxylate carrier.

antiporter is an electroneutral system that normally exchanges extracellular sodium with intracellular H^+, has a stoichiometry of 1:1 and it is present in almost all vertebrate cells studied up to now [Montrose & Murer 1988]. Due to its properties it is an important system involved in protecting the cell against an acid load. Its activity is inhibited by amiloride and its analogues (notably 5,N-substituted analogues such as ethylisopropylamiloride) [Frelin et al 1987, Benos 1988]. At normal pHi the antiporter is quiescent, while at more acid pHi the transporter is activated by an internal allosteric modifier site [Aronson et al 1982]. Antiporter activation without intracellular acidification can be achieved by several factors. The activity of the Na/H antiporter is reported to be modulated by diverse stimuli including growth factors, hormones, mitogenic lectins, fertilization (for eggs), diacylglycerol, tumour promoters, vanadate, intracellular Ca^{2+} and osmotic shrinking [Moolenaar et al 1983, Grinstein & Rothstein 1986, Simchowitz 1985a, 1985b, Grinstein 1988]. Inhibition of the transporter has been shown to occur by treating cells with parathyroid hormone and cyclic AMP [Pollock et al 1986, Kohn et al 1985, Reuss & Peterson 1985]. The activity of the antiporter is determined, at least partially, genetically [Lifton et al 1991]. There are 4 isoforms identified to date [Clark & Limbird 1991, Igarashi et al 1991, Orlowsky et al 1992]. The antiporter is reported to be regulated via phosphorylation as shown by Sardet et al [1990]. Modulation of the antiporter on protein phosphorylation by alters the pH dependence of the internal modifier site thus adjusting the set point of activation. Identification, isolation and cloning of the antiporter has recently been achieved [Pouyssegur et al 1984, Franchi et al 1986, L'Alleiman et al 1984, Sardet et al 1989, Sardet et al 1990, Bianchini et al 1991]. There are several extensive reviews on the antiporter [Grinstein & Rothstein 1986, Grinstein 1988, Grinstein et al 1989a, 1989b, Grinstein & Dixon 1989, Mahnensmith & Aroson 1985].

Alterations in Na/H antiporter activity have been reported in hypertension , diabetes mellitus, cancer, renal acid-base disorders and organ hypertrophy [Cohn et al 1982a,

1982b, Harris et al 1984, Manhnensmith & Aronson 1985, Nord et al 1985, Livne et al 1987, Ng et al 1988, 1989, 1990a, 1990b, Trevisan et al 1989, Nosadi et al 1989, Canessa 1991].

In this thesis fluorescence techniques were employed to study leucocyte pHi, using the pH-sensitive dye bis (carboxyethyl) carboxyfluorescein (BCECF) [Tsien 1989]. Na/H antiporter activity was measured as the ethyl-isopropyl amiloride (EIPA)-sensitive and sodium-dependent alterations in pHi. Patients with chronic renal failure undergoing regular dialysis treatment were studied. They were separated into two groups: diabetic and non-diabetic patients. It has been suggested previously that patients with insulin dependent diabetic nephropathy with a elevated activity of the Na/H antiporter would represent a subgroup with a predisposition for the development of complications including nephropathy. This interesting observation raises the question of what is happening in the chronic renal failure diabetic and non-diabetic population on dialysis.

INSULIN DEPENDENT DIABETES MELLITUS (IDDM) AND DIABETIC NEPHROPATHY

Diabetes is a disease characterized by lack of insulin or ineffective insulin action. It is classified into insulin dependent diabetes mellitus (IDDM) and non insulin dependent diabetes mellitus (NIDDM). IDDM has a strong association with certain HLA types and the destruction of pancreatic ß cells by immune processes. In IDDM the genetic influences are not crucial and the environmental factors are not clear, but viruses and toxins have been suggested. NIDDM is much commoner than IDDM and the understanding of its cause is far from been reached, but genetic mechanisms are probably important. The work in this thesis will only include patients with IDDM.

One possible complication of diabetes is diabetic nephropathy, and in patients with

IDDM it usually presents as persistent proteinuria after 7-10 years of diabetes. Only 30-50% of the patients with IDDM will develop nephropathy suggesting that there is a subset of patients at risk. Once persistent proteinuria is present the usual course is deterioration of renal function, and after 6 months to 15 years patients may reach end stage renal failure with uraemic syndrome and the need for renal replacement therapy. Patients without overt nephropathy may have urinary albumin excretion subclinically elevated (microalbuminuria). Microalbuminuria is predictive of later proteinuria. Until the stage of microalbuminuria it is believed that therapeutic intervention can reverse the process; unfortunately the same is not true for the later stages of the disease. The earliest nephrological alteration that can be detected in diabetic nephropathy is an increment in the glomerular filtration rate and this may be implicated in the pathophysiological mechanism of renal insult [Drury et al 1989].

Could Na/H antiporter activity be a clinical marker for early detection of patients at risk? This question is still unresolved!

The leucocyte Na/H antiporter Vmax is raised in patients with insulin dependent diabetes mellitus with albuminuria(IDDM) in association with a more alkaline intracellular pH (pHi) [Ng et al 1990a, 1990b]. The behaviour of the Na/H antiporter in other cell types or in human cells, when end stage renal failure is present, is unknown. The leucocyte has proven to be a good model to study the Na/H antiporter [Grinstein et al 1985 ,1986; Ng & Bomford 1989, Ng & Dudley 1989, Ng et al 1990a, 1990b]. Insulin dependent diabetes mellitus patients develop albuminuria in the early stages of nephropathy, and are the population at risk of end stage kidney disease. A higher Vmax of the Na/H antiporter is present in patients with IDDM and albuminuria, but not in patients with IDDM without nephropathy [Ng et al 1990b], suggesting that the antiporter activity could be a possible genetic marker to predict which patients are at a higher risk of developing uraemia. Studies of the RBC Na/Li countertransporter, perhaps an analogous system,

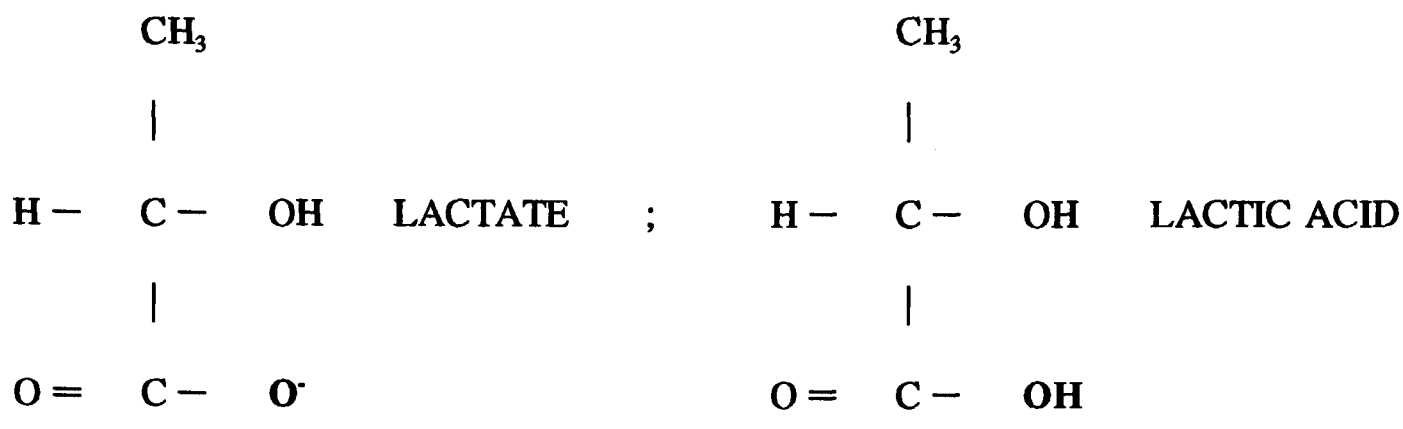
have provided similar conclusions [Krowlewski et al 1988, Mangili et al 1988, Carr et al 1990]. If the Na/H antiporter activity is a marker for the patients who will develop nephropathy one would expect a higher activity in patients with end stage diabetic nephropathy.

1.2.1.3. LACTATE TRANSPORT

Metabolic production of lactic acid tends to acidify cells, in those cell types actively undergoing glycolysis, such as red blood cells, skeletal and cardiac muscle, and cancer cells. Accumulation of lactic acid is an intracellular stress, and there is a requirement for the cell to transport the final metabolic product of glycolysis out of the cell [Denton & Halestrap 1979].

As mentioned above, renal failure is associated with metabolic and acid-base changes which have been suggested to involve cellular disturbances. Lactate transport is an obvious candidate to be studied, because changes in lactate transport could have consequences for cell function. Accordingly, in this thesis, lactate transport has been investigated in erythrocytes from uraemic patients.

Lactate is a short-chain aliphatic monocarboxylate. In this chemical group lactate and pyruvate are physiologically important. Monocarboxylates are the anions of weak and rather lipophilic acids that can permeate membranes by nonionic diffusion of the undissociated acid via the lipid domain of the membrane [Green 1949, Deuticke 1982, Halestrap 1976]. Lactic acid is an important intermediate of the different pathways involved in the metabolism of carbohydrates, proteins and lipids. Under anaerobic conditions, such as in exercise when glycolysis is the main source of energy, lactic acid accumulates and it is vital that lactate is transported out of the cell. The pK_a for lactic acid is 3.86, so at physiological pHs the concentration of undissociated acids is very small [Halestrap & Poole 1989].



The current view is that lactate transport across the cell membrane is via a combination of transfer of the non ionized form, transfer via the anion exchanger and via a monocarboxylate carrier system (MC) [Dubinsky & Racker 1978, Leeks et al 1978, Deuticke 1980, 1982, 1989, Deuticke et al 1978, 1982, Halestrap & Poole 1989, Halestrap et al 1990, Poole et al 1989, 1990]. Detailed studies in human or animal models at 37°C are lacking, but at lower temperatures it is felt that the monocarboxylate system is the principal system responsible for this flux. In the present thesis the experiments were carried out at 37°C as the physiologically relevant temperature. Special attention has been paid to solving the problems of rapid transport, to look at fast fluxes at this temperature. Transport inhibitors have been used to try and dissect lactate uptake into its component parts so that the monocarboxylate carrier, in particular, could be identified and lactate transport examined in renal disease.

Lactate transport across the cell membrane via diffusion of the free acid, anion exchanger and via an monocarboxylate carrier is illustrated in figure 1.6 [Halestrap 1976, Deuticke et al 1982, Deuticke 1989].

At physiological pH the concentration of non-ionized lactic acid is too low to account for the observed rates of lactate transport [Halestrap & Poole 1989]. In erythrocytes lactate transport by the transporter designated anion exchanger (band 3) and monocarboxylate carrier (MC) has been demonstrated using inhibitors of the different systems [Halestrap 1976, Deuticke

1989, Dubinsky & Racker 1978].

The monocarboxylate system can transport monocarboxylate anions. Net movement and electrical neutrality is maintained by exchange of H^+ (or cotransport of OH^-) [Spencer & Lehninger 1976, Dubinsky & Racker 1978]. Evidence for the presence of a monocarboxylate carrier was first presented by Halestrap and Denton [1974, 1975], using cinnamic acid derivatives, such as α-Cyano-4-hydroxycinnamate (CHC), on the transport of monocarboxylates in mitochondria and red blood cells.

Lactate uptake was shown to follow Michaelis Menten kinetics and the kinetic parameters were dependent on temperature and pH [Halestrap 1976, Dubinsky & Racker 1978]. Inhibition of transport by CHC is competitive.

The presence of a specific monocarboxylate transporter has also been demonstrated in several other cell types such as Ehrlich ascites cells, cardiac cells, liver cells, skeletal muscle, smooth muscle, intestine, placenta and kidney tubules [Spencer & Lehninger 1976, Trosper & Philipson 1987,1989, Lupo et al 1990, Edlund & Halestrap 1988, Koch et al 1981, De hemptinne et al 1983, Mason & Thomas 1985, Fishbein 1986, Kutchai et al 1978, Kastendieck & Moll 1977, Moll et al 1980, Mengual et al 1983, Jorgensen & Sheikh 1984, Lamers 1975].

It is thus established how the transporter works at low temperature and at rather unphysiological conditions. The questions to be answered concern lactate fluxes at 37°C (physiological temperature) and how fast lactate can be transported by the red blood cells under such conditions. This is important in situations such as exercise and in sickle cell disease, when the lactic acid produced by muscle needs to be buffered and transported. The characterization of the lactate transfer in renal failure is also important for comparison with other transporter activity and the potential role that alterations could play in the pathophysiology of this syndrome.

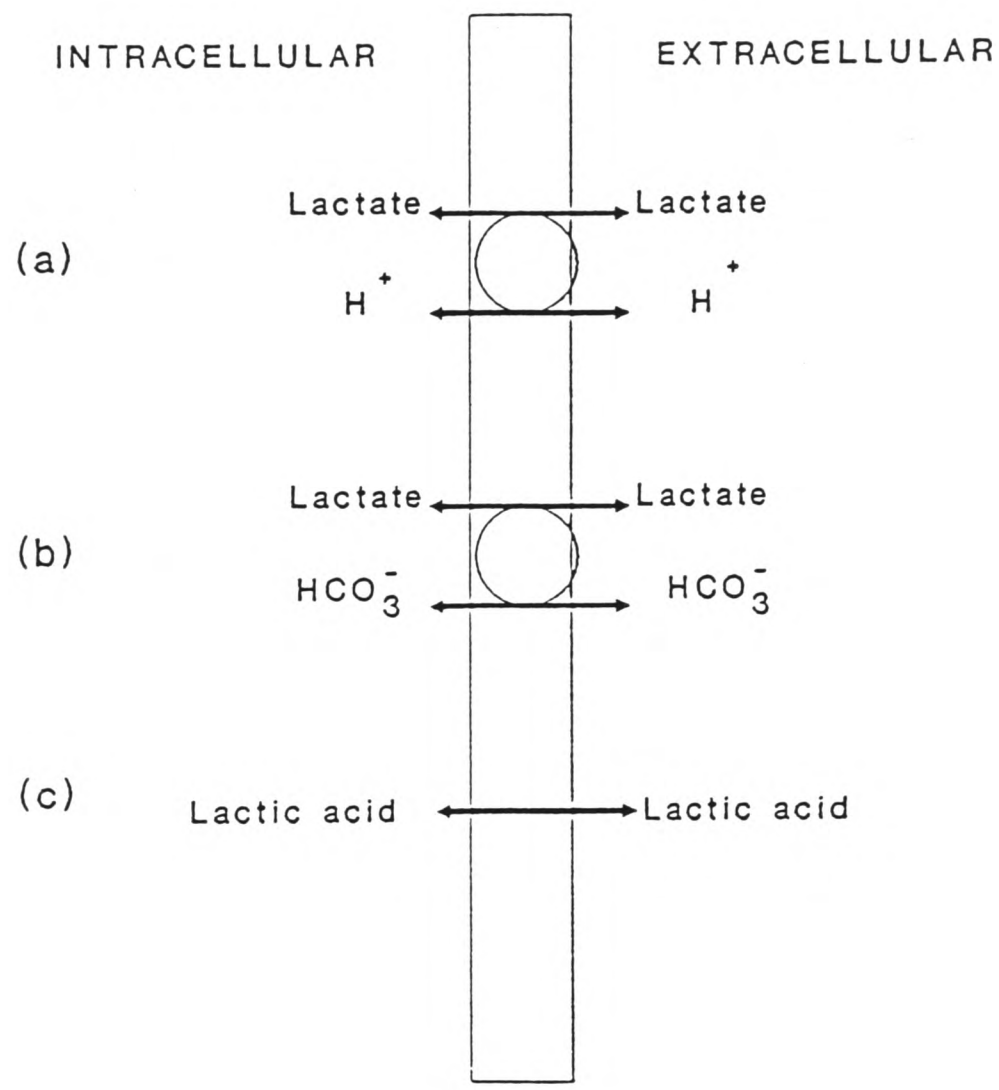


FIGURE 1.6: Lactate transport across the cell membrane. (a) Monocarboxylate carrier (cotransport of protons with lactate or lactate-lactate exchange (b) Anion-exchanger (antiport) (c) Diffusion

1.2.2. ATHEROSCLEROTIC VASCULAR DISEASE. TREATMENT WITH SIMVASTATIN

This study in patients with atherosclerotic vascular disease aims to look at the effects of lowered blood cholesterol concentration on membrane transport function and membrane cholesterol content.

Atherosclerosis is a widely prevalent arterial lesion characterized by infiltration or retention of lipids in the vessel walls, with proliferation and necrosis of connective tissue, alterations which result in compromise to the integrity of the vessel wall and lumen [Woolf 1990]. Its aetiology is not completely understood, but it is multifactorial with both genetic and environmental factors. It is the major cause of death in Western society and several risk factors have been identified: hyperlipidaemia, hypertension, smoking, diabetes and family history. Manifestation of the disease includes coronary artery disease, cerebral vascular events and peripheral artery disease [Galton & Thompson 1990, Ball & Mann 1988].

The participation of elevated plasma cholesterol and lipids levels as a risk factor for atherosclerotic vascular disease has been demonstrated in several studies [Martin et al 1986, Ball & Mann 1988]. It is now recognized that reduction in plasma cholesterol levels with diet or drug treatment is beneficial in reducing and retarding the progression of the vascular lesions [Watts et al 1992]. Despite extensive epidemiological evidence for risk factors associated with atherosclerosis, the mechanisms underlying this disease are poorly understood.

Chapter 6 aims to examine the possible participation of cell membrane properties in the genesis of atherosclerosis. The transfer of lithium across the cell membrane via the erythrocyte Na/Li countertransporter was the model selected. Several diseases are potentially atherogenic, such as hypertension, hyperlipidaemia and diabetes mellitus. In such diseases there

are reports of increased activity of the Na/Li countertransport system. In some instances, such as for diabetes, the activity of the erythrocyte Na/Li countertransport is reported to be higher in patients who will develop complications like nephropathy and cardiovascular events [Krolewsky et al 1988, Mangilini et al 1988, Carr et al 1990, Nosadi et al 1991, Semplicini et al 1992, Faria et al 1992]. Plasma cholesterol and triglyceride levels are correlated with both the Na/Li countertransport and with atherosclerotic vascular events [Hunt et al 1986, 1989, 1991, Corrocher et al 1985]. If the transporter is involved in the pathogenesis of atherosclerosis, and is in part regulated by membrane lipids, lipid-lowering treatment might result in modification of the transporter activity. This hypothesis will be tested.

1.2.2.1. LITHIUM TRANSPORT

Lithium is the smallest of the alkali cations, group IA of the periodic table, and has one positive charge when in solution [Ehrlich & Diamond 1980]. Only trace amounts of lithium are present in the human body and its use in medicine started as a substitute for sodium ions. Lithium salts are one of the main treatments for manic-depressive illness. Cade in 1949 first described the use of lithium salts to control and prevent manic attacks in a patient [Cade 1949]. Lithium is toxic, and therapeutic and toxic levels are very close with a low margin of safety, so monitoring its concentration is important [Abdu-Saleh 1988]. Interest in the membrane transport properties of lithium started around 1973 when Mendels & Frazer found that red blood cell lithium concentration was one third of the plasma concentration, and suggested that red cell lithium concentration and membrane properties could correlate with the clinical status of the patients treated with lithium [Mendels & Frazer 1973, 1974]. That was a surprising finding considering that the expected ratio for a monovalent cation in the red blood cell would be around 1.2,

considering the Nerst equation and the erythrocyte membrane potential of -10mV [Tosteson 1981]. An uphill extrusion system was then suspected [Haas et al 1975; Duhm et al 1976], and extensive studies on the mechanisms of erythrocyte lithium transport have been performed [Duhm et al 1976, Duhm & Becker 1977 a,b,c, 1979; Pandey et al 1978a,b ; Sarkadi et al 1978; Ehrlich & Diamond 1979].

There are several erythrocyte membrane transport systems for lithium identified and illustrated in figure 1.7. Lithium can be transported via a "leak" pathway, Na/Li countertransport system [Haas et al 1975], a bicarbonate-dependent pathway [Duhm & Becker 1977a, Funder et al 1978], Na/K ATPase [Beauge & del Campillo 1976, Dunham & Senyk 1977, Pandey et al 1978], the choline carrier [Martin 1972] and Li-K cotransport [Canessa et al 1982].

Studies by Ehrlich and Diamond showed that only 3 of the systems are important physiologically. Influx of lithium is 70 % via a "leak" pathway and 30 % via the bicarbonate-sensitive pathway (as $Li^{+}CO_3^{-}$ on the anion exchanger). Efflux is largely (75%) via the Na/Li countertransporter and 25 % as a "leak" [Ehrlich & Diamond 1979].

Haas et al and Duhm et al independently demonstrated that lithium was extruded from the red blood cell via an uphill mechanism, driven by the sodium electrochemical gradient maintained by the Na/K ATPase [Haas et al 1975; Duhm et al 1976]. This transport system was later characterized in more detail. The Na/Li countertransporter performs electroneutral exchange of sodium with lithium with a stoichiometry of 1:1 [Sarkadi et al 1978]. The system is ouabain-insensitive, but can be inhibited by several other substances such as phloretin, frusemide, quinine and quinidine [Pandey et al 1978]. The energy for the transport is derived from the sodium gradient maintained by the sodium pump. Lithium does not seem to play any physiological role, so is surprising that erythrocytes have a system for lithium transport. In fact it has been suggested that under normal condition the transporter functions as the Na/Na exchanger [Pandey et al 1978,

Sarkardi et al 1978]. Inhibitin is an inhibitor of both the Na/Na exchanger and Na/Li countertransport providing further evidence that they may be actually the same protein [Morgan et al 1989]. Na/Li countertransport is the main mechanism responsible for the maintenance of the RBC-plasma lithium concentration ratio in individuals receiving lithium therapy. Na/Li transport activity shows saturation with increased Na and Li and follows Michaelis-Menten kinetics [Sakardi et al 1978]. The mechanism of the countertransport is consecutive (figure 1.3) [Hannaert & Garay 1986]. Kinetic analysis shows that the concentrations need to activate half-maximally the system (k_m) are: internal Na and Li 9.0 and 0.5 mM, respectively, external Na and Li 25 and 1.5 mM, respectively [Sakardi et al 1978]. The k_m is very constant between individuals, but the maximal capacity of transport (V_{max}) varies enormously between individuals. These values have been questioned recently [Rutherford et al 1992]. The activity of the transporter is, at least in part, controlled by genetic mechanisms as suggested by studies with relatives of a manic-depressive patient [Pandey et al 1978] and hypertensive families [Hasstedt et al 1988].

Studies on the effects of lithium in psychiatric diseases were the starting point for the demonstration of Na/Li countertransport alterations in diseases. Mendels & Frazer found that the RBC lithium concentration was a better predictor of rat brain lithium concentration than the plasma concentration [1973]. They also showed that the red blood cell lithium concentration was a better predictor of the clinical status and response to lithium therapy in manic patients. Studies suggested that the interindividual variability had a genetic component [Dorus et al 1975, 1983 Mendlewicz et al 1978, Pandey et al 1978].

In the 80's interest in the system in relation to disease was again stimulated when Canessa detected a higher activity of the Na-Li countertransport in patients with essential hypertension and their relatives [Canessa et al 1980]. Numerous other studies have since confirmed a higher rate of Na/Li exchange in patients with essential hypertension and those at risk of

hypertensive cardiovascular complications [Canali et al 1981, Cusi et al 1981, Trevisan et al 1981, Adragna et al 1982, Woods et al 1982, Clegg et al 1982, Williams et al 1983, Cooper et al 1983, Trevisan et al 1983, Turner et al 1985, Norling et al 1986, Beuckelmann & Erdmann 1986, Morgan et al 1986, Turner et al 1987, Weder & Schork 1989, Laurenzi & Trevisan 1989]. Physiological perturbations, such as pregnancy, can temporarily alter Na/Li countertransport [Worley et al 1982, Smith et al 1982, Levy et al 1986a]. Environmental factors such as exercise training can also influence the activity of the countertransporter [Adragna et al 1985]. Obesity, body weight and alcohol consumption [Miilunpalo et al 1985, Trevisan et al 1983, Turner et al 1985, 1987, McDonald et al 1988] affect the activity of the Na/Li countertransporter, as also due hyperlipidaemia and change in plasma lipids composition [Worley et al 1982, Hunt et al 1986]. Recently, interest has been focused on the higher activity of the countertransporter in patients with Insulin Dependent Diabetes Mellitus (IDDM) at risk of complications such as nephropathy [Krolewsky et al 1988, Mangili et al 1988, Carr et al 1990] and cardiovascular complications [Semplicini et al 1992, Nosadi et al 1989, 1991]. Transport abnormalities of this system have also been described in patients with uraemia and thyroid diseases [Woods et al 1983a, Brent et al 1989]. Countertransport activity is also partially determined by race and gender [Okpaku et al 1980, Ibsen et al 1982, Weder et al 1984, Canessa et al 1984, Turner et al 1985, Bunker et al 1987, Laurenzi & Trevisan 1989].

In summary there are apparent genetic and environmental features involved in the regulation of the Na/Li countertransporter, and a potential physiopathological role in atherogenic disorders. We looked at the modulation of this transporter after treatment with simvastatin (a HMG CoA reductase inhibitor inducing reduction of plasma cholesterol levels) in a longitudinal fashion as part of a placebo-controlled study, using the patient as his own control.

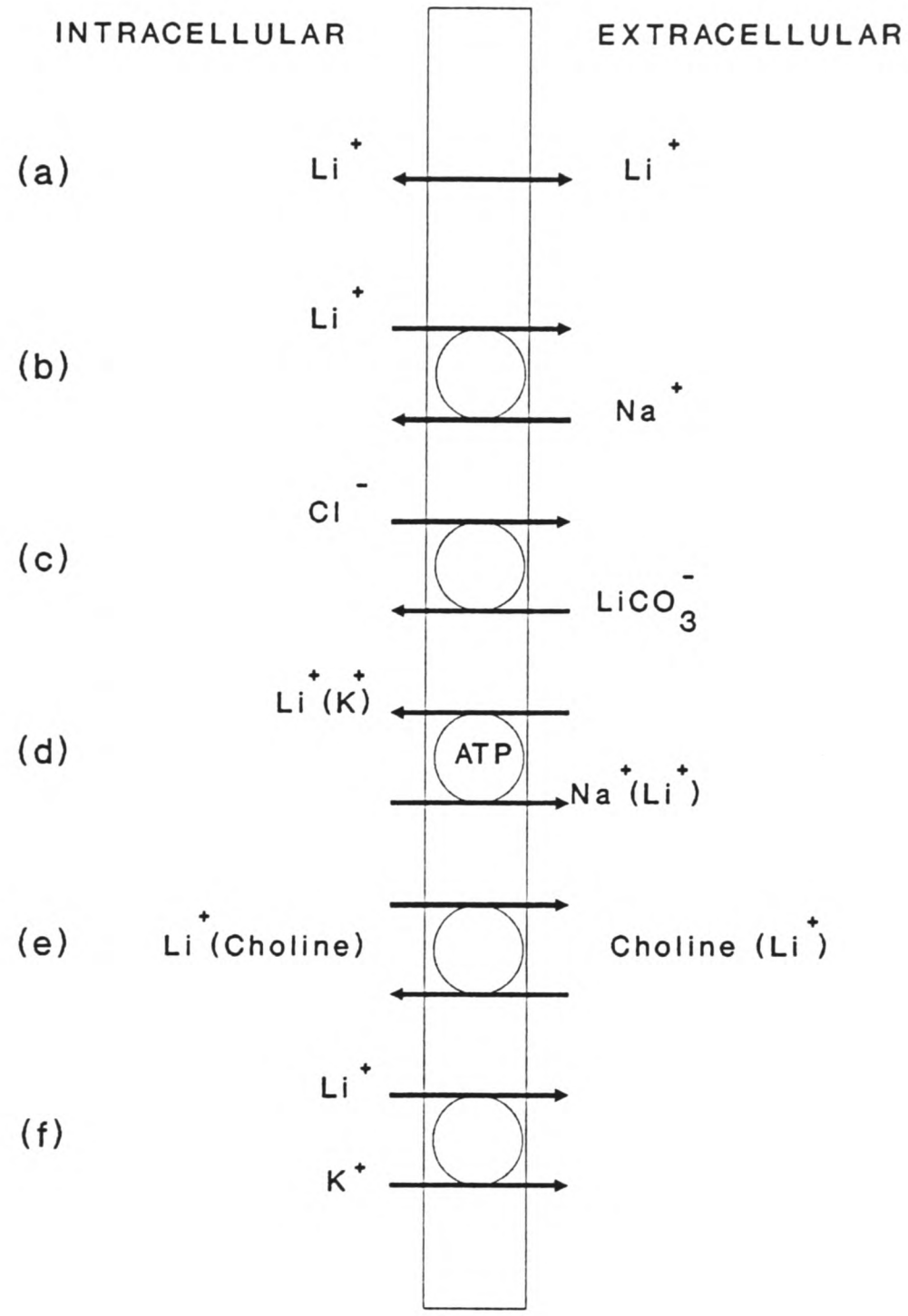


FIGURE 1.7: Lithium transport systems in human red blood cells. (a) Leak, (b) Na/Li countertransport, (c) Anion exchanger, (d) Na/K ATPase, (e) Choline carrier, (f) Li/K cotransport.

1.3. STRUCTURE OF THE THESIS

Following this introduction the chapters on the individual studies performed will be presented. Considering the diverse techniques employed to measure the different transporters, no general material and methods chapter will be presented. The methods are described in the individual chapters

CHAPTER 2

ERYTHROCYTE CHOLINE UPTAKE IN CHRONIC RENAL FAILURE AND RENAL TRANSPLANTATION

The present chapter describes an investigation of erythrocyte choline carrier activity in patients with chronic renal failure (CRF) following kidney transplantation. A longitudinal study has been carried out in which choline transport activity was analyzed before and periodically after kidney transplantation. Changes of the transporter with time were observed and compared with graft function.

As discussed in the Introduction (chapter 1), the mechanisms of the changes of the transporter activity in uraemia are not understood and it is possible that they are secondary to uraemic toxins, circulating plasma factor(s) or to alterations of the transporter during erythropoiesis. The present study was designed to examine the relationship between renal function and transporter activity in more detail further. The intention was to evaluate longitudinal changes in the choline transporter activity as the renal function changes due to the transplanted kidney.

This chapter also includes experiments on choline transport in red cells of different ages and on patients with CRF before renal replacement therapy becomes necessary.

2.1. CHOLINE UPTAKE / MATERIAL AND METHODS / PATIENTS

2.1.1. MATERIAL AND SOLUTIONS

Measurements of the activity of the choline carrier in erythrocytes were performed using a solution containing (in mM): NaCl 150, KCl 5, MOPS 10, Glucose 5, pH 7.4 with tris

base (Saline) and a $MgCl_2$ wash solution containing (in mM): $MgCl_2$ 107, MOPS 10, pH 7.4 with tris base (Wash solution).

Choline chloride was from Sigma Chemical Co. (Poole, Dorset, UK) and was recrystallized by dissolving in hot ethanol and recrystallization started by placing the solution on ice. Crystals were then separated by filtering using paper filter on an Erlenmeyer funnel under suction, and washed with diethyl ether. Ether was eliminated by evaporation in a desiccator under vacuum for a period of approximately 12 hours. Because of deliquescence, a solution of about 1 molar was made, the osmolarity was measured, and the concentration adjusted accordingly [Fervenza 1990]. Choline solutions used in the flux experiments were prepared in saline from the stock solution. Radioisotopic choline was from Amersham International plc (Amersham Place Little Chalfont, Buckinghamshire, UK) as [Methyl-^{14}C] choline chloride in aqueous solution with 2% ethanol at specific activity of 55 mCi/mmol.

Density gradient separation of young and old red cell fractions used Percoll (Sigma Chemical Co.). Concentrated (10 x) Histidine Buffered Saline, containing (in mM) NaCl 1500 and histidine 100 at pH 7.55, was used to prepare an Iso-osmotic Percoll Stock solution by adding 1 part (v/v) of 10 times concentrated histidine buffered saline to 9 parts of Percoll. Iso-osmotic histidine buffered saline was obtained by diluting 1 part of the 10 times concentrated histidine buffered saline in 10 parts of distilled water.

All the chemicals used throughout this thesis were Analar grade, unless otherwise stated.

2.1.2. CELL PREPARATION

In all the studies blood was obtained by peripheral venepuncture, unless stated

otherwise. Anticoagulation was by adding 15-20 units of heparin per ml of blood or by using commercially available vacutainers with lithium heparin. Blood was used one or two hours and was kept on ice. Cells were separated from plasma by centrifugation at 4°C (5 min, 3000 g), sufficient to sediment the cells. Plasma, buffy coat and upper layer of red cells were removed by careful aspiration with a vacuum pump, in order to eliminate platelets, white cells and reticulocytes as far as possible [Young & Ellory 1982]. The red cells were then washed with saline or a "washing solution" in accordance with the experimental protocol. Three washes in 20 vol medium were usually employed by resuspending the cells, centrifuging and discarding the solution repeatedly.

For choline flux measurements 3-10 ml of blood was obtained from normal control or patients.

2.1.2.1. RED CELL QUANTIFICATION

Packed cell volumes were determined spectrophotometrically using Drabkins reagent (cyanomethehemoglobin method). Triplicate samples of 100 µl of red cell suspension were added to 3 tubes of 4.8 ml of Drabkins solution and readings were made after at least 10 minutes. Haemoglobin was determined by spectrophotometry using a Perkin Elmer, lambda 5, UV/Vis spectrophotometer reading the optical density (absorbance) at a wavelength of 540 nm. To convert haemoglobin to haematocrit the following formula was used:

$$Hct = \frac{Abs \,.\, (TV/\ V\ RBC)}{CF} \qquad (2.1)$$

where Hct: Haematocrit, Abs: Absorbance or optical density at 540 nm, TV: Total volume, ie, Drabkin's solution and rbc suspension (4.9 ml), V RBC: volume of red blood cells added to the

Drabkin's reagent (0.1 ml), (TV / V RBC) being the dilution factor and CF: calibration factor (247).

The calibration factor used is standard for the laboratory, but it was also confirmed by the experiments illustrated in figure 2.1, in which absorbance is plotted as a function of the haematocrit of a blood sample after successive dilutions using a microhaematocrit centrifuge to measure red cell pellet directly. The regression line for Hct/Abs obtained has a slope of 19.8. Applying this value to the above formula we obtain:

$$\frac{Hct}{Abs} = \frac{(TV/\ V\ RBC)}{CF} \qquad (2.2)$$

$$CF = \frac{4.9}{19.8} = 0.247 \qquad (2.3)$$

Standard microhaematocrit glass tubes were filled by capillarity with a well mixed cell suspension and sealed with clay sealant. The tubes were placed in the microcentrifuge and spun down for five minutes. Haematocrit readings were made using commercially available haematocrit reading scales.

These methods of red cell quantification are widely used and accepted for measuring red cell volume. If necessary red cells can also be counted by means of a haemocytometer or by Coulter counter. To evaluate haemoglobin concentration the same method as above can be used and haemoglobin standards used to compared with absorbance. An estimation of the RBC indices can be obtained using the formulae described below:

$$MCV = \frac{Hct}{\text{Red blood cell (in millions per } \mu l) \times 1000} = fl \qquad (2.4)$$

$$MCH = \frac{\text{Haemoglobin concentration (g/l)}}{\text{Red blood cell (in millions per } \mu l) \times 1000} = pg \qquad (2.5)$$

$$MCHC = \frac{\text{Haemoglobin concentration (g/l)}}{Hct} = g/l \qquad (2.6)$$

MCV being mean cell volume, MCH: mean cell haemoglobin and MCHC: mean cell haemoglobin concentration.

2.1.2.2. CELL SEPARATION ACCORDING TO CELL AGE

It is known that the density of red blood cells increases as the cell ages, so most age-separation methods use centrifugation techniques which separate cells depending on their density. The density (specific gravity) of red blood cells varies from 1.085 to 1.110 from the youngest to the oldest [Wolowyk 1982]. In the present studies Percoll density gradient separation [Wolowyk 1982, Ellory & Wolowyk 1979] or differential centrifugation in saline [Hall & Ellory 1986] methods were employed to obtain light and dense cell fractions.

PERCOLL DENSITY GRADIENT

The method used for separation of cells according to cell age involved the use of a self-generated continuous density gradient of a colloidal silica solution, Percoll, which has been described in detail by Wolowyk [1982] and will be briefly summarized.

Iso-osmotic Percoll stock solution was diluted to a density of 1.1 g/dl by addition of 17 volumes of isotonic buffered saline solution to 83 volumes of the Iso-osmotic Percoll stock solution. A self-generated continuous gradient was obtained by spinning 30 ml of 1.1 g/dl Percoll with a fixed angle rotor at 24000 g for 15 minutes at 20°C. Centrifuge brakes were kept off to avoid disturbing the gradients during rotor deceleration. The density gradient was checked by means of coloured density marker beads.

Washed red blood cells were resuspended to a haematocrit of approximately 15-20 % with histidine-buffered saline, and 5 ml of the blood suspension layered carefully onto each of the gradient tubes. Tubes were then spun at 800 g for 10 minutes at room temperature using a swing out-rotor.

The top layers, with the younger cells, and the bottom layers were retrieved carefully by means of aspiration. The middle gradient layer was discarded. The cells were washed five times with cold saline and choline uptake measured.

A disadvantage of this method is the small proportion of young cells present in a normal individual. Large volumes of blood plus several gradient tubes are needed. In order to evaluate the kinetic parameters of choline transport (V_{max} and K_m) in the young and old cell fractions, patients with haemochromatosis who were polycythaemic with high a reticulocyte count (5-8 %) were employed. In these patients the separation method employed was differential centrifugation (repeated-centrifugation) in saline [Hall & Ellory 1986, Tucker & Young 1980].

DIFFERENTIAL CENTRIFUGATION IN SALINE

The cells used in those experiments were kindly prepared by Ms. S. Ody. Briefly, blood was washed by repeated suspension and centrifugation (800 g, 15 min) of the cells in about

5 vol. of ice-cold saline. Care was taken to remove leucocytes from the top of the cell pellet following each centrifugation step. Washed cells were suspended in approximately equal volume of saline, then centrifuged for 1 h (800 g, 4°C). The top one third fraction was harvested, pooled between tubes and the procedure was repeated three times successively in order to obtain the young cell fraction. The procedure was repeated to obtain old dense cells but using the bottom fractions of the tubes. The young cell fraction had 10-23 % reticulocytes while the old cell fraction had less than 0.2 % reticulocytes, as assessed by using New Methylene Blue staining [Dacie & Lewis 1991].

2.1.3. RADIOISOTOPIC FLUXES

Membrane transport studies involve the assessment of the transfer and distribution of a substrate from one side to the other side of the cell membrane. This can be achieved by several methods including spectrophotometry, nuclear magnetic resonance, fluorescence, alterations in cell volume or pH and others. For the choline transport experiments, radioactively-labelled substrate was employed (also employed on the lactate experiments, chapter 4).

Radioactive isotopes are a very sensitive and versatile way of detecting and measuring transport of different substrates, when used in conjunction with cell separation techniques [Young & Ellory 1982], and the energy emission can be easily detected by means of liquid scintillation spectroscopy. The isotopes usually have the same chemical and biological properties as the unlabelled substrate, despite a different atomic mass, allowing them to be used as markers or tracers for that substrate. They were used in the present thesis to measure unidirectional fluxes. For choline the tracer was obtained as ^{14}C labelled choline chloride.

2.1.4. TEMPERATURE

Fluxes were performed at 37°C using a shaking waterbath or alternatively gently mixing the cell suspension frequently. Fluxes were started by transferring the tubes from ice to the waterbath and they were stopped at the appropriate time by transferring the tubes back to ice and waiting two minutes before spinning the cells down. It has been shown by Young and Ellory [1982] that temperature equilibration is reached within two minutes by this method.

2.1.5. CHOLINE TRANSPORT

Choline uptake was performed using the method of Fervenza et al [1991b]. Erythrocytes were obtained as described above and washed three times with saline. Cells were then resuspended at a haematocrit near 5% in 1 ml saline containing 250 μM choline and tracer amounts of [^{14}C]-choline. Uptake was started by transferring the samples from ice to a water bath at 37°C. After 5 minutes incubation, fluxes were stopped on ice for two minutes. Cells were separated by rapid centrifugation (14000 g, 15 sec) in an Eppendorf microfuge, the supernatant discarded, and 3 rapid washes with ice cold magnesium chloride solution carried out, to eliminate all the extracellular radiolabelled choline. Cells were lysed with 0.5 ml of Triton X-100 (0.1% in water) and protein precipitated by adding 0.5 ml of 5% trichloroacetic acid. After 10 minutes centrifugation in a microfuge, the clear supernatant containing the released intracellular choline content was transferred to a vial with Pico-fluor 40 scintillation fluid and the ^{14}C activity counted by β-scintillation spectroscopy. Sample haematocrit were determined by the cyanomethaemoglobin method.

Time courses of uptake were performed with an extracellular choline concentration

of 250 μM and samples incubated for varied periods of time. Concentration dependence experiments were executed by incubating the cells for five minutes at several extracellular choline concentrations.

2.1.6. PLASMA CREATININE

Plasma creatinine concentration in CRF patients was determined using an autoanalyser (American Monitor Perspective Analyzer), with the routine biochemistry samples by the laboratory staff, Oxford Renal and Transplant Unit at the Churchill Hospital. The choline flux measurements were performed without knowledge of the creatinine values.

2.1.7. PATIENTS FOR CHOLINE UPTAKE STUDIES

2.1.7.1 CHRONIC RENAL FAILURE

Choline uptake V_{max} and renal function were assessed in a group of 11 chronic renal failure patients with varied degree of renal impairment, who have never received dialysis treatment. These patients were selected from patients attending the weekly pre-dialysis clinic of the Oxford Renal Unit, and are described in table 2.1.

TABLE 2.1.
CHRONIC RENAL FAILURE PATIENTS (PRE-DIALYSIS)

	MEAN (SEM)	RANGE
Number of patients (n): 11		
Male/Female (n) 9/2		
Age	56 (3.7)	39-77
Creatinine (μM)	262 (42)	119-533
Urea (mM)	14.7 (1.9)	5.7-27.8
Sodium (mM)	141 (1.4)	130-147
Potassium (mM)	4.4 (0.2)	2.9-5.5
Haemoglobin (g/dl)	12.3 (0.6)	9.7-17.2
MCV (fl)	90.5 (0.8)	87-95.6

2.1.7.2. KIDNEY TRANSPLANTATION

The longitudinal study in patients following renal transplantation involved patients from the Oxford Transplant Centre. Choline uptake V_{max} and plasma creatinine levels were measured in 10 patients immediately prior to transplantation, several times during the first two months and less frequently afterwards up to a period of 6 months. Six were haemodialysis patients (HD), 2 were on continuous ambulatory peritoneal dialysis (CAPD), and 2 were pre-dialysis patients. Aetiology of the renal failure was glomerulonephritis in four, pyelonephritis in 2, drug induced nephropathy in 1 and unknown aetiology in 3. Clinical details of the patients are included in table 2.2.

TABLE 2.2.
CHRONIC RENAL FAILURE
DATA BEFORE TRANSPLANT

		MEAN (SEM)	RANGE
Number of patients (n): 10			
Haemodialysis	6		
CAPD	2		
Predialysis	2		
Age (years)		45.8 (4.7)	(21-64)
Male/Female (n)	5/5		
Initial results			
Creatinine (μM)		860.5 (74)	(532-1211)
Urea (mM)		25 (2.9)	(13.6-40.1)
Sodium (mM)		138.9 (1.5)	(131-147)
Potassium (mM)		5.0 (0.3)	(3.8-6.9)
Haemoglobin (g/dl)		9.2 (0.8)	(6.1-13.7)
MCV (fl)		92.9 (5)	(85.2-102.2)

Assessment of the renal function was via plasma creatinine levels. Patients were followed for a period of up to 6 months. One patient that had an unsuccessful kidney transplant was followed until graft nephrectomy was performed. In one patient the initial choline uptake levels were not determined. The overall results are shown as the average values for the following intervals: 1-7, 8-14, 15-21, 22-28, 29-42, and 43-56 days during the first two months and monthly afterwards.

2.2.RESULTS

2.2.1. CHOLINE UPTAKE IN NORMAL RED BLOOD CELLS

Normal human red blood cells were used to characterize erythrocyte choline uptake. Figure 2.2 shows the time course of choline uptake using saline buffer containing an

extracellular choline concentration of 250 μM. Influx was measured at several intervals up to 10 min, and the flux was shown to increase in a linear manner during this period. On that basis a 5 minute period was considered adequate to estimate the initial unidirectional influx rates for choline.

2.2.2. CHOLINE UPTAKE IN CHRONIC RENAL FAILURE

Uptake of choline in patients with chronic renal failure prior to dialysis is shown in figure 2.3. In the figure data from patients with chronic renal failure, without symptoms of uraemia, and not requiring renal replacement therapy are shown. The degree of renal impairment was variable, with plasma creatinine concentrations ranging from 119 to 514 μM. Choline uptake correlated significantly with the renal function (Spearman correlation coefficient: 0.6, $p < 0.05$). These results are in agreement with the previous published results on patients with variable degrees of renal function as a result of renal transplantation [Fervenza 1991b].

2.2.3. CHOLINE UPTAKE FOLLOWING RENAL TRANSPLANTATION

Initial mean plasma creatinine was 860 μM (SEM: 74) with a rapid decline after renal transplantation reaching a level of 140 μM (SEM: 11) at the sixth month. Significant decline in creatinine levels occurred in the first two weeks and this is shown in figure 2.4. Maximum mean choline influx rate was 72 μmol/l cells.h (SEM: 6) pre-transplant. Values for normal control is lower [Fervenza et al 1991b]. A significant decline was seen in the first week, as illustrated in figure 2.5. The fall in choline transport rate and in plasma creatinine levels are very similar with a mean half-time of decline to its final value of 4.0 and 3.8 days, for choline uptake V_{max} and

plasma creatinine concentration respectively. Another way of looking at the data was by plotting mean plasma creatinine levels and mean choline influx V_{max} together, as in figure 2.6, giving a significant correlation (Spearman correlation coefficient r^2:0.743, $p<0.01$).

The analysis of the data from each individual patient also provides extra information. The patient described in figure 2.7 had acute deterioration of renal function at the second week post transplant with marked increase in plasma creatinine levels, improving after treatment with 3 pulses of methylprednisolone. Interestingly, the choline uptake V_{max} also showed similar changes, with increases during the rejection period. Figure 2.8 demonstrates another patient presenting with an initial improvement going onto a moderate degree of renal impairment and subsequent graft failure. Follow up was continued until graft nephrectomy was performed. Again the changes in choline transport are similar to the changes in the creatinine levels and when the graft failed choline uptake returned to its pre-transplant levels. It must be said that in one of the patients despite a successful transplant, choline V_{max} decreased only during the first two weeks post-transplant.

2.2.4. CHOLINE UPTAKE IN YOUNG AND OLD ERYTHROCYTES

Choline uptake was measured in young and old erythrocyte fractions. The data shown in figure 2.9 was obtained from four normal donors using Percoll density gradient separated cells. The mean V_{max} in the young erythrocyte fraction was 38.8 (SEM:5.5) μmol/l cells.h, higher than the older cell fraction with 25.8 (SEM:3.7) μmol/l cells.h ($p<0.019$, t test).

The yield of cells from the Percoll gradient was small, so more detailed studies were done using blood obtained from polycythaemic haemochromatosis patients who were frequently venesected. Those patients had a large number of circulating reticulocytes and young

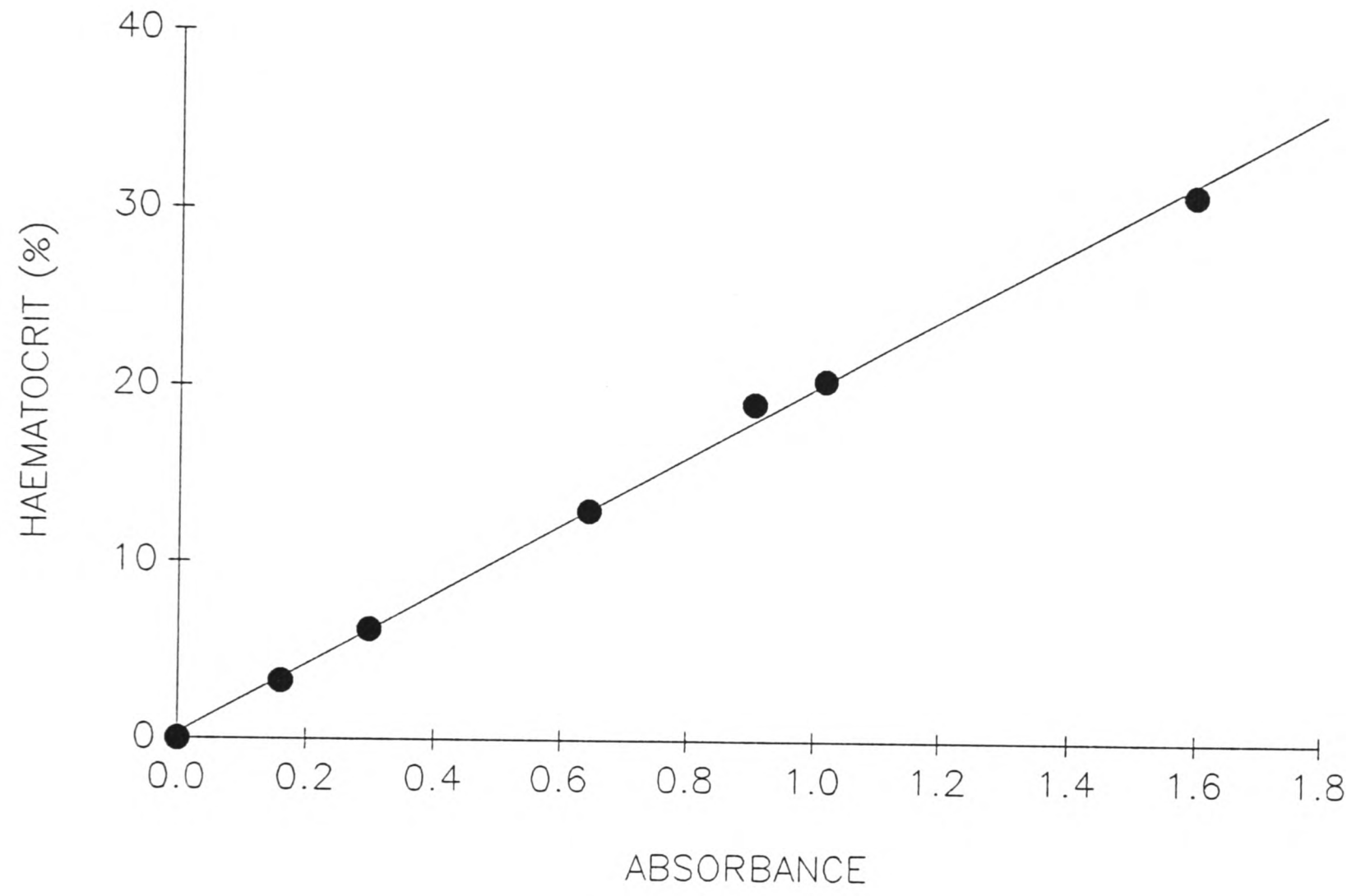


FIGURE 2.1: Plot of haematocrit as a function of absorbance of haemoglobin determined spectrophotometrically with the cyanomethehemoglobin method. See text for discussion.

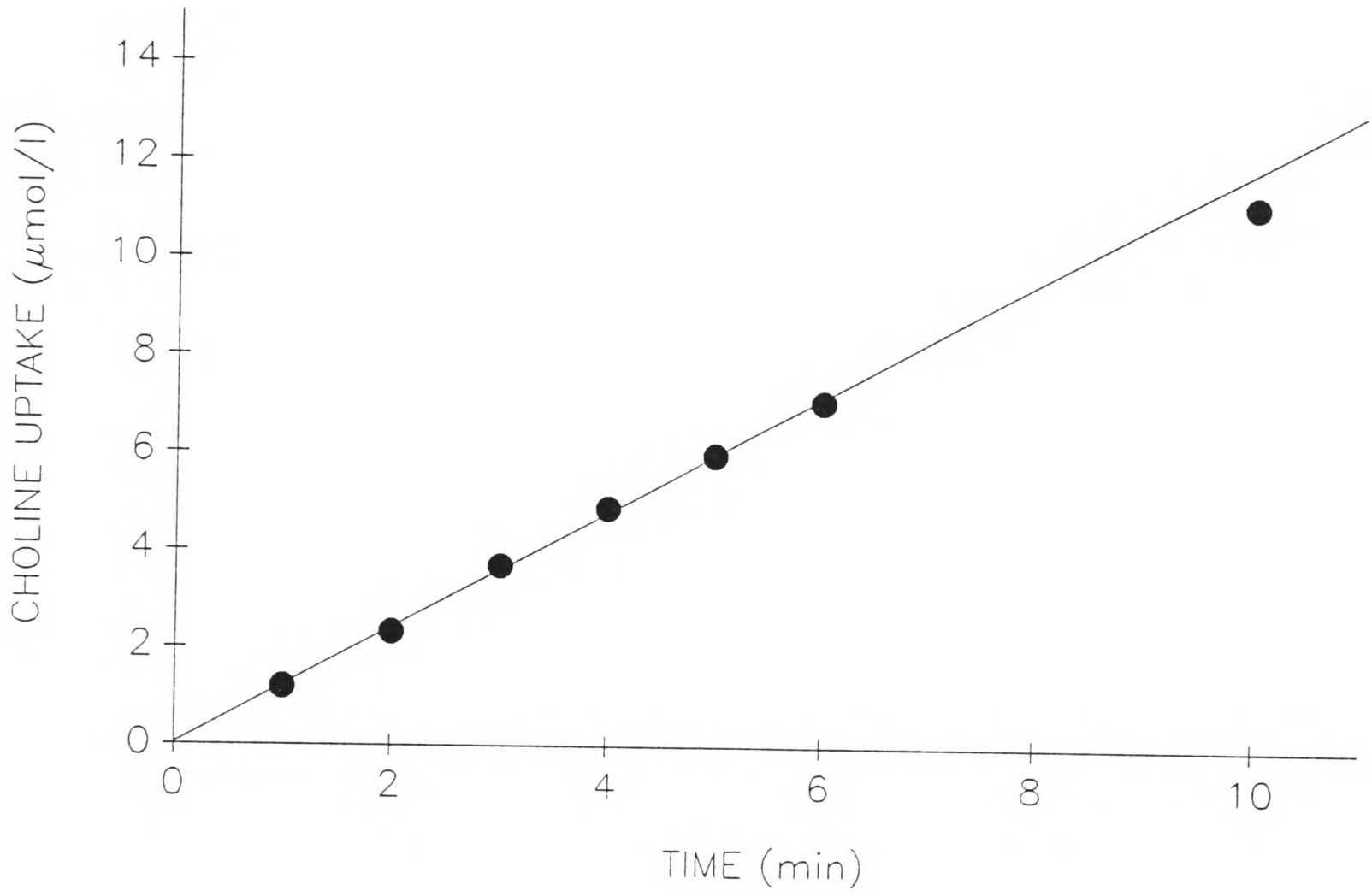


FIGURE 2.2: Time course of choline uptake in normal erythrocytes. Uptake is linear for a period of up to 10 min.

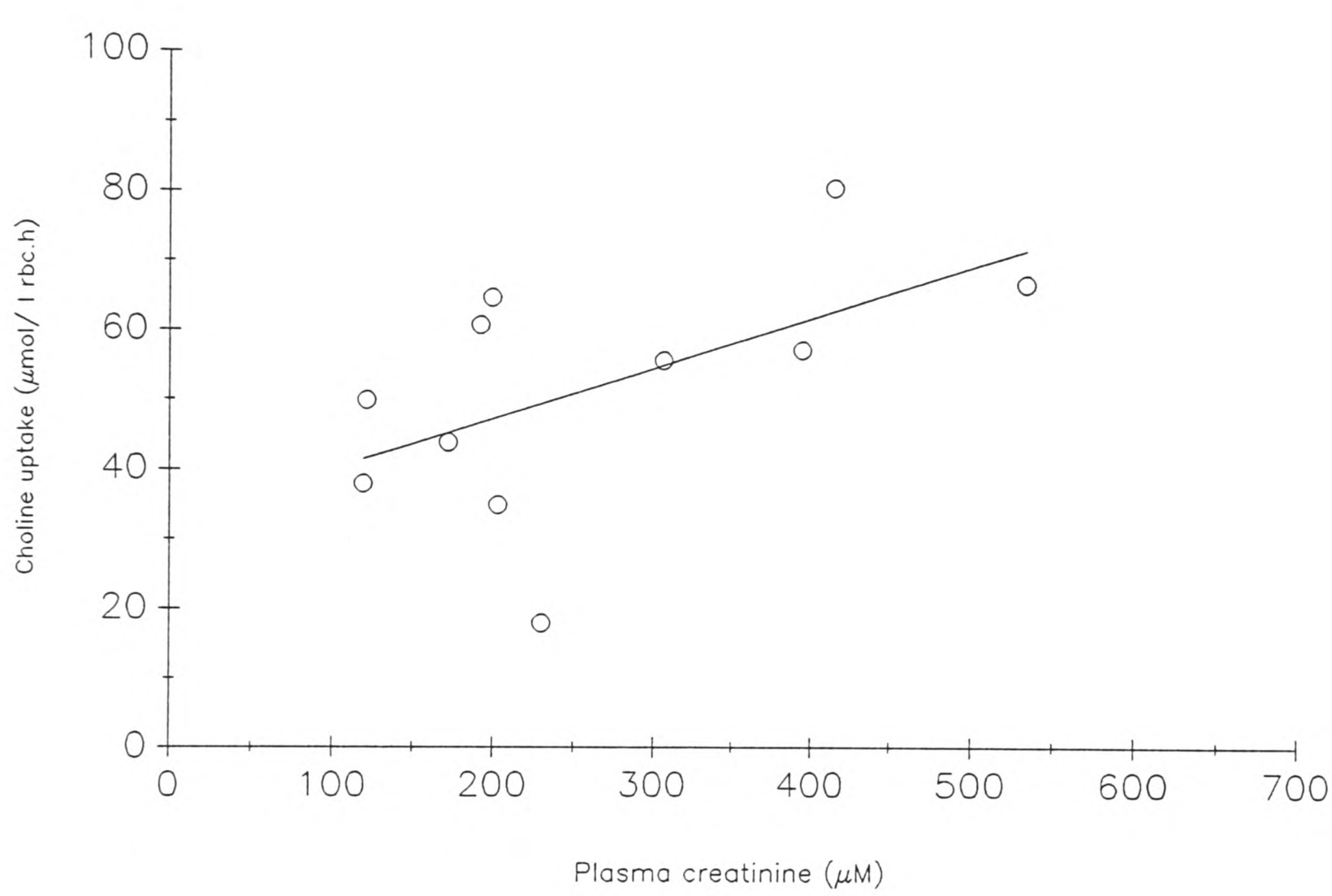


FIGURE 2.3: Choline uptake in erythrocytes from chronic renal failure patients who have never been dialyzed. Uptake is shown as a function of plasma creatinine. A significant correlation is present (Spearman correlation coefficient (r: 0.6, $p<0.05$, n:11).

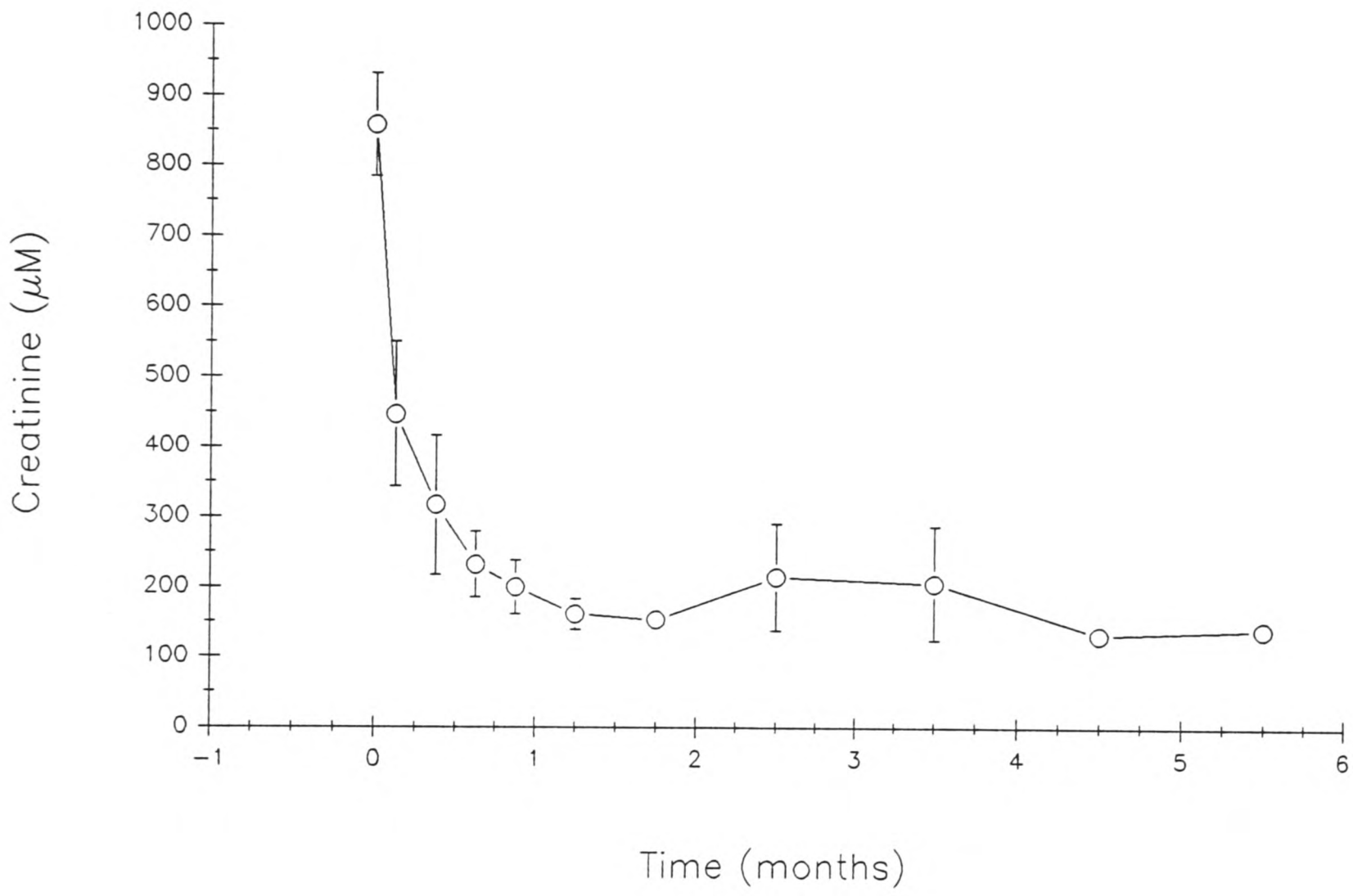


FIGURE 2.4: Mean (SEM) plasma creatinine concentrations before (time 0) and after renal transplantation (n: 10).

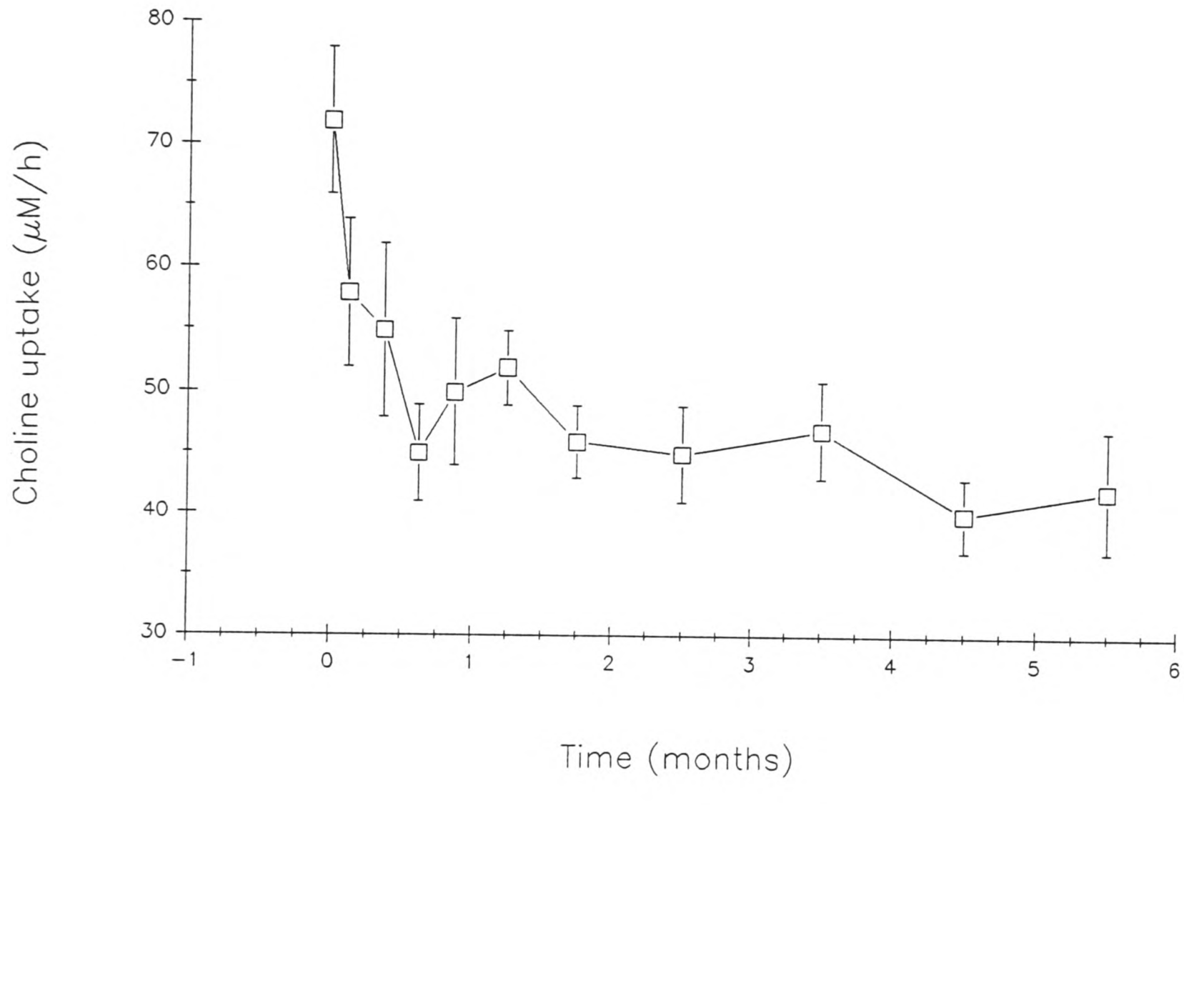


FIGURE 2.5: Mean (SEM) erythrocyte choline uptake rates before (time 0) and after renal transplantation (n:10).

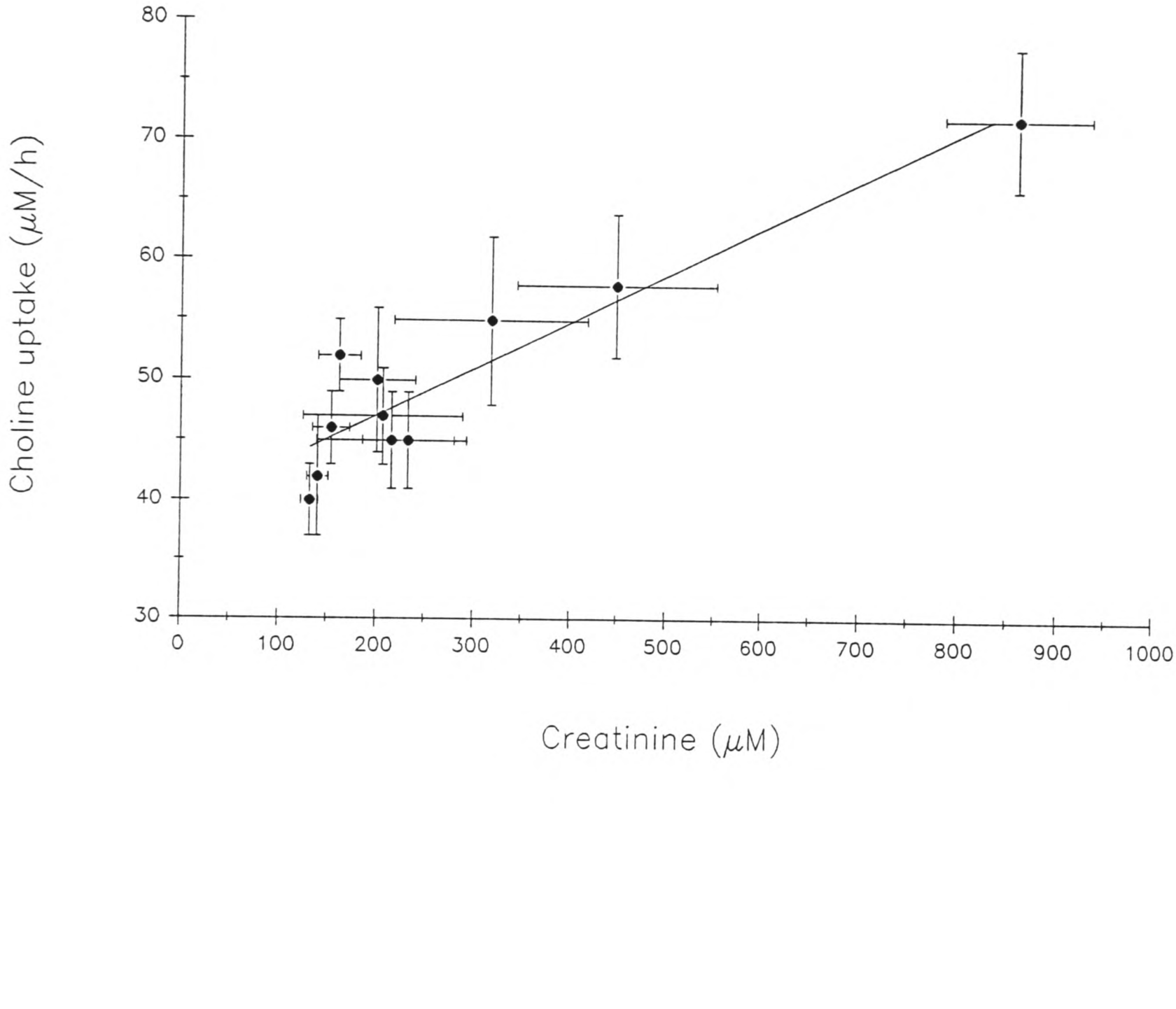


FIGURE 2.6: Mean choline uptake rates as a function of mean plasma creatinine concentration. A significant correlation is present (Spearman correlation coefficient, r^2: 0.743, $p<0.01$, n10). Error bars represent SEM.

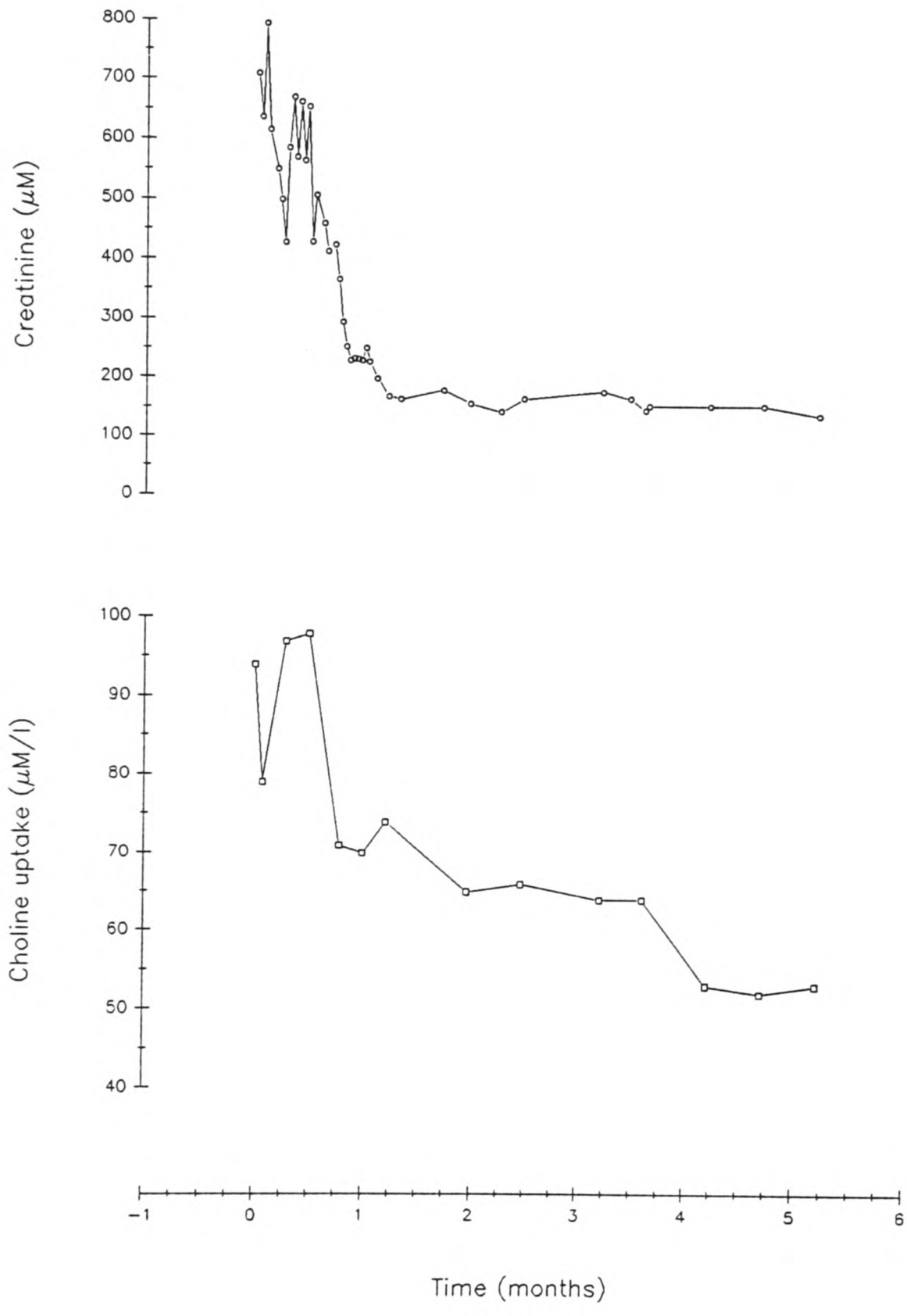


FIGURE 2.7: Plasma creatinine concentration (top graph) and choline uptake rates (bottom graph) before (time 0) and after renal transplantation in patient 1. Choline uptake changes parallel the acute modifications in renal function during graft rejection.

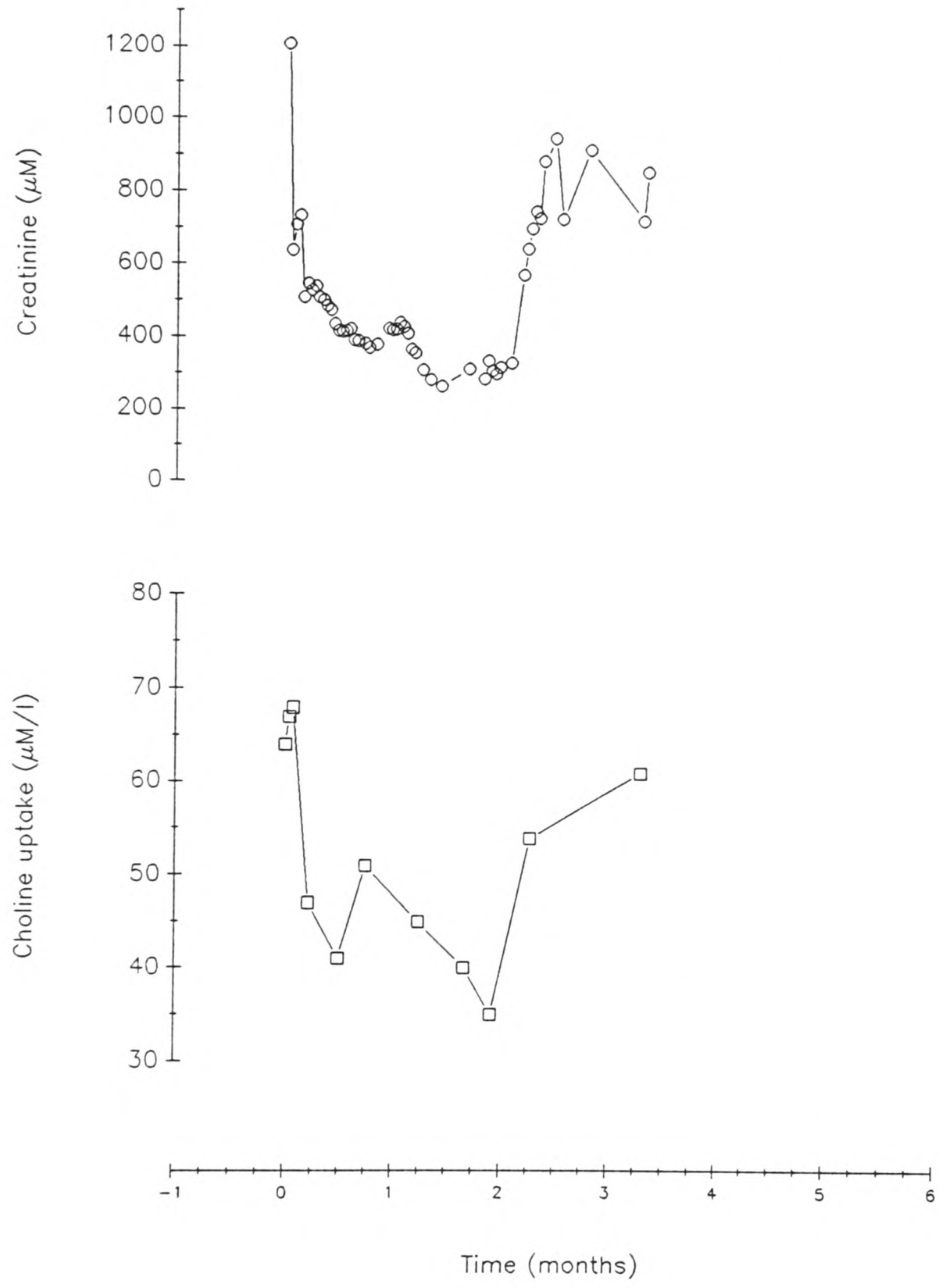


FIGURE 2.8: Patient 8 presented with irreversible graft failure. Choline uptake alters in a similar fashion to renal function.

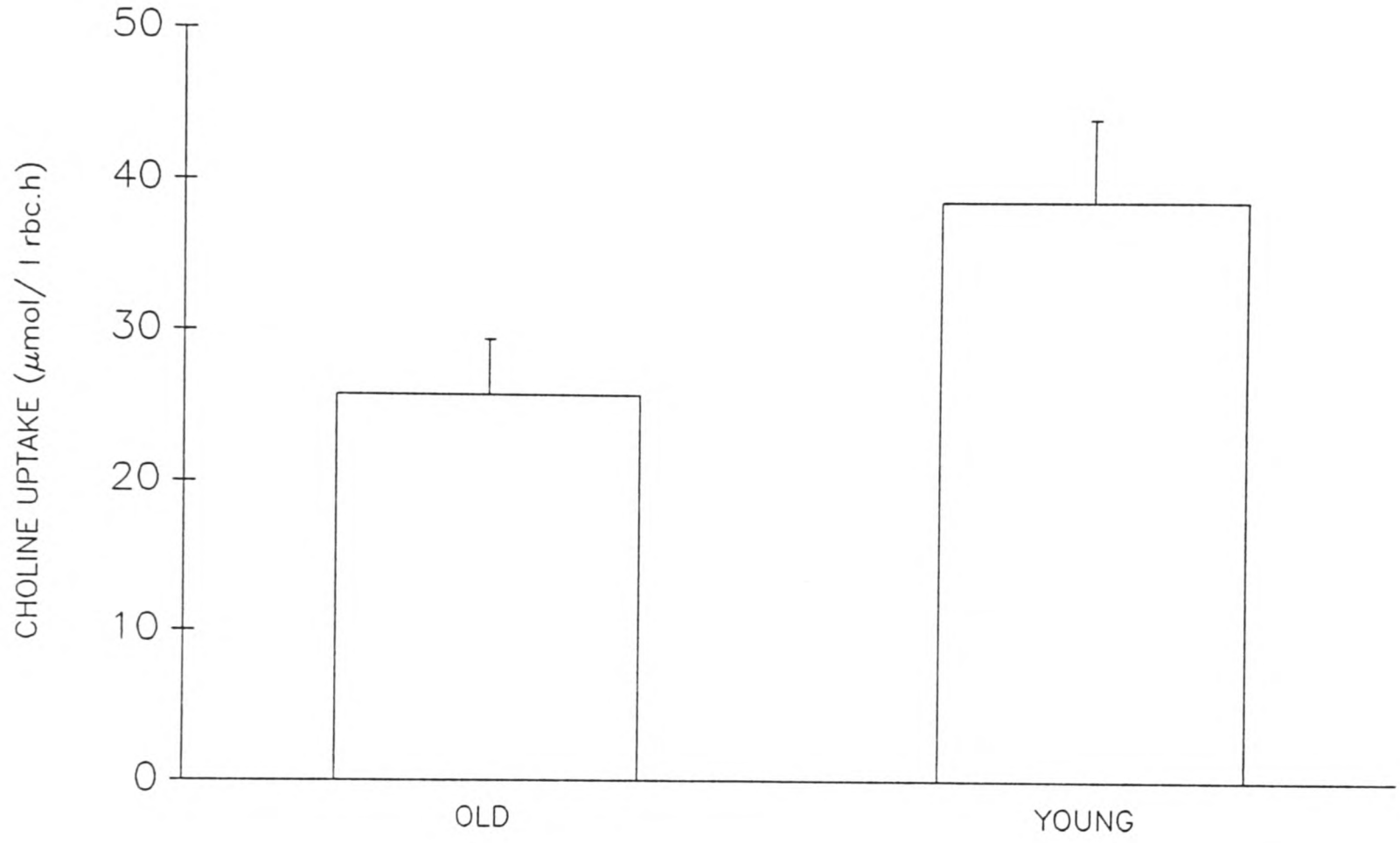


FIGURE 2.9: Choline uptake in young and old cell fractions from normal subjects. Histogram of choline uptake V_{max} in old and young erythrocyte fractions from normal donors (n:4, $p<0.02$, t test).

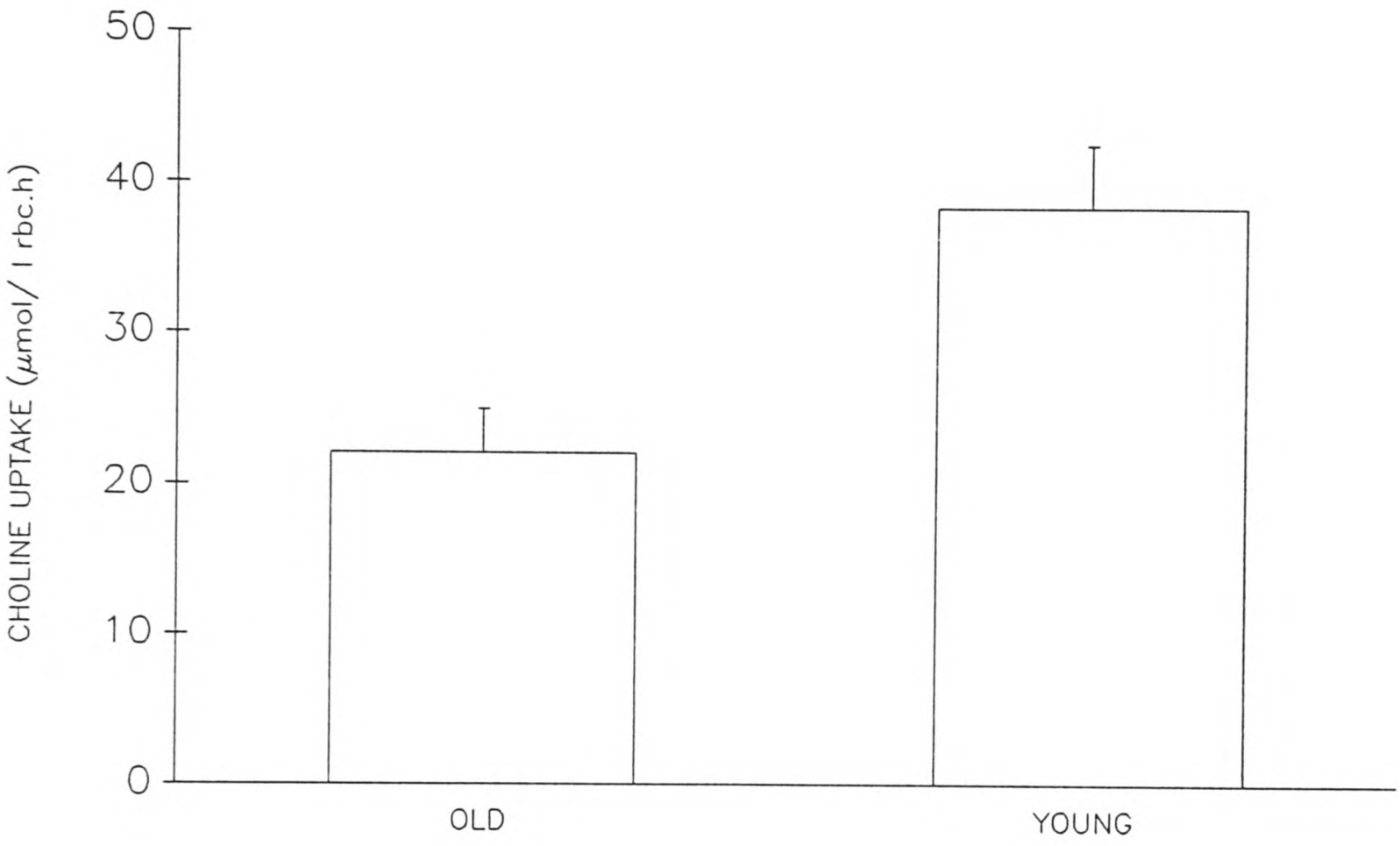


FIGURE 2.10: Choline uptake V_{max} in young and old cell fractions from polycythaemic patients (n: 3, $p<0.05$, t test).

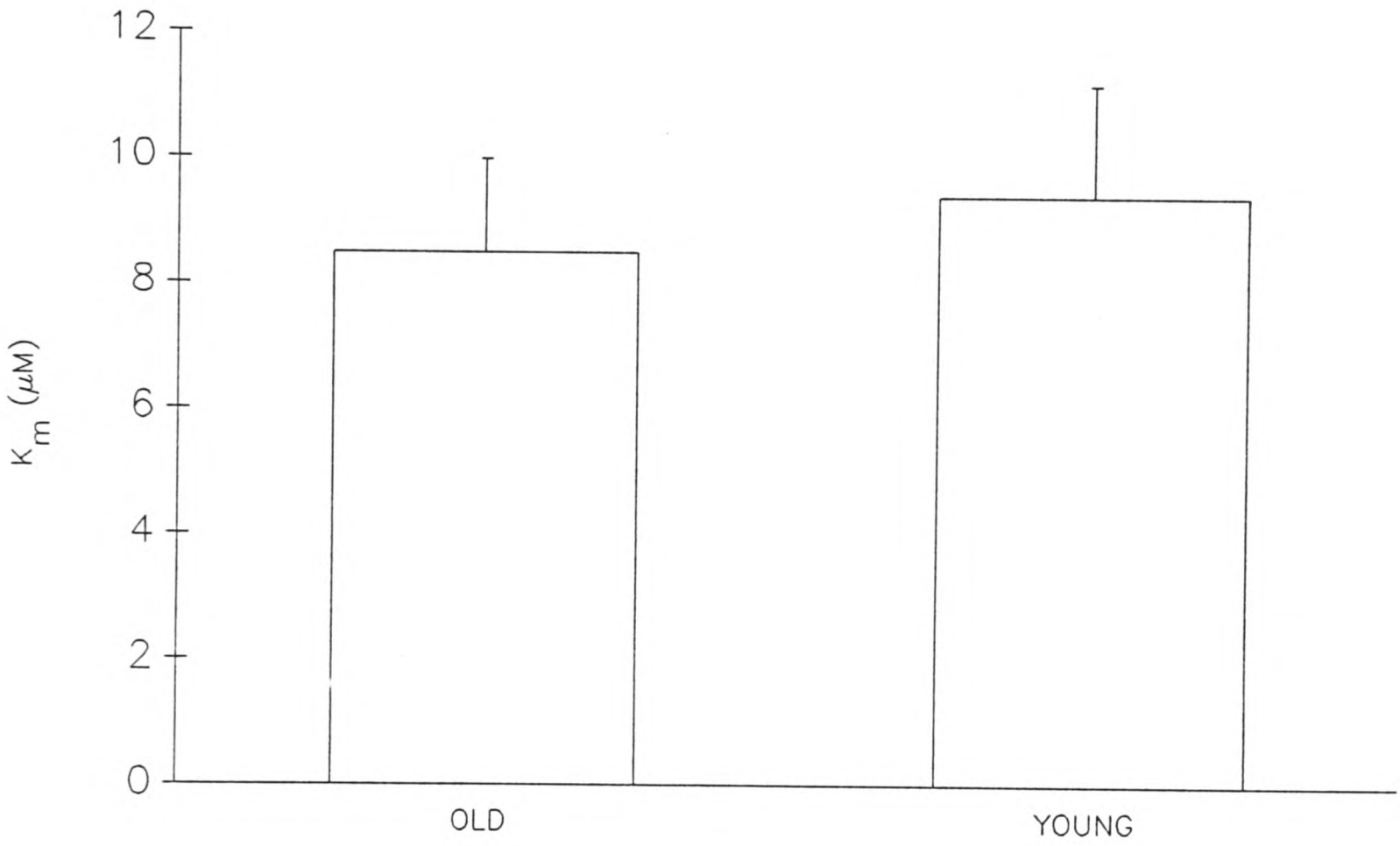


FIGURE 2.11: Choline uptake K_m in young and old cell fractions from polycythaemic patients. For the reticulocyte-enriched cells K_m was 9.4 (SEM 1.8) μM, not different from the old cells at 8.5 (SEM 1.5) μM (n:3, NS, t test).

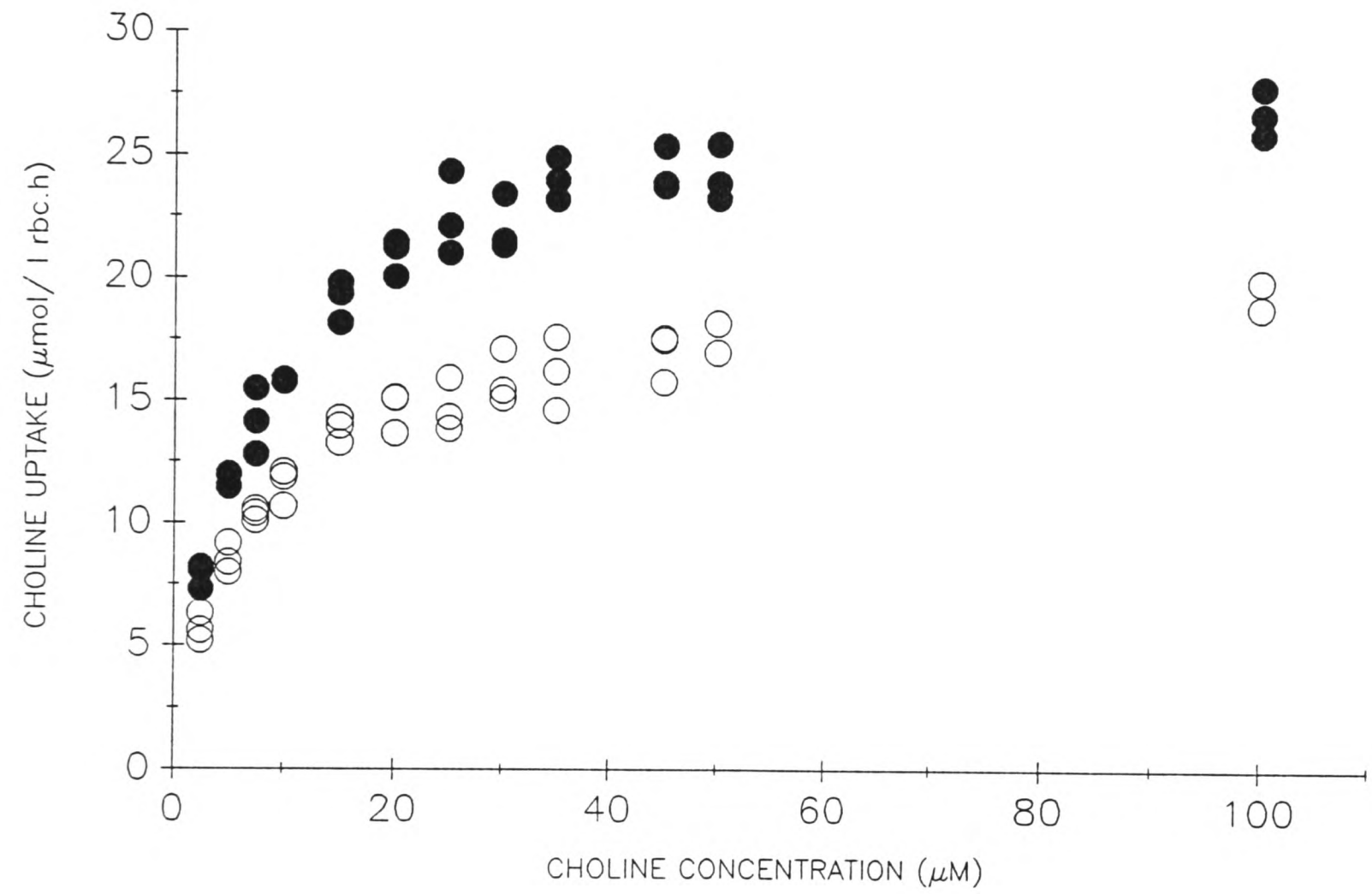


FIGURE 2.12: Concentration dependence of choline uptake in red cells from one polycythaemic patient. For the reticulocyte-enriched cell fraction K_m and V_{max} were 11.5 μM and 33 μmol/1.h, respectively. For the old cell fraction K_m was 8.8 μM V_{max} 21 μmol/1.h.

cells. The cells were separated by differential centrifugation in saline. With this method a large volumes were obtained of bulk for the old and the reticulocyte rich populations. In these patients young cells had a higher choline transport V_{max} of 38.4 (SEM: 4.2) μmol/l cells.h compared with 22.1 (SEM: 2.9) μmol/l cells.h for the old cell fraction (n: 3, $p<0.05$, t-test, figure 2.10). The K_m was not different in the 2 groups as shown in figure 2.11. An experiment for a single subject is illustrated in figure 2.12. The figure shows the plot of choline uptake as a function of with extracellular concentration, showing substrate saturation for both cell fractions.

2.3. DISCUSSION

This investigation was designed as a longitudinal study to assess the influence of renal transplantation and recovery of renal function in erythrocyte choline transport from CRF patients. It enabled choline transport to be followed as a time course after renal transplant, and its connection with renal function modifications. The aim was to try to answer the questions: What happens with the choline uptake after a kidney transplant? How quickly does the transporter function change? Is this due to a change in the membrane itself, or is this secondary to a plasma factor?

Membrane transport abnormalities in uraemia were reviewed in the introduction. The results in this chapter show that the abnormal erythrocyte choline transport in CRF recovers its normal function following a successful renal transplant. The recovery parallels the changes in renal function. Again it is stressed that while membrane transport changes are usually subtle in uraemia, erythrocyte choline transport is grossly abnormal and perhaps the most dramatic membrane transport alteration in uraemia.

Choline is usually derived from the diet or from endogenous sources [Zeisel et

al 1991]. Acara [1975] and Rennick [1974] have studied the metabolism of choline in several species. Choline, an essential nutrient, participates in several processes, is phosphorylated and enters membranes as phospholipids [Haudrich et al 1975, Ancelin & Vial 1989], is oxidized to betaine in the liver and kidneys since it is an important methyl donor [Acara 1975, Rennick 1974], and in neurons it is incorporated into acetylcholine. The plasma levels of choline are maintained within the range of 8-20 μM [Bligh 1952, Zeisel et al 1991, Greenwald et al 1985] and the kidneys and liver are important in maintaining its level constant [Acara 1975, Rennick 1974, Acara & Rennick 1973, Gardiner & Paton 1972].

Choline transport, despite some kinetic complexities, is well characterized in erythrocytes [Devés & Krupka 1978, 1979a,b,c, 1981a,b, 1983, 1986,1987, Krupka & Devés 1980a,b, 1988]. The erythrocyte choline carrier is a high affinity system capable of generating net fluxes of choline or choline-choline exchange fluxes [Martin 1977, Devés & Krupka 1979a,b]. The transporter does not have any known physiological role, since choline is not metabolized or incorporated to the erythrocyte [Askari 1966, Martin 1968], so it was surprising to find marked alterations in association with uraemia. Studies of the kinetics of this carrier in renal failure patients have shown that the maximal capacity of transport, V_{max}, and the affinity were higher in red cells of patients on dialysis compared with normal controls. The mean V_{max} for transport in dialysis patients was 2-3 times higher and not overlapping with normal controls. Renal transplant patients with functionally different grafts had a wide range of choline uptake rates, and a positive correlation was present between creatinine clearance and choline influx V_{max} [Fervenza et al 1991b]. The abnormalities of the erythrocyte choline transporter in uraemia were clear, significant and consistent [Fervenza et al 1990a, 1991b, Bibby et al 1990, Talbot et al 1992]. The present results provide further insights into its possible mechanisms.

The most immediate thought in a disease associated with accumulation of

metabolites is that the higher choline V_{max} could be due to raised intracellular choline or "uraemic toxins" that could be acting as a carrier substrate.

Rennick, Acara et al [1976] studied plasma choline during dialysis. They showed that the levels of choline are reduced at 2 hours of dialysis, but recovered to predialysis levels by 6 hours. This suggests that mechanisms were being activated to produce homeostasis of plasma choline concentration. Higher choline concentrations in renal failure may be due to reduced metabolism and extraction of choline via the kidneys, or to abnormal incorporation into acetylcholine or phospholipid [Rennick et al 1976]. Other metabolites, such as dimethylamine and trimethylamine, which show increased plasma concentrations in renal failure could also be involved. The high concentrations of these aliphatic amines in renal failure derive from the increased gut flora transforming dietary choline and phospholipids [Simenhoff et al 1976]. These compounds are potential substrates for the choline carrier, and could cause trans-stimulation. However, the possibility of trans-stimulation effects causing the elevation of the fluxes in CRF appears very unlikely, since that choline uptake was abnormally elevated even in experiments performed after cells were repeatedly washed to obtain zero trans conditions [cf Fervenza et al 1991b]. It seems rather that a factor(s) is acting upon the transporter protein itself or upon its regulatory mechanisms.

Erythrocyte choline transport can be modified by changes in intra- and extracellular pH [Devés et al 1986]. However the pH shifts required are far greater than those seen in chronic renal failure.

Anaemia is one of the clinical features of renal failure, and its etiology is multifactorial. Factors involved include lack of erythropoietin synthesis, blood loss, iron deficiency, aluminium intoxication and haemolysis [Eschbach 1989,1989a, Erslev 1970]. Erythrocyte survival time is reduced from 120 days, depending on the severity of uraemia,

resulting in a larger proportion of circulating young circulating red blood cells [Eschbach 1989,1989a]. It is conceivable that this could also have effect on the overall membrane transport activities. In fact some membrane transport systems alter their activities as the cell ages [Hall & Ellory 1986, Uney et al 1985]. Kaji and coworkers have suggested that a higher number of younger erythrocytes may have masked the defect in the activity of the Na/K pump in uraemia in some studies, considering that a younger cell population has a higher number of Na pumps/cell [Kaji & Kahn 1987]. If conditions are controlled for cell age, it has been shown that the youngest uraemic cells have fewer Na/K pumps than the youngest normal cells [Cheng et al 1984]. The evidence presented in this thesis militates against the possibility of cell age having an effect on the overall expression of choline transport activity in renal failure. First, red cell fractions with different densities showed to have an enhanced maximal capacity for choline transport in younger cells, with no difference in affinity (fig 2.9 to 2.12). In uraemic patients both V_{max} and K_m were abnormally elevated. Second, after transplantation, increased erythropoietin will be present from the kidney graft, with production and release of new red cells into the circulation from the bone marrow. If the effect was due to cell age, choline uptake after transplantation should increase and not decrease - this is clearly not the case (fig 2.5). Third, the half-time for choline uptake recovery was much faster than any significant modification in red cell population (figure 2.7).

A plasma factor, perhaps a digitalis-like substance, has been suggested as the cause of the inhibition of the Na-K ATPase in chronic renal failure [Cole et al 1968, Graves et al 1983]. Lipids have also been described as potential candidates. Non-esterified fatty acids (NEFA) are increased in the erythrocyte membrane of uraemic patients and evidence suggests that NEFA can inhibit the Na,K ATPase in vitro [Kelly et al 1989]. Other uraemic toxins could also be involved in those changes. The effects could be secondary to a plasma factor affecting circulating erythrocytes by acting upon specific sites at the membrane, or alternatively the

influence of uraemia on cell maturation during erythropoiesis inducing structural changes. Immediately after renal transplantation there is an improvement in the renal function and we demonstrated that the recovery of the choline V_{max} towards normal values was parallel with the changes in creatinine. During a reversible decline in renal function, treated with methylprednisolone, the choline uptake was temporarily increased (figure 3.8). The changes in the choline transport system were fast and accompanied the changes in renal function. This suggested that circulating plasma factors, that were rapidly cleared by a functioning kidney, were involved. If the changes were during cell maturation (when erythrocyte membrane synthesis occurs), with intrinsic structural membrane changes, one would expect slower recovery due to the erythrocyte life span. If a circulating factor is involved it must interact with the membrane strongly, otherwise it would be washed away during the initial cell wash in procedures prior to flux measurements.

Altered physical membrane properties of uraemic cells have the potential to influence cell membrane function [Viljoen et al 1991, Bleiber et al 1980, Komidori et al 1985]. Evidence that cell membrane protein function is extensively altered in uraemia, includes changes in different tissues, different transport systems [table 1.5] and receptor function [Ruiz et al 1990]. These widespread membrane changes also appear to be specific, considering that some transport systems are inhibited, while other are activated or unaltered. Altered transport could result from abnormal lipid composition [Bleiber et al 1980, Komidori et al 1985, Kelly et al 1989], reduced fluidity [Komidori et al 1985] or other changed membrane physical properties associated with uraemia and/or dialysis [Viljoen et al 1991].

The significance, clinical importance and mechanisms of the alterations are not clear. Several clinical features of the uraemic syndrome are successfully reversed by renal transplantation. Some membrane alterations present in chronic renal failure such as Na,K ATPase inhibition, are also corrected following a renal transplant [Fervenza et al 1989a]. It has been

suggested that changes in intracellular sodium appear to be related to the severity of uraemia [Welt et al 1964, 1967, Kaji & Kahn 1987], but previously a correlation with the clinical evolution of uraemia has not been demonstrated with any alteration in membrane function. Data in this thesis illustrate that the maximum transport capacity of the choline transporter (V_{max}) was elevated in uraemic patients. Choline influx V_{max} values presented here were similar to those of a previous study of the choline transport system in CRF [Fervenza et al 1991b]. Choline uptake rates also correlated with renal function in pre-dialysis chronic renal failure patients (fig 3.4). This was similar to the association present in patients with functional transplants. This agreement suggests an influence of the degree of the renal impairment rather than other factors in transplantation such as immunosuppressive treatment.

Choline uptake has also been studied in Alzheimer's disease (AD). In this neurodegenerative disorder red cell choline transport is reported to be higher,lower or unaltered [Kanof et al 1985, Glen et al 1981, Miller et al 1986, Butterfield et al 1985, Friedman et al 1981]. These conflicting results could be due to different methodological approaches and different intracellular choline concentrations. In chronic renal failure red cell choline uptake is high even under zero-trans conditions, (that is when cell choline content is depleted) [Fervenza et al 1991], suggesting that there is an effect on the carrier itself. The changes in AD are not unequivocal but one could be tempted to compare this neurological disease with uraemia. It is perhaps reasonable to say that choline transport may be involved in the mechanisms of some manifestations of renal failure. Rennick et al [1976] in fact have shown a correlation of plasma choline concentration with nerve conduction velocity in renal failure, an interesting association that deserves further investigation. Work by Simenhoff and coworkers suggested that dimethylamine and trimethylamine are toxic products in uraemia [Simenhoff et al 1963, 1976, 1977, 1978, Simmenhoff 1975]. Those aliphatic amines are derived from endogenous and exogenous sources

[Simenhoff et al 1963, 1977, 1976, 1978, Simenhoff 1975]. Choline and phosphatidylcholine from the diet are transformed by the gut bacterial flora [Simenhoff et al 1976] and amines accumulate. Treatment with antibiotics is associated with reduced levels of aliphatic amines and with a symptomatic improvement on the manifestations of uraemia [1976]. There is also a correlation with plasma urea [Simenhoff al 1963]. An interesting hypothesis by Lee et al [1991] raised the possibility that toxic effects of urea on metabolism are counteracted by methylamines. These aspects can not be addressed directly in the present work, but it is interesting to speculate that alterations in choline transport and metabolism could well be an adaptative response of the cell to the uraemic milieu.

The possible clinical implications of the abnormal choline transport, depend largely on whether the findings in red cells can be extended to other cell types. Choline transport is important in the nervous system, endothelial cells and others [Estrada et al 1990, Bevan & Kinne 1990, Haga & Noda 1973, Yamamura & Snyder 1973, Kuhar & Zarbin 1978, Suzuki et al 1987, Vyas & O'Regan 1985]. Cultured bovine cerebral endothelial cells have a saturable choline transport system that shows substrate affinity similar to that of the erythrocyte choline transport system [Estrada et al 1990]. The sensitivity of the endothelial cell and erythrocyte systems to inhibition by choline analogues is also similar. The endothelial cell transporter may control access of choline to the cerebral fluid and abnormalities could affect acetylcholine production [Estrada et al 1990]. Neuronal choline uptake involves two distinct systems [Martin 1977], one similar to the erythrocyte system and the other a Na-dependent system with a higher capacity [Haga & Noda 1973, Yamamura & Snyder 1973, Kuhar & Zarbin 1978]. A complex and unexplained characteristic of the erythrocyte choline carrier is the irreversible inhibition of transport caused by long term treatment with lithium salts used in the treatment of bipolar illness [Martin 1977, Beilharz et al 1986, Mallinger et al 1984, Jope et al 1980, Hunt et al 1984, Uney

et al 1985]. The effects of uraemia on choline transport in other cell types have not been examined yet.

Finally, abnormal choline transport is also present in malaria infected erythrocytes [Ancelin et al 1991, Kirk et al 1991]. Increased permeability to several substrates such as amino acids, sugars, nucleosides and ions is present in plasmodium-infected red cells. Recently a novel malaria-induced erythrocyte choline pathway, not saturable to choline concentrations of up to 500 μM, and distinct both kinetically and pharmacologically from the endogenous choline carrier has been described [Kirk et al 1992]. These disturbances of choline transport are distinct from the one present in uraemia.

2.4. SUMMARY : CHOLINE TRANSPORT

In conclusion we have shown that the changes in the choline transport system present in uraemia are reversible on renal transplantation, that maximum rates of red cell choline uptake recover rapidly and follow the modifications of plasma creatinine concentration. There is some evidence to suggest that a circulating factor could be involved. This is an example where a clear demonstration of a defect in membrane function can be related to the clinical aspects of a disease. The exact clinical importance of these findings is not clear yet and further studies are required. Choline transport abnormalities in other tissues from uraemic patients or animals, such as brain and muscle, should be sought.

The modifications cannot be explained by the production of young cells from the bone marrow of the kidney recipient. Young erythrocytes have a more active choline transporter. It is suggested that circulating factors are involved in the aetiology of these changes, acting by modifying the properties of the cell membrane.

CHAPTER 3

SODIUM HYDROGEN ANTIPORTER ACTIVITY IN CHRONIC RENAL FAILURE AND DIABETIC NEPHROPATHY

The present chapter will describe experiments on the intracellular pH, and activity of the sodium-hydrogen antiporter (Na/H antiporter) in leucocytes of patients with chronic renal failure (CRF) with and without diabetic nephropathy.

CRF causes systemic acidosis by an impairment of the urinary acidification mechanisms and the renal homeostatic responses to acidosis [Warnock 1988, Madias & Kraut 1989, Dubose 1984]. It is also characterized by several derangements of cell function [Eknoyan & Knochel 1984]. The changes in cellular pH homeostatic mechanisms are not well understood in CRF. Intracellular pH (pHi) regulation in uraemia has not received much attention, and it is possible that derangements of pHi homeostasis may underlie some pathological conditions in this syndrome.

Insulin dependent diabetes mellitus patients develop albuminuria in the early stages of nephropathy [Drury et al 1989, Selby et al 1990], and those are the ones at risk of end stage kidney disease [Viberti et al 1982]. A higher Vmax of the leucocyte Na/H antiporter is present in patients with IDDM and albuminuria, but not in IDDM without nephropathy [Ng et al 1990b]. This fact lead to the suggestion that the antiporter activity is a possible genetic marker to predict which patients are at a higher risk of developing advanced nephropathy [Ng et al 1990b]. Studies of the RBC Na/Li countertransporter, perhaps an analogous system, have provided similar conclusions [Krolewski et al 1988, Mangili et al 1988, Carr et al 1990]. These

observations lead us to suspect that the Na/H antiporter would be increased in activity in end stage kidney nephropathy secondary to diabetic nephropathy. Until now no work has examined the leucocyte Na/H antiporter activity in uraemia and end stage diabetic nephropathy. These facts are summarized in a schematic form in the figure 3.1.

3.1. LEUCOCYTE pHi AND Na/H ANTIPORTER. MATERIAL AND METHODS / PATIENTS.

3.1.1. MATERIAL AND SOLUTIONS

Nigericin, monensin, fatty-acid-free bovine serum albumin (BSA), dextran, and tissue culture fluid (TC199) were from Sigma Chemical Co, Poole, Dorset, UK. TC199 was buffered with 15 mmol/l 4-(2-hydroxyethyl)-1-piperazine-enthanesulphonic acid (HEPES) and was nominally bicarbonate-free. Its composition was adjusted to NaCl 140 mmol/l, KCl 5.5 mmol/l (pH 7.4). Ethylisopropyl amiloride (EIPA) was a gift from Dr. Alexander Bearn, of Merck, Sharpe and Dohme, Rahway, New Jersey, USA. Bis(carboxyethyl) carboxy-fluorescein acetoxymethyl ester (BCECF-AM) was from Cambridge Bioscience, Cambridge, UK. All the other chemicals were Analar grade.

Isotonic NaCl buffer had the following composition (in mmol/l): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, HEPES 15, and BSA 1 g/l (pH 7.4 with tris base). Isotonic KCl buffer was composed of (in mmol/l): KCl 140, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, HEPES 15 (pH 6.0). Nigericin and monensin were prepared in isotonic KCl buffer from a stock solution at 4 and 10 mmol/l respectively, in absolute alcohol.

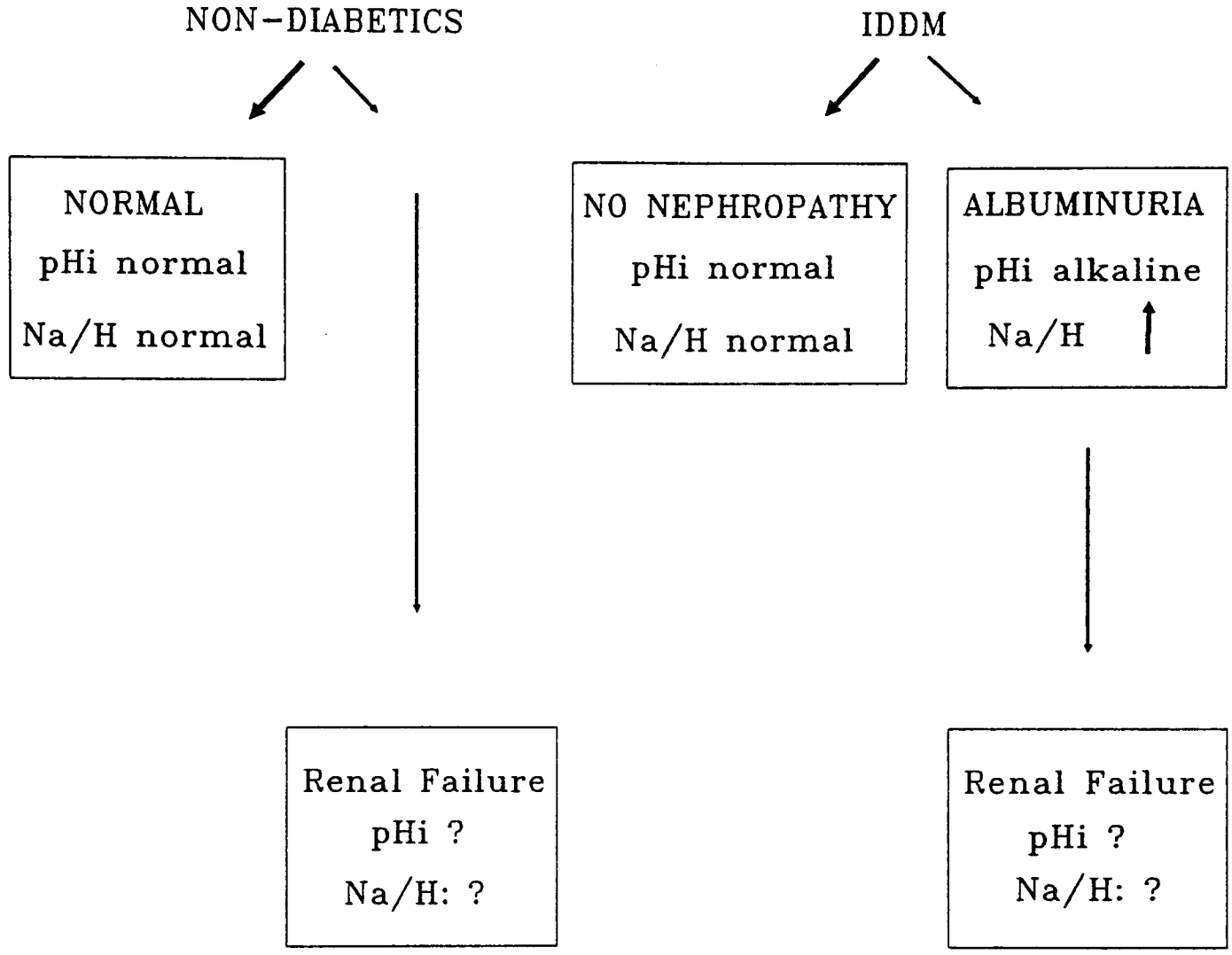


FIGURE 3.1: Diagram summarizing the known findings for the Na/H antiporter activity and intracellular pH in type I (insulin dependent) diabetic and non-diabetic patients. The present study is designed to look at patients with renal failure with and without diabetic nephropathy.

3.1.2. WHITE CELL PREPARATION

Studies of pHi and activity of the Na/H antiporter were performed using white blood cells. Leucocytes are readily available and are very convenient to use with fluorescent indicators to assess pHi.

LEUCOCYTES

Leucocyte separation from blood or bone marrow can be carried out by density gradient centrifugation methods as described in a series of papers by Böyum [1968] and by dextran sedimentation as described by Baron and Ahmed [1969].

The Böyum method involves liquid phases where a suspension of cells is layered onto separation fluid of relatively high density. The outcome of the separation is determined by the sedimentation rates of the cells in the two phases. The Böyum method involves a combination of low viscosity and high density medium (Isopaque) with a solution of high viscosity and low density (methylcellulose, dextran and ficoll) [Böyum 1968]. At present it is easily done employing a commercially available medium such as Histopaque (Sigma Chemical Co.), that contains a mixture of ficoll and sodium diatriazoate. Those methods were used to isolate a specific population of leucocytes, such as lymphocytes.

To obtain a total whole population of leucocytes, dextran sedimentation of erythrocytes, centrifugation and rapid lysis of red blood cells [Baron & Ahmed 1969] was used. Heparinized blood (20-30 ml) was obtained from peripheral venepuncture or from the vascular access in haemodialysis patients. Blood was kept at room temperature and used immediately. Frothing was avoided during collection and manipulation. Dextran solution was prepared at 0.12 g in 10 ml of TC199 and placed in universal tubes. Ten ml of blood was added to each tube,

mixed by inversion (10-20 x), and placed on a stand at an angle of 45°. Any frothing was removed by aspiration with a pasteur pipette and around 30 minutes allowed for the erythrocytes to sediment to the bottom of the tube. Plasma with leucocytes, some erythrocytes and platelets was removed, without disturbing the sedimented layer of red blood cells. This plasma was centrifuged at about 300 g for 10 minutes. The cell pellet containing leucocytes was collected and vortexed with 15 ml of distilled water for 15 seconds, to produce a hypoosmotic shock and lyse any residual red blood cells. Osmolality was restored by the addition of 5 ml of a 4 times concentrated TC199 solution. The cells were then washed twice with TC199 with centrifugation at low speed for 10 minutes to allow removal of platelets from the supernatant (300g). Leucocytes were then used according to each experimental protocol.

3.1.3. INTRACELLULAR pH AND Na/H ANTIPORTER MEASUREMENTS

3.1.3.1. METHODS OF MEASUREMENTS

Various methods are available to measure intracellular pH. There are several reviews on the subject [Roos & Boron 1981, Vaughan-Jones 1988, Okerlund & Gillies 1988, Russell 1988, Tsien 1989, Rink et al 1982, Boron 1987]. They include distribution of weak acid and bases, nuclear magnetic resonance (NMR), micro-electrodes and optical methods using fluorescent indicators.

Intracellular pH can be measured using dyes that change colour or fluorescence with changes in pH. They produce easy and accurate measurements of steady state pHi, as well as monitoring rapid pH changes [Roos & Boron 1981]. In the present thesis we used the fluorescent probe BCECF.

Fluorescent probes usually have a spectrum dependent on pH. They have several advantages and can be incorporated into the intact cell without damage to the membrane. They measure ion activity or free concentration [Tsien 1989]. Fluorescence is proportional to dye protonation so that the higher the pH the greater the fluorescence intensity.

The most popular indicators are derivatives of fluorescein, as is the case for BCECF (figure 3.2). It has a pKa of 6.97-6.99 [Rink et al 1982, Bright et al 1987], and its fluorescence is pH dependent. For excitation and emission the peak wavelengths in which the fluorescence is pH dependent are 502 nm and 528 nm [Tsien 1989]. There is a wavelength (439 nm) at which the excitation efficiency is independent of pH, i.e. at which all the spectra at different pHs cross (isoexcitation point). This is a very useful characteristic since the ratio of the excitation efficiency at a pH-sensitive wavelength to that at the isoexcitation point (502 nm/439 nm) gives a measure of pH that cancels out dye concentration, optical path length and cell concentration [Tsien 1989]. For a illustration of the BCECF spectra see figure 3.3.

Loading with the fluorescent dye is achieved by using the hydrolysable ester form, acetoxymethylester- BCECF-AM, which is permeant to cell membrane. In the cell cytosol this form is cleaved by esterases and the impermeant pH-sensitive form BCECF is released [Roos and Boron 1981, Tsien 1989]. Calibration can be carried out in situ by subjecting cells to a known internal pH with the use of a K/H ionophore, such as nigericin [Thomas 1979]. Backleak of dye is easily determined by centrifuging the cells and measuring the fluorescence in the supernatant [Roos and Boron 1981]. Corrections should be made for the intrinsic fluorescent properties of the tissue being used, autofluorescence.

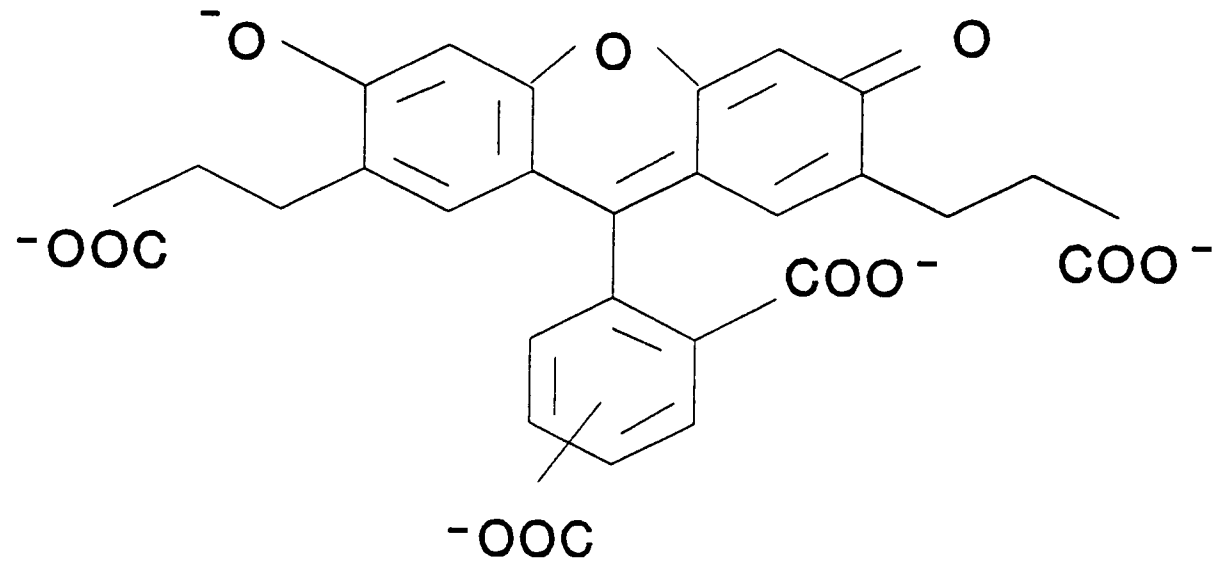


FIGURE 3.2: BCECF molecule.

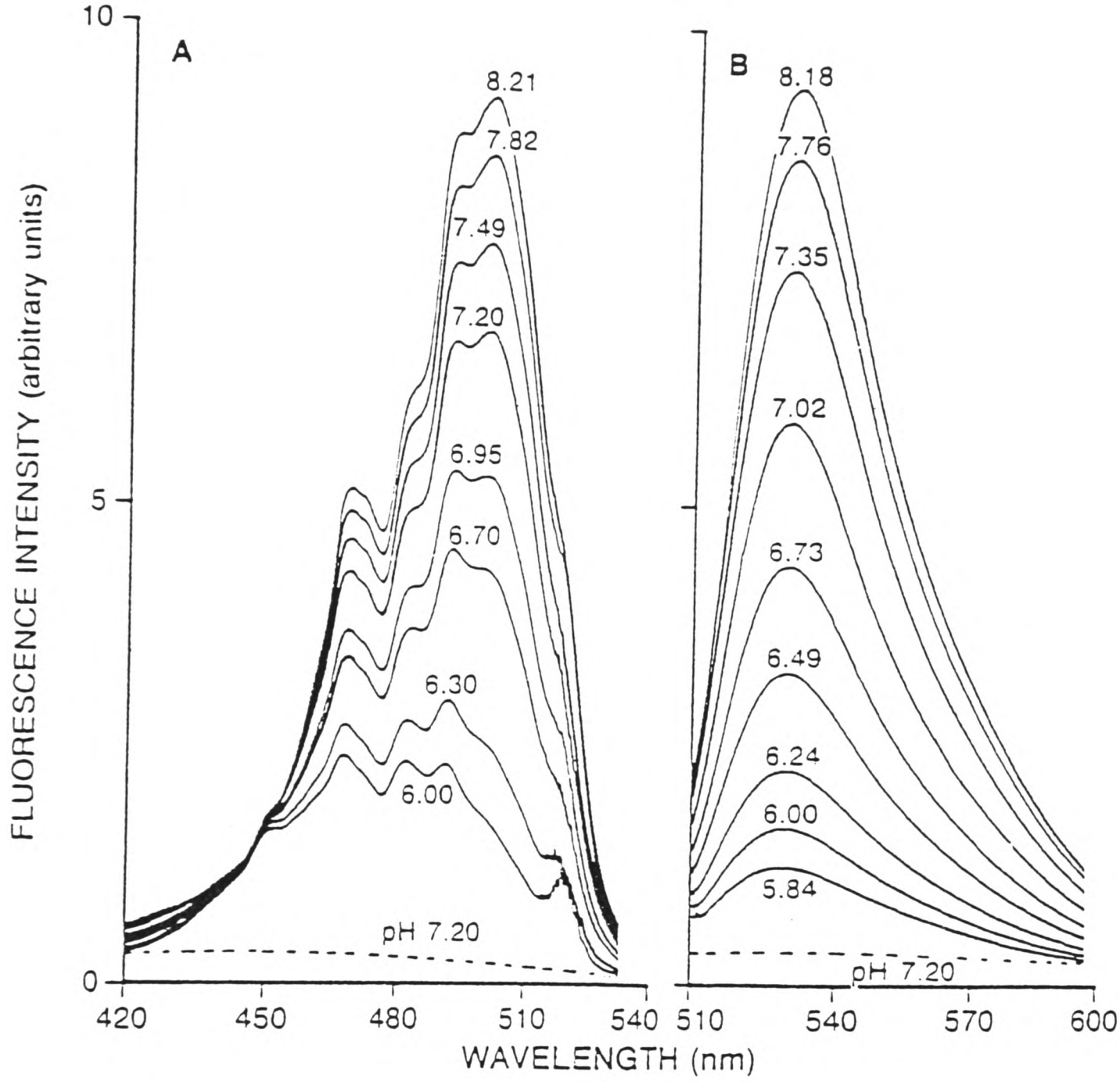


FIGURE 3.3: Representation of the BCECF fluorescence spectra. Excitation (left, recorded at emission of 525 nm) and emission (right, recorded with excitation at 500 nm), spectra of BCECF (solid lines) and an equimolar concentration of BCECF-AM (broken lines) are shown [reproduced from Grinstein et al 1989 by kind permission of Academic Press].

3.1.3.2. Na/H ANTIPORTER

Studies of the Na/H antiporter activity use the fluorescent dye to assess changes in pHi and hence measure Na-dependent H efflux. Determination of the proportion of the fluxes via the Na/H antiporter can be achieved with the inhibitor amiloride or some of its more specific derivatives like ethyl isopropyl amiloride [Frelin et al 1987, Benos 1988]. Alternatively the Na-dependent pH changes reflect the antiporter activity [Grinstein 1988, Bock & Marsh 1988].

In this work, all the solutions and TC199 were bicarbonate-free to enable the study of the Na/H antiporter without contributions from other pH regulation mechanisms such as HCO^-_3/ anion exchangers [Simchowitz & Ross 1985, Simchowitz & Weer 1986, Simchowitz et al 1985, Simchowitz & Davis 1990, Goldsmith et al 1990].

3.1.3.3. EXPERIMENTAL PROTOCOL

BCECF LOADING

Cells were resuspended and incubated at 37°C for 30 minutes with the esterified pH-sensitive fluorescent dye BCECF-AM, final concentration 10^{-5} moles/l. The leucocytes were then washed 3 times and resuspended in TC199 and kept at room temperature for 20 minutes (30 minute for lymphoblasts) to achieve deesterification of BCECF-AM intracellularly, before any measurements were made. Intracellular pH determinations immediately after dye loading give lower values due to the continuous de-esterification of BCECF-AM.

INTRACELLULAR pH DETERMINATIONS

Intracellular pH under resting conditions was measured after washing the cells in an isosmotic NaCl buffer containing BSA (1 g/l).

The fluorescence of the intracellular BCECF was measured on a Perkin-Elmer luminescence spectrometer (LS-5B) equipped with a magnetic stirrer and a thermostatically-warmed cuvette holder. Excitation wavelength was at 500 and 439 nm (slit width 2.5 nm) and the emission wavelength was set at 530 nm (slit width 10 nm). Fluorescence ratio data (500/439 nm) were converted to pH units by constructing a calibration curve for every experiment in the pH range 6.0 to 7.8. Calibration employed a double ionophore technique (nigericin and monensin) [Ng & Dudley 1989]. To construct the calibration curve from pH 7.8 to 6.0, cells were suspended in an isotonic KCl buffer containing nigericin and monensin, but no albumin was used to remove the ionophores from the membrane. Starting from a pH of 7.8 in one cuvette and 6.8 in another, pH was progressively changed by sequential addition of MES and then two minutes equilibration, when sequential readings of the fluorescence ratio and of the cuvette cell suspension pH by means of an pH electrode were made. The plot of the pH against fluorescence ratio gave a curve fitted by a non linear least squares technique. The fluorescence ratio results were converted to pH using the following equation:

$$F = A+B(10^{pH-pK}) / (1+10^{pH-pK})$$

where F was the fluorescence ratio (500/439), A and B were constants obtained from the curve and pK was the pK of the intracellular trapped BCECF [Ng et al 1990a].

Dye leakage from the cells contributed less than 6% of the total fluorescence after 10 minutes of incubation, the time by which the readings had been made [Ng

and Dudley 1989].

Rapid changes in the fluorescence were recorded on a disk and processed for subsequent analysis of the data with a microcomputer. Excitation at 500 nm was measured with a long (4s) integration of the fluorescence from excitation at 439 nm at the end of each experiment. At 439 nm, the isoexcitation point, fluorescence remained constant throughout the experimental period.

INTRACELLULAR pH CLAMPING

Cells were washed once, resuspended and incubated at 37°C for 5 minutes in isotonic KCl buffer containing nigericin 2 μmol/l and monensin 5 μmol/l, at pH values of 6.0 [Ng et al 1990b, Baron & Ahmed 1969]. The monensin (Na/H ionophore) depleted the cell of Na and the nigericin (H/K ionophore) set pHi equal to extracellular pH. In the presence of both ionophores the extracellular and intracellular pH should be equal, and as there was no external Na in the KCl buffer the intracellular Na would fall to very low levels. Cells were then spun down, resuspended and washed in the same KCl buffer (pH 6.0), but containing non-esterified fatty acid free bovine serum albumin (1g/l) and without ionophores. The albumin binds the nigericin and monensin and extracts them from the cell membrane. With this procedure the pHi was clamped at pH=6.0 and the maximum capacity of the Na/H antiporter can be assessed.

Na/H ANTIPORTER V_{max}

When pHi is near 6.0, the leucocyte Na/H antiporter is operating near its maximal transport capacity [Ng & Bomford 1989]. The maximal capacity (Vmax) of the antiporter was thus

measured with the pHi clamped at 6.0 as above. The cells were then resuspended in "isotonic KCl buffer" containing 1 g/l BSA. The initial rate of change of intracellular pH after rapid addition of external sodium (final concentration: 133 mmol/l) was measured in the absence and presence of 10^{-5} mol/l EIPA. The EIPA-sensitive rate of change of intracellular pH was taken to represent the Na/H antiporter activity. The product of buffering power measured at pH 6.0 and the EIPA-sensitive rate of change of pHi allowed Na/H antiporter activity to be expressed in $mmol.l^{-1}.min^{-1}$ of H^+ efflux.

In the experiments using lymphoblasts a sodium-free isotonic solution containing N-methyl-D-glucamine (NMDG) was used instead of EIPA and the difference in rate of change of pHi after adding NaCl and that after adding NMDG represents the Na/H antiporter Vmax. The rate of change of pHi after adding NMDG or adding NaCl in the presence of EIPA was identical under the present experimental conditions.

BUFFERING CAPACITY

Intracellular buffering power varies with pHi [Ng & Dudley 1989] and was measured at both resting pHi and when pHi was 6.0, by recording the change in the intracellular pH after the addition of extracellular $(NH_4)_2SO_4$ (1 to 16 mmol/l final concentration) [Ng & Dudley 1989]. If the pKa of NH_3 inside and outside the cells is assumed to be the same, and only NH_3 crosses the cell membrane rapidly and causes a step change in internal pH, then it is possible to determine the buffering power of cells loaded to different internal pH values following the calculation from the Henderson Hasselbalch equation:

$$\text{Buffering power} = \frac{(\text{change in } [NH_4^+]_{in}}{(\text{change in internal pH})}$$

and the change in $[NH_4^+]_{in}$ is defined as follows:

$$\frac{\text{total base concentration} \times 10^{pHo-pHa}}{(1+10^{pHo-pHa})}$$

in which pHo and pHa are the pH of the buffer and the intracellular pH respectively after adding the $(NH_4)_2SO_4$ and the units are mmol/l.pH unit. The pK_a of NH_3 is 8.89. The total base concentration is the concentration of ammonium added to the solution in mmol/l.

These methods were developed by Dr. Leong L Ng at the Department of Clinical Pharmacology and Sheikh Rashid Diabetes Unit at the Radcliffe Infirmary in Oxford.

3.1.4. PATIENTS

Thirteen chronic renal failure patients without diabetes (CRF), and eight chronic renal failure patients with insulin-dependent diabetes and end-stage diabetic nephropathy (CRF-DM) on haemodialysis treatment were evaluated. None of them had essential hypertension. These patients were compared with 25 normal controls.

The average age of CRF patients was 55 years (range: 21 to 77) with a male/ female ratio of 7/6. The aetiology of renal failure was pyelonephritis (3), IgA nephropathy (1), polycystic kidneys (1), glomerulonephritis (1), scleroderma (1), and unknown (6). The CRF-DM patients had an average age of 51 (range: 35 to 68) and 6 were male. The results of intracellular pH determinations, buffering capacity and Na-H antiporter Vmax measurements were compared with the findings in 25 normal controls [Mean age: 42 (range: 18 to 68); M/F: 12/13].

Mean plasma creatinine was 843 μmol/l in the CRF patients, 802 μmol/l in the

CRF-DM patients and 83 μmol/l in the control group. Clinical and laboratory details of the subjects studied are shown in table 3.1.

Mean blood pH was not significantly different in the two groups 7.36 (SD: 0.05) in CRF and 7.33 (SD 0.03) in CRF-DM (F: 1.47, df: 1,19, p>0.05, NS).

TABLE 3.1
CLINICAL DATA

	CONTROL	CRF	CRF-DM
Number	25	13	8
Age(years)			
Mean	42	55	51
Range	18-68	21-77	35-68
Male/Female(n)	12/13	7/6	6/2
Time on HD(years)			
Mean		3.5	3.4
Range		0.2-13	0.1-10
Duration of DM(years)			
Mean			27
Range			18-34
Creatinine(μmol/l)			
Mean	83	843	802
Range	60-108	558-1094	501-1191
Urea(mmol/l)			
Mean		24	26
Range		14.5-38.5	7.2-43.7
Sodium(mmol/l)			
Mean	141	138	136
Range	138-144	133-146	133-141
Potassium(mmol/l)			
Mean	4.2	5.1	5.5
Range	3.7-4.7	3.5-6.6	4.3-7
Glucose(mmol/l)			
Mean	5.1	5.5	9.5
Range	4.6-6.3	4.2-7.2	5.2-16.3
Haemoglobin(g/dl)			
Mean	14.1	8.1	7.0
Range	12.5-16.4	5.9-11.2	4.8-8.6
Blood pH			
Mean		7.36	7.33
Range		7.29-7.42	7.29-7.39
Blood HCO_3.(mmol/l)			
Mean		19.1	21.7
Range		14.3-22.6	18.7-27.3

3.2. RESULTS

3.2.1. INTRACELLULAR pH MEASUREMENTS, BUFFERING CAPACITY and Na-DEPENDENT H^+ EFFLUX.

Figures 3.4 and 3.5 illustrate typical results for intracellular pH measurements. After the dye loading, cells were allowed 20-30 minutes until complete deesterification of BCECF-AM was achieved. Immediate measurements of pH after loading gives lower values of pHi.

3.2.2. INTRACELLULAR pH

The results for intracellular pH at resting conditions are illustrated in figure 3.6. Data from Insulin Dependent Diabetes Mellitus patients (IDDM) from Ng et al [1990b] are also included for comparison and were collected at the same period of time as the present study. One way analysis of variance (ANOVA) shows that the mean intracellular pH of the groups is significantly different (f: 18.60, df: 3, 61, $p<0.001$). The mean value for the normal controls was 7.43 ± 0.09. Patients with chronic renal failure on dialysis treatment (CRF) were significantly more acidic, with a mean pHi of 7.34 ± 0.05 (t: 2.73, df: 61, $p=0.004$). The end stage renal failure patients with diabetes mellitus (CRF-DM) showed a normal leucocyte pHi of 7.42 ± 0.07 compared to controls (t: 0.29, df: 61, $p<0.05$, NS) even though they had a systemic metabolic acidosis. The group with diabetes and early nephropathy (IDDM) had a markedly more alkaline mean intracellular pH of 7.59 ± 0.14 compared with normal controls (t: -5.27, df: 61, $p<0.004$) or with CRF-DM (t: -4.09, df: 61, $p<0.004$). It is therefore apparent that the patients with diabetes mellitus tended to have a higher intracellular pH than non-diabetic patients, with normal or

impaired renal function.

3.2.3. BUFFERING CAPACITY

Analysis of the resting buffering capacity is illustrated in figure 3.7. Buffering capacity had a considerable individual variability with mean (SEM) values of 14.76 ± 0.96 mmoles/l.pH unit for normal controls, 13.24 ± 0.66 for CRF, 10.86 ± 1.95 for CRF-DM and 10.72 ± 1.61 for IDDM. ANOVA showed no significant difference between the groups (F: 2,48, $p>0.05$, NS).

3.2.4. Na/H ANTIPORTER ACTIVITY

Figure 3.8 demonstrates the Na/H antiporter V_{max} for the different groups of patients. It is apparent that the mean maximal capacity of transport was very similar in three groups (Controls, CRF and CRF-DM), with a control V_{max} of 55.2 mmol/ l. min (SD: 8.8), CRF 56.5 (SD: 9.9) and CRF-DM 56.8 (SD 12.8). The difference between the groups shown in the ANOVA (F: 9.33, df: 3,61, $p< 0.001$) is due to the significantly higher V_{max} in the IDDM patients (IDDM 73.8 mmol/l.min; t: -4.90 (xControl), t: -3.85 (xCRF), t:-3.24 (x CRF-DM), $p<0.0$ 04).

3.2.5. BLOOD pH

Blood pH was measured in the patients with renal failure with or without diabetes. Mean blood pH was not significantly different being 7.36 (SD: 0.05) in CRF and 7.33 (SD 0.03) in CRF-DM (F: 1.47, df: 1,19, $p>0.05$, NS), as shown in figure 3.9.

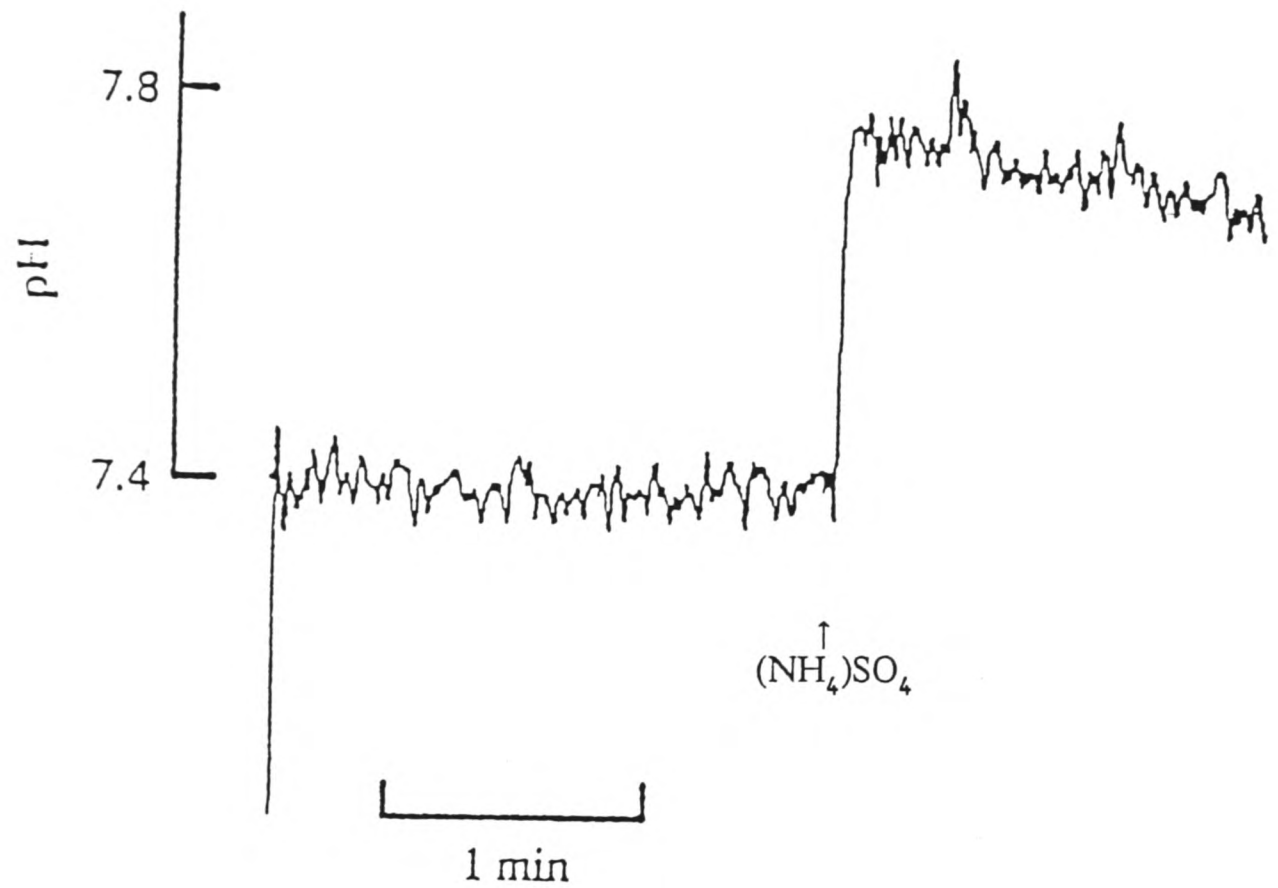


FIGURE 3.4: Leucocyte intracellular pH. The change in pHi from its resting values results from the addition of $(NH_4)SO_4$ [figure kindly provided by Dr. LL Ng].

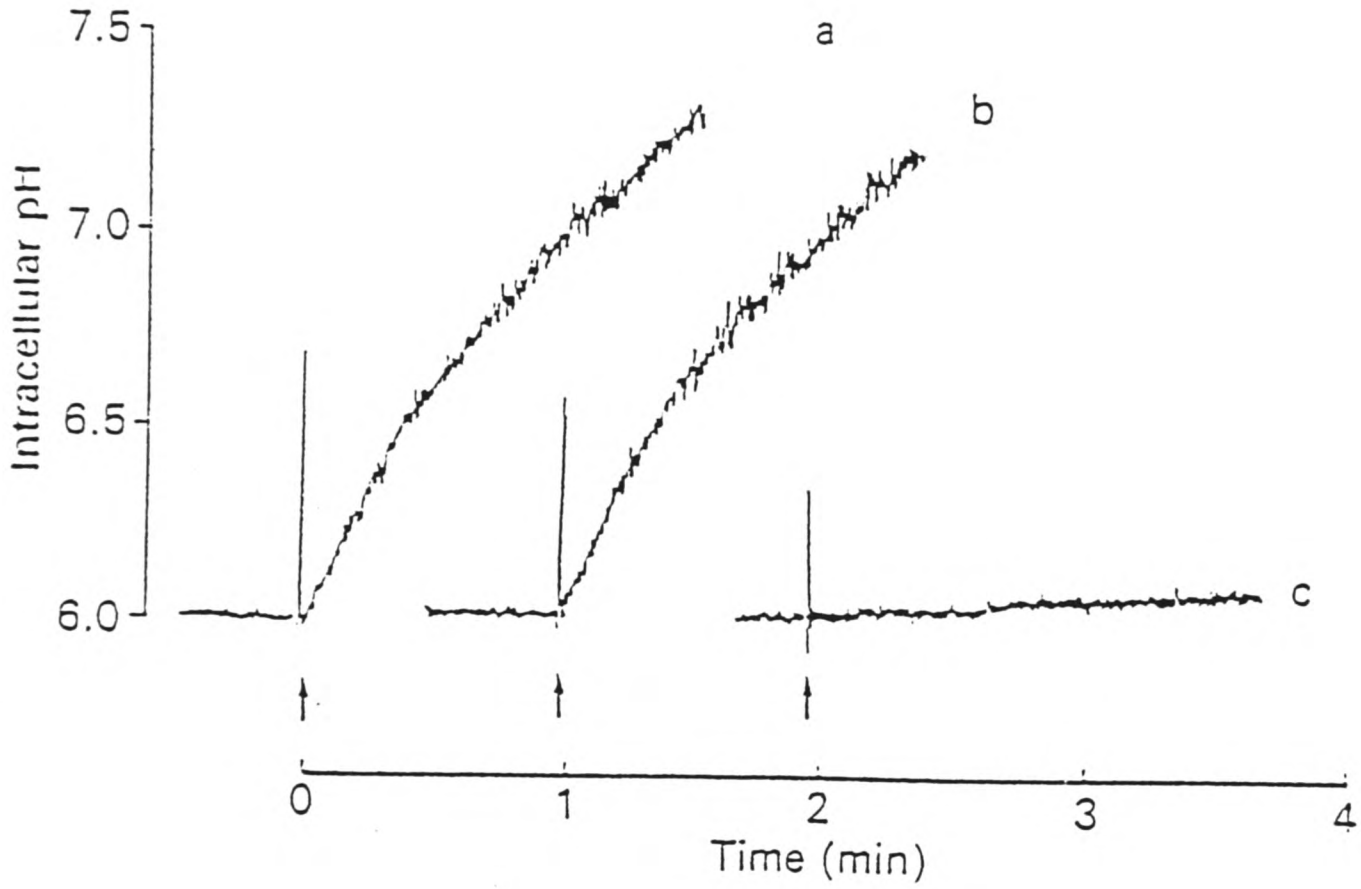


FIGURE 3.5: Leucocyte pHi changes due to H^+ efflux. Leucocytes with pHi clamped at 6.0 (see text) exhibit alkalinization following the addition of extracellular NaCl in the absence (a,b) and presence (c) of EIPA. The EIPA sensitive efflux is mediated via the Na/H antiporter [figure kindly provided by Dr. LL Ng].

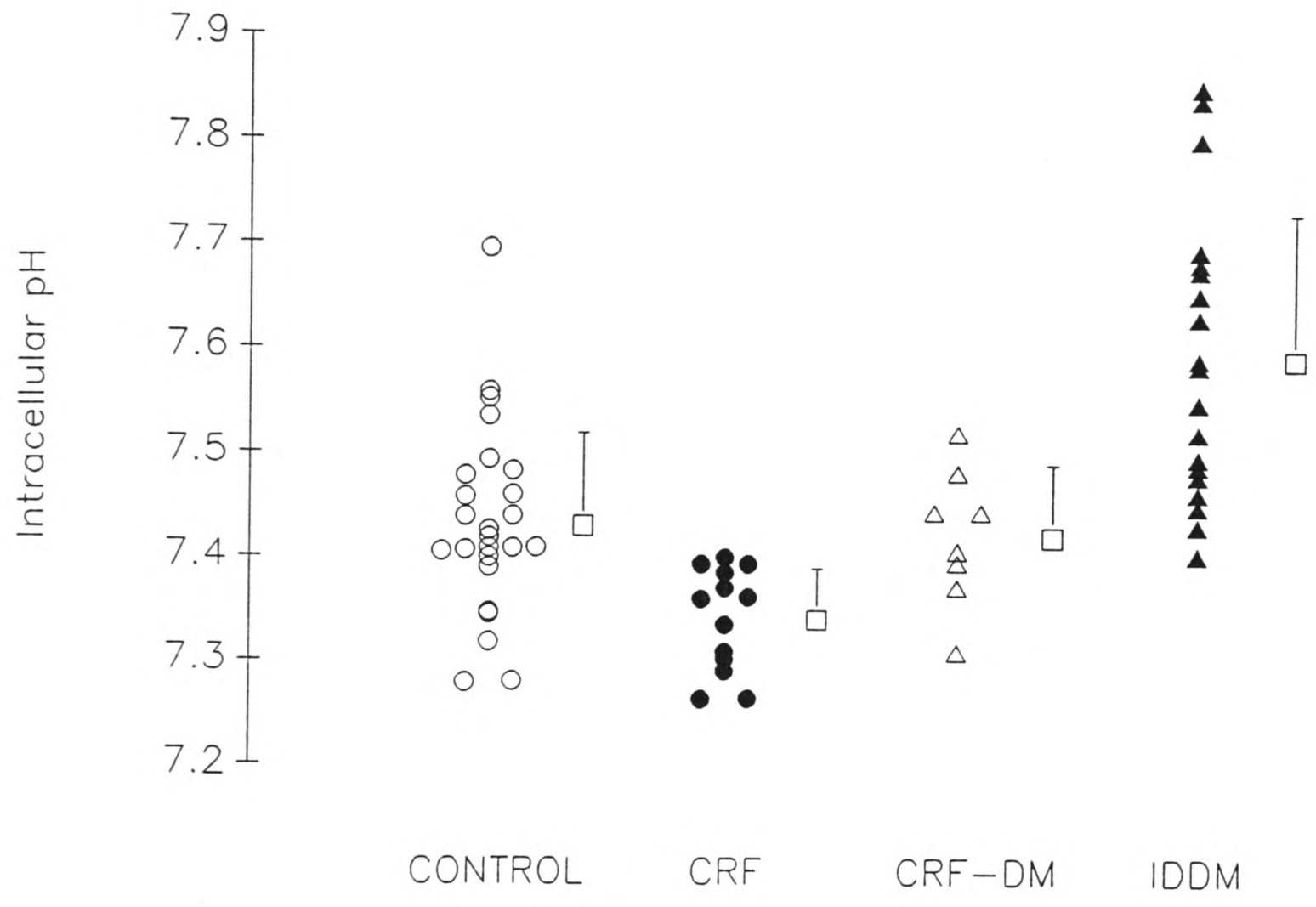


FIGURE 3.6: Measurements of leucocyte pHi in the four groups of patients. Each individual point is the mean of duplicate measurements. The mean and standard deviation is shown for each group. Control: Normal subjects, CRF: Chronic renal failure patients, CRF-DM: End stage diabetic (insulin dependent) nephropathy, IDDM: Insulin dependent diabetic patients with albuminuria.

CRF patients exhibit significantly more acid pHi , while IDDM patients values are significantly more alkaline.

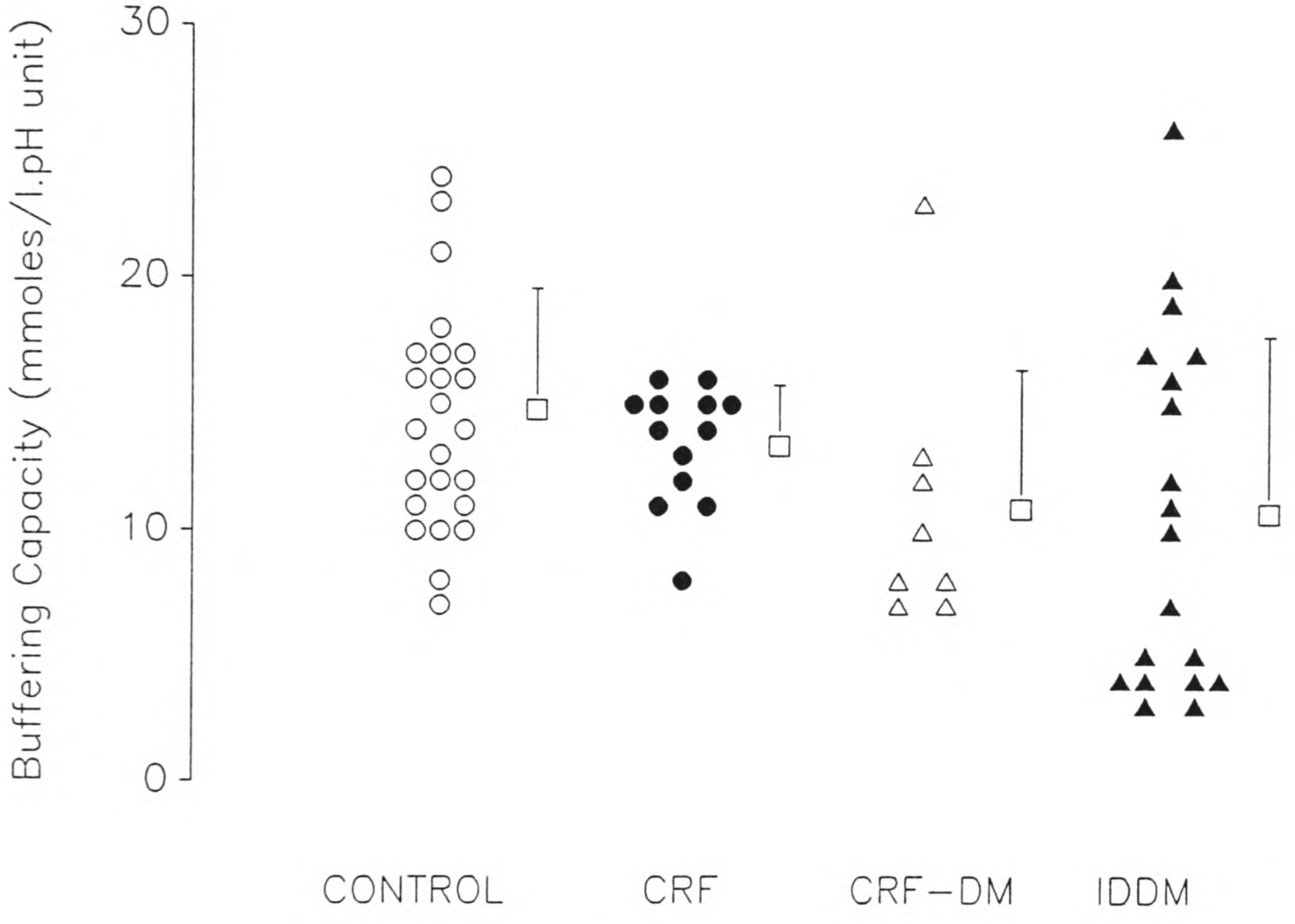


FIGURE 3.7: Measurements of leucocyte buffering capacity measured at resting values of intracellular pHi. Each individual point is the mean of duplicate measurements. The mean and standard deviation is shown for each group. Control: Normal subjects, CRF: Chronic renal failure patients, CRF-DM: End stage diabetic (insulin dependent) nephropathy, IDDM: Insulin dependent diabetic patients with albuminuria.
No significant difference is present.

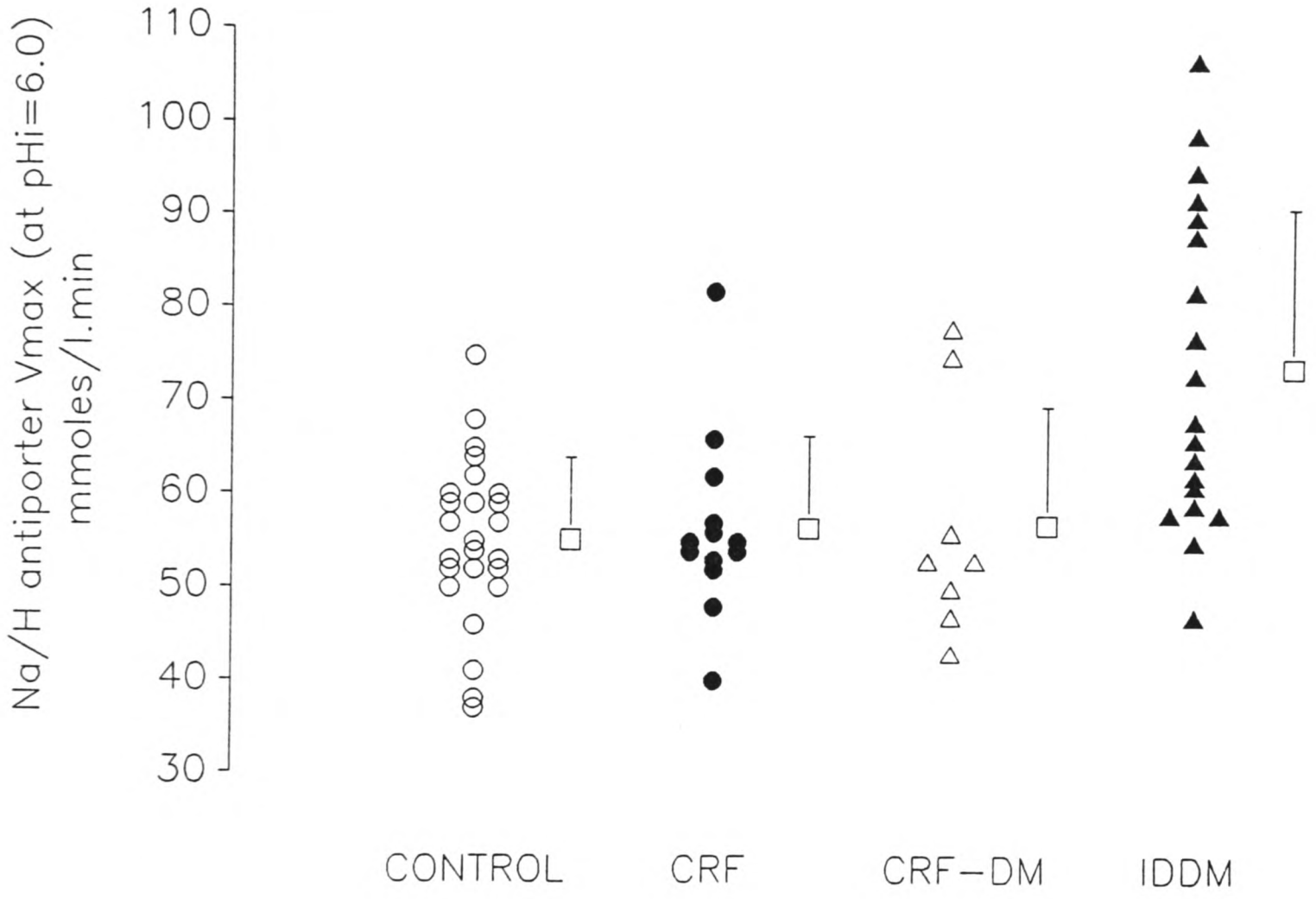


FIGURE 3.8: Measurements of leucocyte Na/H antiporter in the four groups of patients. Each individual point is the mean of duplicate or triplicate measurements. The mean and standard deviation is shown for each group. Control: Normal subjects, CRF: Chronic renal failure patients, CRF-DM: End stage diabetic (insulin dependent) nephropathy, IDDM: Insulin dependent diabetic patients with albuminuria.
Only IDDM exhibit a significantly more active Na/H antiporter.

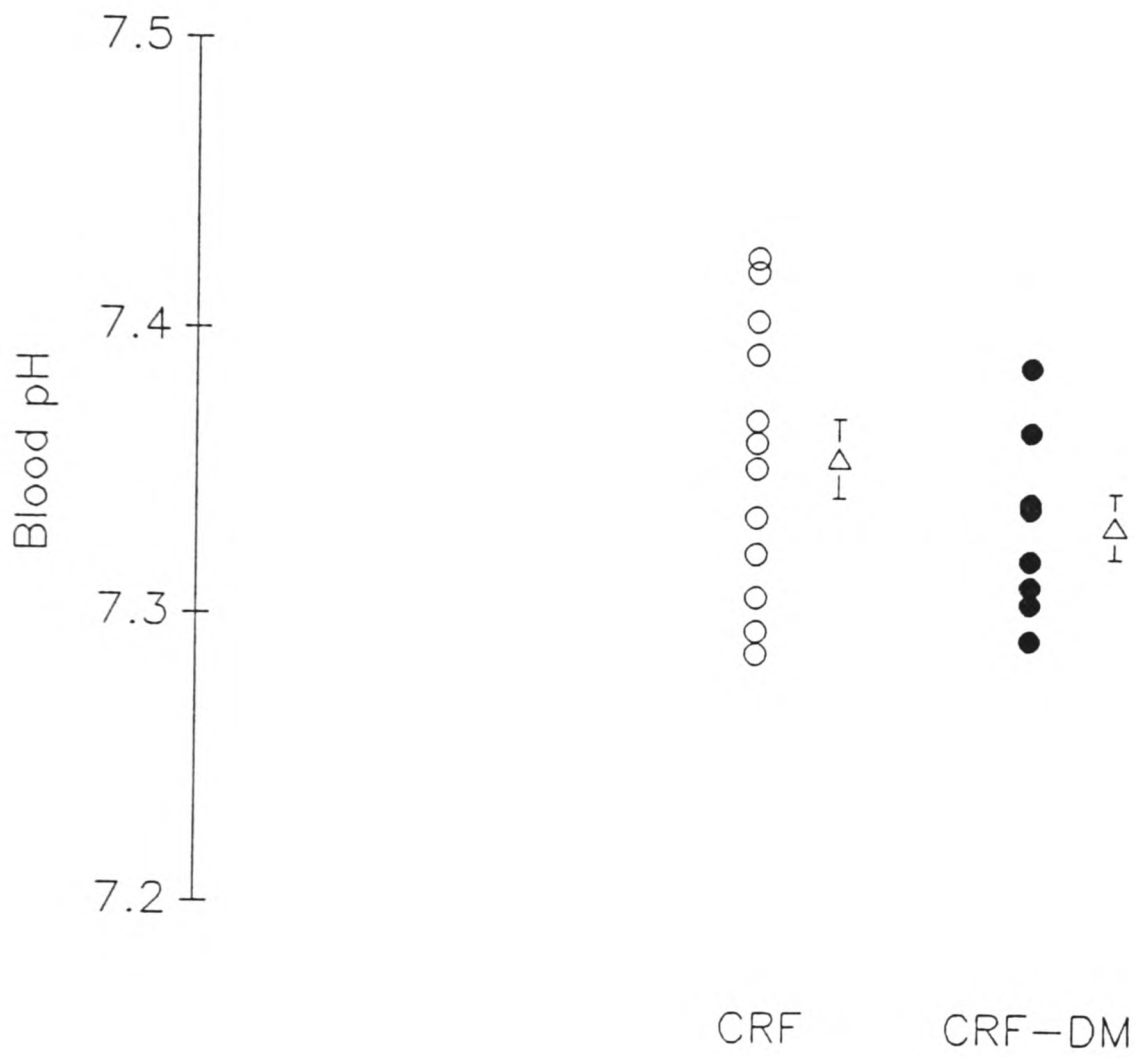


FIGURE 3.9: Blood pH was measured in patients with renal failure. No significant difference was present between the patients with or without diabetes.

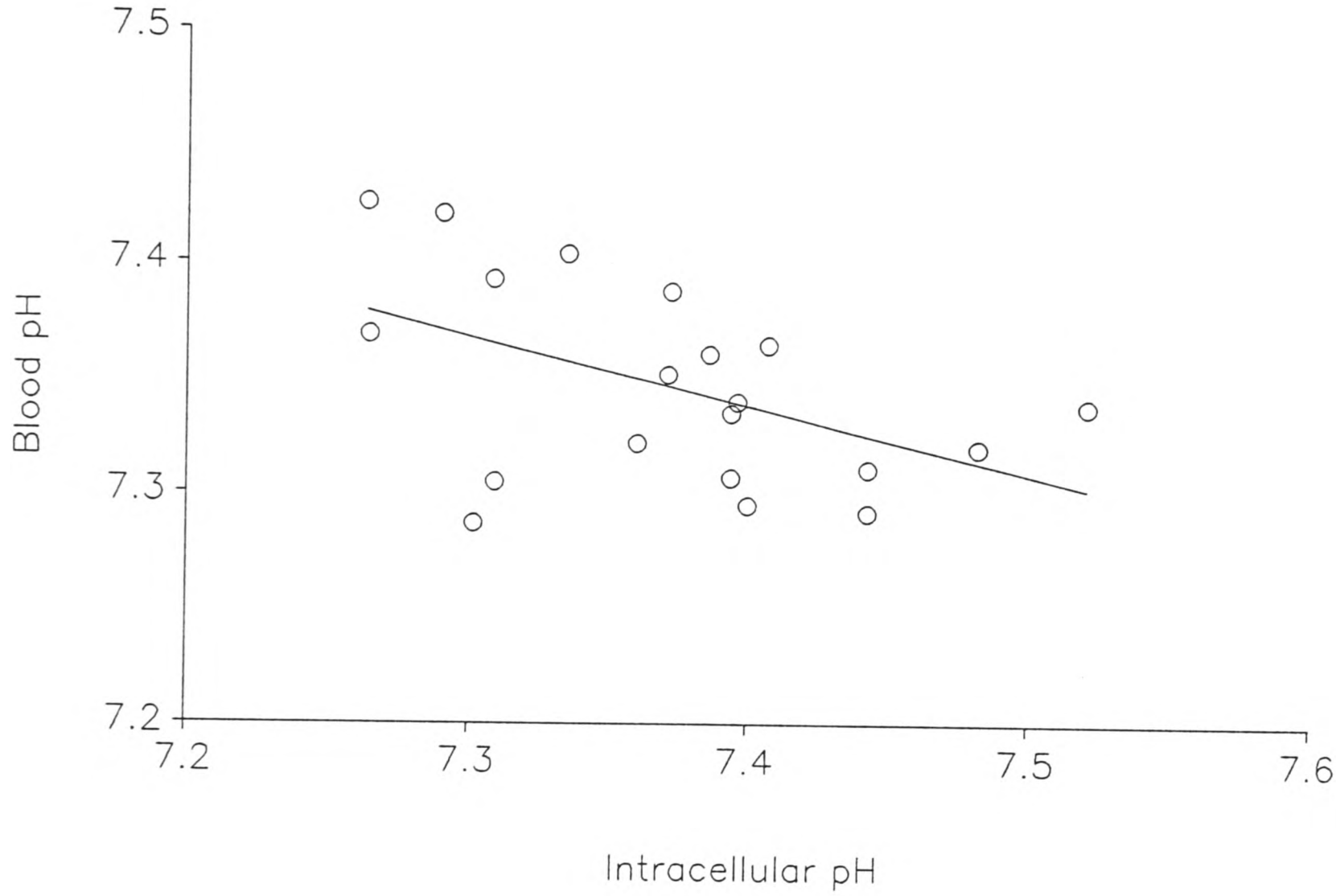


FIGURE 3.10: Blood pH and Intracellular pHi. A negative correlation was present between blood pH and pHi (Spearman correlation coefficient= r: -0.5, $p<0.05$).

3.2.6. INTRACELLULAR pH AND BLOOD pH

Individual values for blood pH and intracellular pH are examined in figure 3.10. A surprising finding was the presence of a significant negative correlation, i.e. patients with the markedly acidotic plasma showed a more alkaline intracellular pH. No correlation could be shown between blood pH and V_{max} of the Na/H antiporter, or between buffering capacity and blood pH, within the CRF and CRF-DM groups.

3.3. DISCUSSION

Although metabolic acidosis is one of the main characteristics of the uraemic syndrome few studies have looked at the regulation of pHi in uraemic cells. Previous studies have used different techniques and models. DMO (dimethyl oxazolidinedione) was employed to measure whole body pHi in uraemic rats [Rothe et al 1984] and uraemic patients [Tizzianello et al 1977], and also to study muscle pHi [Guisado et al 1977, Irvine & Dow 1966]. Total CO_2 was used to evaluate muscle pHi [Canale et al 1986] and ^{31}P NMR to study human red blood cells [Monti et al 1987,1989]. These studies showed that pHi in renal failure was not different from controls except in red blood cells [Monti et al 1987, 1989] and in one human muscle study [Canale et al 1986]. The present paper is the first study using BCECF in leucocytes to investigate pHi in renal failure.

Our finding of a lower pHi in leucocytes in chronic renal failure agrees with the work of Monti in red blood cells [Monti et al 1987, 1989] and of Canale in muscle [1986]. The findings of a lower intracellular pH may have important cellular consequences. Protein breakdown is one of the results of metabolic acidosis and also occurs in renal failure [May et al 1987, 1986].

Grinstein and others have shown that some immune deficiency mechanisms may be related to a lower pHi [1988a, 1988b]. Immunodepression is a well known feature of CRF [Bonomini et al 1988], and it is possible that a lower leucocyte pHi is one of the factors contributing to this.

The major finding in this work was that patients on haemodialysis for end-stage renal failure have a normal mean leucocyte Na/H antiporter V_{max}. This was true for patients with diabetic nephropathy and for non-diabetic subjects. Insulin-dependent diabetic patients with early nephropathy (defined as having albuminuria without a reduced glomerular filtration rate) are reported to show significant elevated leucocyte Na/H antiporter activity [Ng et al 1990b]. These patients also have increased Na/H antiporter activity in cultured fibroblasts [Trevisan et al 1989]. Similar increases in activity of the erythrocyte Na/Li countertransport system have been observed in diabetics with early nephropathy [Krowlewski et al 1988, Mangili et al 1988, Carr et al 1990]. It has been speculated that this cluster of abnormalities of Na/H and Na/Li transport may represent a genetically-determined marker for the risk of nephropathy in diabetes mellitus [Ng et al 1990b, Krowlewski et al 1988, Mangili et al 1988, Carr et al 1990]. Similar countertransport abnormalities are reported in essential hypertension [Ng et al 1989], and in family members of diabetics with either nephropathy and hypertension [Walker et al 1990, Canessa et al 1980].

If these countertransport changes are determined genetically, then patients with end stage diabetic nephropathy might be expected to show enhanced leucocyte Na/H antiporter V_{max}. This study has shown that they do not. It has recently been reported that erythrocyte Na/Li countertransport is normal in certain patients with end-stage diabetic nephropathy and that this group as a whole do not exhibit the same clear increase in mean Na/Li countertransport seen in early diabetic nephropathy [Carr et al 1990]. The countertransport changes seen in early diabetic nephropathy appear to ameliorate in diabetic patients with end-stage renal failure. This suggests that non-genetic factors may play a significant role in determining Na/H and Na/Li countetransport

activity. One possibility is that uraemia itself is such a factor which might inhibit Na/H antiport. However the data here from non-diabetic patients with renal failure shows no inhibition. It is not known whether ion transporters in diabetic subjects are more susceptible to modulation by a "uraemic" factors. Perhaps it should be pointed out that the activity of the antiporter measured at an intracellular pH of 6.0 provides an approximation of the maximum rates of Na/H antiporter.

Studies of Na/H antiporter activity have been reported in membrane vesicles isolated from rat, rabbit, and canine proximal tubules both in control conditions and after induction of renal failure in a remnant kidney model [Cohn et al 1982a,b, Nord et al 1985, Harris et al 1984]. There was enhanced activity associated with renal failure. This finding may have been organ specific and related to the compensatory renal hypertrophy occurring in this model of uraemia. The data presented in this chapter do not support the hypothesis of widespread increase in Na/H antiporter activity in renal failure.

The measurements reported here for leucocyte intracellular pH were made in bicarbonate-free solutions. Accordingly, pH values in vivo are likely to be somewhat different and to be influenced by plasma bicarbonate concentrations and by the activity of the anion-exchange protein. Most studies of intracellular pH in renal failure have reported normal values [Rothe et al 1984, Tizianello et al 1977, Irvine & Dow 1966], although there are reports of intracellular acidosis in human erythrocytes and in human skeletal muscle in uraemia [Canale et al 1986, Monti et al 1987]. In diabetic patients with early nephropathy the mean intracellular leucocyte pH (in bicarbonate-free media) is increased with respect to controls and with respect to diabetes without evidence of nephropathy [Ng et al 1990b]. This abnormality is consistent with the enhanced Na/H antiport V_{max} which could cause cell alkalinization by increased proton efflux. The reduced mean intracellular pH measured here in the non-diabetic CRF group cannot be explained on this basis, as these patients had a normal mean Na/H antiport V_{max}. Changes in the set-point for activation

of the Na/H antiporter could occur in the CRF group without alterations of V_{max}. Such changes might account for alterations in pHi, and this possibility is not excluded by the current experiments. The measured intracellular acidosis did not show a positive correlation with plasma pH, and so does not appears to be secondary to plasma acidosis. It is of interest that diabetic nephropathy may be associated with increased intracellular pH (whether comparing patients with normal glomerular filtration rate or comparing patients with end-stage renal failure) but the significance of these findings is unknown at present.

Despite the lower pHi in CRF the Vmax of the Na/H antiporter was unchanged when one might have expected a higher rate of transport. In canine brush border vesicles the Na/H exchanger exhibits increased activity in renal failure [Cohn et al 1982a,b]. Specialized epithelial cells may behave differently from white cells. Also studies on vesicles may not reflect pump activity in intact cells, as intracellular regulators may have been disturbed during vesicle preparation. The Na/H antiporter has an intracellular allosteric modifier binding site [Aronson et al 1982] and its activity is regulated by pHi with a set point. The transporter can also be regulated by phosphorylation under the action of a number of agonists [Sardet et al 1990]. Leucocyte pHi is reported to be invariant with extracellular pH in the range of 6.8-7.4 [Levin et al 1976, Deutsch et al 1984]. Uraemic toxins could inhibit the response to intracellular acidosis as, for example, parathormone (PTH) has been demonstrated to have an inhibitory effect on the antiporter [Pollock et al 1986, Miller & Pollock 1987]. We do not have plasma PTH values from all the patients, but plasma alkaline phosphatase measurements from CRF and DM-CRF groups did not correlate with the Vmax of the Na/H antiporter (data not shown).

Raised intracellular sodium has been reported in uraemic leucocytes [Edmondson et al 1975]. This could have reduced H efflux via the Na/H antiporter due to competition between intracellular Na and H for the transporter. This is unlikely in the present experiments as the

monensin used in the pH-clamping technique reduces intracellular Na^+ to very low levels, preventing inhibition of the antiporter by intracellular Na^+ binding [Ng & Bomford 1989]. However we cannot exclude a more chronic effect of raised intracellular sodium on Na/H antiporter activity.

The activity of the Na/H antiporter was higher in patients with IDDM with early nephropathy and pHi was raised in this group [Ng et al 1990a, 1990b]. A similar result has been reported in fibroblasts from IDDM patients with nephropathy [Trevisan et al 1989]. As with the Na/Li countertransport system [Krolewski et al 1988, Mangili et al 1988], perhaps an analogous transporter, these alterations have been attributed to genetic factors. In this study we have demonstrated a different behaviour of the transporter and pHi in the diabetic patients with end stage renal failure on dialysis treatment. These patients do not have a raised antiporter Vmax.

These findings demonstrate abnormal behaviour of the pHi homeostatic mechanisms in the uraemic syndrome, not entirely attributable to changes in the Vmax of the Na/H antiporter, and not attributable to abnormal plasma pH. Uraemia may have an inhibitory effect on the different mechanisms of cellular pHi control, and this could explain the contrasting results in IDDM and CRF-DM. The effect could be secondary to a circulating factor or an influence of the "uraemic environment" where the precursor cells develop and differentiate, resulting in changes of membrane protein expression or in the synthesis of the antiporter. Altered membrane lipids have been suggested to affect other transporters in uraemia [Kelly et al 1989] and lipid changes could be involved. This issue is not yet resolved but various membrane transport systems have been shown to have altered kinetics in renal failure including the Na/K ATPase and amino acid transporters [Ellory et al 1988c, Fervenza et al 1988, 1989a, 1990]. Our studies in uraemic diabetic and non-diabetic subjects suggest no alteration in the Vmax of the antiporter compared to normal controls. The differences in intracellular pH between the uraemic and non-

uraemic subjects in both diabetic and non-diabetic groups might be explained by alterations in the Km or cooperativity of the antiporter internal proton binding sites.

One could argue that the lower pHi in uraemia was secondary to a lower blood pH. However, as shown in figure 3.9, pHi in the uraemic patients showed a negative correlation with blood pH. In other words the patients with systemic acidosis had a higher value of pHi. There was no correlation between blood pH and either Vmax or buffering capacity.

The intracellular buffering capacity at rest was not different in the various groups. A higher buffering capacity could be expected if pHi falls below 7 [Ng & Dudley 1989]. All the pHi values measured in the uraemic patients (diabetic and non-diabetic) were above 7.0. Other pHi regulating mechanisms are present in leucocytes such as Cl/HCO_3 exchange [Simchowitz & Ross 1985], but the contribution of Cl/HCO_3 exchange in our experiments was minimal as cells were studied in nominally bicarbonate free media.

The absence of bicarbonate in the present experiments enabled the study of the Na/H antiporter without interference from bicarbonate-chloride exchange. In vivo, pH values and buffering capacity would be infiuenced by bicarbonate [Goldsmith & Hilton 1992, Thomas 1989, Syme et al 1991]. In leucocytes bicarbonate-dependent systems participate in the regulation of pHi [Schimchowitz & Roos 1985, Mason et al 1989, Schimchowitz et al 1991]. The acid extruding Na-dependent $Cl\text{-}HCO_3^-$ exchanger has not been demonstrated in leucocytes yet. In previous experiments, the presence of such a mechanism was not found in leucocytes [Ng-personal communication]. In a cell line (U937 human leukaemic cells) Na-dependent $Cl\text{-}HCO_3^-$ exchange has been demonstrated, but surprisingly the same cells lack the Na-independent $Cl\text{-}HCO_3^-$ system [Ladoux et al 1987]. It is probable that leucocytes share similarities with mesangial and smooth muscle cells where bicarbonate-dependent mechanisms are largely responsible for the steady state intracellular pH [Ganz et al 1989, Kikeri et al 1990, Kahn et al 1990]. In the presence of

bicarbonate, acid extrusion from vascular smooth muscle is mostly due to the Na/H antiporter at low pHi values, while Na-dependent Cl-HCO_3^- exchange predominates at resting pHi [Kikeri et al 1990, Kahn et al 1990]. The relative contributions of these mechanisms to pH regulation in leucocytes are unclear. Growth factors and other factors have been shown to influence both bicarbonate-dependent and -independent acid-base transporters [Ganz et al 1989, Kikeri et al 1990, Hoog et al 1991]. In leucocytes phorbol esters have an inhibitory effect on the Cl^-/HCO_3^- exchanger, while they stimulate Na/H exchange [Hogg et al 1991]. In vivo studies on rat skeletal muscle have suggested that bicarbonate is crucial in regulating resting intracellular pHi, with Na/H antiporter activity increased in spontaneously hypertensive rats (SHR), but no evidence for a difference in bicarbonate handling between SHR and Wistar-Kyoto rats [Syme et al 1991]. The present study has not examined the influence of bicarbonate-dependent mechanisms in uraemia; it has looked at the only pHi regulatory mechanism that seems to work without bicarbonate [Thomas 1989].

We can conclude that the pHi regulating mechanisms in uraemia are complex. Non diabetic haemodialysis patients have a low mean leucocyte pHi. The higher pHi secondary to activation of the Na/H antiporter in IDDM with early nephropathy is no longer present when they develop end-stage kidney disease and are on haemodialysis treatment. Nevertheless, the pHi of IDDM patients on dialysis is higher than that of non-diabetic uraemic patients.

3.4. CONCLUSION

CRF patients have a lower leucocyte pHi when measured under bicarbonate-free conditions. The activity of the Na/H antiporter is not altered in chronic renal failure. The higher activity of the Na/H antiporter present is IDDM patients with early nephropathy is not present in patients with end stage diabetic nephropathy. Figure 3.11 summarizes the findings.

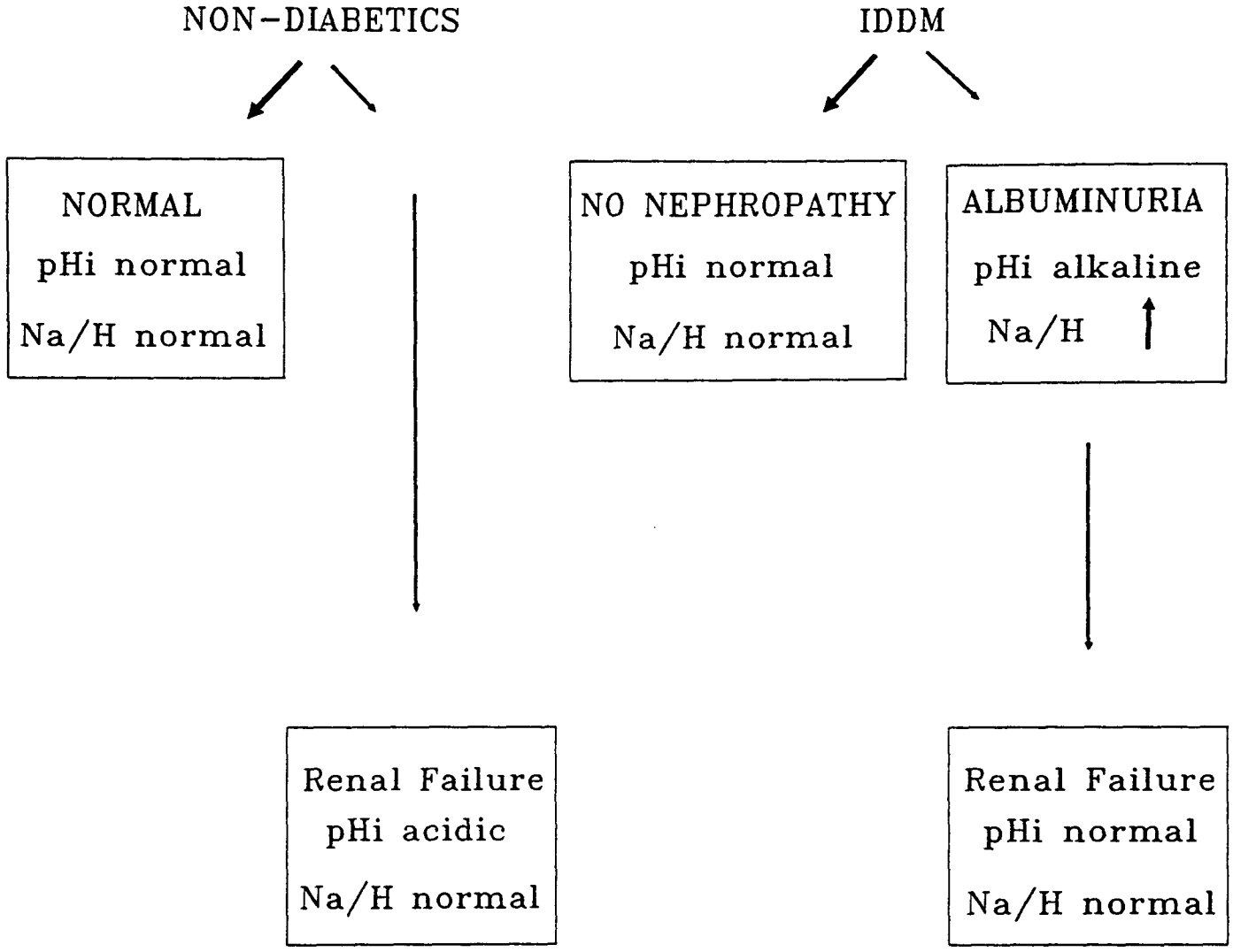


FIGURE 3.11: Diagram with the answers to the question of figure 3.1.

CHAPTER 4

ERYTHROCYTE LACTATE UPTAKE UNDER PHYSIOLOGICAL CONDITIONS AND IN CHRONIC RENAL FAILURE

The work presented in this chapter represents a study of erythrocyte lactate transport in patients with chronic renal failure (CRF). The attention directed towards this transport in uraemia derives from our interest in examining the widespread changes in membrane transport present in uraemic patients. Lactate transport has not been examined in CRF previously. The chapter will also describe the characterization of lactate uptake in erythrocytes under conditions close to physiological. When the study on CRF was being planned it became clear that there was a lack of information on the transport of lactate at physiological temperatures, so some effort was concentrated on characterizing the rates of transport at normal conditions of body temperature. Information on erythrocyte lactate transport via the monocarboxylate carrier, anion exchanger (band 3) and via diffusion of the undissociated acid is available from various extensive studies, but they have usually being performed at low temperatures (0-25°) [Deuticke et al 1978, 1982; Deuticke 1980, 1982, 1989; Halestrap 1976; Halestrap et al 1990; Leeks & Halestrap 1978, 1979; Halestrap & Poole 1989; Poole et al 1989, 1990; Dubinsky & Racker 1978; Juel et al 1990; Fishbein 1986; Fishbein et al 1988a, 1988b; Edlund & Halestrap 1988; Johnson et al 1980].

The uptake of lactate in erythrocytes is very important under stimulated anaerobic metabolism, such as during exercise, and red cell lactate accumulation is a factor in buffering and transporting lactic acid away from exercising muscle [Fishbein 1986].

In renal failure blood lactate concentration has been reported to be either normal,

or elevated [Diesel et al 1990, Askanjii & Sacks 1991, Savig et al 1991]. The rise in blood lactate after exercise has also been reported to be abnormal in uraemia. The results have been controversial, either presenting more marked [Parrish 1981, Parrish et al 1990] or reduced [Savig et al 1991, Diesel et al 1990] blood lactate concentrations after exercise.

Chronic renal failure patients [Capaldo et al 1990] and acutely uraemic dogs [Cianciarruso et al 1991] have been reported to have normal blood lactate levels and hepatic lactate liver uptake; however, splanchnic lactate uptake was abnormal after glucose infusion. Glucose infusion in controls resulted in net hepatic lactate output, while uraemics had net splanchnic lactate uptake [Capaldo et al 1990, Cianciarruso et al 1991].

Finally in uraemia abnormal lactate transport could play a role in the pathogenesis of acid-base disturbances.

The methodology for the study is presented next.

4.1. METHODS AND PATIENTS FOR LACTATE WORK

4.1.1. MATERIAL AND SOLUTIONS

Lactate experiments used a glucose-free saline solution with the following composition (in mmol/l): NaCl 150, KCl 5, $MgCl_2$ 1, MOPS 10 and pH 7.4 with Tris base. L-Lactic acid, α-cyano-4-hydroxycinnamate (CHC), bumetanide, 4,4'-diisothiocyanostillbene-2,2'-disulfonate (DIDS), 4-acetamido-4'-isothiocyanostillbene-2,2'-disulfonate (SITS) and *p*-chloromercuribenzene sulfonate (PCMBS) were obtained from Sigma Chemical Company (Poole, Dorset, UK). L-[^{14}C] lactic acid (specific activity of 154-160 mCi/mmol) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Lactate solutions were prepared from a 40

mM stock of L-lactic acid in saline with pH adjusted to 7.4. CHC and bumetanide had to be dissolved initially using tris base. Stock solutions of CHC (30 mM), bumetanide (1 mM), SITS or DIDS (2 mM) were prepared in the saline medium and the pH adjusted to 7.4.

Cell separation with oil was achieved by using dibutyl phthalate (DBT), from Sigma Chemical Company.

4.1.2. CELL PREPARATION

Cells were prepared as for the choline experiments (chapter 2), but a glucose-free saline was used to wash and incubate the cells, in order to avoid glycolysis and production of lactic acid. All washes were at 37°C and the cells were incubated with saline at 37°C for 30 minutes in order to deplete intracellular lactate. Cells were resuspended at an haematocrit of 16 % and kept on ice. The final haematocrit during the fluxes was around 8%.

4.1.3. LACTATE UPTAKE PROTOCOL

For lactate fluxes at 37°C, techniques that allow very fast measurements of the transport of the labelled substrate had to be employed. With flux rates of a few seconds it is essential to be able to stop the fluxes as quickly as possible. This can be achieved by inhibition of transport by diluting the cell suspension with a cold medium containing inhibitors of lactate transport (CHC or PCMBS), and finally by separation of the cells by centrifugation through oil.

4.1.3.1. LACTATE UPTAKE

Before starting flux measurements, cells (obtained as in chapter 2) were washed once more, resuspended in saline (or saline with the required inhibitor) and incubated at 37°C for ten minutes.

Assessment of transport required mixing the cells with the substrate and sample at timed intervals. Unless otherwise stated, extracellular lactate concentration during each flux measurement was 1 mM, i.e. close to physiological resting concentrations. Fluxes were started by adding and mixing 0.2 ml of red blood cell suspension to the "flux tubes", which contained 0.2 ml of saline with the labelled L-lactate at twice the desired final concentration. Tracer amounts of ^{14}C-Lactate were present. Flux was stopped by quickly transferring a 0.2 ml aliquot to a cold "stop tube" already placed in a microfuge and then spinning for 15 seconds. The stop tubes were kept on ice and contained 0.5 ml of dibutyl phthalate and 0.5 ml of 6 mM CHC in saline, or 10 μM PCMBS. For the measurements two workers were needed, one to perform the actual fluxes and the other to perform the timing (seconds). The supernatant was aspirated first, followed by the oil layer and the walls of the Eppendorf tube cleaned using a cotton swab. Triton X-100 (0.1 % v/v in water) was used to lyse the cells and TCA 5 % (w/v) to precipitate proteins. Activity of ^{14}C was counted by β-scintillation spectroscopy.

Uptake was represented as μmol/(l.sec) and the values obtained from the slope of the regression line calculated from four samples taken at times under 15 sec (4,8,11 and 15 sec for example). The y axis intercept of the regression line for fluxes up to 15 sec was used as a measure of extracellular space contamination (vide section 4.1.3.2).

4.1.3.2. ADJUSTMENT FOR EXTRACELLULAR CONTAMINATION

In the stop technique as employed the cells were spun through oil (DBT). Despite dilution of the cell suspension with ice cold saline and inhibitors, some of the radiolabelled marker was trapped in the extracellular space within the cell pellet. If this was not taken into consideration flux measurements would be artificially elevated. The y-intercept of the regression line, at time zero, of the measurements up to 15 seconds was taken as an estimate of the extracellular contamination, allowing correction for the actual fluxes by subtracting this value. Figure 4.1.a and .b illustrates a representative example in which a time course was performed with or without inhibitors. All the regression lines intercept at the same point on the y axis as seen in figure 4.1.a. When this figure was subtracted the corrected true values for the fluxes can be plotted in figure 4.1.b.

As an alternative, fluxes could be measured as rapidly as feasible (3-4 sec) at 0°C in the presence of inhibitors. In this case, the radioactive label detected in the cell pellet represented the trapped extracellular label or membrane-bound contamination.

4.1.3.3. TEMPERATURE EFFECT

Different temperatures were compared using a water-bath to incubate cells at 37°C, room temperature and 0°C on ice. Most experiments were performed at 37°C in order to estimate the fluxes close to physiological conditions.

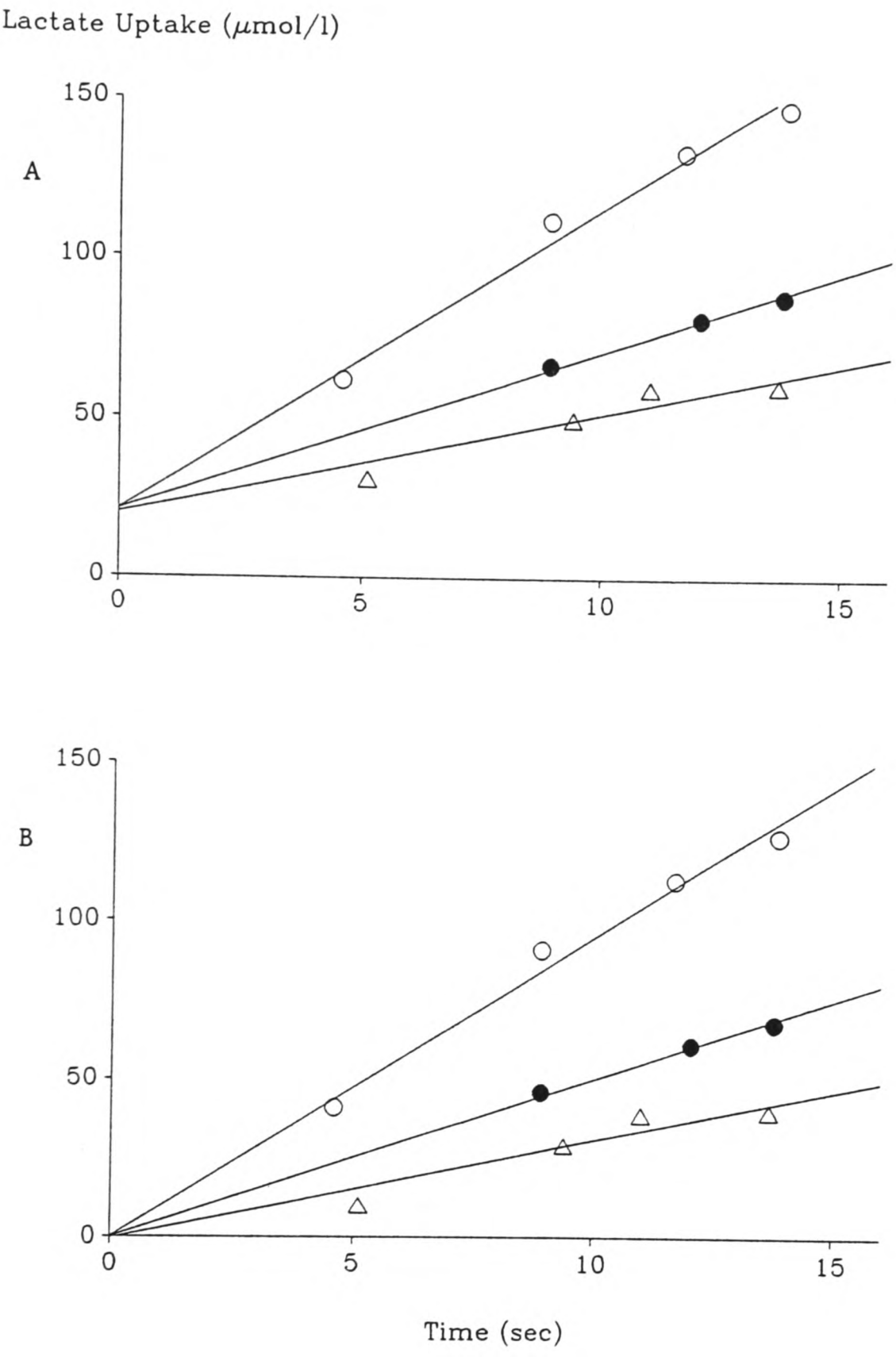


FIGURE 4.1: Time course of erythrocyte lactate uptake. (a) The intercept of the regression lines at the y axis represents extracellular contamination with the radiolabel, (b) Corrected true fluxes. Open circles: total flux, closed circles: flux in the presence of 200 μM bumetanide, open triagles: fluxes in the presence of CHC 3 mM.

4.1.3.4. INHIBITORS

Inhibitors used include SITS, DIDS, CHC, bumetanide and PCMBS. The inhibitors were chosen for their reported efficiency in inhibiting the monocarboxylate carrier and/or band 3 [Halestrap 1976, Leeks & Halestrap 1979, Deuticke 1989, Deuticke et al 1982, Gunn 1985, Wieth & Brahm 1985]. The concentration of inhibitor used will be stated for each experiment. Lactate was 1 mM final concentration, unless stated otherwise. Extracellular contamination in some experiments was taken as the trapped counts at 0°C, following sampling as quickly as possible.

4.1.3.5. LACTATE UPTAKE IN PLASMA

Plasma was used in some experiments in order to simulate physiological conditions. After venesection blood was kept on ice for 10 minutes and cells separated by centrifugation at 2°C. Plasma was saved and kept on ice; one sample was used to measure lactate plasma levels. Immediately before measurements, red cells were suspended using non-labelled plasma with a packed cell volume of about 16 %, and incubated at 37°C. Fluxes were started by adding and mixing 0.1 ml of red blood cell suspension in plasma to the flux tubes, which consisted of 0.1 ml of plasma with tracer amounts of ^{14}C-lactic acid at 37°C. Aliquots of plasma with the added radiolabel had been taken as a standard for scintillation counting. At appropriate time intervals the flux was stopped by transferring an 0.1 ml aliquot to a cold stop tube already placed on a microfuge for immediate centrifugation. Stop tubes were kept on ice and contained 0.5 ml of dibutyl phthalate and 0.5 ml of 6 mM CHC in saline. Time courses were performed.

4.1.3.6. PATIENTS

Chronic renal failure patients on dialysis treatment at the Oxford Renal Unit, Churchill Hospital, were selected for the experiments. Their clinical characteristics are summarized in Table 4.1. The etiology of renal failure were Reflux Nephropathy, Henoch Schonlein Purpura, Adult polycystic kidney disease and 2 with obstructive uropathy.

TABLE 4.1
CHRONIC RENAL FAILURE PATIENTS - CLINICAL DATA

	MEAN	RANGE
SEX	M 3/F 2	
AGE	48	21-74
CREATININE (μM)	697	418-1026
GLUCOSE (mM)	5.12	4.2-7.1
POTASSIUM (mM)	4.6	3.6-6.1
SODIUM (mM)	138	132-142
HAEMOGLOBIN (g/dl)	11.1	6.5-15.3
MCV (fl)	87.9	63.3-107.9

4.2. RESULTS

4.2.1. TIME COURSE AND TEMPERATURE DEPENDENCE

Lactate uptake at three different temperatures is shown in figure 4.2. There was a marked temperature effect on the rates of lactate uptake and this was one of the reasons leading other authors to perform lactate studies at low temperatures. After 5 minutes lactate was 457, 242

and 24 μmol/l when measured at 37, 20 and 0°C respectively, at an extracellular concentration of lactate of 1 mM.

Fluxes performed over a period of up to 20 seconds were linear with time, as shown in figure 4.3. Measurements of the fluxes for a period up to 15 seconds are adequate for estimation of the initial rates of transport. In the graph the initial rates of lactate uptake are 6.8, 2.1 and 0.3 μmol/l.sec respectively at 37, 20 and 0°C.

All the other experiments were performed by measuring the initial rate of lactate uptake at 37°C.

4.2.2. CONCENTRATION DEPENDENCE

Concentration dependence studies showed that total lactate influx displayed saturation kinetics. Lactate uptake shown in figure 4.4. was an example of data from one individual who at 37°C gave a V_{max} of 43 μmol/l.sec and a K_m of 4 mM.

4.2.3. TIME COURSE WITH INHIBITORS OF LACTATE TRANSPORT

It is reported that α-cyano-4-hydroxycinnamate (CHC) is a "specific" inhibitor of the lactate carrier [Halestrap 1976]. The literature suggests that the rest of the fluxes, ie CHC-resistant, are mainly mediated by band 3. Accordingly the experiments shown in figure 4.5 were performed. These results were initially surprising since there was a similar inhibition of the fluxes with either 6 mM CHC or 400 μM DIDS, and less inhibition by bumetanide (another band 3 inhibitor) [Gunn 1985, Wieth & Brahm 1985].

The initial rates of lactate uptake in several normal volunteers were inhibited as

shown in figure 4.6. SITS and CHC, at the specified concentrations, inhibit similar fractions of the fluxes while SITS and bumetanide are less effective in inhibiting the fluxes.

To examine the effects of each inhibitor, concentration curves were performed.

4.2.4. CONCENTRATION DEPENDENCE OF INHIBITORS ON LACTATE TRANSPORT

Inhibition of lactate transport (extracellular lactate concentration: 1 mM) by CHC, SITS, DIDS, bumetanide and PCMBS is shown in figures 4.7 to 4.11.

PCMBS seemed to be the most effective inhibitor of lactate uptake with 50% maximal inhibition at 2 μM. At higher concentrations, PCMBS inhibited 90 % of the total lactate flux at 5 μM. All the other inhibitors had variable but significant effects on lactate fluxes. CHC was able to inhibit up to 20 % of the total lactate fluxes with half-maximal inhibition at approximately 0.5 mM. The anion exchange inhibitors SITS, DIDS and bumetanide had less effective inhibitory capacity.

4.2.5. PLASMA

To mimic physiological conditions, uptake was measured in autologous plasma instead of saline. Tracer amounts of radiolabel were added to plasma, which had its lactate concentration measured. Lactate uptake from plasma of four individuals into erythrocytes had an initial rate of 8.8 μmol/l.sec (SEM 1.6), with uptake of 430 (SEM 98) μmol/l.sec (range 304-625) after 5 minutes. These results are comparable to the values obtained using saline. A representative time curve is illustrated in figure 4.12.

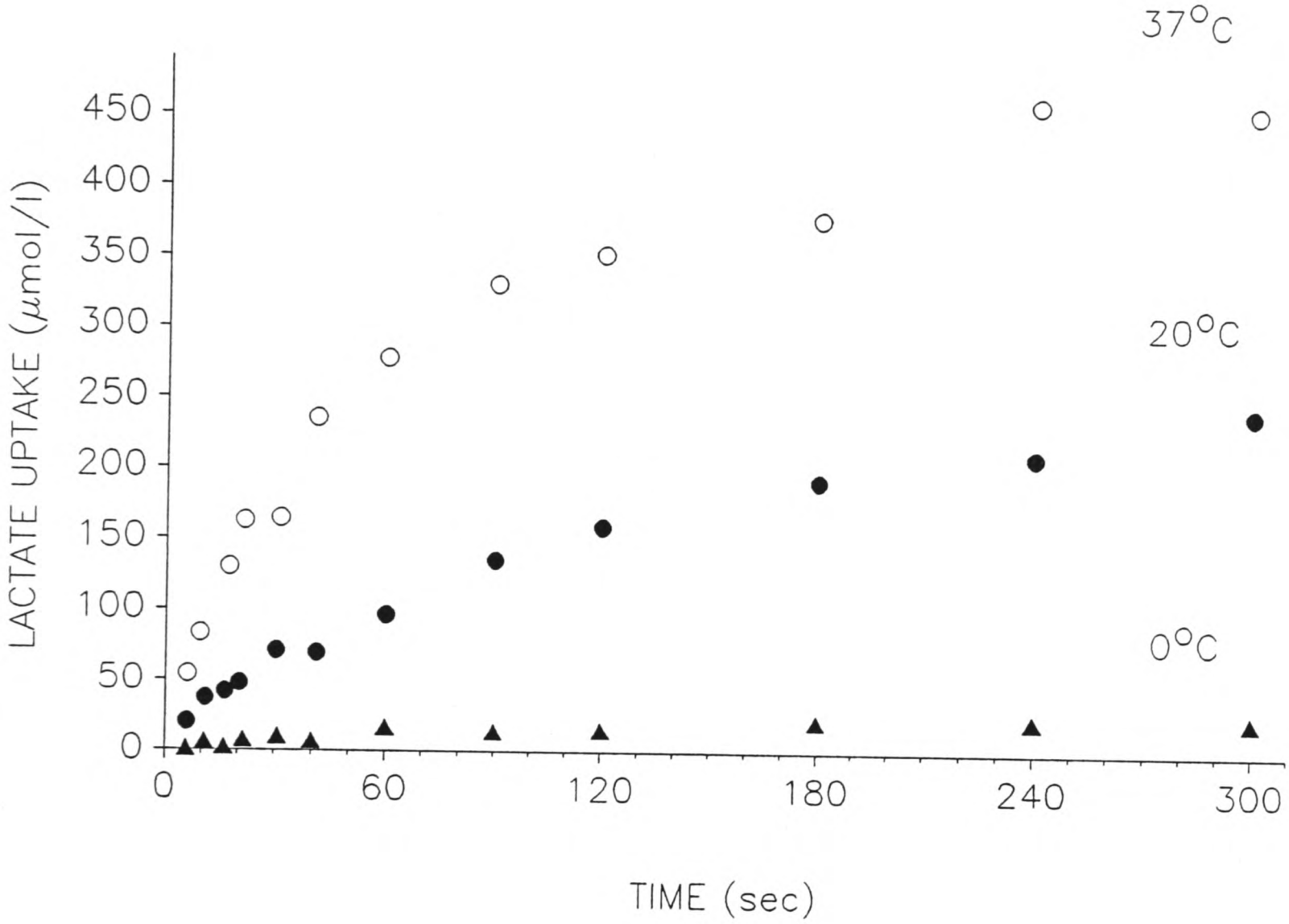


FIGURE 4.2: Time course of lactate uptake measured at different temperatures (extracellular lactate: 1 mM). There is a marked temperature dependence.

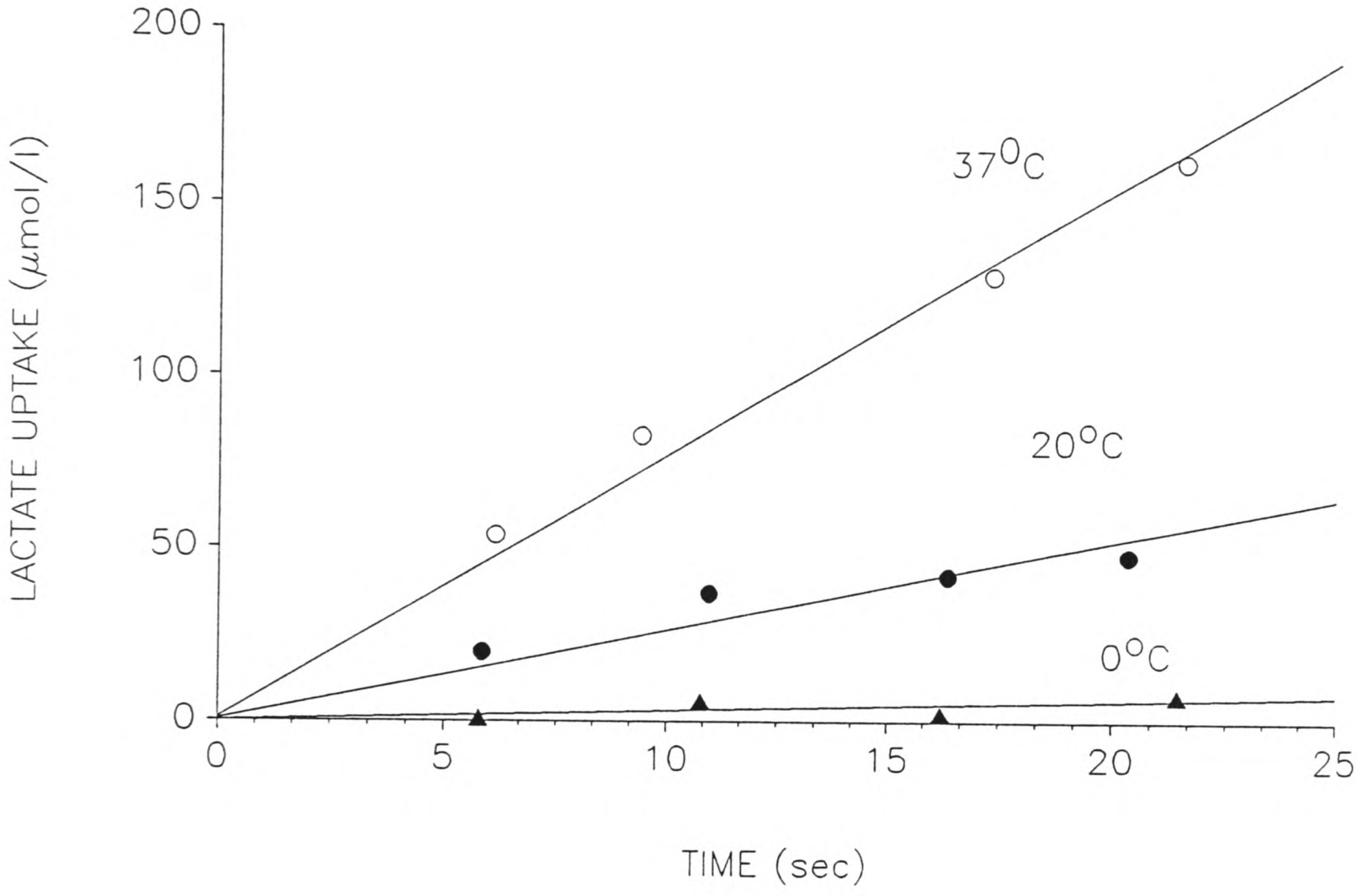


FIGURE 4.3: Time course of lactate uptake showing linearity of the initial fluxes for a period up to 20 seconds. Extracellular lactate concentration was 1 mM.

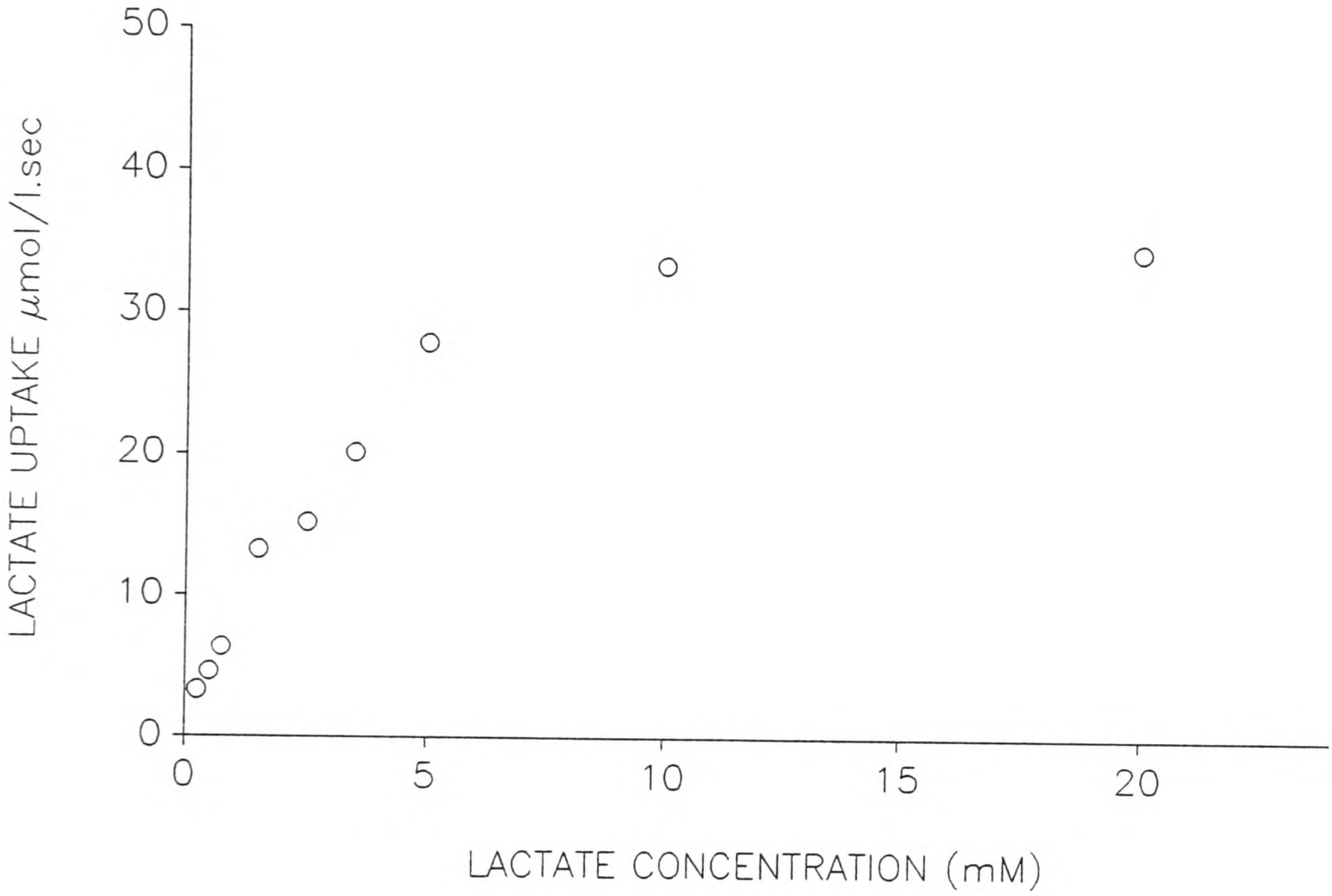


FIGURE 4.4: Total lactate uptake as a function of extracellular lactate concentration. Uptake follows saturation kinetics.

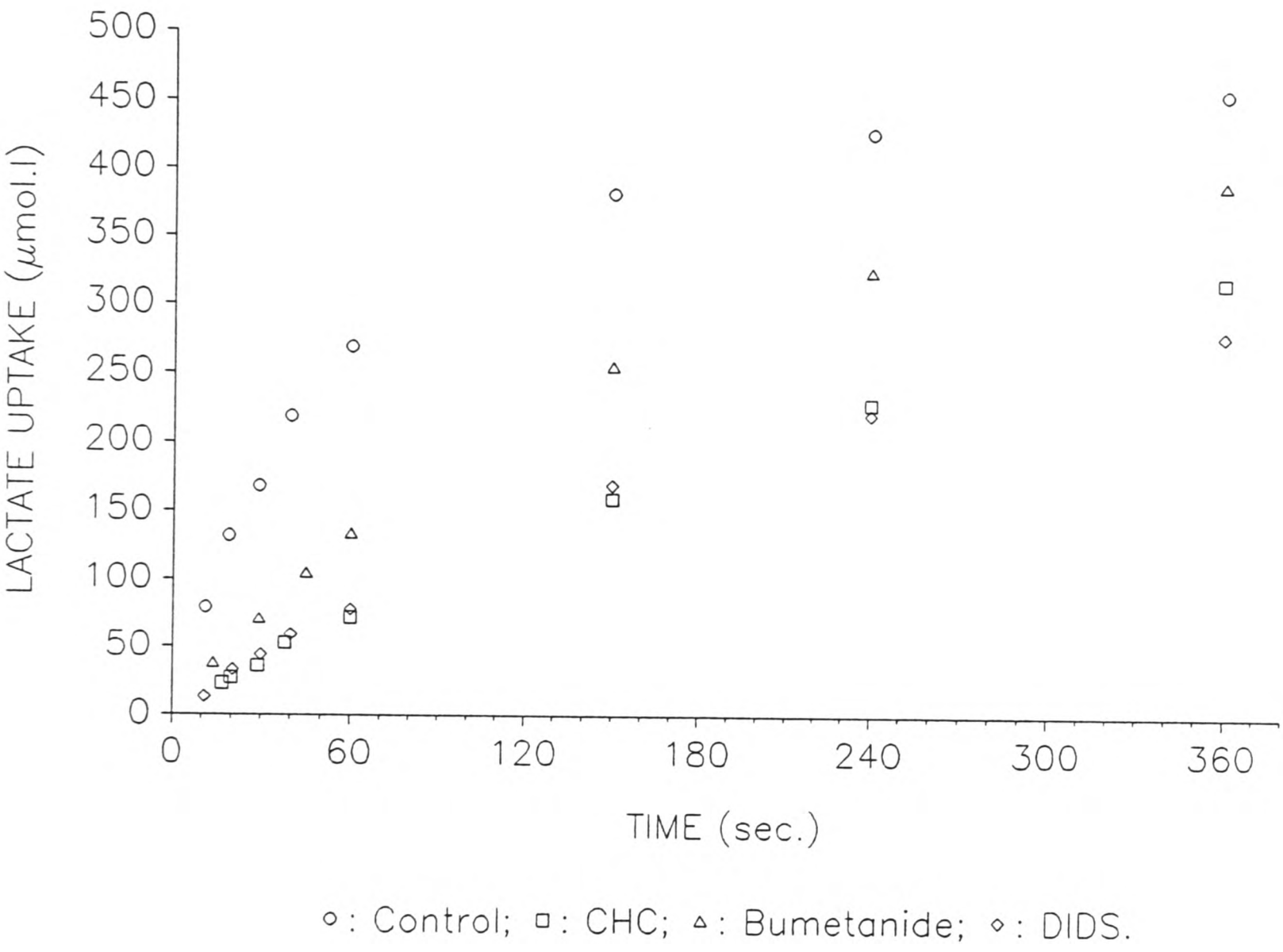


FIGURE 4.5: Lactate uptake as a function of time. Extracellular lactate concentration was 1 mM. Inhibitors used: bumetanide 300 μM, DIDS: 400 μM, CHC: 6 mM.

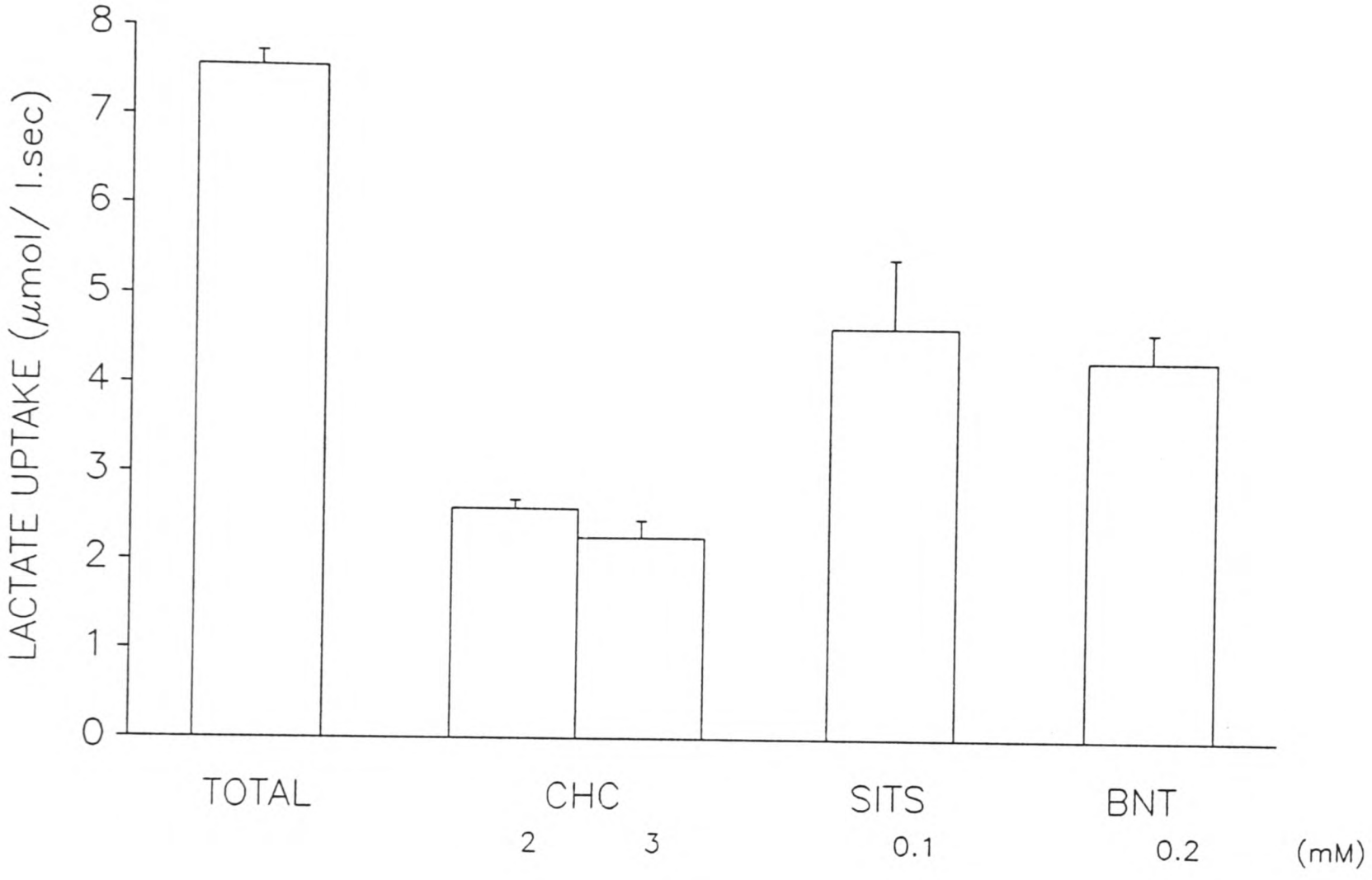


FIGURE 4.6: Inhibition of lactate uptake at 1 mM extracellular concentration. Total: fluxes in the absence of inhibitor (n:28), CHC: Fluxes in the presence of CHC 2 (n:2) or 3 mM (n:7), SITS: Fluxes in the presence of SITS 0.1 mM (n:3), BNT: Bumetanide 0.2 mM present (n:7).

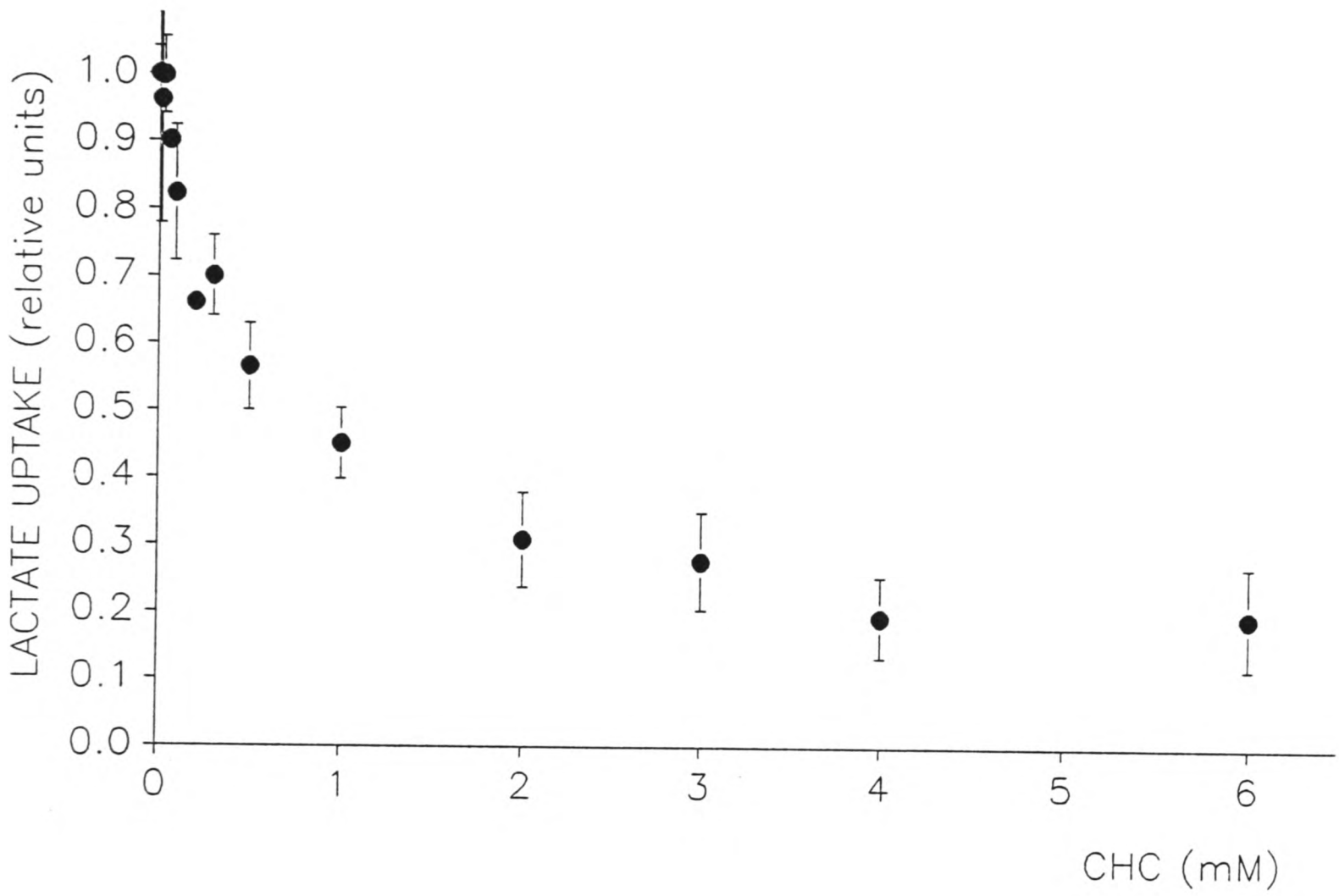


FIGURE 4.7: Concentration dependence of inhibition of lactate uptake by CHC (n:4). Extracellular lactate concentration: 1 mM.

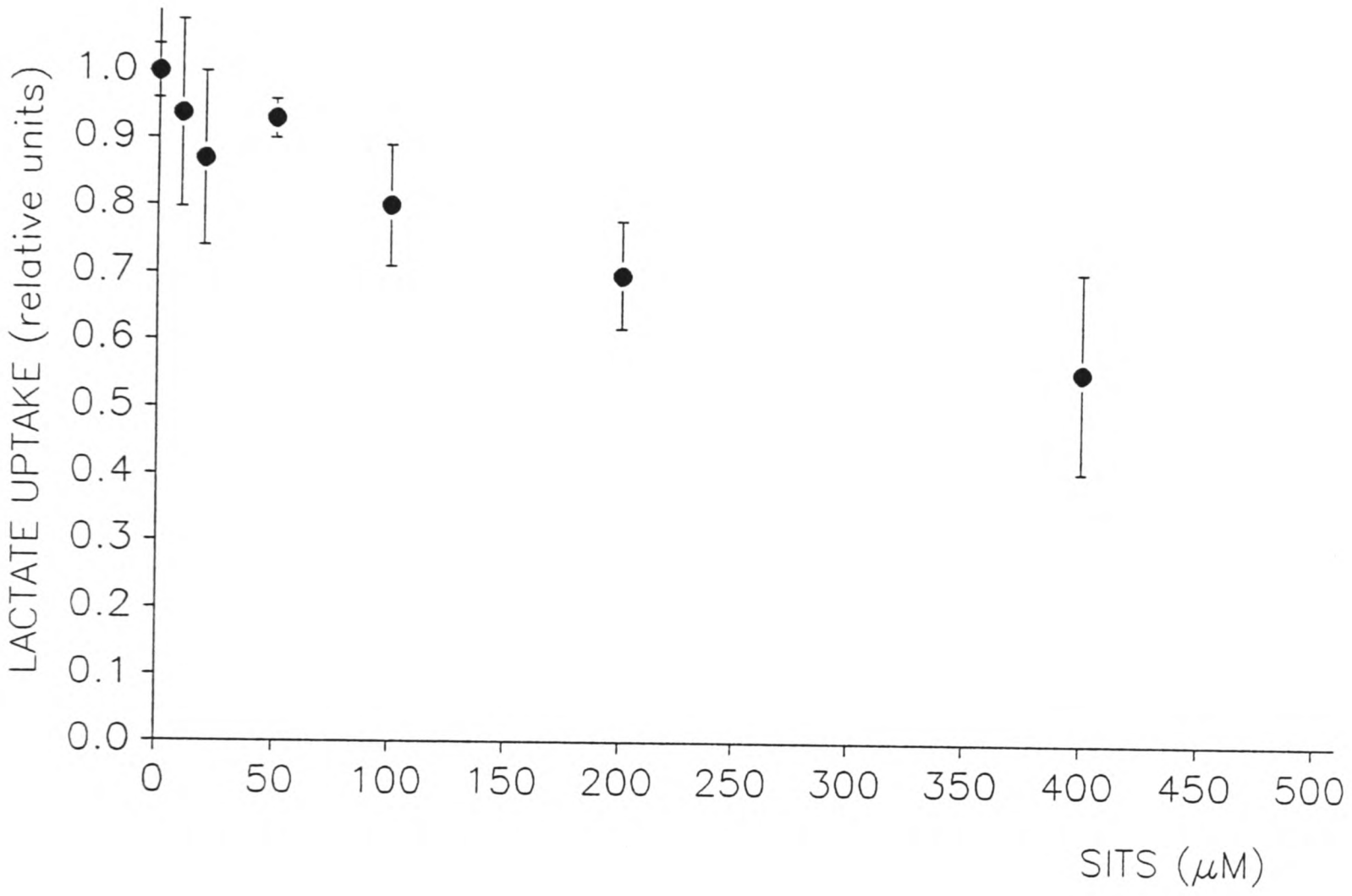


FIGURE 4.8: Concentration dependence of inhibition of lactate uptake by SITS (n:4). Extracellular lactate concentration: 1 mM.

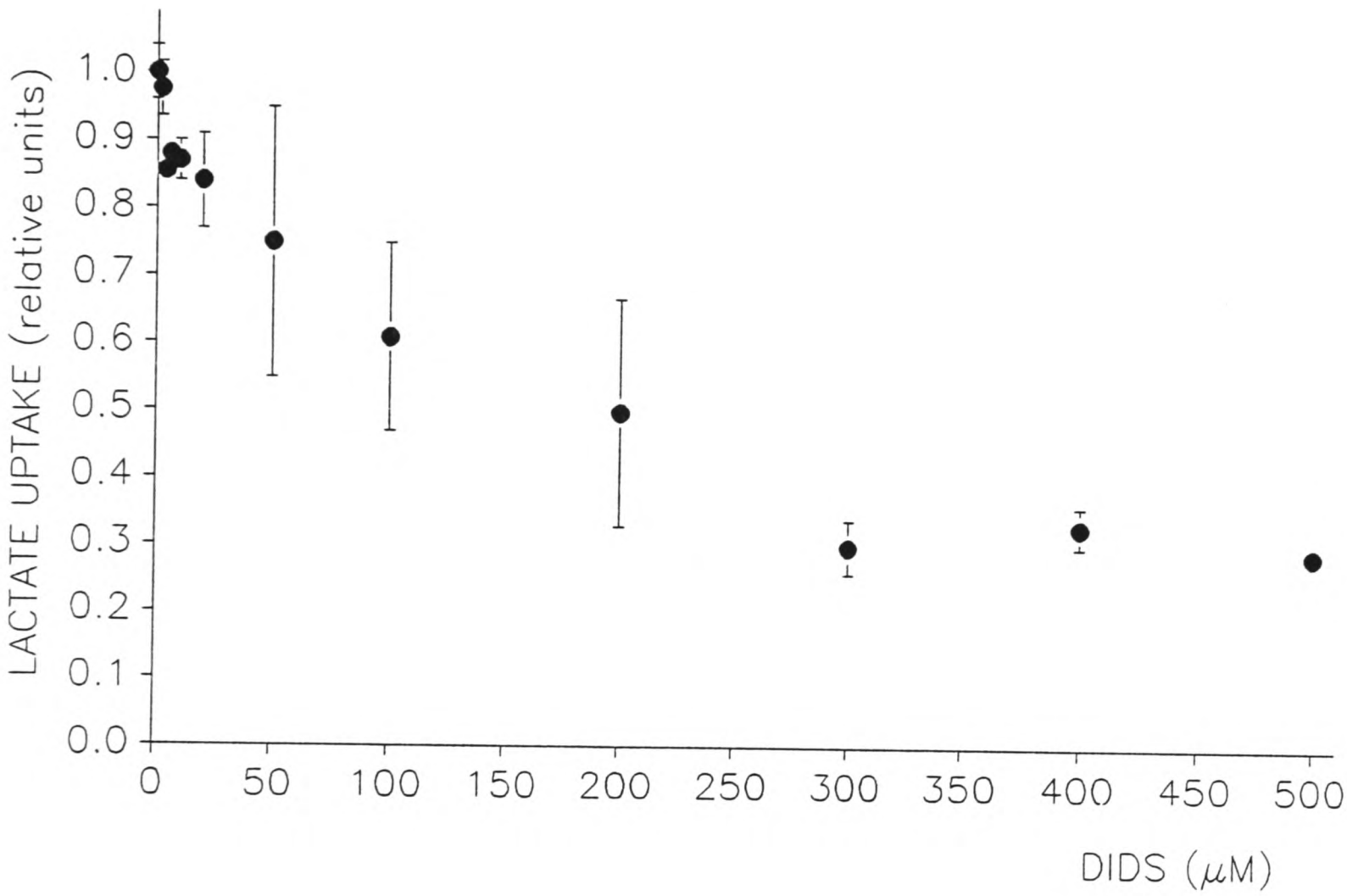


FIGURE 4.9: Concentration dependence of inhibition of lactate uptake by DIDS (n:5). Extracellular lactate concentration: 1 mM.

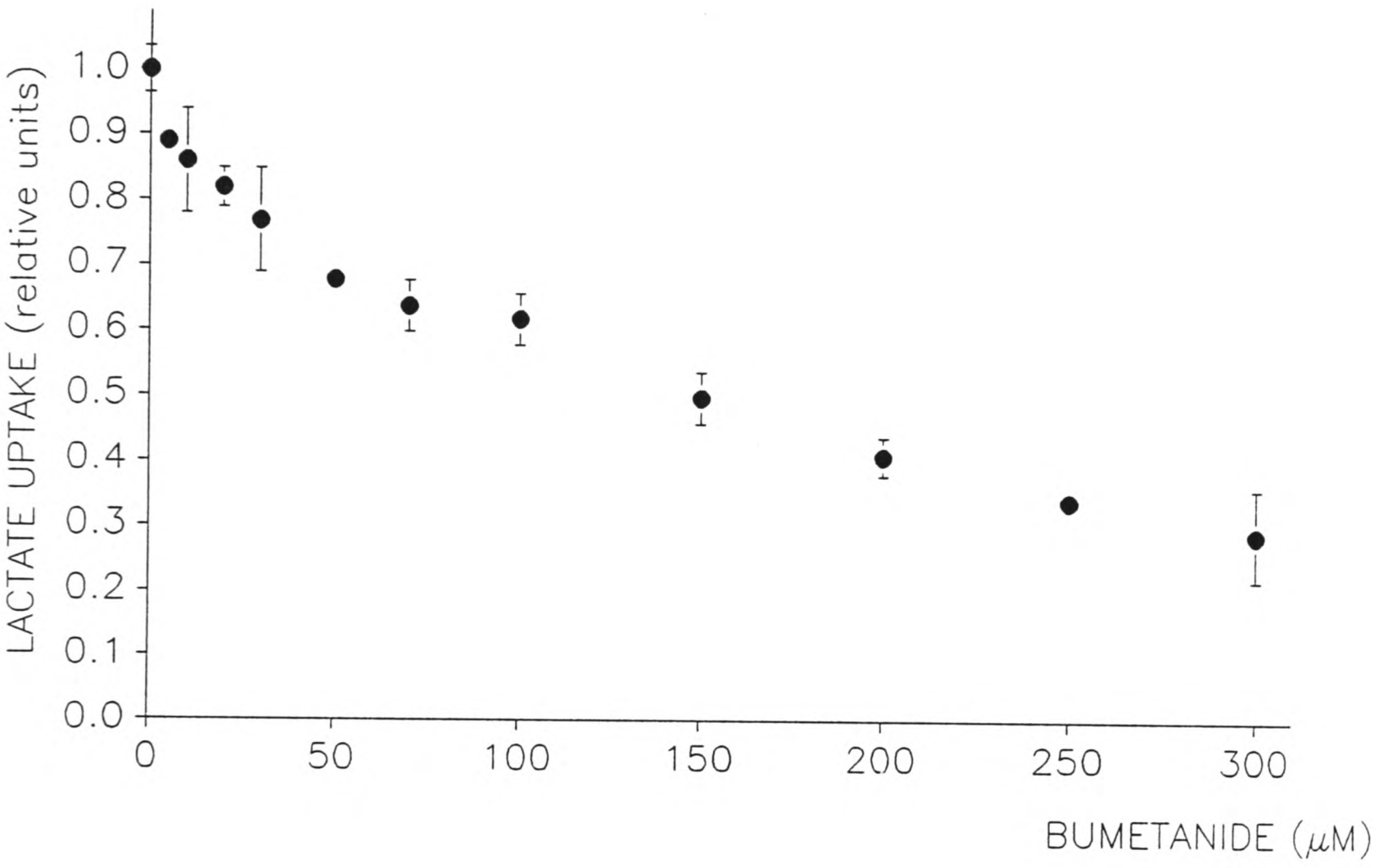


FIGURE 4.10: Concentration dependence of inhibition of lactate uptake by Bumetanide (n:4). Extracellular lactate concentration: 1 mM.

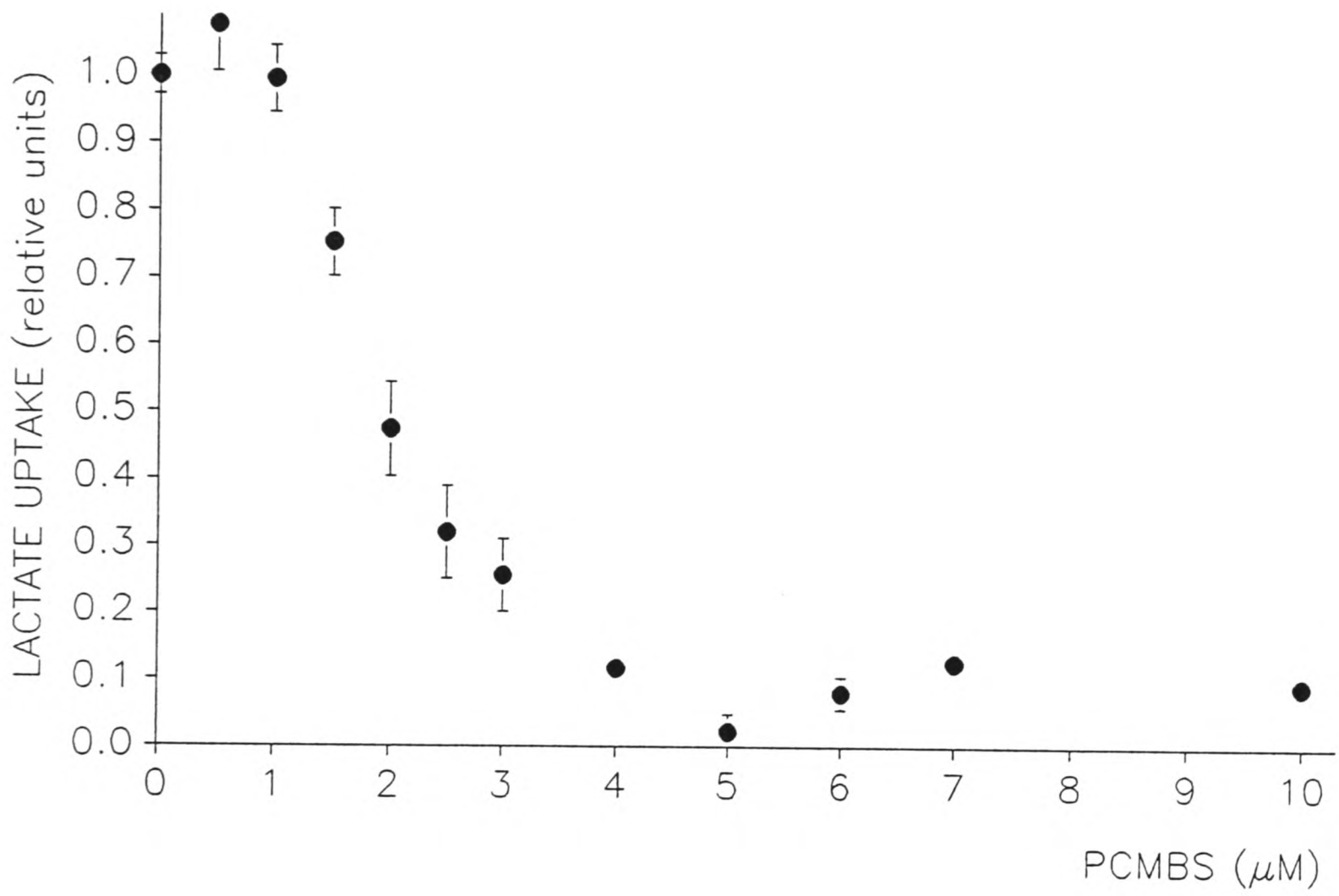


FIGURE 4.11: Concentration dependence of inhibition of lactate uptake by PCMBS (n:6). Extracellular lactate concentration: 1 mM.

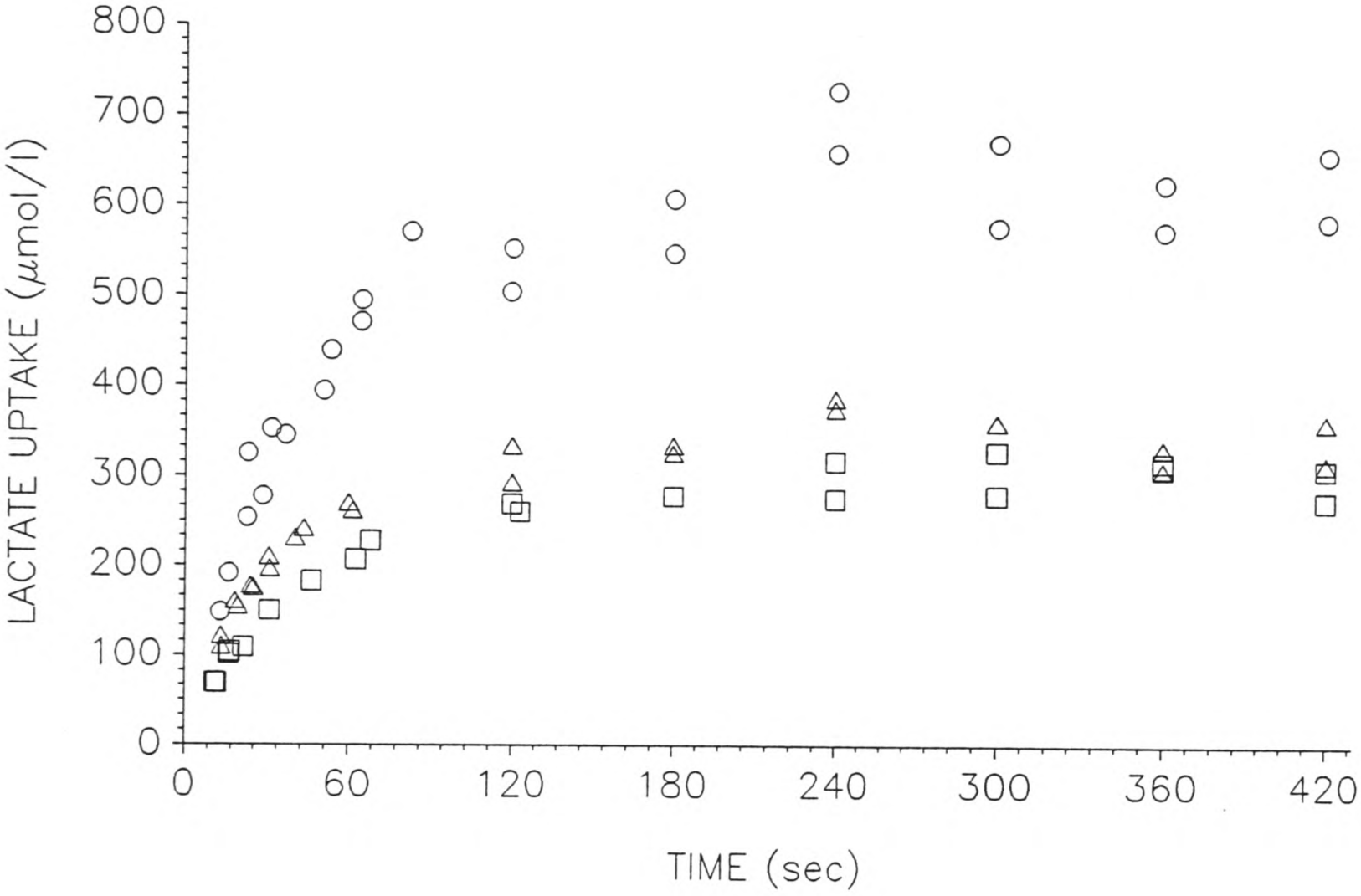


FIGURE 4.12: Lactate uptake in autologous plasma from 3 normal subjects Plasma lactate concentration was 1.63 mM (circle), 0.63 (square) ar 1.07 (triangle).

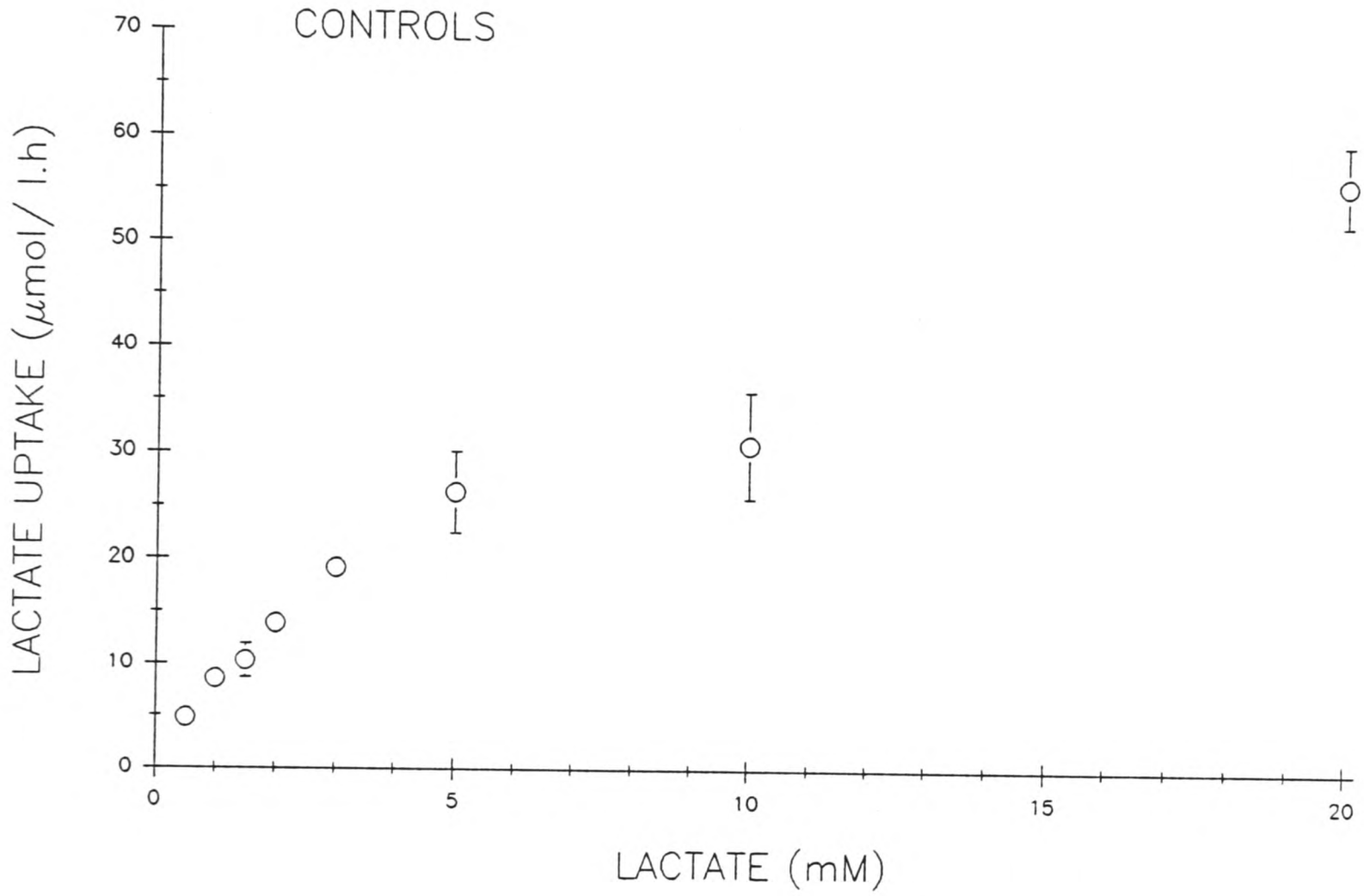


FIGURE 4.13: Concentration dependence of lactate uptake in 5 nor controls at 37°C.

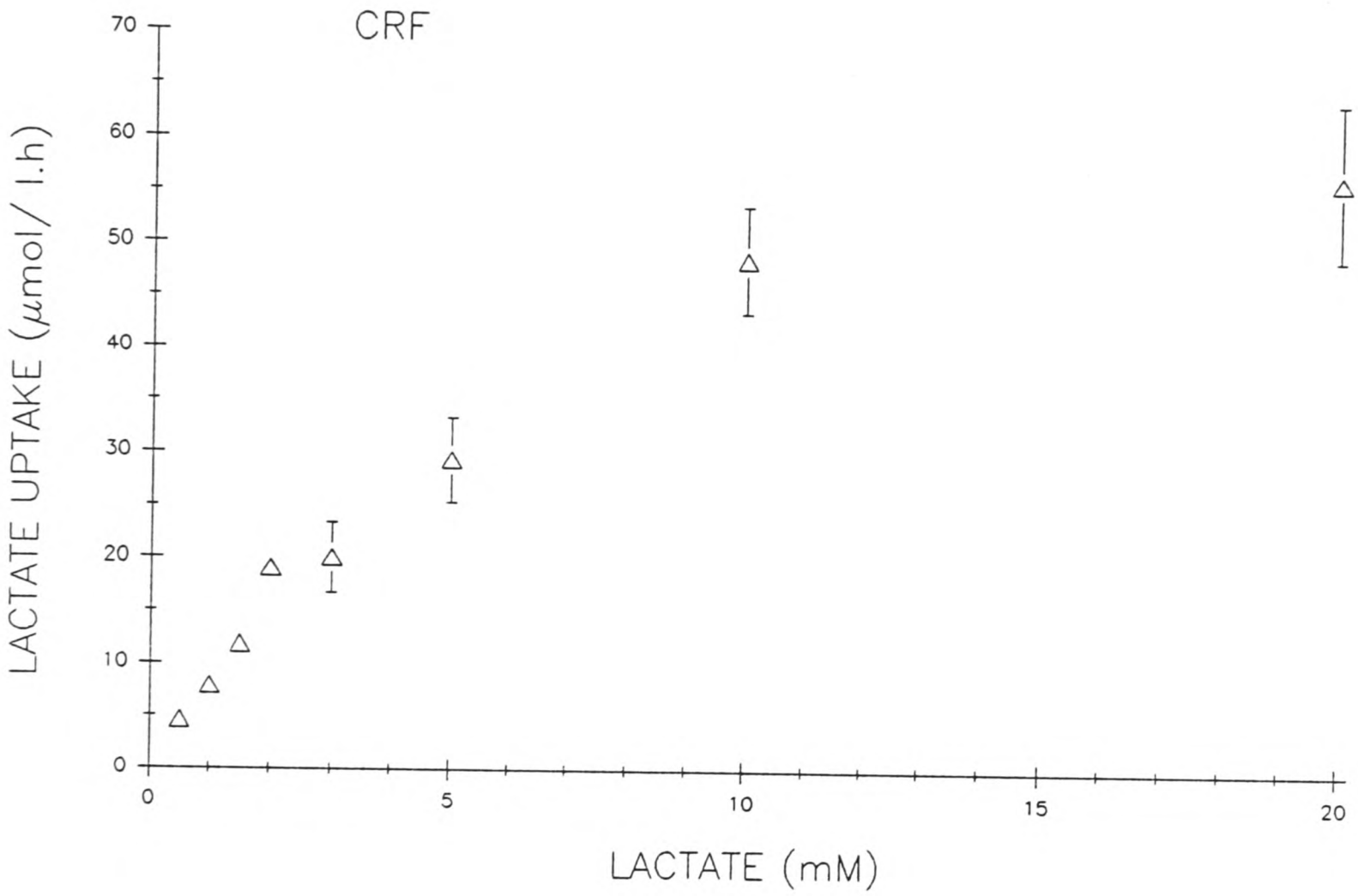


FIGURE 4.14: Concentration dependence of lactate uptake in 5 patients at 37°C.

4.2.6. CHRONIC RENAL FAILURE

Lactate uptake was determined in 5 patients with CRF on haemodialysis, and compared with 5 healthy controls. Initial rates of uptake were measured at 37°C with extracellular lactate concentrations varying from 0.5 to 20 mM over 5-15 s. The results in figures 4.13 and 4.14 show that the mean maximum rate of lactate uptake in CRF was 81 μmol/ l.sec (SEM: 9), with a similar V_{max} of 83 (SEM: 10) in the control group. Mean K_m values were 8.0 mM (SEM: 1.9) and 11.5 (SEM: 2.6) for CRF and controls , respectively (NS).

4.3. DISCUSSION

This chapter presented two main observations. First, it demonstrated the activity of the lactate transporter under physiological conditions of temperature and pH. Second, it showed that lactate erythrocyte uptake in patients with chronic renal failure on dialysis treatment was similar to normal controls.

ERYTHROCYTE LACTATE UPTAKE UNDER PHYSIOLOGICAL CONDITIONS OF TEMPERATURE

Lactate is a small carboxylic acid and under normal pH is usually an anion. The movement of physiologically important anions across the red cell membrane is more difficult to measure than that of cations, because membrane anion transport is so much faster than that of cations. Most studies employ some means of slowing the rate of ion transport, such as cooling the cell suspension, or substituting a larger anion, e.g., sulphate [Clark 1988].

In order to study erythrocyte lactate fluxes in disease states it was necessary first to characterize the transport at normal temperature conditions. Biochemically lactate is formed from the reduction of pyruvate. Pyruvate is an important intermediate of the major catabolic and anabolic pathways [Denton & Halestrap 1979], participating in the major metabolic pathways for carbohydrates, lipids and proteins. The breakdown of glucose results in the production of lactate and protons and its accumulation would cause metabolic acidosis with consequent alterations in cell function [Alberti & Cuthbert 1982]. Glycolysis is the main metabolic pathway in the production of lactate, occurring chiefly under anaerobiosis as seen in anoxia or exercising skeletal muscle. Red cells do not have mitochondria and the principal source of energy is from glycolysis with resulting lactate formation [Denton & Halestrap 1979]. The lactate produced in glycolyzing cells is released into the blood and then extracted by the liver, kidney, heart or resting skeletal muscle where it is metabolized again into glucose. Specific transport systems enable the rapid translocation of these metabolites across the membranes of different types of cells [Spencer & Lehninger 1976].

The main anion transporter in erythrocytes is the anion exchanger protein (band 3). The affinity of band 3 for monocarboxylates is very low, and the K_m values are high [Halestrap 1976, Deuticke 1982]. As discussed previously three main pathways for lactate transport have been identified.

Lactate fluxes are fast and special techniques have to be employed in order to measure the linear initial rate of transport. In experiments using rapid cell separation through oil, associated with a cold inhibitor-stop method, information of the activity of the transporter at 37°C can be obtained. Lactate-monocarboxylate exchange operates in a faster mode than the net flux of lactate in exchange with protons, but in these studies cells were present at zero-trans conditions for lactate [Dubinsky & Racker 1978]. Concern about the possible effect of metabolism on the

measured transport rates has been expressed [Halestrap et al 1990]. L-lactate is not further metabolized in erythrocytes. Its interconversion by lactate dehydrogenase, which catalyses the reduction/oxidation of lactate and pyruvate, is the major metabolic complication [Deuticke 1982]. No inhibitor of this enzyme was present in our experiments, and this complication would be of more concern in efflux experiments [Deuticke 1982]. To minimize this factor the fluxes were measured in saline without glucose, i.e. no substrate for the glycolytic pathway present in the red cells was available. The fluxes were also performed with the cells depleted of their lactate content as described in the methods section. The fluxes at 37°C were shown to be linear for at least 20 seconds, representing the equivalent of a first order reaction and a non-significant backflux component.

The transport via a monocarboxylate carrier was first confirmed by the use of cinammate and its derivatives as inhibitors of the lactate and pyruvate transport in mitochondria and erythrocytes by A Halestrap [Halestrap 1976, 1975, Halestrap & Denton 1974]. This carrier has since been identified and characterized in several different tissues.

The results seen in the present work are in agreement with previous descriptions that lactate uptake is largely via the monocarboxylate carrier. The inhibition of a significant proportion of lactate uptake by various inhibitors of the anion exchange protein, band 3, led to an initial proposition that larger contribution of the anion exchanger to the fluxes occurred at 37°C. That is not the case as is shown by the very effective inhibition achieved with PCMBS, which does not inhibit fluxes via band 3 [Deuticke 1989]. This confirms the participation of the monocarboxylate carrier in about 90 % of the lactate uptake, values similar to that seen at lower temperatures [Deuticke 1982]. PCMBS is a slowly penetrating organomercurial that acts almost instantaneously when added to the cells, suggesting an action on an extracellular sulphydryl site [Deuticke et al 1978, Dubinsky & Racker 1978, Johnson et al 1980]. Although CHC has been

proposed as a specific inhibitor of the monocarboxylate carrier, the present study suggests PCMBS is a better inhibitor to measure the MC fraction of the fluxes.

It is difficult to compare the kinetic parameters in the present thesis with those in the literature, since no previous measurements of lactate transport in erythrocytes have been carried out at 37°C. Furthermore the rates of transport are markedly influenced by the experimental conditions and pH. Still, some indirect evidence supports the present findings. Fishbein [1986] has shown that erythrocyte lactate efflux at 20°C has a Km of 7.2 mM and a Vmax of 17 μmol/l.sec (1.02 mM/min). Extrapolation of his data for lactate efflux at 37°C, applying estimated values of lactate carrier activation energy [Dubinsky & Racker 1978], resulted in values for Vmax of 133 μmol/l.sec (8 mM/min) [Fishbein 1978]. Results for lactate fluxes by other author are illustrated in table 4.2, for comparison.

TABLE 4.2
ERYTHROCYTE LACTATE FLUXES

REFERENCE	T (°C)	K_m (mM)	V_{max} (μmol/l.sec)
FISHBEIN 1976	20	7.2	17
DUBINSKY & RACKER(1978)	25	13.4	55
DEUTICKE 1982	20	10.5	14.5
	10	3	1.5
	5	0.33	1.7
HALESTRAP 1976	10	9.14	19.5
LEEKS & HALESTRAP(1978)	10	8.07	13.9
	25	18.6	7.61
		18	11.3
PRESENT STUDY	*37*	*11.5*	*83*

The activity of the transporter also varies between species and cell types [Edlund & Halestrap 1988, Deuticke 1989, Leeks & Halestrap 1979, Poole et al 1989, Halestrap et al 1990, Lupo et al 1990, Watt et al 1988, Fafournoux et al 1985, Jennings & Adams-Lackey 1982, Trosper & Philipson 1989, 1987, Monson et al 1982, Spencer & Lehninger 1976].

The main function of the lactate transporter is to export lactate (in skeletal muscle), the major organic acid that accumulates inside the cell. The erythrocyte obtains most of its energy from glycolysis and needs a rapid transporter of lactate. In muscles high levels of lactate occur mainly at exercise and an important role of the carrier is to participate in muscle lactate extrusion. Blood cells contain a third of the aqueous volume of blood, and the rapid uptake of lactate exported from muscle would dilute the rise in plasma lactate and facilitate efflux from muscle [Fishbein 1986]. At the level of the liver the release of lactate to hepatocytes would help gluconeogenesis [Denton & Halestrap 1979]. As has been proposed for amino-acids [Christensen 1982,1990], erythrocytes then play a role in the interorgan distribution of lactate.

ERYTHROCYTE LACTATE TRANSPORT IN URAEMIA

Metabolism of carbohydrates is abnormal in uraemic patients, with significant alterations related to a peripheral resistance to the action of insulin [Alvestrand et al 1989, DeFronzo et al 1982, Mak 1989]. Anaemia of renal failure can provoke a hypoxic stimulus perhaps as a stimulus for anaerobic glycolysis. As described in the introduction of this chapter, lactate levels in uraemia are reported to be either normal or elevated and there is also conflicting evidence on lactate levels following exercise. The experiments described in this chapter shows that erythrocyte transport of lactate in chronic renal failure is normal. It does not appear that abnormal lactate transport is a key factor in uraemic cell damage. This is an important result, in which the

selectivity of the abnormalities of membrane transport in uraemia is confirmed. An normal lactate transport is important in preventing further metabolic derangements in renal failure.

Study of lactate transport in diseases has been of interest. A deficiency of monocarboxylate carrier in patients with muscular pain [Fishbein 1986], and the existence of naturally occurring inhibitors of the mitochondrial and red cell monocarboxylate carrier in phenylketonuria (phenylpyruvate) and maple syrup disease (α-ketoisocaproate) induces severe metabolic abnormalities [Denton & Halestrap 1979]. Also in starvation and diabetes, due to the ability of the monocarboxylate carrier to transport ketone bodies its role is important [Denton & Halestrap 1979]. In these conditions the lactate carrier participates in the mechanisms of disease. That is not the case for renal failure patients.

CHAPTER 5

MEMBRANE CHOLESTEROL MODULATION AND THE EFFECTS ON MEMBRANE TRANSPORT

The present chapter presents "in vitro" experiments on the modulation of membrane transport processes by changes in membrane cholesterol. Cholesterol was altered by cell incubation with liposomes of defined composition. The objective was to analyze the direct effect of altered membrane cholesterol on the activity of some of the transport systems studied in this thesis in the context of disease processes.

Membrane lipid composition is known to modulate membrane function [Yaegle 1989, Deuticke & Haest 1987, Clandinin 1991, Cabantchik et al 1986, Spector & Yorek 1985, Luly 1989, Carruthers & Melchior 1988, Lucio et al 1991]. The significance of this modulation for cell physiology in health and disease is not completely clear, but this process may be important in pathological states [Clandinin et al 1991]. As mentioned previously blood lipids and cholesterol are very much involved in concepts of certain diseases. It is clear now that there is an association between plasma cholesterol levels and mortality and morbidity, mainly in the context of cardiovascular disease [Ball & Mann 1988]. The association of low cholesterol and increased risk of neoplasia (or other non cardiovascular deaths) is controversial, but has been reported [Ball & Mann 1988]. The diseases in focus in the present thesis: chronic renal failure, diabetes and atherosclerotic vascular disease all have clinical associations with changes in blood lipids [Lindner et al 1974, Martin et al 1986, Gibbons 1986, Betteridge 1989, Mulec et al 1990, D'Amico & Sanna 1991, Hargreaves et al 1991]. They also have abnormal erythrocyte membrane lipid composition reported [Bleiber et al 1980, Baldini et al 1989, Wood et al 1984, Michalak et al

1988]. Further association between dyslipidaemia and hypertension has been reported in some individuals [Williams et al 1988, Hunt et al 1989, 1991].

Sodium-lithium countertransport in the erythrocyte has been examined in cholesterol-depleted cells and compared with control cells. The study of Na/Li countertransport was a preliminary study to the investigation on patients with atherosclerosis treated with lipid-lowering therapy, which will be described in the next chapter. Sodium-hydrogen antiporter activities and choline transport have been studied in cells enriched and depleted in cholesterol.

5.1. MATERIAL AND METHODS

5.1.1. MATERIAL AND SOLUTIONS

L α-phosphatidylcholine (from egg yolk), cholesterol (from porcine liver), tissue culture medium 199 without sodium bicarbonate (TC199) and glutamine were obtained from Sigma Chemical Company, Dorset, Poole, UK. TC199 was buffered with 15 mmol/l 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and was nominally bicarbonate-free; its composition was adjusted to (in mmol/l) Na 140, K 5.5 (pH 7.4 with tris base). Chloroform and methanol were from BDH Limited, Atherstone, UK; 2-propanol and n-hexane (HPLC grade) from Aldrich Chemical Company, Gillingham, UK; and nitrogen (N_2) from BOC Ltd, Brentford, Middlesex, UK. RPMI and foetal bovine serum were from Flow Laboratories, Rickmansworth, Herts, UK. All other chemicals were Analar grade.

Sonication was performed using an Astell Scientific bath sonicator for the phosphatidylcholine liposomes employed for erythrocyte cholesterol depletion in the Na/Li countertransport experiments. An MSE ultrasonic power unit with a 9mm diameter probe tip was

used to prepare all the other liposomes.

Liposomes and control solutions had Penicillin 50 UI/ml and Streptomycin 50 µg/ml added to avoid bacterial growth under prolonged incubation periods.

Other chemicals and equipment are described in the relevant chapters of this thesis on each of the transporters.

5.1.2. LIPOSOME PREPARATION AND CELL CHOLESTEROL MODULATION

Modification of cell cholesterol was achieved with the use of liposomes. Cholesterol depletion was performed with phosphatidylcholine liposomes (PC liposomes), while enrichment was achieved with cholesterol-phosphatidylcholine liposomes (PCCH liposomes).

5.1.2.1. LIPOSOMES FOR CHOLESTEROL DEPLETION AND Na/Li COUNTERTRANSPORT

One ml of a lecithin solution (phosphatidylcholine 100mg/ml in chloroform/methanol) was evaporated to dryness under nitrogen in a round-bottomed glass and then aspirated under vacuum. The lipids were resuspended in 20 ml saline (lecithin 5 mg/ ml of saline) and sonicated for 30 min twice with a interval of 15 min for refrigeration. Liposomes were cooled and used immediately. The saline had the following composition: NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, Glucose 5 mM, MOPS 10 mM, pH 7.4 with tris base. $MgCl_2$ was included to prevent haemolysis [JC Ellory, FJ Lucio Cazana, BM Hendry & C Harvey: personal communication].

Incubation of erythrocytes with PC liposomes or control saline was performed overnight (≈ 12 hours). For each ml of packed cells a total of 100 mg of lecithin was used. Packed erythrocytes were resuspended with the liposome suspension (1:20, V/V), incubated and mixed constantly by a rotating mixer in a water bath at 37°C. A control cell suspension was submitted to the same procedures, but in saline without liposomes. This method was modified after others [e.g. Claret et al 1978, Shinitzky & Inbar 1974]. After the incubation period, the cells were spun down at 1500 g for 15 min, and the supernatant and top layer of cells discarded. Subsequently cells were washed five times with ice cold saline and centrifuged at 3000 g. Two or three aliquots of red cell suspension were separated for haematocrit and cholesterol content measurements. The cells were then used for measurements.

5.1.2.2. LIPOSOMES FOR CHOLESTEROL MODULATION OF THE Na/H ANTIPORTER

Liposomes were prepared with some modifications from previous methods [Claret et al 1978, Shinitzky & Inbar 1974]. Lecithin alone (240 mg) (PC liposomes), or with cholesterol (480 mg) was dissolved in chloroform-methanol (2:1,v/v) (PCCH liposomes) were evaporated to dryness under nitrogen in a round-bottomed glass vessel. Complete dryness was achieved by aspiration under vacuum for 2 hours. The lipids were dispersed in 16 ml of "isotonic KCl buffer" (chapter 3) and sonicated using an MSE Ultrasonic Power Unit. Sonication was carried out using a 9 mm diameter probe tip at maximum energy output while cooling the dispersion with ice. After sonication of PC liposomes for 60 min and PCCH liposomes for 120 min, the suspensions were centrifuged at 4°C for 40 min at 100,000g using an MSE Europa Ultracentrifuge (Model 55M) with an MSE 8x35 ml titanium angle rotor. The supernatants containing the liposomes were then

saved for the experiments and kept at 4°C to be used in the next 24 hours.

Depletion or enrichment of membrane cholesterol in lymphoblasts was achieved by incubating 8 to 16 x10^6 lymphoblasts per ml in PC or PCCH liposomes at 37°C using a water bath, while the control cells were incubated in the same isotonic KCl buffer without the liposomes. The medium was changed every 2 hours of incubation. At times 0, 2, 4 and 6 hours of incubation, samples were taken and the cells washed 3 times with TC199. One aliquot was taken and used for measuring the pHi and the Vmax of the Na/H antiporter, as described in chapter 3. The other aliquot was used to count cells with a Coulter Counter (Coulter Electronics, Luton, UK), and to measure the membrane cholesterol content (see below). The medium was changed every 2 hours of incubation. Cell viability was higher than 95% after 6 hours of incubation when tested by trypan blue exclusion.

5.1.2.3. LIPOSOMES FOR CHOLESTEROL MODULATION OF THE CHOLINE TRANSPORT

Liposomes for all the other experiments were prepared as described for the experiments with lymphoblasts. The liposomes obtained in that way with more intense sonication are smaller, and more effective at achieving faster cell cholesterol changes. The saline comprised NaCl 150 mM, KCl 5.36 mM, $MgCl_2$ 1 mM, MOPS 15 mM, Glucose 10 mM, pH adjusted to 7.4 with NaOH. Phosphatidylcholine liposomes for cholesterol depletion were submitted to sonication with an initial concentration of 7.6 mg of PC/ml of saline and the mixed cholesterol-phosphatidylcholine liposomes had 15.3-7.6 mg/ml of saline. The incubation under the different conditions was performed at a packed cell volume of approximately 7 %.

5.1.3. CHOLESTEROL EXTRACTION AND MEASUREMENTS

Lipid extraction was in chloroform/isopropanol (7:11) following which the solution was dried under nitrogen and the residue resuspended in n-hexane for analysis of cholesterol by normal phase isocratic high-performance liquid chromatography (HPLC). The solvent phase was 97% n-hexane 3% isopropanol with a 5 μm Microsorb Silica column. An internal standard of progesterone was used and the system was calibrated with pure cholesterol standards. Prior to the lipid separation the lymphoblasts were counted using a Coulter Counter and erythrocytes had the packed cell volume measured. The cholesterol values were comparative and cell cholesterol results are expressed as percentage of change from the sample taken at control time zero. Measurements of cholesterol were kindly done by Dr. Francisco J Lucio Cazana and Prof. Bruce M Hendry.

5.1.4. WHITE CELLS FOR THE CHOLESTEROL MODULATION EXPERIMENTS

5.1.4.1. LYMPHOCYTES

Lymphocytes were isolated using Histopaque-1077 with a method similar to Böyum [1968]. Histopaque 1077 density is 1.077. Sixty ml of blood were obtained, mixed with 40 ml of TC199 and a 20 ml aliquot carefully layered over 5 ml of Histopaque 1077 in universal tubes. Lymphocytes and other mononuclear cells will be held at the plasma-histopaque interface, while RBC and granulocytes will gravitate to the bottom, after centrifugation at 300 g for 30 minutes. Any residual red cells were eliminated by hypotonic shock as described for dextran sedimentation in chapter 3.

5.1.4.2. LYMPHOBLASTS

Modulation of membrane cholesterol and its effects on intracellular pH and Na/H antiporter activity was performed by using cultured Epstein Barr Virus modified lymphocytes, lymphoblasts. These experiments require a large yield of cells representing large volumes of blood, if lymphocytes were to be separated. Lymphoblasts growing in culture were used as an alternative.

The lymphoblast tissue culture manipulation and cell feeding was kindly performed by Dr. L.L. Ng who provided the cells for the experiments. The following conditions were used: transformed human lymphocytes by Epstein-Barr virus were cultured in RPMI medium containing 10% foetal bovine serum, 1 mmol/l glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 24 mmol/l $NaHCO_3$ (pH 7.4 with 5% CO_2 in air). These lymphoblasts were fed every 2 days, keeping the cell density between 0.5 and 1 x 10^6/ml. The cell viability as judged by trypan blue exclusion was over 98%.

After retrieval from the culture, lymphoblasts were washed three times and then used accordingly for each experimental protocol.

5.1.5. Na/Li COUNTERTRANSPORT

The activity of the erythrocyte Na/Li countertransport has been reported to be altered in conditions such as essential hypertension [Canessa et al 1980], insulin dependent diabetic patients with a higher risk of developing diabetic complications, uraemia and hyperlipidaemia [Woods et al 1983, Hunt et al 1986, Krolewski et al 1988, Mangili et al 1988]. All these conditions are implicated in a higher risk of developing vascular disease. Erythrocyte cholesterol modulation was performed to evaluate any possible influence on the countertransport activity. The methods for assessing its activity are discussed next.

5.1.5.1. CELL PREPARATION

Blood preparation and cell quantification is described in detail in chapter 2. Usually 10 to 20 ml of blood was required from normal controls or patients, kept on ice and experiments were performed within 1 to 2 hours. Packed red cells were washed 3-5 times with ice cold $MgCl_2$ wash solution ($MgCl_2$ 107 mM, MOPS 10 mM pH 7.4 tris base).

5.1.5.2. GLASSWARE CARE

Special care had to be taken with the cleaning of the glassware used to prepare and keep the solutions used in the Na/Li countertransporter experiments. Any undesired contamination, e.g. traces of ions and detergent, can have a major effect on the measurements using atomic absorption or emission spectrophotometry. In those experiments all the glassware as submitted to the following cleaning procedures:

After standard cleaning in an automatic glass washing machine, glassware was washed with ethanol, the excess was discarded and immediately nitric acid was added to the tube and the reaction was allowed to continue until all the brown fumes had gone. Four washes with deionized water were then given and the items placed in a drier.

Due to the chemical reaction between the alcohol and nitric acid, toxic fumes were emitted so the procedures were carried out on a fume cupboard with special care and protection due to risk of explosion and the corrosive characteristics of the chemicals and the reaction.

5.1.5.3. IONIC MEASUREMENTS

Intracellular sodium, potassium and lithium measurements were done using flame photometry (IL Flame Photometer, Instrumentation laboratory IL 943) with caesium as an internal standard. Cell samples for electrolyte measurements were separated and washed three times (10000 g, 15 sec) with ice cold $MgCl_2$ wash solution using an Eppendorf microfuge. Triplicate samples were taken to estimate the haematocrit. After the last wash the supernatant was aspirated and discarded. The remaining cell pellet was then lysed by adding 0.5 ml Triton X-100 (0.1 %, w/v). TCA 5% (w/v) was added to precipitate the protein, and the tube was spun in a microfuge (10000 g) for 10 minutes. The clear supernatant was then aspirated and used for electrolyte analysis.

Potassium and lithium were analyzed in the standard mode of the flame photometer. Measurements of intracellular sodium used the prediluted mode with a standard containing Na 1.4 mM, K 0.05 mM, Li 0.01 mM and Cs 1.5 mM. Lithium in the efflux medium was measured by means of atomic absorption spectrophotometry (Atomic absorption spectrophotometer-ATOMSPEK, Hilger & Watts H1170) using a slit width of 30 nm and wavelength of 670.8 nm. Standards contained lithium chloride (0 to 100 μM) made up in the same Na-containing or Na-free solution used for the efflux experiments. Alternatively lithium was measured by means of atomic emission spectrophotometry using a Pye Unican spectrophotometer using the same standards as above. A mixture of acetylene and compressed air was used to fuel the flame.

Extreme care was needed in cleaning the burning slot, monitoring aspiration rates and checking the calibration for every individual experiment.

Electrolytes are expressed as mmoles/l rbc and intracellular concentration was calculated using the formula below (mmoles/ l rbc):

$$[ION]_i = (x \, . \, TV) / Hct \qquad (5.1)$$

where $[ION]_i$ is intracellular concentration of ion; x: measured concentration in mmoles/l; TV: total volume (ml) in the tube, that is) 0.5 ml Triton X-100, 0.5 ml TCA and cell volume (Hct); Hct: Haematocrit.

The initial protocol employed a wash solution contained sucrose and glucose (75 mM $MgCl_2$, 85 mM sucrose and 10 mM glucose) [Canessa et al 1980]. This solution caused frequent difficulties when used in the atomic absorption/emission spectrophotometer, even when sucrose was replaced in the solution with higher concentrations of $MgCl_2$. The flame would get contaminated very quickly, needing frequent interruptions to clean the flame slit. Magnesium-salt-containing media provided unstable readings in another study using flame emission spectrophotometry [Ibsen et al 1982].

5.1.5.4. Na/Li COUNTERTRANSPORT ASSAY

Sodium-lithium countertransport was measured with a modified method based on Canessa et al [Canessa et al 1980; Trevisan et al 1982; Smith et al 1982].

All the solutions were prepared using deionized water. After erythrocytes were obtained and washed as above with ice cold $MgCl_2$ wash medium, cells were then resuspended at approximately 10 % haematocrit, and two 1 ml samples taken into two Eppendorf tubes for measurements of intracellular electrolytes. The tubes were spun down with a microfuge (10000 g, 15 sec), supernatant discarded and cells lysed with 0.5 ml Triton X-100 and proteins precipitated by adding 0.5 ml TCA. After centrifugation the clear supernatant was saved for later measurements of intracellular sodium, potassium and lithium. Three 100 µl aliquots of cell

suspension were used to estimate haemoglobin content.

To load the erythrocytes with lithium the cells were suspended at an haematocrit of approximately 10% in the lithium loading solution at 37° for 3 hours in a shaking water bath. Lithium loading solution consisted of (in mmol/l) 150 LiCl, 10 Glucose and MOPS 10 at pH 7.4 with tris base. The lithium loaded cells were then washed with ice-cold washing solution (containing in mmol/l: 107 $MgCl_2$, 10 MOPS at pH 7.4 adjusted with tris base) (3000 g, 5 min) five times to eliminate extracellular lithium. At the last wash cells were resuspended at approximately 10 % haematocrit and samples taken for measurement of intracellular electrolyte content and haemoglobin as described above.

To measure Na/Li countertransport, lithium efflux was measured in a sodium-free and a sodium-enriched solution containing ouabain. The Na-free solution consisted of (in mmol/l) N-Methyl-D-Glucamine (NMDG) 150, Glucose 10, MOPS 10 and ouabain 0.1, pH 7.4 with 30% HCl. Na-enriched solution consisted of (in mmol/l) NaCl 150, glucose 10, MOPS 10, ouabain 0.1, pH 7.4 with tris base. The difference between the two represents the Na-dependent ouabain-insensitive lithium efflux, or Na/Li countertransport. Lithium-loaded cells were divided into two aliquots and resuspended in ice-cold either Na-enriched or Na-free solutions. Three 100 µl samples from each were used to estimate haemoglobin. The suspension was then divided between 8 Eppendorf tubes and kept on ice. Fluxes were started at time zero by placing the tubes in a water bath at 37°C. At time 0, 20, 40 and 60 minutes tubes were transferred to ice and kept for two minutes to stop the fluxes. Then tubes were spun down in a microfuge (10000g, 15 sec) and the clear supernatant was saved for measurement of the lithium content by means of atomic absorption or emission flame spectrophotometry.

This protocol meets the conditions necessary for approximate estimation of the maximum rate of Na/Li exchange. In order to produce maximal Na/Li countertransport, it is

necessary to maintain saturating concentrations of the exchanger substrates at both membrane surfaces [Tosteson et al 1981, Ibsen et al 1982]. Under these conditions the internal concentration of lithium and external sodium concentration are at least 10 and 6 times higher, respectively, than that necessary to half-activate exchange. However, recently those values have been questioned [Rutherford et al 1992,a,b]

Lithium efflux in mmoles/l rbc.h was computed from the linear regression of lithium loss (mmoles/l) as a function of time (h). The difference between the lithium efflux in the sodium containing solution and the sodium free solution represents the Na/Li countertransport. One example is shown on figure 5.1.

5.1.6. Na/H ANTIPORTER

Methods for measuring pHi and Na/H antiporter activity are described in Chapter 3. Na replacement with N-Methyl-D-Glucamine (NMDG) is used instead of inhibition by EIPA to measure the Na-independent H flux. The difference between efflux in either Na or NMDG media is taken as an approximation of Na/H antiporter activity, V_{max}. The use of EIPA or NMDG gave identical results.

5.1.7. CHOLINE

The protocol for choline uptake measurements is described in chapter 2.

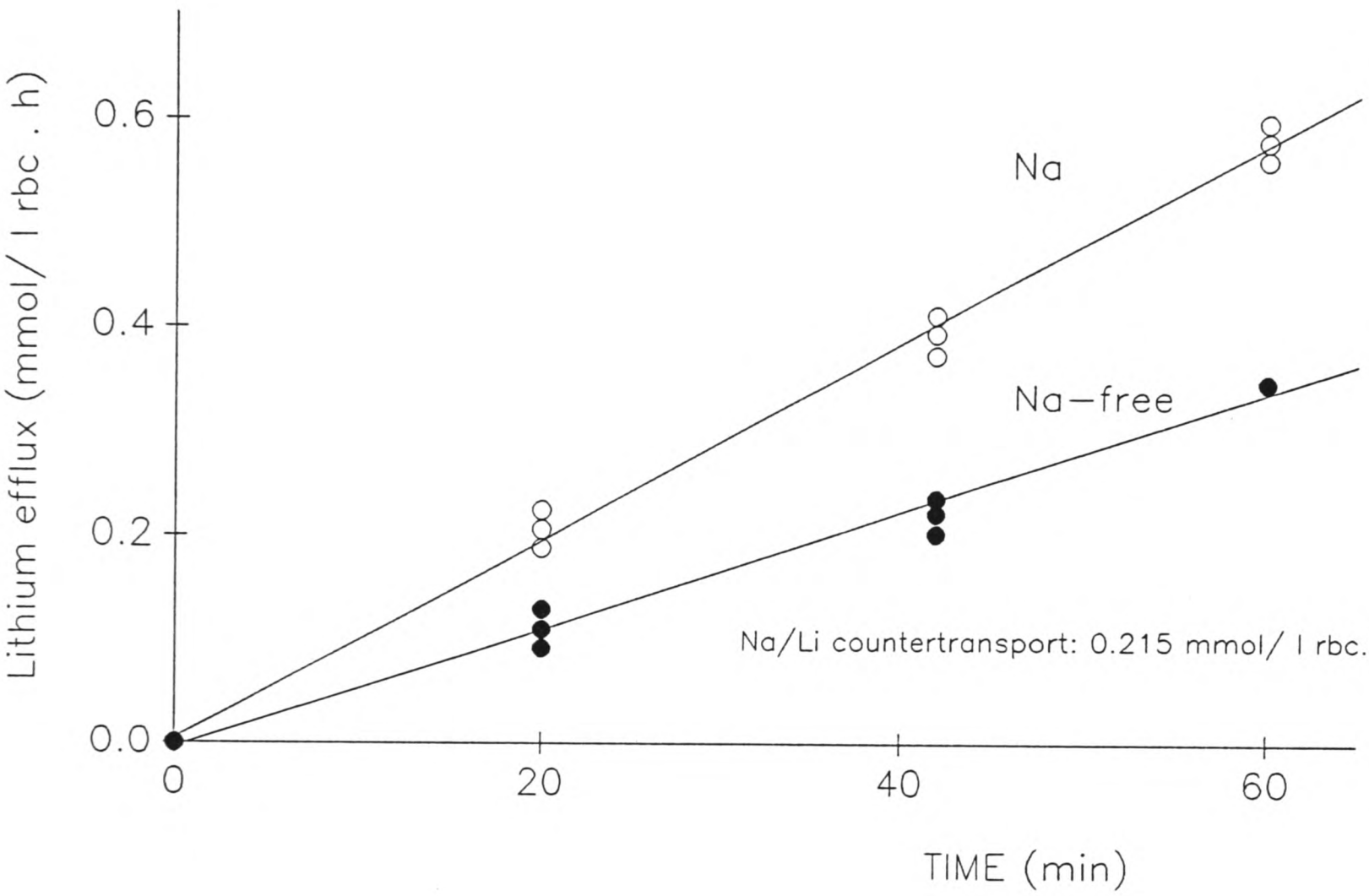


FIGURE 5.1: Na/Li countertransport in one subject. Lithium efflux is represented as a function of time in a Na-containing and a Na-free medium. The Na/Li countertransport is the difference in the efflux between the two conditions.

5.2.RESULTS

5.2.1. ERYTHROCYTE SODIUM-LITHIUM COUNTERTRANSPORT

5.2.1.1. ERYTHROCYTE CHOLESTEROL MODULATION

Depletion of cell membrane cholesterol was achieved with the use of phosphatidylcholine liposomes. Figure 5.2 shows that depletion of cholesterol can be achieved successfully after a few hours incubation and it is effective in depleting membrane cholesterol up to about 12 hours incubation.

We found that in some incubations the liposome to cell concentration ratio can influence the ability of the liposomes to modulate cell cholesterol.

5.1.2.2. ERYTHROCYTE SODIUM LITHIUM COUNTERTRANSPORT

Membrane cholesterol was depleted in red cells from 10 normal subjects (control). Mean membrane cholesterol content was 1.92 (SEM 0.30) μmoles/ml cells after depletion, compared with 3.11 (SEM 0.18) in control cells from the same blood, as shown in figure 5.3 ($p<0.01$, t test).

Lithium loading in the cholesterol-depleted cells achieved a mean value of 8.3 (SEM 1.7) mmoles/ l cells, and 6.0 (SEM 1.4) in the control group. Mean Na/Li countertransport activity was 273 (SEM 49) μmoles/l cells.h in cholesterol-depleted cells, compared with 342 (SEM 53) μmoles/l. h in the matched control ($p<0.01$, t test, figure 5.4).

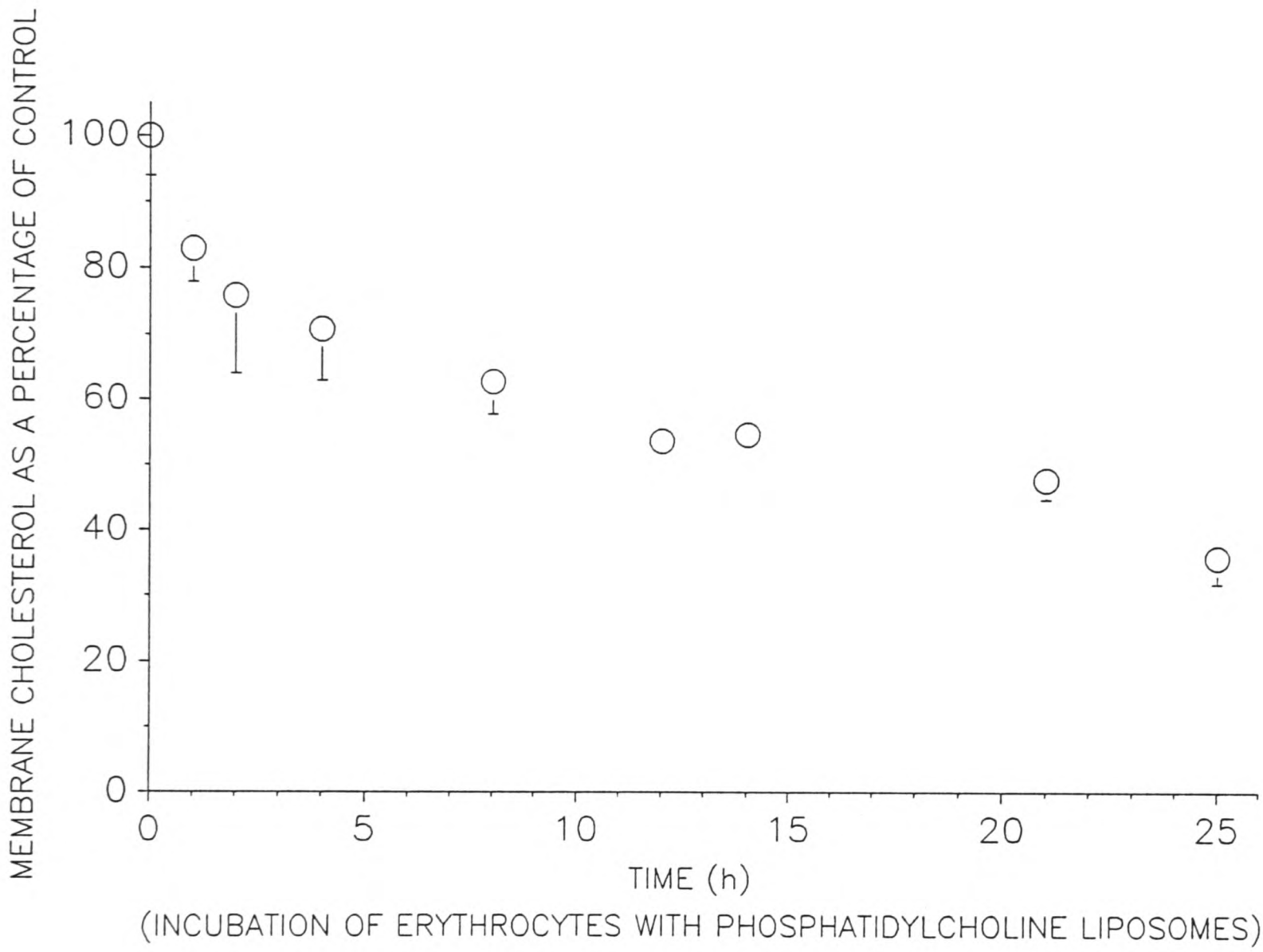


FIGURE 5.2: Erythrocyte membrane cholesterol changes after incubation with phosphatidylcholine liposomes. The liposomes in this experiments were prepared as described for the Na/Li countertransport experiments.

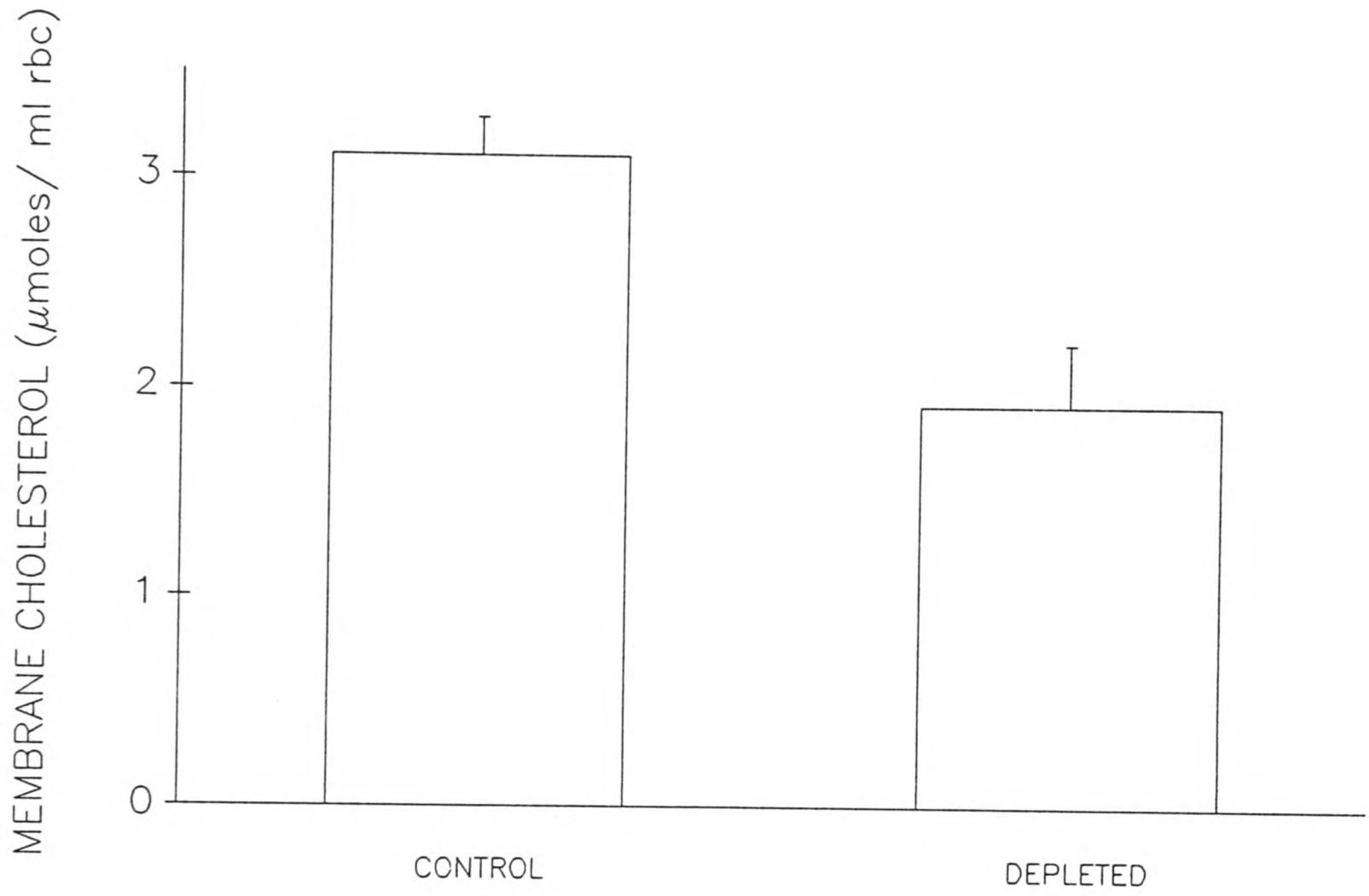


FIGURE 5.3: Erythrocyte cholesterol changes after phosphatidylcholine liposomes for the Na/Li countertransport experiments. Significnt depletion was obtained ($p<0.01$).

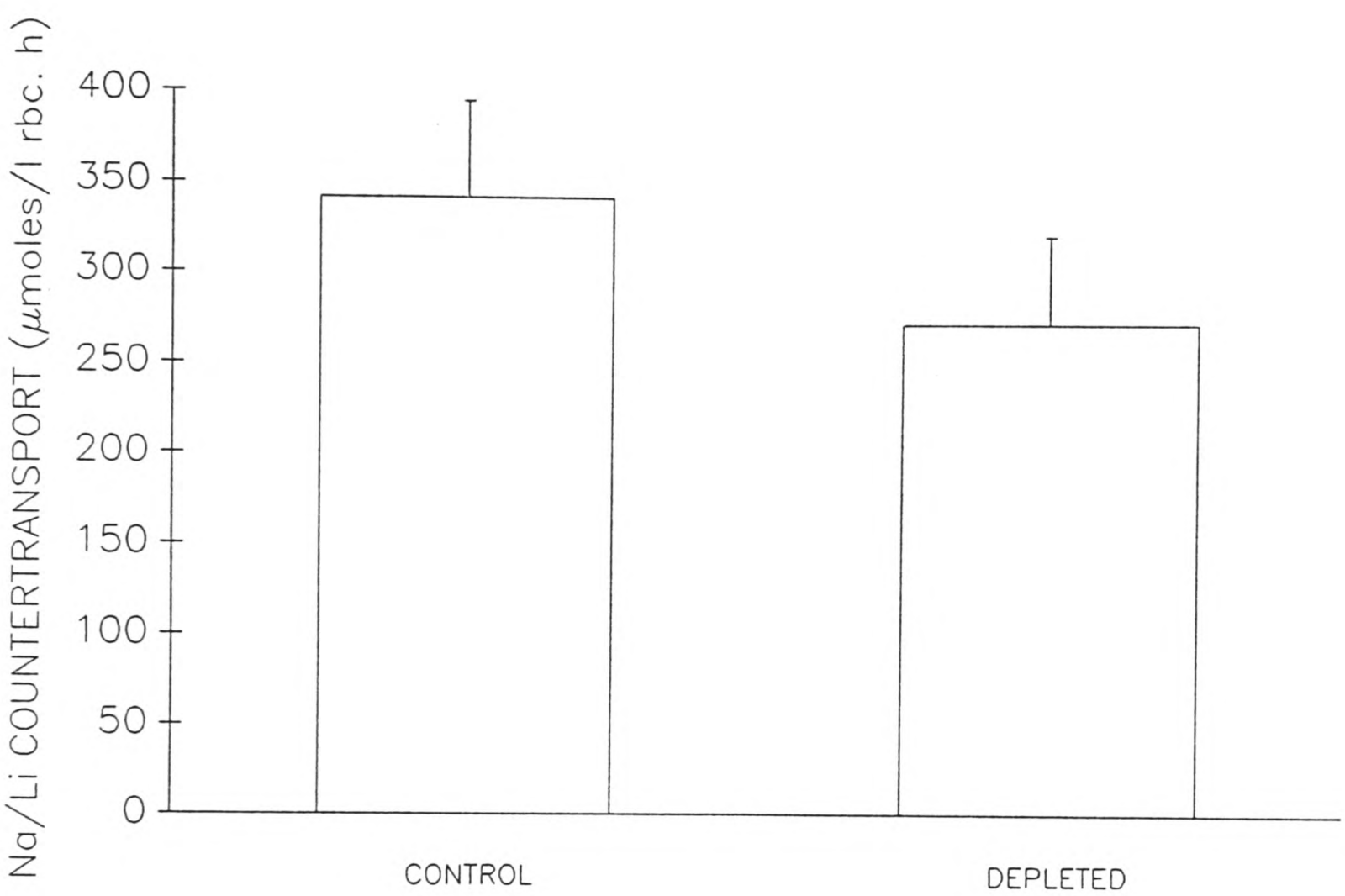


FIGURE 5.4: Na/Li countertransport activity in control and cholesterol-depleted erythrocytes. There is a significant reduction in the transporter activity following cholesterol depletion ($p<0.01$).

5.2.2. SODIUM HYDROGEN ANTIPORTER ACTIVITY IN WHITE BLOOD CELLS

Initial experiments used lymphocytes, but to obtain sufficient cell numbers for these studies cultured EBV-modified lymphocyte (lymphoblasts) was used.

5.2.2.1. LYMPHOCYTE CHOLESTEROL MODULATION

Attempts to use the lymphocyte as a model to study cholesterol modulation of the Na/H antiporter activity were not very successful. Possible reasons for this include varied cell yield obtained, incubations at 0-4°C, and the low concentration of cholesterol in the phosphatidylcholine-cholesterol liposomes employed initially. Sonication with a bath sonicator resulted in inappropriate liposomes to employ with white cells.

5.2.2.2. LYMPHOBLAST CHOLESTEROL MODULATION

Cell membrane cholesterol modulation of lymphoblasts by liposomes is shown in Figure 5.5 and 5.6. Cholesterol levels during the incubation period are given as the percentage change from the mean value at time zero. Control cells did not change their cholesterol content significantly during the incubation period and lymphoblast incubation with liposomes at 37°C proved to be a effective method of modulating cell cholesterol. Cells incubated with PC liposomes had a progressive depletion of cholesterol up to 46% at 6 hours. Cholesterol enrichment with PCCH liposomes was rapid in the first 2 hours, with a subsequent slower rate of increase of cholesterol. Cell cholesterol enrichment was very marked, doubling after 6 hours of incubation.

When compared with control cells, the differences were highly significant ($p<0.001$, n:3).

Initially, incubations were performed at low temperature as suggested by Shinitski & Inbar [1974]. Figure 5.5 shows that the modulation of lymphoblast cholesterol was not successfully achieved at low temperatures, in contrast to what was obtained at 37°C.

5.2.2.3. INTRACELLULAR pH

In the control cells intracellular pH fell progressively with time from the initial value of 7.24 ± 0.02 to a final value at 6 hours of 7.05 ± 0.01. In contrast the cholesterol-depleted cells showed a very modest drop in pH, the final value being 7.17 ± 0.02. Finally the cholesterol-enriched cells showed a marked drop in pH, with time to a final value of 6.98 ± 0.01. The differences between control and the cholesterol-depleted and enriched groups were significant ($p<0.005$, n:8), as shown in Figure 5.7. This experiment under bicarbonate-free conditions shows that cholesterol-depleted cells were able to regulate pHi better than the other groups and it is likely that the Na/H antiporter was the mechanism involved.

5.2.2.4. Na/H ANTIPORTER ACTIVITY

In the control group the activity of the Na/H antiporter (defined as Na dependent H^+ efflux at pH 6.0) was maintained throughout the incubation. A significant ($p<0.001$, n:8) increase in the Na/H antiporter Vmax of cholesterol-depleted cells occurred after 2 hours of incubation , and the increased flux was maintained throughout the experiment. The effect on the antiporter activity was very marked ($p<0.001$, n:8) in the cholesterol-enriched cells, with almost complete inhibition of the Na/H antiporter activity. These changes are illustrated in Figure 5.8.

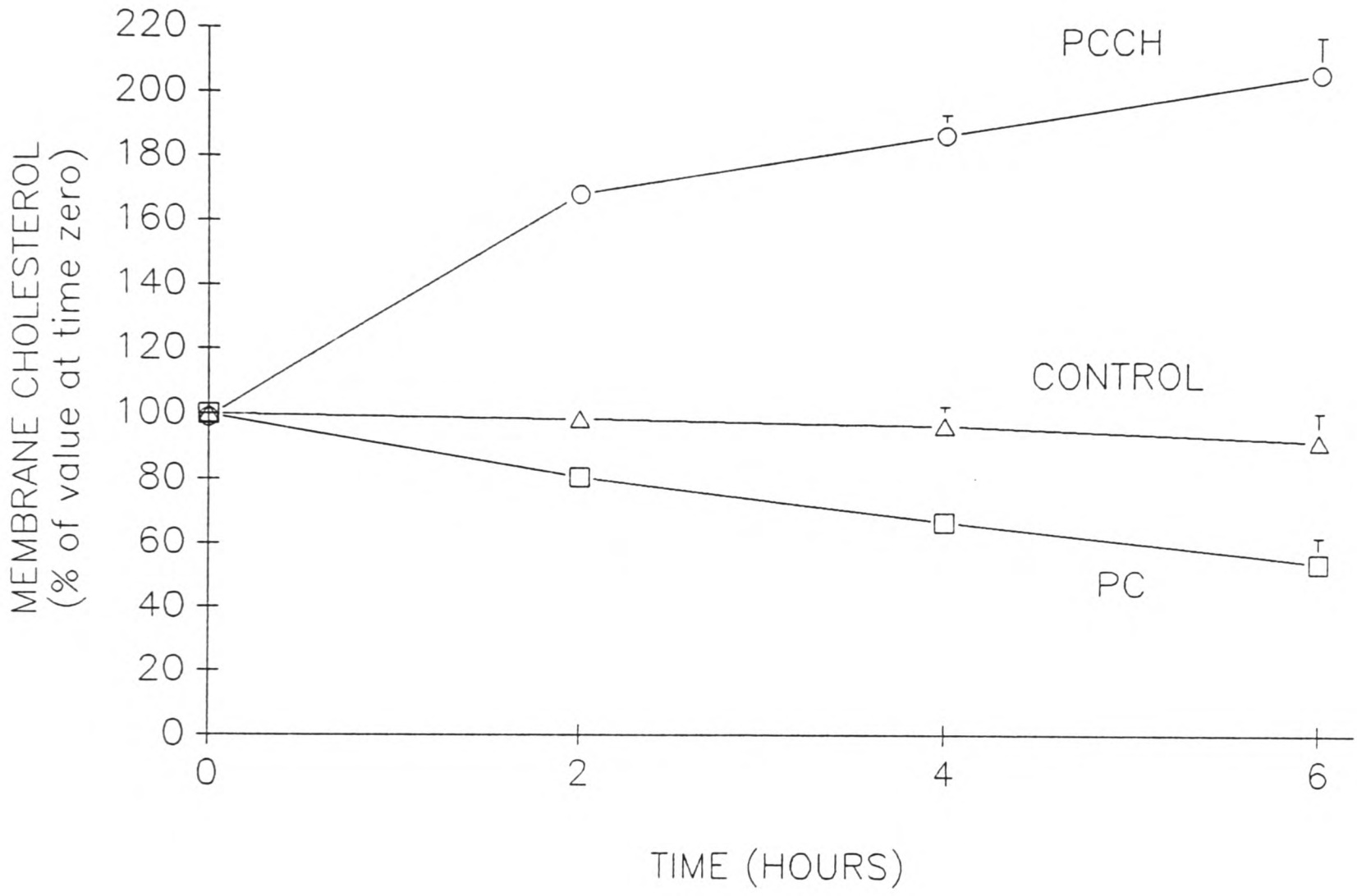


FIGURE 5.5: Lymphoblast cholesterol content is shown as the percentage of change from time zero. Incubation performed at 37°C. PCCH: represents the cells incubated with phosphatidylcholine-cholesterol liposomes, PC: cells incubated with phosphatidylcholine liposomes, Control: Cells incubated without liposomes.

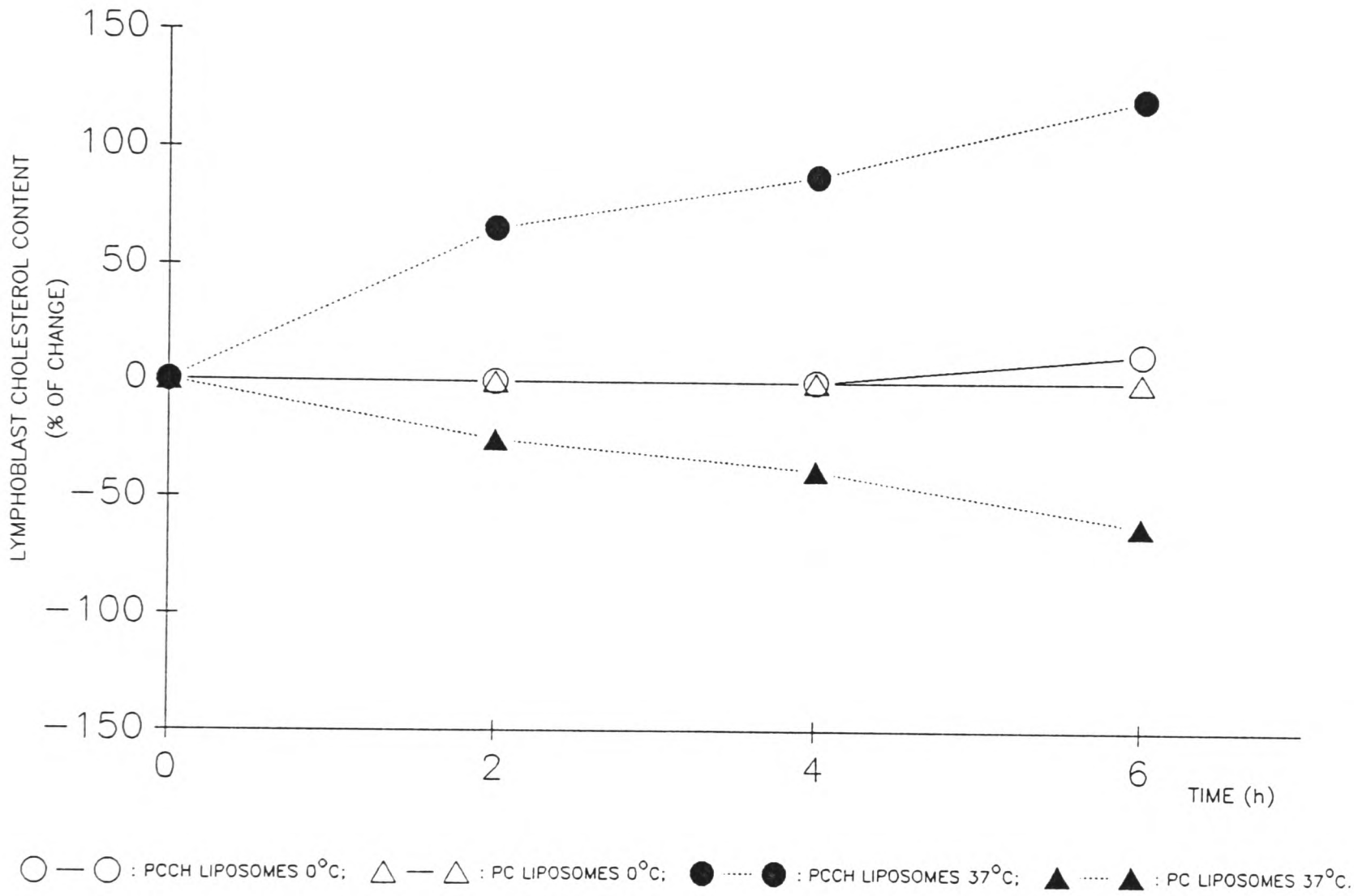


FIGURE 5.6: Lymphoblast cholesterol content is shown as the percentage change from time zero. Incubation performed at 37°C or 0°C. No changes were obtained by incubation at 0°C.

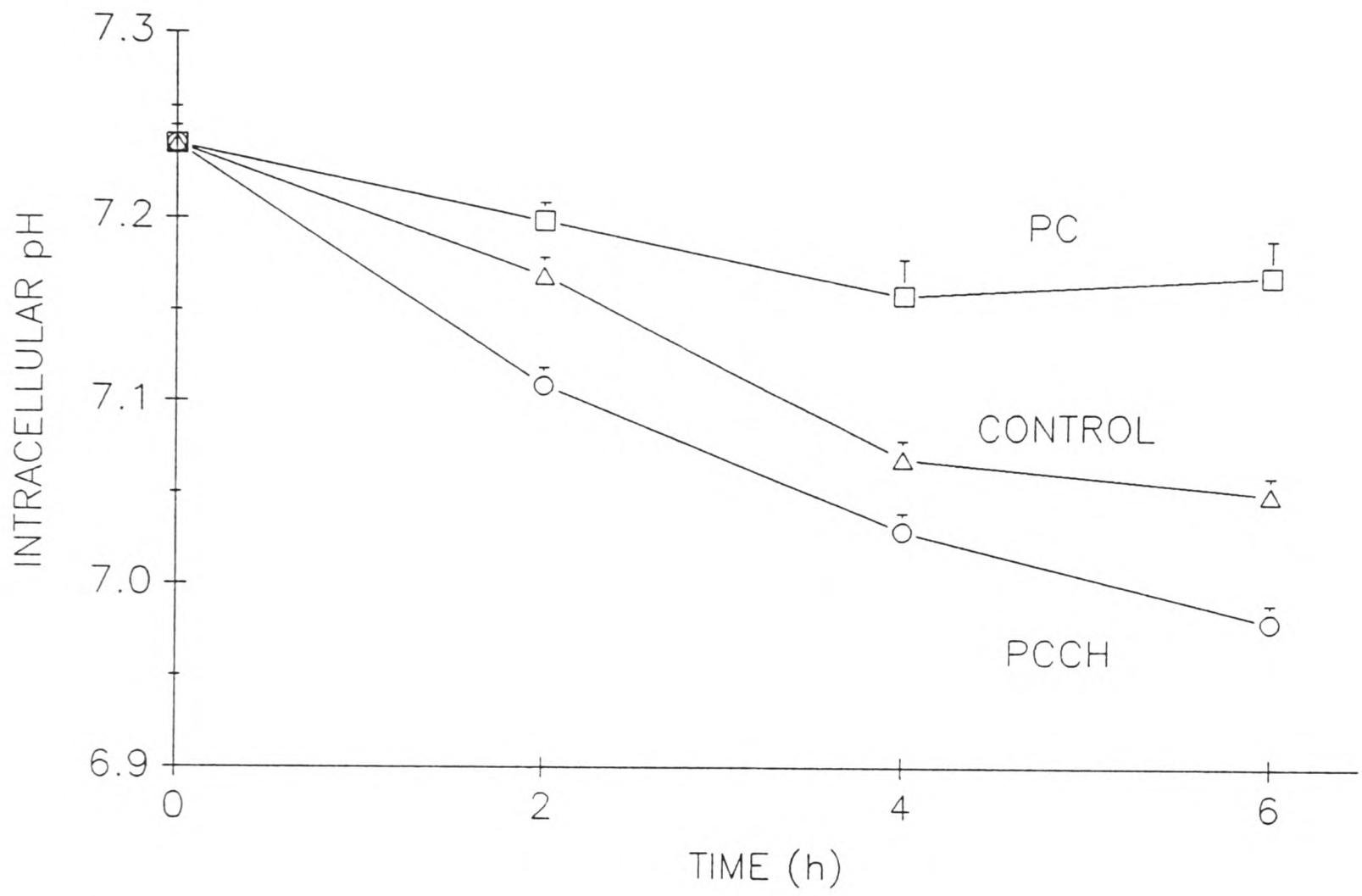


FIGURE 5.7: Lymphoblast mean pHi is plotted against time of incubation with phosphatidylcholine-cholesterol liposomes (PCCH), phosphatidylcholine liposomes (PC), or KCl buffer (Control).

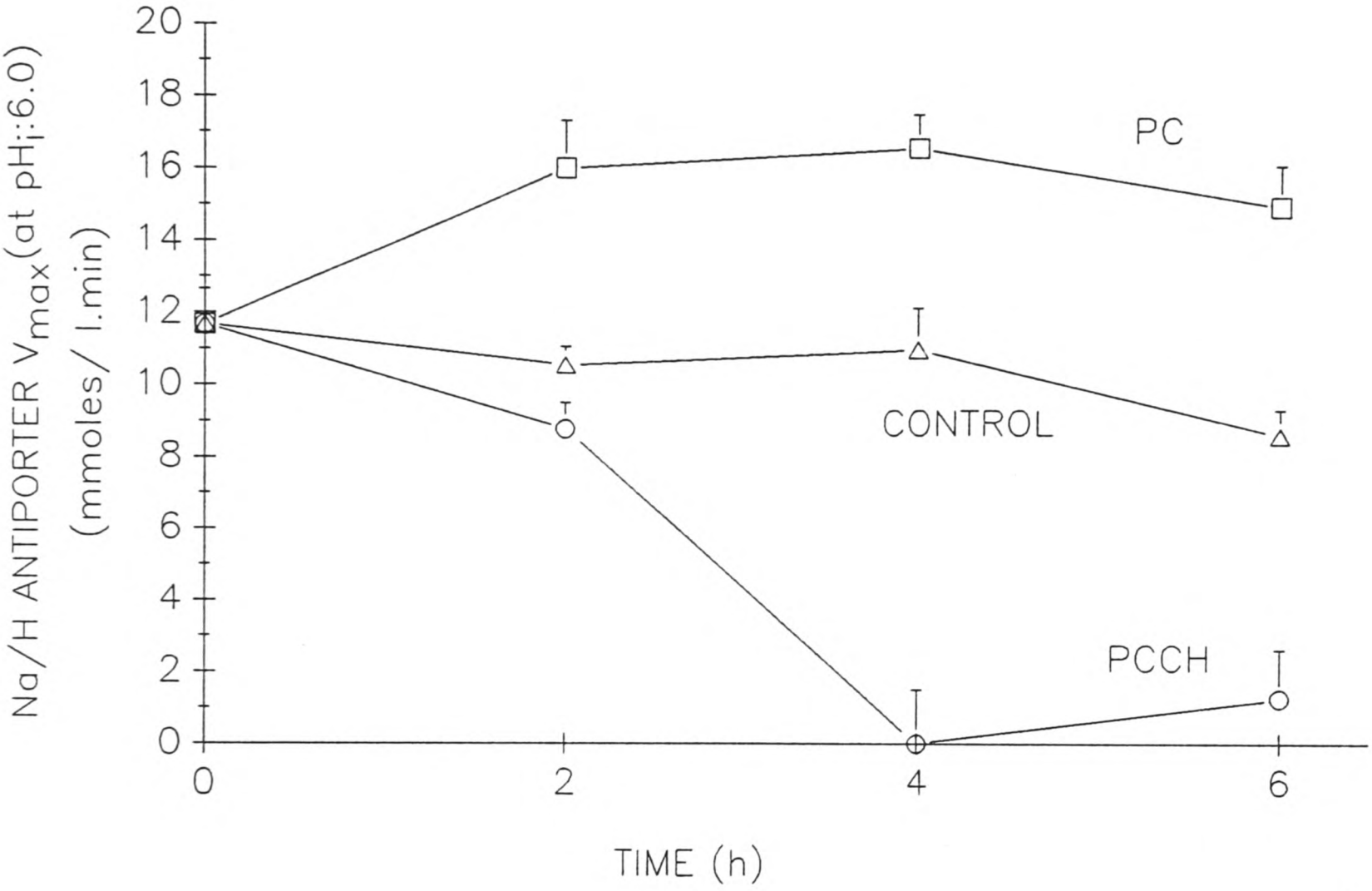


FIGURE 5.8: Mean Na/H antiporter activity is displayed as a function of time. PCCH: represents the cells incubated with phosphatidylcholine-cholesterol liposomes, PC: cells incubated with phosphatidylcholine liposomes, Control: Cells incubated without liposomes.

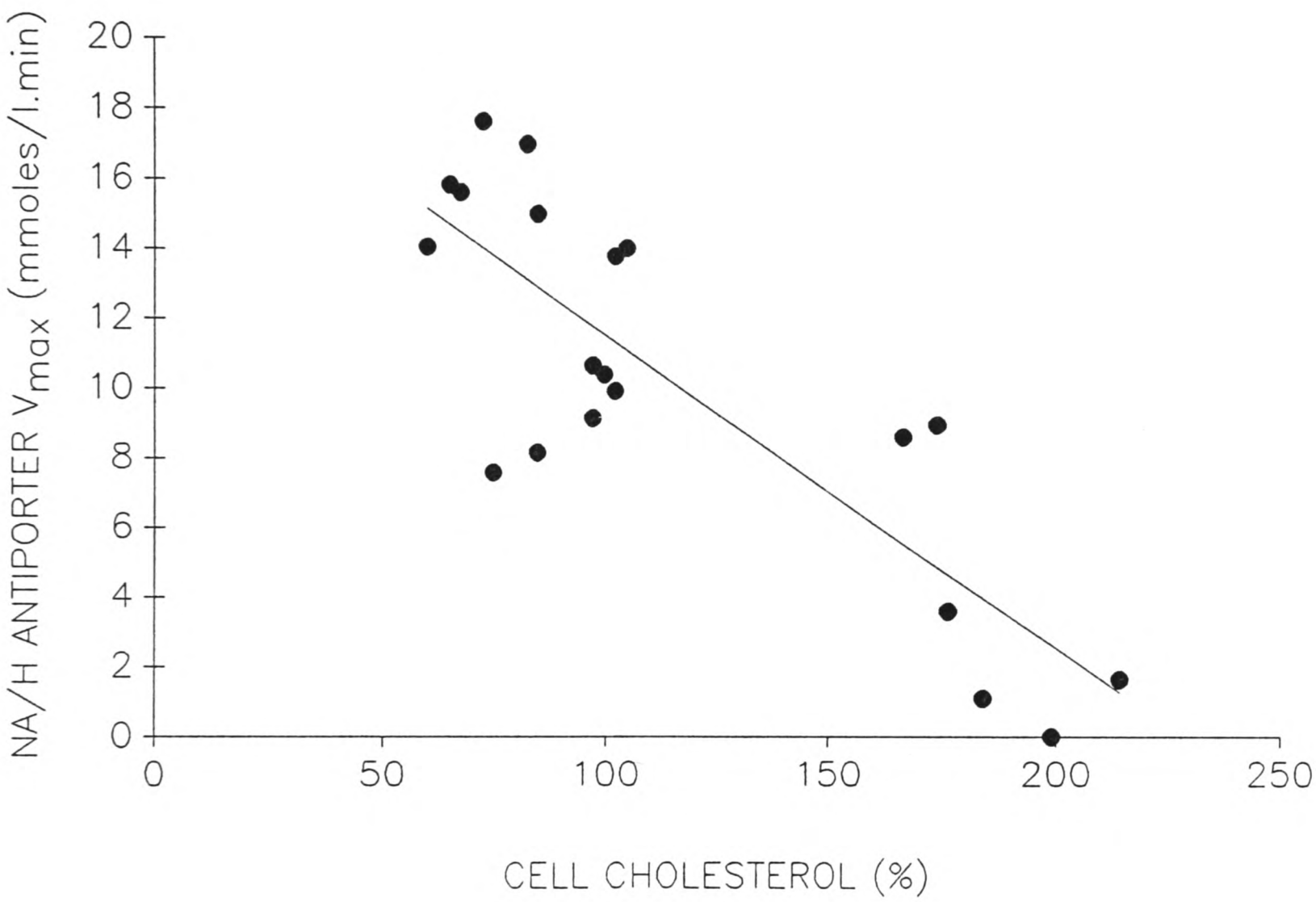


FIGURE 5.9: Relationship between lymphoblast Na/H antiporter activity and cell cholesterol content is shown. Significant correlation is present (r: -0.821, $p<0.001$). Data from two separate lymphoblast suspensions are included. Each point is quadruplicate measurements of cholesterol and Na/H antiporter activity as described in the text.

Figure 5.9. shows data where the Na/H antiporter activity and the corresponding cell cholesterol content were analyzed in the same experiment. A significant correlation between the activity of the Na/H antiporter and cell cholesterol content was found (Spearman correlation coefficient: -0.821, $p<0.001$, n:20). Data from all time points for each of the three groups of cells are plotted together in this figure.

As mentioned above, incubation of lymphoblasts with the liposomes of the same composition at 4°C did not change the cholesterol content even up to 6 hours of incubation. The pHi and Na/H antiporter activity was also not altered significantly. This confirms that changes in Na/H antiporter activity were due to cholesterol modulation.

5.2.3. CHOLINE

The activity of the choline transporter was also examined in red blood cells following modification of membrane cholesterol.

5.2.3.1. ERYTHROCYTE CHOLINE TRANSPORTER

Figure 5.10 illustrates choline transport in the cholesterol-depleted, control and cholesterol-enriched red cells. There is no difference in the rate of choline uptake between these three groups. Depleted cells had a choline uptake V_{max} of 11 (SEM 0.7) µmol/l.h and K_m of 15 µM (SEM 2.8). Control cells had choline uptake V_{max} of 12.2 (SEM 0.6) µmol/l.h and K_m of 10 µM (SEM 1.5). A V_{max} of 12.4 (SEM 0.7) µmol/l.h and K_m of 12.7 µM (SEM 2.3) for

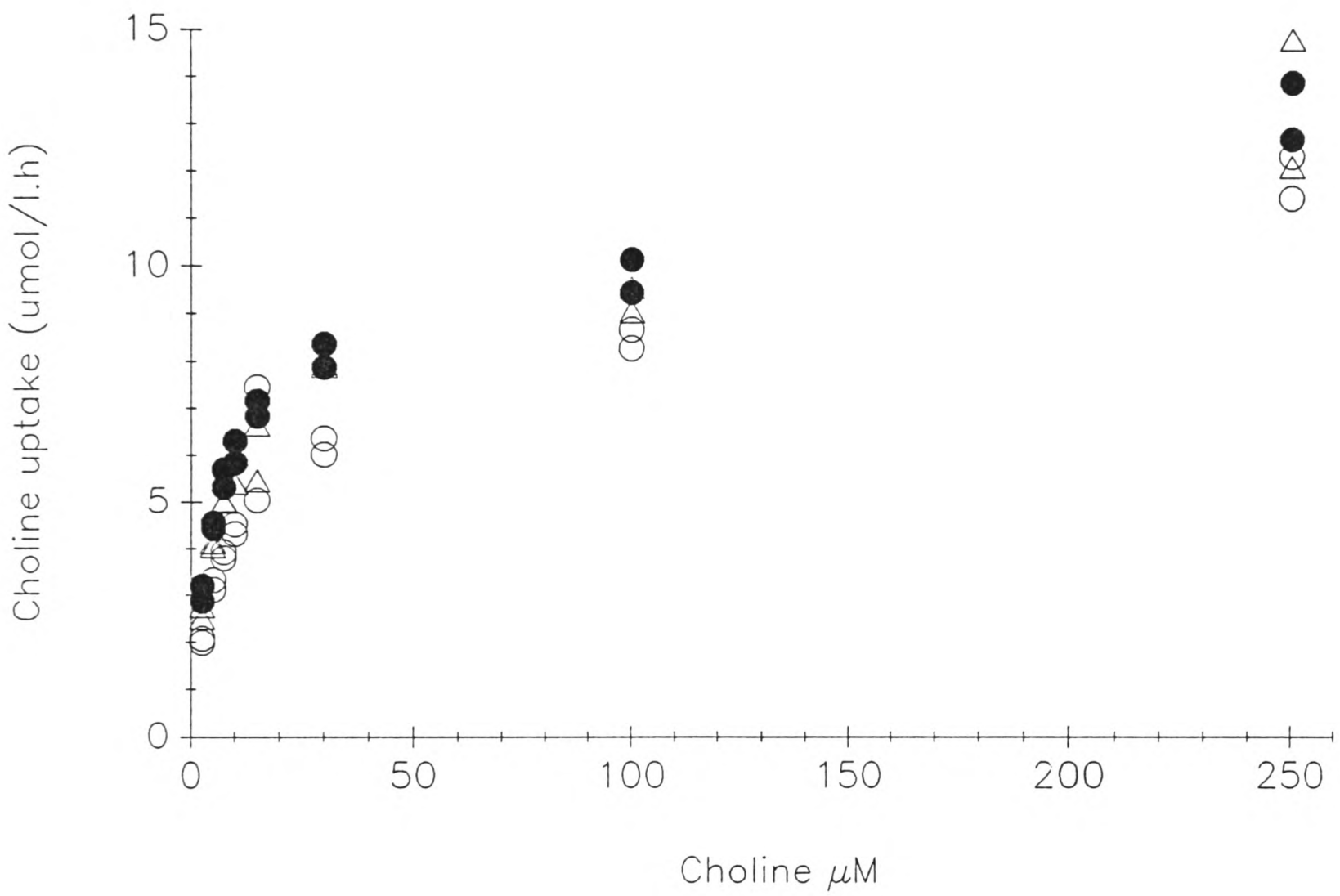


FIGURE 5.10: Choline uptake is shown as a function of extracellular choline concentration for 3 groups of cells. Open circles: cholesterol-depleted cells, Closed circles: control cells, Open triangles: cholesterol-enriched cells. No significant difference is seen.

cholesterol-enriched cells. In these experiments cholesterol depletion of 44 % and enrichment of 40 % were achieved, compared to control cells.

5.3. DISCUSSION

Results reported in this chapter concentrate on the effect of cholesterol modulation on membrane transport activity. Erythrocyte Na/Li countertransport and lymphoblast Na/H antiporter activities are shown to alter after changes in cholesterol content. In contrast, no significant change in the choline uptake in erythrocytes could be detected by either increasing and reducing cholesterol content.

CHOLESTEROL AND MEMBRANE TRANSPORT

Interactions between membrane proteins and their lipid environment are important in determining membrane function. Membrane cholesterol influences the fluidity of the membrane, regulates the activity of ion pumps and may be required for the normal function of essential membrane enzymes [Yeagle 1989]. In general, the mechanism of membrane lipid effects on transport involves the physical and chemical characteristics of the bilayer. Membrane fluidity, phospholipid composition, acyl chain carbon number, degree of saturation/desaturation, bilayer thickness, the nature of the head group and its charge have all been implicated directly in regulation of membrane protein function [Deuticke & Haest 1987, Yeagle 1989]. The concept of an annulus of specific lipid for membrane proteins, suggests a microenvironment in which lipids could influence proteins independently and not necessarily respond to change in bulk lipid properties such as fluidity [Clandinin et al 1991]. Some lipids act as cofactors or allosteric regulators. Interactions with cytoskeletal elements by membrane lipids and proteins also participate

in modulating membrane function. It is clear that membrane lipids, including cholesterol, participate in the regulation of membrane function. The lipid bilayer not only provides a physical boundary to the cell essential for cell survival [Yeagle 1989] but also interacts with the other membrane elements. How alterations in the membrane composition participate in pathological changes is still unclear.

The cholesterol effect on membrane transporter has been studied in several systems [Reviewed in Deuticke & Haest 1987, Yeagle 1989]. ***Activation*** by cholesterol is shown for the erythrocyte monocarboxylate carrier and passive Ca uptake, Na-dependent gamma-aminobutyrate uptake in synaptic plasma membranes and the erythrocyte glucose transporter [Masiak & LeFevre 1974, Neyses et al 1975, Gunze et al 1980, Yuli et al 1981, North & Fleischer 1983]. ***No effect*** of cholesterol on calcium pump activity is probably due to the absence of cholesterol from its lipid annulus [Marsh 1985, Bennett et al 1980]. Other systems that are insensitive to cholesterol are Na and K channels in squid axons and choline uptake in synaptic plasma membranes [Steele et al 1981, North & Fleischer 1983]. ***Inhibition*** of the erythrocyte Na/K/Cl cotransport, reconstituted glucose transporter, l-lactose and l-arabinose transport in human erythrocytes by cholesterol have been reported [Wiley & Cooper 1975, Grunze et al 1980, Jackson & Morgan 1982, Connolly et al 1985].

The effects of cholesterol on the Na/K pump are interesting and seem to be biphasic for cholesterol depletion with both inhibition and activation depending on the magnitude of cholesterol modification [Lucio et al 1991]. A biphasic response is also seen in rat medulla membrane vesicles [Yeagle et al 1988], and in studies on the glucose transporter in human erythrocyte and 3t3 mouse fibroblasts [Yuli et al 1981, Carruthers & Melchior 1988]. The erythrocyte anion transporter (band 3 protein) activity is suppressed by raised cholesterol levels, while being insensitive to cholesterol depletion [Grunze et al 1980, Köhne et al 1981, Jackson &

Morgan 1982, Gregg & Reithmeier 1983, Rooney et al 1984].

The results are therefore complex depending on the type of cell studied and the range over which cholesterol concentration is altered [Deuticke & Haest 1987, Yeagle 1989].

PRESENT RESULTS AND LIPIDS IN DISEASE

The interest in the present results is that the activity of both the Na/Li and the Na/H antiporter have been associated with plasma lipid changes in several clinical conditions including hypertension, diabetes, pregnancy, obesity, exercise and hyperlipidaemia.

Hypertension has been studied extensively from the point of view of membrane transport, and in fact this disease has received most of the attention in recent years. The most consistent abnormalities involve the Na/Li countertransport or the Na/H antiporter [Canessa et al 1980, Duhm & Behr 1986, Clegg et al 1982, Morgan et al 1986, Turner et al 1987, Carr et al 1989, 1989a,b, Woods et al 1982, Trevisan et al 1983, Weder et al 1986, 1991, 1991a, Hunt et al 1986, Beucklelmann D & Erdmann 1984, 1986, Cusi et al 1981, Brugara et al 1983, Smith et al 1984, Cooper et al 1983, Weder et al 1991]. Postnov has even suggested that hypertension could be referred as a membranopathy [1990, Postnov & Orlov 1985].

Studies in a large population of hypertensive subjects suggested that Na/Li transporter activity may in part be explained by plasma lipid levels. Although there are some discrepancies, cholesterol and triglyceride composition seem to be correlated most frequently with transport activity. In pregnancy and after exercise Na/Li activity is altered, an effect probably secondary to changes in lipid composition in the blood. Certainly, membrane lipid alterations are not a unique factor by which to explain all the changes associated with hypertension, but there are strong suggestions that they may contribute to its pathogenesis. Other transporters have also been reported as abnormal in hypertension including the Na-KCl cotransporter [Garay et al 1982], Na-K

ATPase [Hilton 1986] and calcium pump [Postnov 1990].

The results presented here show a definite alteration in transport function with cholesterol modulation. However the importance and role of these changes is more complex to establish. The Na/Li countertransport and Na/H antiporter have been suggested to be similar transporters [Mahnesmith & Aronson 1985]. However they are unlikely to be the same considering the different affinity for inhibitors. Reduced levels of cholesterol lower the activity of the Na/Li countertransport in erythrocytes, but activate Na/H antiporter activity in lymphoblasts.

Na/Li COUNTERTRANSPORT AND MEMBRANE LIPID ALTERATIONS.

If the Na/Li countertransport participates in the pathology of disease and cholesterol modulation interferes with its activity, there may be potential therapeutic implications. At present genetic determinants cannot be modified, but the environmental factors such as blood lipids can.

This study shows a reduction in the activity of the transporter after depleting membrane cholesterol. The mechanism of this reduction is not clear, but previous work using cholesteryl hemisuccinate failed to demonstrate any significant change in Na/Li countertransport [Levy et al 1986]. This suggests that mechanisms other than membrane fluidity may be playing a role and that the effects are specific for each sterol species. More recently [Engelmann & Duhm 1991] Na/Li countertransport activity was examined after cholesterol modulation and it was suggested that cholesterol enrichment reduces Na/Li exchange rates with depletion having the opposite effect. The results seem to be significant statistically, but the magnitude of the changes are small. Our results are at variance with their findings. It is possible that methodological differences could explain the discrepancy, including the different lipid species employed. Levy et al used cholesteryl hemisuccinate and not cholesterol, while Engelmann & Duhm used liposomes

with dipamitoyl phosphatidylcholine.

As mentioned earlier the activity of the Na/Li countertransport has been correlated with plasma lipid composition. If plasma cholesterol directly influences membrane cholesterol levels, it could be suggested that lower plasma cholesterol would induce reduced Na/Li countertransport activity. This is discussed further in the next chapter.

Na/H ANTIPORTER AND MEMBRANE LIPID ALTERATIONS.

Modulation of Na/H antiporter activity in cultured cells by several growth factors, hormones and other factors has been described [Grinstein & Rothstein 1986, Sardet et al 1990]. The antiporter activity is abnormal in some pathological states including hypertension and insulin dependent diabetes mellitus (IDDM) [Ng & Dudley 1989, Ng et al 1990a, 1990b].

In this study we have used cultured lymphoblasts as a model system to study Na/H exchange. Liposome treatment was an effective way to alter cell cholesterol. Cholesterol-depleted cells had a smaller change in pHi during incubation for up to 6 hours, an interesting finding considering that the cells were incubated in KCl buffer in the absence of bicarbonate, conditions where neither the Na/H antiporter nor any anion/HCO^-_3 exchangers are operating. As a result, intracellular protons produced by metabolism would accumulate with time, accounting for the gradual intracellular acidification of the control cells in isotonic KCl buffer. The subsequent measurement of pHi in Na^+-containing buffers would reflect an alteration of pHi in the alkaline direction by activation of Na/H exchange. Thus, in cells depleted of cholesterol (PC liposomes), the progressive fall in pHi with time was less than that of control cells, while in those enriched with cholesterol (PCCH liposomes) pHi fell more rapidly. This means that PC-treated cells (when they were resuspended in Na^+- containing buffers) were able to dispose of the intracellular metabolic acid produced better than control cells, and the control cells better than

PCCH-treated cells. As bicarbonate was absent from the NaCl buffer, it is likely that Na/H exchange was enhanced in PC-treated cells and depressed in PCCH treated cells.

Direct confirmation of these deductions comes by measuring Na/H exchange, clamping pHi to a level near the maximal transport capacity of the antiporter. Higher cholesterol levels reduced the Vmax of the antiporter while lower cholesterol levels enhanced its activity. The cholesterol effect on transporters can be due to membrane fluidity changes, conformational modifications, direct lipid-protein interactions or other interactions with membrane components [Deuticke & Haest 1987, Yeagle 1989, Spector & Yorek 1985]. Previous studies from Dudeja and coworkers using rat colonic brush-border membrane vesicles suggest a correlation between the activity of the Na/H antiporter and membrane fluidity, and the fluidity changes could be secondary to changes in cholesterol/phospholipid ratio [Brasitus et al 1986, Dudeja et al 1986, 1987a, 1987b]. Our studies in intact cells are in agreement with these findings on membrane vesicles, although we have used liposomes to modulate the cholesterol content directly.

One possible caveat to be considered in the present case is that the changes result directly from an alteration in phosphatidylcholine produced by incubation with liposomes. This is unlikely since both the cholesterol-depleted and the enriched lymphoblasts were equally exposed to phosphatidylcholine, so the effect is far more likely to be due to cholesterol modulation.

In insulin-dependent diabetes mellitus (IDDM) the cholesterol:phospholipid ratio in erythrocytes has been reported to be decreased [Baldini et al 1989], although this finding is controversial [Bryszewska et al 1986]. The Vmax of the antiporter in leucocytes and fibroblasts is enhanced in IDDM with albuminuria,and this is considered to be a familial effect [Ng et al 1990 a, 1990b]. Our results raise the possibility that altered membrane lipid composition may play a role in the abnormal membrane transport and cell physiology of cells from IDDM patients.

Shinitzky and Inbar have demonstrated in rat lymphocytes that membrane

cholesterol levels are higher than in lymphoma cells and have suggested that the malignancy of lymphoma cells is inhibited by increasing the levels of cholesterol in the plasma membrane, with a possible relationship between control of growth and membrane fluidity [1974]. This hypothesis is consistent with our findings if the pivotal role of Na/H antiporter activity in cell growth and proliferation is considered [Madshus 1988].

In conclusion, this study has demonstrated that the activity of the lymphoblast Na/H antiporter can be dramatically modulated by membrane cholesterol content and supports the idea that membrane cholesterol levels may play a role in some of the alterations in cellular function in disease.

Since the present study was performed, further evidence to support the importance of membrane lipids in the activity of the Na/H antiporter activity has been published [Ng & Davies 1991, 1992]. HMG CoA reductase inhibitors (simvastatin and 25-hydroxycholesterol) reduced the activity of the antiporter, and the effects were prevented by addition of mevalonate thus bypassing the inhibition by simvastatin [Ng & Davies 1991]. Pravastatin produced similar effects [Ng & Davies 1992]. It was hypothesized that the abnormalities could also result from anomalous control of the mevalonate pathway.

CHOLINE TRANSPORT AND MEMBRANE LIPID ALTERATIONS.

Choline uptake in erythrocytes was unaffected by modulation of cell cholesterol. It has been demonstrated previously that cholesterol has no effect on choline uptake in synaptic plasma membranes [North & Fleischer 1983]. However the choline transporter in this preparation is different from the transporter described in red blood cells. The lack of modulation of choline transport by cholesterol argues against a possible effect of cholesterol or membrane fluidity changes, causing the abnormally elevated choline uptake seen in patients with chronic renal failure

(chapter 2). However, it does not exclude the possible effect of other lipid membrane components or plasma lipids affecting the transporter activity - this has still to be tested.

5.5. CONCLUSION

The main conclusion from this chapter is that membrane transport can be modulated by membrane cholesterol. The sensitivity of transporters to cholesterol is specific for each transport system. The possibility of affecting membrane function by modulating its lipid content, or its metabolic pathways, is an exciting field of investigation considering the potential therapeutic implications.

Lipid-lipid, lipid-protein, protein-protein and lipid(protein)-cytoskeleton interactions are complex. This study has looked at only one component of the lipid bilayer (cholesterol) and further studies are necessary to understand the full implications of these findings.

The next chapter will describe the study of Na/Li countertransport activity following in vivo modification of plasma cholesterol.

CHAPTER 6

SODIUM LITHIUM COUNTERTRANSPORT IN ARTERIOSCLEROSIS AND FOLLOWING TREATMENT WITH SIMVASTATIN

The preceding chapter demonstrated that changes in membrane transport systems can occur secondary to modification of the membrane cholesterol content. Experiments performed under *in vitro* and relatively "controlled" conditions provide much of the information applied to understanding the mechanisms of normal physiology and modifications in disease. Despite this, it is sometimes difficult to extrapolate from the *in vitro* to the *in vivo* situation.

The present longitudinal study was designed to evaluate the activity of the sodium-lithium countertransport (Na/Li countertransport) system in erythrocytes of patients with atherosclerotic vascular disease before and after treatment with simvastatin, an inhibitor of the HMG-CoA reductase, which results in reduced synthesis of cholesterol with lower levels of plasma cholesterol [Aronson & Ng 1990, Maher & Thompson 1990, Alberts 1990, Slater & MacDonald 1988, Walker 1988]. The population of patients studied was recruited when they attended the clinic of the Oxford Cholesterol Study (OCS) [Keech AC 1990]. This study, of the effect of simvastatin treatment on plasma lipid levels with a long term follow-up of patients with vascular disease, offered us the opportunity to examine effects on the Na/Li countertransport after alteration of the lipid profile of the patients.

The idea behind the project is illustrated in Figure 6.1. Several factors have been described as being of risk in the development of atherosclerotic vascular disease, including hypertension and hyperlipidaemia [Samuelson 1988, Hulley 1988, Mann & Ball 1988, Martin et

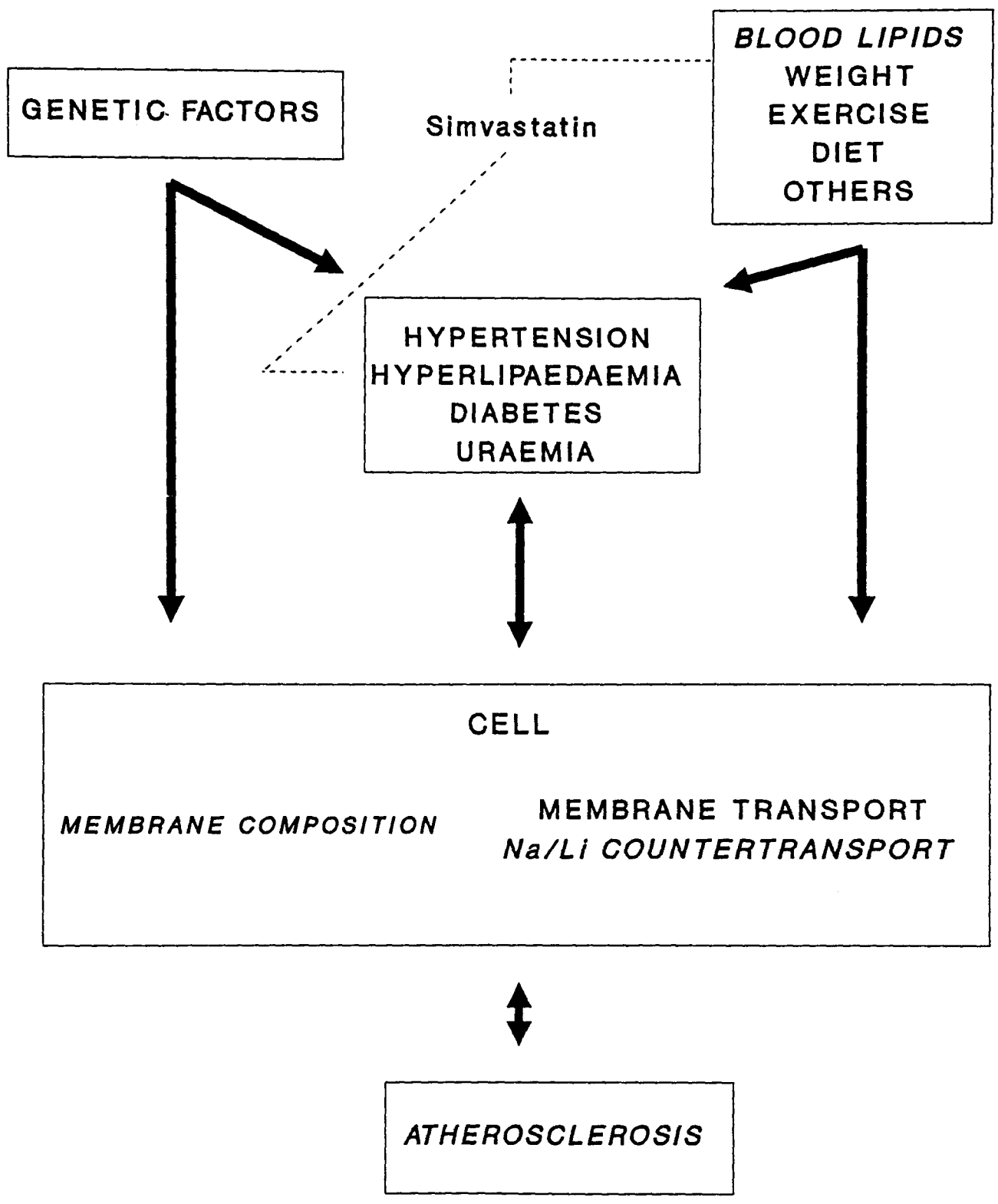


FIGURE 6.1: Schematic representation of the hypothesis behind the study in the present chapter. Genetic and environmental factors interact to determine cell composition, cell function and risk or manifestations of disease. Simvastatin is an effective treatment to reduce plasma cholesterol. Reducing plasma cholesterol in patients reduces the risk of atherosclerotic vascular complications. The possible interaction with the cell membrane was examined by studying membrane cholesterol composition and Na/Li countertransport activity.

al 1986, Garber et al 1989]. Diseases such as diabetes and uraemia are also associated with a higher risk of vascular alterations [Steiner 1988, Linder et al 1974, Velez & Henrich 1984, Betteridge 1990]. A common element of these atherogenic pathologies is alterations in cell membrane properties, as demonstrated by the abnormal activity of the Na/Li countertransport [Canessa et al 1980, Hunt et al 1986, Krolewski et al 1989, Trevisan et al 1986]. This system is not only altered in pathological states, but also can be modified in physiological conditions such as pregnancy perhaps due to altered plasma cholesterol levels [Worley et al 1982, Macphail et al 1990]. Determinants of the transport rate via the Na/Li system include genetic factors, diet, gender, age, exercise, weight and blood lipids [Dorus et al 1980, Williams et al 1983, Behr et al 1985, Beuckelmann & Erdmann 1984, Duhm & Behr 1986, Smith et al 1982, Worley et al 1982, Cooper et al 1983, Duhm et al 1982, Turner et al 1985, Clegg et al 1982, Adragna et al 1985, Ehrlich et al 1983, Turner & Michels 1991, Nosadi et al 1991, Hunt et al 1991, Hassttedt et al 1988]. It is possible that the mechanism of action of such determinants involves alteration in cell composition and consequently function. The inheritance of the transporters may involve gene/s that control the membrane exchange protein per se or that determine other factors influencing Na/Li countertransport. They also could be altered by exogenous factors [Hunt et al 1991, Duhm 1989]. The objective of the present study is to evaluate the activity of the Na/Li countertransporter and determine membrane cholesterol levels in patients with atherosclerotic vascular disease before and after significant reduction in their plasma lipids with simvastatin treatment.

Treatment with simvastatin is expected to decrease plasma cholesterol by 20-45 % [Grundy 1988]. Cholesterol has been reported to exchange very quickly between the plasma pool and the erythrocyte cell membrane [Dawidowicz 1987, see ch 5], so a modification of the membrane cholesterol content would be expected as blood cholesterol decreases. The activity of the transporter was tested in the patients before, and at 2 and 5 months after treatment with

simvastatin. Concomitantly plasma and cell membrane cholesterol were evaluated in the same patients. We hypothesized that treatment with simvastatin would decrease the membrane cholesterol levels with consequent reduction in the Na/li countertransport.

6.1. MATERIAL AND METHODS

6.1.1. METHODS

The methodology employed for the study of the Na/Li countertransport in erythrocytes from the patients of the OCS, and for membrane cholesterol extraction and measurements are described in the previous chapter (Chapter 5).

6.1.2. PATIENTS

Patients for this study were selected randomly from a larger number being recruited to the Oxford Cholesterol Study (OCS) [Keech 1990]. This is a double-blind, placebo-controlled study of simvastatin effects in patients with a clinical history of atherosclerosis, excluding patients with a total cholesterol of less than 3.5 mmoles/l, and being conducted in preparation for a large mortality trial. The patients were allocated to receive placebo, 20 mg or 40 mg simvastatin in one oral dose per day. Advice on moderating their intake of cholesterol and saturated fats was given. Blood samples were taken during their visits to the OCS clinic prior to initiation of treatment, and after 2 and 5 months treatment. Serum was separated for measurements of total cholesterol, and the erythrocytes used for membrane cholesterol measurements and Na/Li countertransport assays.

6.2. RESULTS

The results are express alterations in cholesterol content as a percentage of change from time zero, that is before any treatment was started. Three groups are present for each condition: The first group is of patients that received placebo (n:10), the second group of patients who received simvastatin at 20 mg daily (n:18), and the third group received simvastatin at 40 mg per day (n:14).

All the results are presented in figure 6.2. The measurements were not different for any of the parameters between 2 and 5 months, and they will be illustrated together.

6.2.1. PLASMA CHOLESTEROL

The effect of treatment with simvastatin on the plasma composition is shown in figure 6.2. No significant change is seen in the plasma cholesterol of the placebo group at the 2 and 5 months follow up. Blood cholesterol is lower in the two groups receiving simvastatin after 2 months and remained lower at the last follow-up. The mean total blood cholesterol has decreased by about 30 %.

6.2.2. RED BLOOD CELL CHOLESTEROL

Mean erythrocyte cholesterol in the sample immediately before treatment was started was 3.2 μmoles/ml cells. It is interesting to notice that there was no significant change in the red blood cell cholesterol content after 2 and 5 months treatment with simvastatin or in the placebo group, despite the fact that there was a significant modification in the total plasma cholesterol levels.

6.2.3. Na/Li COUNTERTRANSPORT

As seen for cell cholesterol, no significant change is present after 2 and 5 months of treatment for all the groups. The mean Na/Li countertransport activity in the initial samples was 0.34 mmoles/l.h.

6.3. DISCUSSION

In the previous chapter it been shown that on depleting erythrocyte membrane cholesterol the activity of the Na/Li countertransport system in erythrocyte is reduced. This is interesting because blood lipid abnormalities and hypertension associated with atherosclerotic vascular disease are associated with high rates of Na/Li countertransport (discussed in chapters 1 and 5). There is an obvious question which arises: are those high transport rates because of high cholesterol content in the cell membrane in those patients? Michalak et al [1988] investigated patients with atherosclerosis and they show a high red cell cholesterol content. Thus, is the high red cell cholesterol and red cell Na/Li countertransport activity related? Could this be a pathophysiological mechanism by which membrane cholesterol is modifying the membrane transport system? To investigate this further patients with atherosclerosis the present study on patients undergoing blood cholesterol reduction by simvastatin has been performed.

A significant reduction of 30 % in plasma cholesterol was obtained with the treatment, but no change in erythrocyte membrane cholesterol or in the activity of Na/Li countertransport occurred. The fact that cholesterol exchanges spontaneously between red cells and plasma has been known for a long period [Hagerman & Gould 1951]. This is a fast exchange, reported to be in the range of minutes or hours [Hagerman & Gould 1951, Eckles et al 1955].

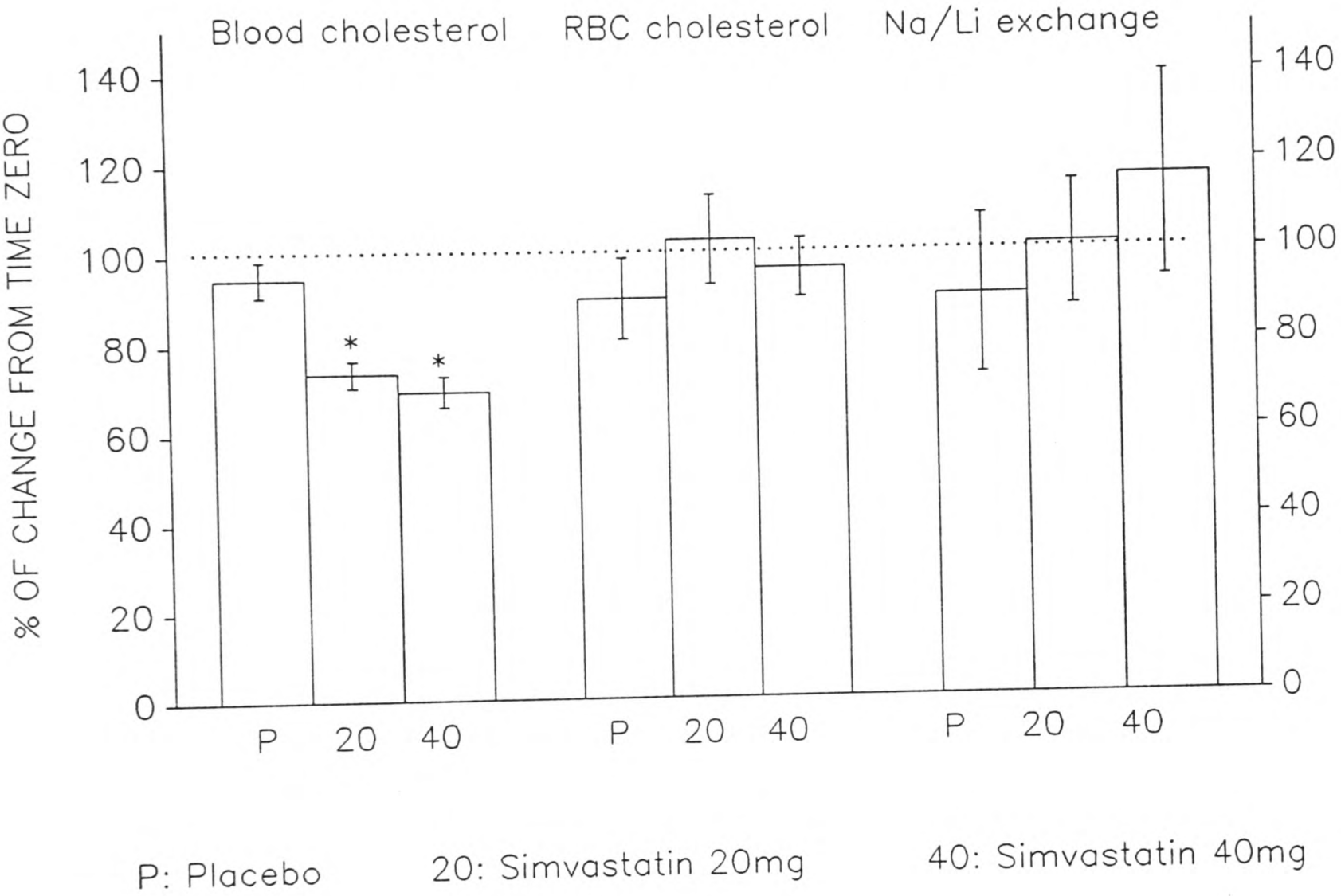


FIGURE 6.2: Effects of treatment with simvastatin on plasma cholesterol, erythrocyte cholesterol nd Na/Li countertransport activity. Percentage of change from time zero is illustrated for all the parameters. Measurements after 2 and 5 months of treatment were not different and the results are plotted together. P corresponds to the placebo-treated. 20 and 40 refers to the simvastatin-treated groups 20 and 40 mg daily respectively (*: $p<0.001$).

Exchange of cholesterol was also demonstrated to occur using phosphatidylcholine-cholesterol dispersions or vesicles. Net movement of cholesterol can occur depending on the properties of the liposomes, and in particular the fractional and physical properties of the dispersion cholesterol [Bruckdorfer et al 1968a, 1968b, 1969, Lange & D'Alessandro 1977, Haran & Shporer 1977, Backer & Dawidowicz 1979]. The mechanism of cholesterol transfer is not completely clear, but there are indications that aqueous diffusion is involved for cholesterol exchange [Dawidowicz 1987]. Whatever the mechanism, equilibration of plasma cholesterol and erythrocyte cholesterol is fast [Dawidowicz 1987]. It is possible that the chemical potential of cholesterol in the blood (or 'free aqueous cholesterol') does not change when the total cholesterol is reduced by about 30% by simvastatin. If the chemical potential did fall, then a reduced erythrocyte membrane cholesterol content would be predicted.

A possible caveat to be considered in the simvastatin study relates to the variation between the groups. The patients for the study were randomly selected from a larger study (OCS). This created a few unexpected difficulties: the distribution of the patients was different in the groups, since we had limited access to the patients' data. There is a wide scatter of interindividual variation in the activity of the Na/Li activity, in agreement with previous investigations. By using each individual as his own control this fact was minimized. Na/Li countertransport activity was not statistically different in any group, but large standard deviations are present. It may be necessary to study much larger populations to confirm the present findings. Despite that, no correlation was present between the changes in the plasma and membrane cholesterol levels and the changes in the activity of the Na/Li countertransporter.

The mechanism by which plasma lipid can be related to membrane transport processes is not known, but it has been thought that they affect transport via altered membrane lipids [Hajem et al 1990, Weder et al 1991]. In a recent study by Carr et al [1990] lipid lowering

therapy (not specified) resulted in changes in the Na/Li countertransport correlated with plasma triglycerides, and not with cholesterol. Dietary modification of fatty acid composition of plasma lipids, also failed to demonstrate modulation of the Na/Li countertransporter activity [Sacks et al 1987]. Studies by Weder's group of the effects of treatment with another HMG-CoA reductase inhibitor, lovastatin, on membrane transport and plasma lipids have also failed to show any alteration in the activity of erythrocyte Na/Li countertransport despite changes in plasma cholesterol [Weder et al 1991]. The same study has indirect evidence that lovastatin treatment reduced the activity of platelet Na/H antiporter. In fact simvastatin has been suggested to reduce only plasma cholesterol, not cholesterol in the cell membrane [Mazzanti et al 1990], and although in *in vitro* studies on lymphoblasts simvastatin reduced the activity of the Na/H antiporter [Ng & Davies 1991] the mechanism of this activation was postulated not to be via cell cholesterol depletion. Lymphoblasts treated with simvastatin actually had a *higher* cholesterol content [Ng & Davies 1991].

The present study cannot confirm the participation of membrane lipids in the *in vivo* regulation of Na/Li countertransport and of its participation in vascular disease. Whether changes in cell cholesterol levels and Na/Li countertransport play a role in the aetiology of vascular disease remains unknown. The hypothesis could not be tested due to the lack of change in cell cholesterol with simvastatin treatment.

CHAPTER 7

GENERAL DISCUSSION

This thesis presents studies of selected membrane transporters in certain disease states. Membrane transport systems, such as the erythrocyte choline carrier, the erythrocyte lactate transporter, the leucocyte Na/H antiporter and the erythrocyte Na/Li countertransport system were studied in relation to chronic renal failure (CRF), diabetic nephropathy and atherosclerosis. The diseases chosen have heterogenous causes and manifestations, affecting several body systems and organs, and their pathophysiological basis is not fully understood. To look at aspects of altered cell function with disease, blood cells were used as a convenient and accessible model system to study membrane transport.

What was the present aim? Most biological processes are genetically determined. Environment will interact with genetic factors and modify the expression of predetermined genes. Disease can result from perturbation of either of these infiuences or can affect their normal interaction. By investigating cell membrane properties it was hoped to be able to identify specific defects that may be present in disease, allowing a better understanding of the pathological basis of disease. An abnormality in transport may serve as a marker for disease or yield a clue to symptoms. Environment can cause abnormal membrane function by itself or by interacting with genetic factors in susceptible subjects. Such alterations have potential implications for diagnosis, research and therapeutics. This thesis attempts to present a general view of several of those influences using certain specific models.

MEMBRANE CHOLESTEROL. WHY SHOULD IT BE STUDIED?

Membrane composition is one of the possible determinants of cellular transport and I have concentrated on one of the major cell membrane components, namely cholesterol. In the Introduction to this thesis, the literature on the effects of membrane cholesterol in regulating membrane transport function was reviewed [eg Yeagle 1987, 1985, 1989, Stubbs & Smith 1984, Spector & Yorek 1985]. There is a continuous transfer of cholesterol between plasma and cell membranes [Lange et al 1980, 1981, Norum et al 1983, Miller 1984, Dawidowicz 1987], and plasma cholesterol levels have been implicated as an etiological factor for complications of the clinical conditions selected for this thesis.

Patients with uraemia have been shown to present with accelerated atherosclerosis [Lindner et al 1974]. It has also been suggested that the altered lipid metabolism in uraemia which results in elevated plasma triglyceride and cholesterol concentrations, and an abnormal lipoprotein profile (high levels IDL and VLDL, decreased levels of HDL) would have a possible atherogenic role in this syndrome [Attman & Alaupovic 1991]. Plasma cholesterol levels in uraemia have also been reported to be lower than controls due to reduced HDL-cholesterol [Hsia et al 1985, Dieplinger et al 1986]. In general HDL-cholesterol is involved in removing cholesterol molecules from the tissues, while LDL-cholesterol has the opposite role. A reduced plasma HDL concentration is a risk factor for cardiovascular disease. In fibroblasts cultured in uraemic plasma a net transport of free cholesterol into the cell was described [Dieplinger et al 1986, Hsia et al 1985]. This suggests that increased flux of free cholesterol from plasma to cell membrane could increase the risk of atherosclerotic vascular disease.

Another atherogenic disease in which plasma lipid levels and metabolism are altered is diabetes [Gibbons 1986, Betteridge 1989]. Arterial disease is a major problem facing

these patients and is responsible for considerable morbidity and mortality [Drury et al 1989]. There is evidence that plasma cholesterol levels correlate with the appearance of atherosclerosis in insulin-dependent diabetes mellitus [Merrin et al 1992]. Diabetic patients free of complications are likely to have fewer disturbances in blood rheology [Tooke 1989]. Further alterations in membrane cholesterol and lipid composition in blood cells have been described in diabetes [Vermes et al 1987, Juhan-Vague et al 1984, Baldini et al 1989, Bryszewska et al 1986].

The association of plasma cholesterol levels with atherosclerosis has been demonstrated in several studies [Martin et al 1986, Hargreaves et al 1991, Garber et al 1989, Feinlieb et al 1979], while erythrocyte cholesterol concentration has been shown to be elevated in this disease [Michalak et al 1988]. Despite such extensive evidence for the pathological role played by the plasma lipids in these diseases, not much attention has been paid to possible cellular abnormalities. The role of cell cholesterol in modulating membrane transport has therefore been analyzed in detail.

URAEMIA AND THE CELL MEMBRANE

The study of membrane transport in renal failure is a fascinating field. The widespread nature of the uraemic manifestations and membrane transport abnormalities has been discussed in the previous chapters. Uraemia is an example of disease in which environmental factors are the basis of the manifestations, as suggested by the uraemic toxins theory [Ringoir et al 1986, Knochel 1984, Vanholder et al 1989]. Reduced kidney function leads to multiple adaptative metabolic changes as homeostasis is perturbed, some substances are accumulated and others have curtailed production [Maher 1989, Eknoyan & Knochel 1984].

Although it is almost 30 years since defects of membrane transport in uraemia

were first demonstrated [Welt et al 1964], the mechanisms are still not understood in detail. There are several interesting points to discuss.

The specificity of the transport defects seen in uraemia is striking. The present study of the erythrocyte choline transporter is an example of a grossly abnormal cellular function. In contrast, the two systems participating in the regulation of cell pH studied in here, the erythrocyte lactate transport and the leucocyte Na/H antiporter, seem to be normal in patients with renal failure. Table 1.5 (Chapter 1) described several other transporters studied previously. Both activation and inhibition of transport can occur, with some transporters remaining unchanged.

The disease-related abnormalities can be organ specific, which is most clearly seen in epithelial tissues, principally the kidneys. Na/H antiporter activity is unchanged in uraemic leucocytes [chapter 3], while most studies demonstrate an activation of the remnant kidney in experimental animal models of uraemia [Cohn et al 1982a,b, 1982a, Nord et al 1985, Harris et al 1988, Salihagic et al 1988, Preisig & Alpern 1991]. This adaptative capacity is impressive, since in an attempt to compensate for the reduced renal mass the kidney increases the activity of the Na/H transporter involved in cell growth, cell proliferation and cellular acid-base homeostasis [Fine et al 1992, 1985a, 1985, Seifter & Harris 1986]. This response is probably organ specific since in other models of kidney hypertrophy activation of the Na/H antiporter can also occur [Sefter & Harris 1986, Harris et al 1986, Dudeja et al 1991]. Kelly et al [1990] have used a similar argument to justify a normal Na/K pump in myocardial cells of uraemic rats with cardiac hypertrophy, when skeletal muscle of the same rats had inhibited pump activity.

Several circulating factors have been postulated as potential causes of membrane transport alterations in renal failure, including the so-called "uraemic toxins". It is logical to expect that in a disorder largely related to an excretory dysfunction, that accumulated substances will create a new environment in which cells will have to operate. The notion of toxaemia has major

therapeutic implications. By dialysis treatment the uraemic environment can be modified and several clinical manifestations are controlled. In fact reducing the levels of accumulated substances is the main aim of dialysis. Improvement of the clinical and biochemical features of renal failure with removal of diffusible substances during dialysis is a strong argument for the circulating factor theory in uraemia [May et al 1991].

In uraemia, suggestions of circulating factors with specific inhibitory activity comes from the early work on the Na/K ATPase by Welt, which showed that the inhibition of the pump was reversible by haemodialysis [Welt 1967] and that uraemic serum could reproduce the defect in normal erythrocytes [Cole et al 1968]. The reversibility of the changes is important, and further studies support the idea of inhibitors of the Na/K pump in uraemic plasma [see chapter 1]. The removal of substances from the plasma by dialysis or transplantation has a beneficial effect in several membrane transport abnormalities, as shown for the Na-K ATPase [Zannad et al 1982, 1985, Izumo et al 1984, Diez et al 1986, Fervenza et al 1989]. Further evidence for a plasma-borne factor came from cross-incubation studies, in which transport is measured in normal cells incubated with uraemic plasma (and vice-versa) or including plasma obtained prior to and after dialysis [Kramer et al 1976, Izumo et al 1984, Fervenza et al 1989]. Circulating factors have also been suggested to affect other transporters such as glucose transport, Na/Li countertransport, and metabolic processes such glucose utilization [Haag-Weber et al 1989, Balestri 1972, McCaleb et al 1984, Woods et al 1983, Morgan & Morgan 1964].

The spectrum of potential toxins is immense, including urea, guanidine, methylguanidine, indoxyl sulphate, myoinositol, hippuric acid, peptide, hormones, β_2 microglobulin, polyamines, amines [Vahholder et al 1989]. The concept of middle molecules, implicated as uraemic toxins, was introduced for substances that behave as having a molecular weight around 350-5000 daltons [Babb et al 1972, Bergström 1989].

Several endocrine systems are altered in patients with renal failure, and hormones can play a role in regulating the function of selective membrane transport systems. In peripheral tissues insulin can stimulate the rate of glucose transport, Na/K ATPase, amino acid uptake and Na/H antiporter [Klip 1988]. Insulin resistance is one of the characteristics of renal failure [Maloff et al 1983, DeFronzo et al 1981, 1983]. It is also present in states such as hypertension, hyperlipidaemia, atherosclerosis and diabetes [Doria et al 1991]. Thyroid function is usually reduced in uraemia [Lim et al 1980], and can affect transporters such as the Na/K ATPase and the Na/H antiporter [Sacktor & Kinsella 1988]. Other hormones with potential to affect transport function are altered in uraemia, parathormone (PTH) for example, and this illustrates how complex the relationship between membrane transport and uraemia is.

Renal transplantation is the most effective way of reversing the uraemic syndrome, and in this thesis advantage was taken of this to provided strong evidence for the association of a transport system with the clinical evolution of renal failure. The dramatic down-regulation of erythrocyte choline uptake parallels the recovery of renal function following a successful kidney graft. The half-time of the changes for both the choline transporter and renal function are similar. The rapid increment and recovery of the choline transport activity during a reversible reduction in graft function supports the concept that a circulating factor may be involved. If this is the case this factor must be rapidly excreted or metabolized by a functioning kidney. It also must interact strongly with the cell membrane considering that in the present assay of red cell choline transport activity washed cells are used. If a "toxin" was only present in the plasma, it would be completely removed by the initial washes - which is not the case.

The uraemic syndrome manifestations may result of alterations on the physical properties of the cell membrane, reflecting altered membrane composition. For example, erythrocyte membrane fluidity, measured by electron spin resonance, is reduced in haemodialyzed

patients, associated with a significant decrease of phosphatidylcholine and change in the molar ratio of phosphatidylcholine to sphingomyelin [Komidori et al 1985]. Red cell deformability is increased in CRF [Viljoen et al 1991]. Erythrocyte membrane phosphatidylethanolamine is elevated and phosphatidylcholine is reduced [Bleiber et al 1980]. An increased content of erythrocyte membrane cholesterol has also been shown in cells of uraemic patients with a reduced content of phosphatidylcholine and sphingomyelin [Peuchant et al 1988].

Thus, modification of the intrinsic characteristics of the membrane could participate in abnormal transport in uraemia. The present study attempted to look at the possible effect of changes in one of the components of the lipid bilayer, cholesterol on membrane transport. Kelly and coworkers [1989] have demonstrated that an increase in the activity of the Na-K pump was correlated with a reduction in nonesterified fatty acid (NEFA) content of the membrane. Fractions in desalted deproteinized plasma extracted by chromatography were shown to exhibit inhibitory activity for the Na/K pump, displaced ouabain from the enzyme and had digitalis-like immunoreactivity [Kelly et al 1985, 1986].

Historically, the concept of endogenous digitalis-like factors (EDLF) developed from studies in animals that received a volume-expanding dose of saline, which suggested a circulating factor was to be responsible for the natriuresis [de Wardener et al 1961]. Later, volume expansion was shown to produce an inhibition of the Na/K ATPase [Gonick et al 1977]. As natriuretic factor does not have an inhibitory effect on the Na/K pump the term EDLF emerged to allow differentiation. EDLF are postulated to play a role in hypertension and renal failure pathogenesis [Haddy et al 1979, Poston et al 1981, Kelly et al 1986]. Such endogenous substances have been identified as NEFA and steroids [Kelly et al 1986, Tamura et al 1987, Lancet editorial 1991, Mathews et al 1991, Ludens et al 1991].

In the present thesis another steroid molecule (cholesterol) was the focus of

attention. It is disappointing to report that when looking at the membrane transport system which shows the most marked alteration in uraemia (choline transport) no modification of activity was detected by enriching or decreasing membrane cholesterol content. On the other hand, marked modulation of the Na/H antiporter occurred on changing cell cholesterol, ie modulation of a transporter known to remain unchanged in renal failure patients. Cholesterol levels have been reported to be abnormal in the plasma of uraemic subjects, but it is not possible to identify a role for membrane cholesterol in the transport changes associated with uraemia. It is, of course, possible that compensatory lipid adaptations occur in cells adapted to different cholesterol levels, as has been shown to be the case in atherosclerotic patients [Mickalak et al 1988]. Other possible lipid bilayer modifications include changes in phospholipid or fatty acid composition; however this has not been addressed in the present study.

Uraemic manifestations can be linked with membrane transport dysfunction. The cellular consequences of an inhibited Na-K ATPase are obvious and basic to several cell properties such as the membrane potential and transport systems depending on Na gradient, i.e. secondary active transport [Cotton et al 1979, Cunningham et al 1971, Bilbrey et al 1973]. The choline transporter again is an important example of a transporter able to affect cellular activity. High plasma choline levels have been related to a reduced nerve conduction velocity in haemodialysis patients [Rennick et al 1976], a significant finding in view of uraemic neuropathy. If abnormal choline transport occurs at the blood-brain barrier and across cerebral endothelial cells, the availability of choline for acetylcholine synthesis in the CNS could be compromised [Estrada et al 1990]. However, this is unlikely since in the nervous system a Na-dependent choline transporter is more important than the Na-independent system similar to the one present in erythrocytes that we have tested [Haga & Noda 1973]. Choline is not metabolized or incorporated into red cells, making erythrocytes a excellent model for the study of choline transport changes [Askari 1966,

Martin 1977]. The significance of choline transport modification in other cells dependent on choline as a precursor of phospholipid and a methyl-group donor will need to be assessed by studies in these cell types [Suzuki et 1987, Zeisel et al 1991]. Choline is derived in part from diet and in the gut it can be metabolized to aliphatic amines, accumulation of which has been shown to be associated with uraemic symptoms [Simenhoff 1975, Simenhoff et al 1963, 1976, 1977, 1978, Zeisel et al 1991]. The possible participation of these amines in the erythrocyte choline transport system has not been determined.

The present thesis also demonstrates that the erythrocyte lactate transporter and leucocyte Na/H antiporter in uraemia are normal. These two systems are important in maintaining the acid-base homeostasis of the cell [Bock & Marsh 1988, Simchowitz & Davis 1990]. In particular the lactate study represents an important negative result. Normal functioning of the monocarboxylate carrier is essential to prevent further metabolic derangement in uraemic cells. Lactate and pyruvate are at the cross-roads of all the major metabolic pathways involving protein, lipids and carbohydrates [Denton & Halestrap 1979]. Adequate lactate transporter function is important in exporting lactic acid from exercising muscle or in other cells, such as erythrocytes, involved in glycolysis [Wasserman & Casaburi 1991, Denton & Halestrap 1979]. A normal uptake is essential for gluconeogenesis and for red cells participating in the interorgan distribution of substances [Relman 1978, Christensen 1975].

It was interesting to find normal Na/H antiporter activity in the presence of a reduced intracellular pH in the uraemic patients. Two facts should be considered. First, despite reduced pHi in uraemia when compared with control conditions, in each of the groups pHi was not low enough to be below the acid threshold, or set point, at which activation of the antiporter would occur [Aronson et al 1982]. Second, it must be kept in mind that the studies aimed to look at the Na/H antiporter in isolation and bicarbonate-free solutions were used throughout the

experiments. The pHi in bicarbonate buffered solutions is likely to be different considering the participation of anion/bicarbonate exchangers in the intracellular acid-base homeostasis [Thomas 1989, Simchowitz & Ross 1985], as discussed in the introduction and in chapter 3.

To sum up, abnormal membrane transport is one of the manifestations of the uraemic syndrome and may be involved in its pathogenesis. Of the factors which influence transport, environmental abnormalities such as the change in profile of plasma composition (e.g. uraemic toxins, blood lipids, hormones) seem to be the most significant determinant. This has been shown by the fact that membrane transport systems are significantly altered by the plasma composition. Eventually, these alterations in the composition of the extracellular fluid may interact with genetic determinants of membrane transport and result in a modification of the expression of membrane transporters. The changes are not only specific for each transport system, but they are also reversible. Interestingly for the choline transporter the changes reverse in a period much shorter than the red cell life span, which suggest that changes are not due to an intrinsic membrane defect during cell maturation. The change can be quickly reversed with variations in renal function, as demonstrated in the transplant patients. Improvement or correction of cellular defects can be achieved by removal of the cells from the uraemic "habitat". The principle behind the dialysis treatment is to modify that environment.

DIABETIC NEPHROPATHY: Na/H ANTIPORTER AS A GENETIC MARKER?

The study on the leucocyte Na/H antiporter included data on patients with end-stage diabetic nephropathy. The interest in examining this particular group of patients results from the extensive data identifying membrane transport via the erythrocyte Na/Li countertransport and leucocyte Na/H antiporter as a marker for a group of patients prone to develop renal and

cardiovascular complications of insulin dependent diabetes mellitus (IDDM).

About a third of patients with IDDM develop advanced nephropathy [Anderson et al 1983, Krolewski et al 1985], and there is a strong association with a predisposition to essential hypertension [Krolewsky et al 1988]. Mangili et al [1988] and Krolewski et al [1988] independently suggested that increased erythrocyte Na/Li countertransport activity, a system abnormal in essential hypertension, could also be a genetic marker for diabetic nephropathy [Canessa et al 1980, Turner et al 1987, Williams et al 1983, Redgrave et al 1989]. The high Na/Li countertransport activity was confirmed by other groups in patients with early nephropathy and the possible genetic link further supported by studies in parents of IDDM patients [Carr et al 1990, Walker et al 1990]. The genetic link has been questioned by a Danish group who suggested other reasons for the familial clustering of diabetic nephropathy [Jensen et al 1990, Borch-Jonhsen et al 1992]. That conclusion has been questioned, since the data supported a high Na/Li countertransport in IDDM with renal involvement [Laffel et al 1991]. Further evidence exists for Na/Li countertransport activity as a marker in studies showing an association of high transporter activity and renal, cardiac and metabolic complications in IDDM and hypertension [Semplicini et al 1989, 1992, Jones et al 1990, Mangili et al 1990, Trevisan et al 1992].

Findings similar to the alterations in erythrocyte Na/Li countertransport were found for the Na/H antiporter in leucocytes, fibroblasts and platelets [Ng et al 1990a, 1990b, Trevisan et al 1989, Halkin et al 1991] of IDDM and hypertensive patients [Ng et al 1988, 1989, Livne et al 1987]. There are functional similarities between the two transporters, but they probably represent different proteins on the basis of inhibitor sensitivity and the weak correlation between the function of the two processes in clinical studies [Pandey et al 1978, Carr et al 1988, Halkin et al 1991, Semplicini et al 1992]. However it is still possible that Na/Li countertransport may be one isoform of the Na/H antiporter [Orlowsky et al 1992, Igarashi et al 1991, Clarck & Limbird

1991]. The protein and gene for the Na/Li countertransport is still to be identified in spite of extensive studies on the Na-H system [Lifton et al 1991, Sardet et al 1989].

The point is that the activity of both systems has been suggested as a potential predictor of the subgroup of diabetics who will develop overt diabetic nephropathy, with a higher activity expected in IDDM uraemic patients. In the present study the activity of the antiporter was normal in cells from uraemic diabetics. If a high Na/H antiporter activity is a marker for early identification of patients who will develop end stage diabetic nephropathy, a high Na/H antiporter activity would be expected in patients with uraemia due to IDDM. In the present thesis it is of particular interest to consider the associations between plasma lipids and Na/H antiporter and Na/Li countertransport system activity [Trevisan et al 1992, Ng & Davies 1992].

It was surprising to find a normal activity of the Na/H antiporter in these patients with renal failure. The elevated Na/H antiporter activity found in leucocytes associated with an alkaline pHi in early diabetic nephropathy is not present when they patients develop uraemia. It is unlikely to be a factor related to uraemia per se; in non-diabetic uraemics Na/H antiporter activity is not altered. The lack of difference in the V_{max} of the Na/Li countertransport between IDDM patients with or without nephropathy has been described [Elving et al 1991, 1992]. This is consistent with the hypothesis of Barzilay et al [1992] that when nephropathy develops late in the course of diabetes other factors, such as hyperglycaemia, could be more significant.

Abnormal physical properties of the membrane in IDDM may be involved in modifying transporter expression. In diabetes erythrocytes has been reported to have reduced membrane fluidity and impaired cell deformability, possibly due to an increased membrane cholesterol/phospholipid ratio [Bryszewska et al 1986, 1990]. Leucocytes from a rat model of diabetes also had a reduced membrane fluidity [Masuda et al 1989]. However there has been one study in which no change in erythrocyte membrane fluidity was present, despite a low cholesterol,

low phospholipid content, reduced cholesterol:phospholipid ratio and changes in the fatty acid composition of the red blood cells [Baldini et al 1989]. Recently, it has been reported that leucocytes from diabetic patients with initial nephropathy fail to present a reduction in activity of the Na/H antiporter activity with raising plasma HDL levels, as seen in normal individuals [Ng & Davies 1992].

ATHEROSCLEROSIS AND SIMVASTATIN TREATMENT

Atherosclerotic vascular disease is, again, a heterogeneous disorder. It is characterized pathologically by lesions in the arteries resulting from accumulation of excess plasma-derived lipid, proliferation of connective tissue forming a fibro-muscular cap and necrosis of connective tissue at the plaque base [Woolf 1990]. There are several potential risk factors, eg hyperlipidaemia, gender, diabetes, hypertension and others [Tunstall-Pedoe & Smith 1990, Brindley & Rolland 1989, Martin et al 1986, Ball & Mann 1988]. The disease can be genetically determined as seen in the case of familial hypercholesterolaemia, in which an autosomal dominant gene is responsible for defective or a reduced number of LDL receptors in the cells [Goldstein & Brown 1983]. Plasma lipids and cholesterol levels are strong determinants of the morbidity and mortality associated with atherosclerosis [Martin et al 1986, Mann & Ball 1988]. Evidence has recently suggested that if plasma cholesterol is reduced with treatment, the lesions of atherosclerosis will also diminish [Watts et al 1992].

We assessed here the possible link between the atherosclerotic process in relation to the cell membrane composition and Na/Li countertransport system. In mature erythrocytes no synthesis of membrane lipids can occur, and the modifications in cell cholesterol content are derived from the exchanges with the plasma lipoproteins [Lange et al 1980, 1981, Dawidowicz

1987].

Proving the hypothesis that changing membrane cholesterol affects the Na/Li countertransport *in vitro* was straightforward. The same cannot be said about the study in which plasma cholesterol was altered *in vivo* by treating atherosclerotic patients with an HMG CoA reductase inhibitor (simvastatin). Plasma cholesterol was effectively lowered by this treatment, but surprisingly, membrane cholesterol levels did not alter. It is clear in the present study that no reduction in membrane cholesterol concentration was obtained with simvastatin treatment.

As described earlier recent studies showed no change or slight elevation in cell cholesterol concentration with HMG CoA reductase inhibitors [Mazzanti et al 1990, Ng & Davies 1991]. It is feasible that by reducing plasma cholesterol, its inhibitory effect on cell cholesterol synthesis will be reduced [Goldstein & Brown 1990, Ng & Davies 1991, 1992]. This effect is a possible explanation for the elevation of cholesterol in membranes of cells capable of metabolizing and synthesizing membrane cholesterol [Norum et al 1983]. The same is not true for mature erythrocytes, in which exchange with plasma lipids is achieved [Dawidowicz 1987], unless the effects occur during reticulocyte maturation.

The lack of change in membrane Na/Li countertransport activity in the simvastatin-treated patients may be related to the stability of the membrane cholesterol in the present study. In other studies lipid-lowering therapy resulted in reduction of erythrocyte Na/Li countertransport activity associated with change in plasma triglycerides, but not with plasma cholesterol [Carr et al 1990, 1990a, 1991]. Lovastatin, another HMG CoA reductase inhibitor, had no effect on the erythrocyte Na/Li countertransport, but actually suppressed platelet Na/H antiporter in the same group of patients [Weder et al 1991c]. This same study was in agreement with the present findings of no correlation between the magnitude of changes in plasma lipids and activity of cation transport. The lack of association may also be due to the use, in the present

study, of total plasma cholesterol as the lipid parameter, which is associated with Na/Li activity in some studies [Hunt et al 1986,Corrocher et al 1985], but not all [De la Sierra et al 1988, McDonald et al 1990]. Perhaps analysis of other lipid fractions, such as HDL, LDL or triglycerides would yield different results from the present study. The reduction of Na/H antiporter activity seen in cultured lymphoblasts treated with HMG CoA reductase inhibitors has been suggested to result not from alterations in membrane cholesterol, but perhaps other products of mevalonate metabolism [Ng & Davies 1991, 1992].

The main finding of the simvastatin study is that cell membrane total cholesterol is not modified by the reduction of plasma cholesterol with simvastatin. The influence of plasma lipids on the activity of Na/Li countertransport does not seem to involve cholesterol cell content.

TRANSPORTERS

It seems unassailable that membrane transport alterations are important in disease. Which alterations are present, which are important, which are significant, which are just an epiphenomenon, which can be used as a marker, and which can be used for therapeutic interventions are all questions to which I do not have a clear answer.

There are some specific disorders in which transport abnormalities are clear cut. Take the example of glucose-galactose malabsorption: a single point gene mutation is responsible for a defective Na-dependent epithelial transporter that can result in a fatal disorder. Now with adequate diagnosis simple dietary measures prevent any serious consequences of the disease [Wright et al 1992].

It would be satisfying if the same could be said for heterogenic and/or polygenic diseases, such as the ones studied in here. Of the transport systems studied in this thesis, the

results for the choline transporter seem to be particularly significant. Its activation is one of the most marked changes described in uraemia and indeed such marked alterations are not usual in most clinical disorders. To assess the significance of the findings, choline transport should be assessed in other tissues such as brain and muscle. There is a potential for the choline transporter to be used as a clinical marker in uraemia, but probably for practical purposes urea, creatinine and clearance studies are more feasible and cheaper. Further characterization of choline transport mechanisms in renal failure should be sought and plasma effects should be assessed by cross-incubation studies with normal and uraemic plasma.

The potential clinical implications of altered choline metabolism have been mentioned above, and are exemplified by the role of choline in peripheral neuropathy [Rennick 1976] and choline metabolites in the clinical manifestations of uraemia [Simenhoff et al 1977,1978, Simenhoff 1975]. Altering bacterial flora with antibiotics, results in reduced accumulation of these potential toxins and improved symptomatology [Simenhoff et al 1976]. Presently it has been demonstrated that there is an association of the clinical evolution of uraemia, reversed by transplantation, and the activity of the erythrocyte choline carrier.

The studies of the acid-base regulatory transport systems, Na/H antiporter and lactate, provide useful information in the patients with chronic renal failure. These are important negative results: characterizing the activity of each transporter in uraemia is of interest in attempting to understand the nature of its metabolic derangements. However, there are factors that deserve further investigation. The reduced pHi in uraemia may eventually suggest that HCO_3^--dependent mechanisms could be altered in the syndrome [Thomas et al 1989]. Bicarbonate is largely responsible for controlling resting intracellular pH in a number of cell types [Thomas 1989, Simchowitz & Ross 1985]. The studies here were performed in bicarbonate free solutions in order to analyze the Na/H antiporter in isolation. The study also cannot exclude a possible abnormality

in the Na/H antiporter substrate affinity in patients with uraemia with or without endstage diabetic nephropathy.

Normal lactate transport is generally important for cellular homeostasis and so was measured in the present thesis in uraemic cells. Additionally a significant contribution of the lactate study was to provide, for the first time, information on lactate transport rates at physiological conditions of temperature. Further studies should consider using bicarbonate containing buffers and varying lactate concentration, considering that in exercise plasma bicarbonate has been shown to be the main buffer for plasma lactate [Wasserman & Casaburi 1991]. A negative association between blood bicarbonate concentration and lactate concentration is present during exercise [Stringer et al 1992]. In fact the present work does report fluxes measured in plasma which will still have significant bicarbonate levels. The fluxes in plasma were not significantly different from fluxes performed in saline.

In this thesis blood cells have been used as models, and all the studies make assumptions that *in vitro* modifications reflect the disturbances present *in vivo*, and that changes in one tissue reflect the changes in other cell types [Swales 1990]. The *in vitro* studies using blood cells are a way towards characterizing membrane disorders in disease. Further investigations *in vivo* are desirable.

Finally the intention of the present thesis was not to study one process, for example uraemia, in detail, but to investigate a number of clinical situations and number of transporters systems, in different cell types to look at a wide range for potential pathophysiological effects. The fact that the changes were found to be selective both in transport system and disease states confirms the utility of the approach for membrane transport but also defines large areas of work for the future, since it seems obvious that many of these situations should be studied in more depth.

REFERENCES

AALKJAER C (1990). Regulation of intracellular pH and its role in vascular smooth muscle function. J Hypertension 8: 197-206.

ABOU-SALEH MT (1988). The relationship between morbidity index and lithium dosage. In: BIRCH N, editor. Lithium: inorganic pharmacology and psychiatric use. IRL Press ltd. Oxford 25-28.

ACARA M (1975). The kidney in regulation of plasma choline in the chicken. Am J Physiol 228: 645-649.

ACARA M & RENNICK B (1973). Regulation of plasma choline by the renal tubule: bidirectional transport of choline. Am J Physiol 225: 1123-1128.

ADRAGNA N, CANESSA ML, SOLOMON H, SLATER E & TOSTESON DC (1982). Red cell lithium-sodium countertransport and sodium-potassium cotransport in patients with essential hypertension. Hypertension 4: 795-804.

ADRAGNA NC, CHANG JL, MOREY MC & WILLIAMS RS (1985). Effect of exercise on cation transport in human red cells. Hypertension 7: 132-139.

AKANJI AO & SACKS S (1991). Effect of acetate on blood metabolites and glucose tolerance during haemodialysis in uraemic non-diabetic and diabetic subjects. Nephron 57: 137-143.

ALBERTI KGMM & CUTHBERT C (1982). The hydrogen ion in normal metabolism: a review. IN: "Metabolic acidosis", Ciba Foundation symposium 87, Pitman Books Ltd., London, 1-19.

ALBERTS AW (1990). Effects of HMG CoA reductase inhibitors on cholesterol synthesis. Drug Invest 2 (S2): 9-17.

ALVESTRAND A, MUJAGIC M, WAJNGOT A & EFENDIC S (1989). Glucose

intolerance in uremic patients: the relative contribuition of impaired β-cell function and insulin resistance. Clin Nephrol 31: 175-183.

ANCELIN ML & VIAL HJ (1989). Regulation of phosphatidylcholine biosynthesis in Plasmodium-infected erythrocytes. Biochim Biophys Acta 1001: 82-89.

ANCELIN ML, PARANT M, THUET MJ, PHILIPPOT & VIAL HJ (1991). Increased permeability to choline in simian erythrocytes after *Plasmodium knowlesi* infection. Biochem J 273: 701-709.

ANDERSEN AR, CHRISTIANSEN JS, ANDERSEN JK, KREINER S & DECKERT T (1983). Diabetic nephropathy in type 1 (insulin-dependent) diabetes, an epidemiological study. Diabetologia 25: 495-501.

APARICIO M, VINCENDEAU P, COMBE C, CAIX J, GIN H, DE PRECIGOUT V, BEZIAN J-H, BOUCHET J-L & POTAUX L (1991). Improvement of leucocytic Na,K-pump activity in uremic patients on low protein diet. Kidney Int 40: 238-242.

ARNOLD WC & HOLLIDAY MA (1979). Tissue resistance to insulin stimulation of amino acid uptake in acutely uremic rats. Kidney Int 16: 124-129.

ARONSON PS, NEE J & SUHM MA (1982). Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. Nature 299: 161-163.

ARONSON JK (1990). Methods for expressing the characteristics of transmembrane ion transport systems. Clin Sci 78: 247-254.

ARONSON JK & NG LL (1990). HMG CoA reductase inhibitors in the treatment of hypercholesterolaemias. Quart J Med 74: 111-115.

ASKARI A (1966). Uptake of some quartenary ammonium ions by human erythrocytes. J Gen Physiol 49: 1147-1160.

ATTMAN P-O & ALAUPOVIC P (1991). Lipid abnormalities in chronic renal

insufficiency. Kidney Int 39 (S31): S16-S23.

BABB AL, FARELL PD, UVELLI DA & SCRIBNER BH (1972). Hemodialyzer evaluation by examination of solute molecular spectra. Trans Am Soc Artif Int Organs 18: 98.

BABB AL, JOHANSEN PJ, STRAND MJ, TENCKHOFF H & SCHRIBNER BH (1973). Bi-directional permeability of the human peritoneum to middle molecules. Proc Eur Dial Transp Assoc 10: 247-262.

BAKER JM & DAWIDOWICZ EA (1979). The rapid transmembrane movement of cholesterol in small unilamellar vesicles. Biochim Biophys Acta 551: 260-270.

BALDINI P, INCERPI S, LAMBERT-GARDINI S, SPINEDI A & LULY P (1989). Membrane lipid alterations and Na-pumping activity in erythrocytes from IDDM and NIDDM subjects. Diabetes 38: 825-831.

BALESTRI PL, RINDI P, BIAGINI M & GIOVANNETTI S (1972). Effects of uraemic serum, urea, creatinine and methylguanidine on glucose metabolism. Clin Sci 42: 395-404.

BALL M & MANN J (1988). Lipids and heart disease. A practical approach. Oxford Medical Publications, Oxford.

BARON DN & AHMED SA (1969). Intracellular concentration of water and of the principal electrolytes determined by analysis of isolated human leucocytes. Clin Sci 37: 205-219.

BARZILAY J, WARRAM JH, BAK M, LAFFEL LMB, CANESSA M & KROLEWSKI AS (1992). Predisposition to hypertension: Risk factor for nephropathy and hypertension in IDDM. Kidney Int 41: 723-730.

BAUDOUIN-LEGROS M & MEYER P (1990). Hypertension and atherosclerosis. J Cardiovasc Pharmacol 15 (s1): s1-s6.

BEHR J, WITAGALL H, LORENZ R, WEBER PC & DUHM J (1985). Red cell Na^+/K^+ transport in various forms of human hypertension. Role of cardiovascular risk factors and plasma

potassium. Klin Wchenschr 63 (S3): 63-65.

BEILHARZ GR, MIDDLEHURST CR, KUCHEL PW, HUNT GE & JOHNSON GFS (1986). An experimental study and computer simulation of the turnover of choline in erythrocyte of patients treated with lithium carbonate. Aust J Exp Biol Med Sci 64: 271-289.

BEAN GH & LOWENSTEIN LM (1978). Choline pathways during normal and stimulated renal growth in rats. J Clin Invest 61: 1551-1554

BENNETT JP, MCGILL KA & WARREN GB (1980). The role of lipids in the functioning of a membrane protein: the sarcoplasmic reticulum calcium pump. Curr Top Membr Transport 14: 127-164.

BENOS DJ (1988). Amiloride: Chemistry, kinetics, and structure-activity relationships. IN: GRINSTEIN S, editor. Na^+/H^+ exchange, CRC Press, Inc., Boca Raton, FL, USA, 121-136.

BERGSTRÖM J (1989). Toxicity of uremia: Physiopathology and clinical signs. Contrib Nephrol 71: 1-9.

BERGSTRÖM J & FÜRST P (1983). Uremic toxins. In: DRUKKER W, PARSONS FM & MAHER MF, editors. Replacement of renal function by dialysis. A textbook of dialysis, Boston, Nijhoff, 354.

BETTERIDGE DJ (1989). Diabetes, lipoprotein metabolism and atherosclerosis. Br Med Bul 45: 285-311

BEUCKELMANN D & ERDMANN E (1984). Exogenous factors influencing the human erythrocyte sodium-lithium cóuntertransport syustem. Eur J Clin Invest 14: 392-397.

BEUCKELMANN D & ERDMANN E (1985). Pertubation of sodium-lithium countertranport in red cells (Letter). N Engl J Med 312: 1193-1194.

BEUCKELMANN D & ERDMANN E (1986). Na^+-Li^+ countertransport and electrolyte composition in erythrocytes of patients with essential hypertension before and after

antihypertensive treatment. Klin Wochenschr 64: 1101-1105.

BEUGE & DEL CAMPILLO (1976). The ATP dependence of a ouabain-sensitive sodium efflux activated by external sodium, potassium and lithium in human red cells. Biochim Biophys Acta 433: 547-554.

BEVAN C & KINNE KH (1990). Choline transport in collecting duct cells isolated from the rat renal inner medulla. Pflügers Arch 417: 324-328.

BIANCHINI L, WOODSIDE M, SARDET C, POUYSSÉGUR J, TAKAI A & GRINSTEIN S (1991). Okadaic acid, a phosphatase inhibitor, induces activation and phosphorylation of the Na^+/H^+ antiport. J Biol Chem 266: 15406-15413.

BIBBY K, MEREDITH D, FERVENZA FC, ELLORY JC & HENDRY BM (1990). Altered membrane transport of choline in renal failure: a comparison of non-dialysed and haemodialysed patients. In: ANDREUCCI VE & DAL CANTON, editors, New therapeutic strategies in nephrology: proceedings of the 3rd international meeting on current therapy in nephrology, Sorrento, Italy, May 27-30: 289-291.

BILBREY GL, CARTER NW, WHITE MG, SCHILLING JF, KNOCHEL JP & BORROTO J (1973). Potassium deficiency in chronic renal failure. Kidney Int 4: 423-430.

BING RF, HEAGERTY AM, THURSTON H & SWALES JD (1986). Ion transport in hypertension: are changes in the cell membrane responsible? Clin Sci 71: 225-230.

BLAUSTEIN MP (1977). Sodium ions, calcium ions, blood pressure regulation and hypertension: a reassessment and a hypothesis. Am J Physiol 232: C165-C173.

BLEIBER R, EGGERT W, REICHMANN & KUNZE D (1980). Erythrocyte and plasma lipids in terminal renal insufficiency. Acta Haemat 63: 117-123.

BLIGH J. The levels of free choline in plasma. J Physiol 117: 234-240, 1952.

BOCK G & MARSH J, editors (1988). Proton passage across cell membranes, Ciba

Foundation Symposium 139, Wiley Interscience publication, Chichester, UK.

BOERO R, GUARENA C, BERTO IM, DEABATE MC, ROSATI C, QUARELLO F & PICCOLI G (1988). Erythrocyte Na,K pump activity and arterial hypertension in uremic dialyzed patients. Kidney Int 34: 691-696.

BOERO R, ESPOSTI D, FABBRI A, GUARENA C, FORNERIS G, QUARELLO F, FUSAROLI M & PICCOLI G (1991). Increased sodium-lithium countertransport activity in red cells of IgA nephropathy patients. Kidney Int 40: 1118-1122.

BONOMINI V, STEFONE S, VANGELISTA A & COSTA AN (1988). Immune system in uremia. In: DAVIDSON AM, editor. Nephrology, London, Bailliere Tindall, 1988, V II, p 1042-1049.

BONTING SL & dE PONT JJHHM (1981). Membrane transport. Elsevier/North-Holland Biochemical Press, Amsterdam, Netherlands. New Comprehensive Biochemistry, Volume 2.

BORCH-JOHNSEN K, NORGAARD K, HOMMEL E, MATHIESEN ER, JENSEN JS, DECKERT T & PARVING H-H (1992). Is diabetic nephropathy an inherited complication? Kidney Int 41: 719-722.

BORON WF (1985). Control of intracellular pH. IN: "The Kidney: Physiology and pathophysiology", Eds: SELDIN DW & GIEBISCH G, Raven Press Ltd., New York, 1417-1439.

BORON WF (1987). Intracellular pH regulation. In: ANDREOLI TE, HOFFMAN JF, FANESTIL DD & SCHULTZ SG, editors. Membrane transport processess in organized systems. Plenum Medical Book Company, New York & London, 39-51.

BORON WF (1989a). Chemistry of buffer equilibria in blood plasma. In: SELDIN DW & GIEBISCH G, editors. The regulation of acid-base balance. Raven Press Ltd., New York, 3-32.

BORON WF (1989b). Cellular buffering and intracellular pH. IN: SELDIN DW & GIEBISCH G, editors. The regulation of acid-base balance. Raven Press Ltd., New York, 33-56.

BÖYUM A (1968). Separation of leucocytes from blood and bone marrow. Scand J Clin Lab Inv 21 (S97): 9-109.

BRASITUS TA, DUDEJA PK, WORMAN HJ & FOSTER ES (1986). The lipid fluidity of rat colonic brush-border membrane vesicles modulates Na/H exchange and osmotic water permeability. Biochim Biophys Acta 855: 16-24.

BRENT GA, CANESSA M & DLUHY RG (1989). Reversible alteraion of red cell lithium-sodium countertransport in patients with thyroid disease. J Clin Endocr Metabol 68: 322-328.

BRICKER NS (1972). On the pathogenesis of the uremic state: an exposition of the "trade-off" hypothesis. N Engl J Med 286: 1093.

BRICKER NS, BOURGOIGNIE JJ & KLAHR S (1970). A humoral inhibitor of sodium transport in uremic serum. Arch Int Med 126: 860.

BRIGGS WA, SILLIX DH, MAHAJAN S & MCDONALD FD (1983). Leukocyte metabolism and function in uremia. Kidney Int 24 (S16): S93-S96.

BRIGHT GR, FISCHER GW, ROGAWSKA J & TAYLOR DL (1984). Fluorescence ratio imaging microscopy: temporal and spatial measurements of cytoplasmic pH. J Cell Biol 104: 1019-1033.

BRINDLEY DN & ROLAND Y (1989). Possible connections between stress, diabetes, obesity, hypertension and altered lipoprotein metabolism that may result in atherosclerosis. Clin Sci 77: 453-461.

BROD J, SCHAEFFER J, HENGSTENBERG JH & KLEINSCHMIDT TG (1984). Investigations on the Na,K-pump in erythrocytes of patients with renal hypertension. Clin sci 66: 351-355.

BRUCKDORFER KR, EDWARDS PA & GREEN C (1968a). Properties of aqueous

dispersions of phospholipids and cholesterol. Eur J Biochem 4: 506-511.

BRUCKDORFER KR, GRAHAM JM & GREEN C (1968b). The incorporation of steroid molecules into lecithin sols, β-lipoprotein and cellular membranes. Eur J Biochem 4: 512-518.

BRUCKDORFER KR, DEMEL RA, GIER JD & VAAN DEENEN LLM (1969). The effect of partial replacement of membranme cholesterol by other steroids on the osmotic fragility and glycerol permeability of erythrocytes. Biochim Biophys Acta 183: 334-345.

BRUGARA C, CORROCHER R, FORONI L, STEINMAYR M, BONFANTI F & DE SANDRE G (1983). Lithium-sodium countertransport in erythrocytes of normal and hypertensive subjects. Relationship with age and plasma renin activity. Hypertension 5: 529-534.

BRYSZEWSKA M, WATALA C & TORZECKA W (1986). Changes in fluidity and composition of erythrocyte membranes and in composition of plasma lipids in type I diabetes. Br J Haematology 62: 111-116.

BRYSZEWSKA M, PRZYBYLSKA M, LEYKO W & CHAPMAN IV (1990). Thermosensitivity and fluidity of human control and diabetic erythrocytes (abstract). European Red Cell Club, Dundee Meeting, July 1990, 41.

BUNKER CH, MALLINGER AG, ADAMS LL & KULLER LH (1987). Red blood cell sodium-lithium countertransport and cardiovascular risk factors in black and white college students. J hypertension 5: 7-15.

BUSA WB & NUCCITELLI R (1984). Metabolic regulation via intracellular pH. Am J Physiol 246: R409-R438.

BUTTERFIELD DA, NICHOLAS MM & MARKESBERY WR (1985). Evidence for an increased rate of choline efflux across erythrocyte membranes in Alzheimer's disease. Neurochemical Res 10: 909-918.

CABANTCHIK ZI, BARUCH D, KEREN-ZUR, ZANGVILL & GINSBURG H (1986).

The modulatory effect of membrane viscosity on structural and functional properties of the anion exchange protein of human erythrocytes. Membrane Biochemistry 6: 197-216.

CADE JFJ (1949). Lithium salts in the treatment of psychotic excitement. Med J Aust 2: 349-352.

CANALE S Del, FIACCADORI E, COFFRINI E, VITALI P, RONDA N, ANTONUCCI C, ARDUINI U, GUARIGLIA A (1986). Uremic acidosis and intracellular buffering. Scand J Urol Nephrol 20: 301-306.

CANALE S Del, FIACCADORI E, GUARIGLIA A, COFFRINI E & BORGHETTI (1986a). Intracellular acid-base and energy metabolism in uremia: a preliminary study of five patients before and during dialysis treatment. In: RINGOIR S, VANHOLDER R & MASSRY SG, editors. Uremic toxins. Series: Advances in experimental medicine and biology, Volume 223, Plenum press, New York, 141-144.

CANALI M, BORGHI L, SANI E, MONTANARI A, NOVARINI A & BORGHETTI (1981). Increased erythrocyte lithium-sodium countertransport in essential hypertension: its relationship to family history of hypertension. Clin Sci 61: 13s-15s.

CANESSA M (1989). Kinetic properties of Na/H exchange and Li/Na, Na/Na, and Na/Li exchanges of human red cells. Methods in Enzymology 173: 176-191.

CANESSA M, ADRAGNA N, SOLOMON HS, CONNOLLY TM & TOSTESON DC (1980). Increased sodium-lithium countertransport in red cells of patients with essential hypertension. N Engl J Med 302: 772-776.

CANESSA M, BIZE I, ADRAGNA N & TOSTESON DC (1982). Cotransport of lithium and potassium in human red cells. J Gen Physiol 80: 149-168.

CANESSA M, SPALVINS A, ADRAGNA N & FALKNER B (1984). Red cell sodium countertransport and cotransport in normotensive and hypertensive blacks. Hypertension 6: 344-

351.

CANESSA M, MORGAN K, GOLDSZER R, MOORE TJ & SPALVINS A (1991). Kinetic abnormalities of the red blood cell sodium-proton exchange in hypertensive patients. Hypertension 17: 340-348.

CAPALDO B, CIANCIARUSO B, NAPOLI R, ANDREUCCI V, KOPPLE JD & SACCA L (1990). Role of the splanchinic tissues in the patogenesis of altered carbohydrate metabolism in patients with chronic renal failure. J Clin Endocr Metabol 70: 127-133.

CARR S, THOMAS T & WILKINSON R (1988). Sodium-lithium countertransport activity and its sensitivity to inhibitors with erythrocyte aging in man. Clin Chim Acta 178: 53-61.

CARR S, THOMAS T, LAKER M & WILKINSON R (1989). Do abnormal plasma lipids explain elevated sodium-lithium countertransport in hypertension? (abstract) Clin Sci 77 (Suppl 21): 10p.

CARR S, THOMAS T, LAKER M & WILKINSON R (1989a). Sodium-lithium countertransport: a genetic marker of hyperlipidaemia and hypertension? (abstract) Clin Sci 77 (Suppl 21): 10p.

CARR S, THOMAS T & WILKINSON R (1989b). Erythrocyte sodium-lithium countertransport in primary and renal hypertension. Eur J Clin Invest 19: 101-106.

CARR S, MBANYA JC, THOMAS T, KEAVEY P, TAYLOR R, ALBERTI KGGMM & WILKINSON R (1990). Increase in glomerular filtration rate in patients with insulin-dependent diabetes and eleveted erythrocyte sodium-lithium countertransport. N Engl J Med 322: 500-505.

CARR S, THOMAS T, LAKER M & WILKINSON R (1990a). Lipid lowering therapy leads to a reduction in sodium-lithium countertransport (abstract). Clin Sci 79 (4): 8p-9p.

CARR S, THOMAS T, LAKER M & WILKINSON R (1991). Lipid lowering therapy leads to a reduction in sodium-lithium countertransport activity. Arteriosclerosis 87: 103-108.

CARRUTHERS A & MELCHIOR DL (1988). Effects of lipid environment on membrane transport: The human erythrocyte sugar transport protein/lipid bilayer system. Ann Rev Physiol 50: 257-271.

CHENG JT, KAHN T & KAJI D (1982). Mechanism of alteration of Na-K pump in erythrocytes from patients with chronic renal failure (CRF) (abstract). Kidney Int 21:228.

CHENG JT, KAHN T & KAJI D (1984). Mechanism of alteration of sodium potassium pump of erythrocytes from patients with chronic renal failure. J Clin Invest 74: 1811-1820.

CHRISTENSEN HN (1975). Biological transport. WA Benjamin, Inc., Reading, Massachusetts, USA.

CHRISTENSEN HN (1982). Interorgan amino acid nutrition. Physiol Reviews 62: 1193-1233.

CHRISTENSEN HN (1990). Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Reviews 70: 43-77.

CIANCIARRUSO B, BELLIZZI V, NAPOLI R, SACCA L & KOPPLE JD (1990). Hepatic uptake and release of glucose, lactate, and aminoacids in acutely uraemic dogs. Metabolism 40: 261-269.

CLANDININ MT, FIELD CJ, HARGREAVES K, MORSON I & ZSIGMOND E (1985). Role of diet fat in subcellular structure and function. Can J Physiol Pharmacol 63, 546-556.

CLANDININ MT, CHEEMA S, FIELD CJ, GARG ML, VENKATRAMAN J & CLANDININ TR (1991). Dietary fat: exogenous determination of membrane structure and cell function. FASEB J 5: 2761-2769.

CLARET M, GARAY R & GIRAUD F (1978). The effect of membrane cholesterol on the sodium pump in red blood cells. J Physiol 274: 247-263.

CLARK MR (1988). Ion transport measurements in red cells. In: SHOHET SB &

MOHANDAS N, editors, "Red Cell Membrane", Series: Methods in Haematology, Volume 19, Churchill Livingstone, London, 237-269.

CLARK JD & LIMBIRD LL (1991). Na-H exchanger subtypes: a predictive review. Am J Physiol 261: C945-C953.

CLARK AS & MITCH WE (1983). Muscle protein turnover and glucose uptake in acutely uremic rats. J Clin Invest 72: 836-845.

CLEGG G, MORGAN DB & DAVIDSON C (1982). The heterogenetity of essential hypertension. Relation between lithium efflux and sodium content of erythrocytes and family hystory of hypertension. Lancet ii: 891-894.

CLEGG G, GLEDHILL J & MORGAN DB (1982a). Lithium efflux and organic anion exchange in the erythrocytes of patients with chronic cholestasis. IRSC, Medical Science 10: 828-829.

COHN DE, HRUSKA KA, KLAHRS S, HAMMERMAN MR (1982a). Increased Na^+/H^+ exchange in brush border vesicles from dogs with chronic renal failure (abstract). Kidney Int 21: 228.

COHN DE, HRUSKA KA, KLAHRS S, HAMMERMAN MR (1982b). Increased Na^+/H^+ exchange in brush border vesicles from dogs with renal failure. Am J Physiol 243: F293-F299.

COLE CH (1973). Decreased ouabain-sensitive adenosine triphosphatase activity in the erythrocyte membrane of patients with chronic renal disease. Clin Sci 45: 775-784.

COLE CH, STEINBERG R & GUTTMANN R (1978). Altered erythrocyte sodium efflux following renal tranplantation. Nephron 20: 248-257.

COLE CH, WELT LG & BALFE JW (1968). Induction of a ouabain-sensitive ATPase defect by uremic plasma. Trans Assoc Amer Phys 81: 213-220.

COLE CH & MALETZ (1975). Changes in erythrocyte membrane ouabain-sensitive

adenosine triphosphatase after renal transplantation. Clin Sci 48: 239-242.

CONNOLLY TJ, CARRUTHERS A & MELCHIOR DL (1985). Effects of bilayer cholesterol on human hexose transport protein activity in synthetic lecithin bilayers. Biochemistry 24: 2865-2873.

COOPER RA (1977). Abnormalities of cell-membrane fluidity in the pathogenesis of disease. N Engl J Med 297: 371-377.

COOPER R, LEGRADY D, NANAS S, TREVISAN M, MANSOUR M, HISTAND P, OSTROW D & STAMLER J (1983). Increased sodium-lithium countertransport in college students with elevated blood pressure. JAMA 249: 1030-1034.

CORNISH-BOWDEN A & WHARTON CW (1988). Enzime kinetics. IRL Press, Eynsham, Oxford, UK.

CORROCHER R, STEINMAYR M, RUZZENENTE O, BRUGNARA C, BERTINATO L, MAZZI M, FURRI C, BONFANTI & de SANDRE (1985). Elevation of red cell sodium-lithium countertransport in hyperlipidaemias. Life Sci 36: 649-655.

CORRY DB, TUCK ML, BRICKMAN AS, YANAGAWA N & LEE DBN (1986). Sodium transport in red blood cells from dialyzed uremic patients. Kidney Int 29: 1197-1202.

CORRY DB, LEE DBN & TUCK ML (1987). A kinetic study of cation transport in erythrocytes from uremic patients. Kidney Int 32: 256-260.

COTTON JR, WOODWARD T, CARTER NW & KNOCHEL JP (1979). Resting skeletal muscle membrane potential as an index of uraemic toxicity. J Clin Invest 63: 501-506.

COTTON JR, WOODWARD T & KNOCHEL JP (1985). Correction of uremia cellular injury with a protein-restricted amino acid-supplemented diet. Am J Kidney Dis 5: 233-236.

CUMBERBATCH M & MORGAN DB (1981). Relations between sodium transport and sodium concentration in human erythrocytes in health and disease. Clin Sci 60: 555-564.

CUNNINGHAM JN, CARTER NW, RECTOR FC & SELDIN DW (1971). Resting transmembrane potential difference of skeletal muscle in normal subjects and severely ill patients. J Clin Investig 50: 49-59.

CUSI D, BARLASSINA C, FERRANDI M, PALAZZI P, CELEGA E & BIANCHI G (1981). Relationship between altered Na^+-K^+ cotransport and Na^+/Li^+ countertransport in the erythrocytes of 'essential' hypertensives patients. Cli Sci 61: 33s-36s.

DACIE JV & LEWIS SM (1991). Practical haematology. Churchill Livingstone, London.

D'AMICO G & SANNA G, editors (1991). Lipid abnormalities in renal disease. Kidney International - Supplement 31.

DAWIDOWICZ EA (1987). Lipid exchange: transmembrane movement, spontaneous movement, and protein-mediated transfer of lipids and cholesterol. Current Topics in Membrane and Transport 29: 175-202.

DEFERRARI G, GARIBOTTO G, ROBAUDO C, SAFFIOTI S, GURRERI G, PAOLETTI E, PASSERONE GC & TIZIANELLO A (1987). Abnormalities in amino acid metabolism in patients with chronic renal failure. Contr Nephrol 55: 11-19.

DEFRONZO RA, ALVESTRAND A, SMITH D, HENDLER R, HENDLER E & WAHREN J (1981). Insulin resistance in uremia. J Clin Invest 67: 563-568.

DEFRONZO RA, HENDLER R & SIMONSON D (1982). Insulin resistance is a prominent feature of insulin-dependent diabetes. Diabetes 31: 795-801.

DEFRONZO RA, SMITH D & ALVESTRAND A (1983). Insulin action in uremia. Kidney Int 24 (S16): S102-S114.

DE HEMPMPTINNE A, MARRANNES R & VANHEEL B (1983). Influence of organic acid s on intracellular pH. Am J Physiol 245: C178-C183.

DE LA SIERRA A, COCA A, AGUILERA MT & URBANO-MARQUEZ A (1988). Na-

Li countertransport in essential hypertension. J Hypertens 6: 931-937.

DENTON RM & HALESTRAP AP (1979). Regulation of pyruvate metabolism in mammalian tissues. Essays in Biochemistry 15: 37-77.

DESANTO NG, TREVISAN M, DECOLLE S, DIMURO M, DECHIARA F, LATTE M, FRANZESE A, IACONO R, CAPASSO G, CAPODICASA G & GIORDANE C (1987). Intraerythrocytic cation metabolism in children with uremia undergoing hemodialysis. J Lab Clin Med 110: 231-236.

DESFORGES JF (1970). Anemia in uremia. Arch Int Med 126: 808-811.

DEUTICKE B (1980). Kinetic properties and substrate specificity of the monocarboxylate carrier in the human erythrocyte membrane. In: Membrane transport in erythrocytes, eds: Lassen UV, Ussing HH & Wieth JO, Musksgaard, Copenhagen: 539-555.

DEUTICKE B (1982). Monocarboxylate transport in erythrocytes. J Membrane Biol 70: 89-103.

DEUTICKE B (1989). Monocarboxylate transport in red blood cells: Kinetics and chemical modification. Methods in Enzymology 173-329.

DEUTICKE B & HAEST CWM (1987). Lipid modulation of transport proteins in vertebrate cell membranes. Ann Rev Physiol 49: 221-235.

DEUTICKE B, RICKERT I & BEYER E (1978). Steroselective, SH-dependent transfer of lactate in mammalian erythrocytes. Bioch Biophys Acta 507: 137-155.

DEUTICKE B, BEYER E & FORST B (1982). Discrimination of three parallel pathways of lactate transport in the human erythocyte membrane by inhibitors and kinetics properties. Bioch Biophys Acta 684: 96-110.

DEUTSCH C, TAYLOR JS & PRICE M (1984). pH homeostasis in human lymphocytes: modulation by ions and mitogen. J Cell Biol 98: 885-894.

DEVÉS R & KRUPKA RM (1978). Testing transport models with substrate and reversible inhibitors. Biochim Biophys Acta 513: 156-172.

DEVÉS R & KRUPKA RM (1979a). The binding and translocation steps in transport as related to substrate structure. A study of the choline carrier of erythrocytes. Biochim Biophys Acta 557: 469-485.

DEVÉS R & KRUPKA RM (1979b). A general kinetic analysis of transport. Tests of the carrier model based on predicted relations among experimental parameters. Biochim Biophys acta 556: 533-547.

DEVÉS R & KRUPKA RM (1979c). A simple experimental approach to the determination of carrier transport parameters for unlabelled substrate analogs. Biochim Biophys Acta 556: 524-532.

DEVÉS R & KRUPKA RM (1981a). Evidence for a two-state mobile carrier mechanism in erythrocyte choline transport: effects of substrate analogs on inactivation of the carrier by N-ethylmaleimide. J Membrane Biol 61: 21-30.

DEVÉS R & KRUPKA RM (1981b). Reaction of internal forms of the choline carrier of erythrocytes with N-ethylmaleimide: evidence for a carrrier conformational change on complex formation. J Membrane Biol 63: 99-103.

DEVÉS R & KRUPKA RM (1983).Apparent noncompetitive inhibition of choline transport in erythrocytes by inhibitors bound at substrate site. J Membrane Biol 74: 183-189.

DEVÉS R, REYES G & KRUPKA RM (1986). The carrier reorientation step in erythrocyet choline transport: pH effects and the involvement of a carrier ionizing group. J Membrane Biol 93: 165-175.

DEVÉS R & KRUPKA RM (1987). Effects on transport of rapidly penetrating competing substrates: activation and inhibition of the choline carrier in erythrocytes by imidazole. J

Membrane Biol 99: 13-23.

de WARDENER HE, MILLS IH, CLAPHAM WG & HAYTER CJ (1961). Studies on the efferent mechanism of the sodium diuresis which follows administration of intravenous saline in the dog. Clin Sci 21: 249-258.

DIEPLINGER H, SCHOENFELD PY & FIELDING CJ (1986). Plasma cholesterol metabolism in end-stage renal disease. Difference between treatment by hemodialysis or peritoneal dialysis. J Clin Invest 77: 1071-1083.

DÍEZ J, VIRTO R, YAP L, DÍAZ-TEJEIRO R, ERRASTI P & PURROY A (1986). Uremia and red blood cell sodium transport. Nephron 43: 155-157.

DIESEL W, NOAKES TD, SWANEPOEL C & LAMBERT M (1990). Isokinetic muscle strenghth predicts maximum exercise tolerance in renal patients on chronic haemodialysis. Am J Kidney Dis 2: 109-114.

DORIA A, FIORETTO P, AVOGARO A, CARRARO A, MOROCUTTI A, TREVISAN R, FRIGATO F, CREPALDI G, VIBERTI G & NOSADINI R (1991). Insulin resistance is associated with high sodium-lithium countertransport in essential hypertension. Am J Physiol 261: E684-E691.

DORUS E, PANDEY GN & DAVIS JM (1975). Genetic determinations of lithium ion. An in vitro and in vivo monozigotic -dizygotic twin study. Arch Gen Psychiatry 32: 1097-1102.

DORUS E, COX NJ, GIBBONS RD, SHAUGHNESSY R, PANDEY GN & CLONINGER R (1983). Lithium ion transport and affective disorders within families of bipolar patients: identification of major locus. Arch Gen Psychiatry 40: 545-552.

DRUML W, KELLY RA, MAY RC & MITCH WE (1988). Abnormal cation transport in uremia. J Clin Invest 81: 1197-1203.

DRUML W, KELLY RA, ENGLAND BK, O'HARA DS & MITCH WE (1990). Effects

of acute and chronic uremia on active cation transport in rat myocardium. Kidney Int 38: 1061-1067.

DRURY PL, WATKINS PJ, VIBERTI GC & WALKER JD (1989). Diabetic nephropathy. Br Med Bull 45: 127-147.

DUBINSKY WP & RACKER E (1978). The mechanism of lactate transport in human erythrocytes. J Membrane Biol 44: 25-36.

DUBOSE TD (1984). Acid-base balance. In: EKNOYAN G & KNOCHEL JP, editors. The systemic consequences of renal failure., Grune & Stratton, Orlando, FL, USA, 421-442.

DUDEJA PK, FOSTER ES, BRASITUS TA (1986). Regulation of Na/H exchange by transmethylation reactions in rat colonic brush-border membranes. Biochim Biophys Acta 859: 61-68.

DUDEJA PK, FOSTER ES, BRASITUS (1987a). Modulation of rat distal colonic brush-border membrane Na-H exchange by dexamethasone: role of lipid fluidity. Biochim Biophys Acta 905: 485-493.

DUDEJA PK, FOSTER ES, DAHIYA R & BRASITUS TA (1987b). Modulation of Na-H exchange by ethinyl estradiol in rat colonic brush-border membrane vesicles. Biochim Biophys Acta 899: 22-228.

DUDEJA PK, WALI RK, KLITZKE A, SITRIN MD & BRASITUS TA (1991). Correction of enhanced Na-H exchange of rat small intestine brush-border membranes in streptozotocin-induced diabetes by insulin or 1,25-dihydroxycholecalciferol. J Clin Invest 87: 1755-1762.

DUHM J (1989). Possible role of plasma lipids in alterations of cell mebrane sodium transport in essential hypertension: patogenetic implications. In: MEYER P & MARCHE P, editors. Blood cells and arteries in hypertension and atherosclerosis. Raven Press, New York, 247-

270.

DUHM J & BECKER BF (1977a). Studies on the lithium transport across the red cell membrane. II. Characterization of ouabain-sensitive and ouabain-insensitive Li^+ transport. Effects of bicarbonate and dipyrydamole. Plügers Archiv 367: 211-219.

DUHM J & BECKER BF (1977b). Studies on the lithium transport across the red cell membrane. III. Factors contributing to the intraindividual variability of the in vitro Li^+ distribuition across the human red cell membrane. Plügers Archiv 368: 203-208.

DUHM J & BECKER BF (1977c). Studies on the lithium transport across the red cell membrane. IV. Interindividual variations of the Na^+-dependent Li^+ countertransport system of human erythrocytes. Plügers Archiv 370: 211-219.

DUHM J & BECKER BF (1979). Studies on lithium transport across the red cell membrane. V. On the nature of the Na-dependent Li countertransport system of mammalian erythrocytes. J Membr Biol 51: 263-286.

DUHM J & BEHR J (1986). Role of exogenous factors in alterations of red cell Na^+-Li^+ exchange and Na^+-K^+ cotransport in essential hypertension, primary hyperaldosteronism, and hypokalemia. Scan J Clin Lab Invest 46 (S 180): 82-95.

DUHM J & GÖBEL BO (1982). Sodium-lithium exchange and sodium-potassium cotransport in human erythrocytes. Part 1: evaluation of a simple uptake test to assess the activity of the two transport systems. Hypertension 4: 468-476.

DUHM J, EISENRIED F, BECKER BF & GREIL W (1976). Studies on the lithium transport across the red cell membrane. I. Li^+ uphill transport by the sodium-dependent Li^+ counter-transport system of human erythrocytes. Plügers Archiv 364: 147-155.

DUHM J, GÖBEL BO, LORENZ R & WEDER PC (1982). Sodium-lithium exchange and sodium-potassium cotransport in human erythrocytes. Part 2: a simple uptake test apllied to

normotensive and hypertensive individuals.Hypertension 4: 477-482.

DUNHAM PB & SENYK O (1977). Lithium efflux through the Na/K pump in human erythrocytes. Proc Nat Acad Sci USA 74: 3099-3103.

DWIGHT JFStJ, MENDES RIBEIRO AC & HENDRY BM (1992). Membrane incorporation of non-esterified fatty acids and effects on the sodium pump of human erythrocytes. Clin Sci 82: 99-104.

DZURIK R, SPUSTOVA V & GERYKOVA M (1986). Pathogenesis and consequences of alteration of glucose metabolism in renal insufficiency. In: RINGOIR S, VANHOLDER R & MASSRY SG, editors. Uremic toxins. Series: Advances in experimental medicine and biology, Volume 223, Plenum press, New York, 105-110.

EARLE K, WALKER J, HILL C & VIBERTI G (1992). Familial clustering of cardiovascular disease in patients with insulin-dependent diabetes and nephropathy. N Engl J Med 326: 673-677.

ECKLES NE, TAYLOR CB, CAMPBELL DJ & GGOULD RJ (1955). The origin of plasma chollesterol and the rates of equilibrium of liver, plasma and erythrocyte cholesterol. J Lab Clin Med 466: 359-371.

EDLUND GL & HALESTRAP AP (1988). The kinetics of transport of lactate and pyruvate into rat hepatocytes. Evidence for the prsence of a specific carrrier similar to that in erythrocytes. Biochm J 249: 117-126.

EDMONDSON RPS, HILTON PJ, JONES NF, PATRICK J, THOMAS RD (1975). Leucocyte sodium transport in uraemia. Clin Sci 49: 213-216.

EDWARDS PAW (1973). Evidence for the carrier model of transport from the inhibition by n-Ethylmaleimide of choline transport across the human red cell membrane. Biochim Biophys Acta 311: 123-140.

EHRLICH BE & DIAMOND JM (1980) Lithium, membranes and manic-depressive illness. J Membrane Biol 52: 187-200.

EHRLICH BE & DIAMOND JM (1979). Lithium fluxes in human erythocytes. Am. J. Physiol. 237 (1): C102-C110.

EHRLICH BE, DIAMOND JM, FRY V & MEIER K (1983). Lithium's inhibition of erythrocyte cation countertransport involves a slow process in the erythrocyte. J Membrane Biol 75: 233-240.

EKNOYAN G & KNOCHEL JP, editors (1984). The systemic consequences of renal failure. Grune & Stratton, Inc. Orlando, FL, USA.

ELLORY JC & LEW VL, editors (1977). Membrane transport in red cells. Academic Press, London, UK.

ELLORY JCE & WOLOWYK MW (1979). A new density gradient technique for age separation of human erythrocytes and reticulocytes. J Physiol 295: 9-10p.

ELLORY JC & YOUNG JD, editors (1982). Red cell membranes a methodological approach. Biological Techniques Series, Academic Press, London.

ELLORY JC, ELFORD BC & NEWBOLD CI (1988a). Introductory remarks: transport mechanisms across cell membranes. Parasitology 96: S5-S9.

ELLORY JC, FERVENZA FC & HENDRY BM (1988b). Human erythrocyte sodium pump function in chronic renal failure (abstract). J Physiol 403: 10p.

ELLORY JC, FERVENZA FC, HARVEY CM & HENDRY BM (1988c). Erythrocyte amino acid transport in patients on haemodialysis (abstract). J Physiol 407: 10p.

ELLORY JC, FERVENZA FC, HARVEY CM & HENDRY BM (1988d). Heterogeneous amino acid exchange in the human erythrocyte membrane (abstract). J Physiol 407: 11p.

ELVING LD, WELTZELS JFM, DE NOBEL & BERDEN JHM (1991). Erythrocyte

sodium-lithium countertransport is not different in type 1 (insulin-dependent) diabetic patients with or without diabetic nephropathy. Diabetologia 34: 126-128.

ELVING LD, WELTZELS JFM, DE PONT JJHHM & BERDEN JHM (1992). Is increased erythrocyte sodium-lithium countertransport a useful marker for diabetic nephropathy? Kidney Int 41: 862-871.

ENGELMANN B & DUHM J (1991). Effect of cholesterol and dipalmitoyl phosphatidylcholine enrichement on the kinetics of Na-Li exchange of human erythrocytes. J Membrane Biol 122: 231-238.

ERSLEV AJ (1970). Anemia of chronic renal disease. Arch Int Med 126: 774-780.

ESCHBACH JW (1989). The anemia of chronic renal failure: pathophysiology and the effects of recombinant erythropoietin. Kidney Int 35: 134-148.

ESCHBACH JW (1989a). Hematological problems of dialysis patients. In: MAHER JF, editor. Replacement of renal function by dialysis, Chapter 40, Kluwer Academic Publishers, Dordrecht, Holland, 851-864.

ESTRADA C, BREADY J, BERLINER J & CANCILLA PA (1990). Choline uptake by cerebral capillary endothelial cells in culture. J Neurochem 54: 1467-1473.

EVANS WH& GRAHAM JM (1989). Membrane Structure and Function. Ed: D Rickwood, IRL Press at Oxford University Press, Oxford, UK.

FAFOURNOUX P, DMIGNE C & REMESY C (1985). Carrier-mediated uptake of lactate in rat hepatocytes. Effects of pH and possible mechanisms for L-lactate transport. J Biol Chem 260: 292-299.

FANESTIL DD (1968). Renal Na-K ATPase relationship to total functional renal mass. Nature 218: 176-177.

FARIA JBL, FRIEDMAN R, TARIQ T & VIBERTI G (1992). Prevalence of raised

sodium-lithium countertransport activity in type 1 diabetic patients. Kidney Int 41: 877-882.

FEINLIEB M, KANNEL WB, TEDESCHI GE, LANDAU TK & GARRISON RJ (1979). The relation of ante-morten characteristics to cardiovascular findings at necropsy: "The Framingham Study". Atherosclerosis 34: 145-157..

FERRANNINI E, BUZZIGOLI G, BONADONNA R, GIORICO MA, OLEGGINI M, GRAZIADEI L, PEDRINELLI R, BRANDI L & BEVILACQUA S (1987). Insulin resistance in essential hypertension. N Engl J Med 317: 350-357.

FERVENZA FC, HENDRY BM, ELLORY JC & OLIVER DO (1988). Altered amino acid transport in erythrocyte from patients on haemodialysis (abstract). Nephrol Dial Transplant 3: 539-540.

FERVENZA FC, HENDRY BM & ELLORY JC (1989a). Effect of dialysis and transplantation on red cell Na pump function in renal failure. Nephron 53: 121-128.

FERVENZA FC, HARVEY CM, HENDRY BM & ELLORY JC (1989b). Increased lysine transport capacity in erythrocyte from patients with chronic renal failure. Clin Sci 76: 419-422.

FERVENZA FC, MEREDITH D, ELLORY JC & HENDRY BM (1990). A study of erythrocyte amino acid transport in patients with chronic renal failure. Nephrol Dial Transplant 5: 594-599.

FERVENZA FC, MEREDITH D, ELLORY JC & HENDRY BM (1990a). Altered membrane transport of choline in haemodialysis patients (abstract). Renal Failure 12: 280.

FERVENZA FC, MEREDITH D, ELLORY JC & HENDRY BM (1991a). Uridine transport in human erythrocytes: data from normal subjects and from patients with renal failure. Experimental Physiology 76: 53-58.

FERVENZA FC, MEREDITH D, ELLORY JC & HENDRY BM (1991b).Abnormal erythrocyte choline transport in patients with chronic renal failure. Clin Sci 80: 137-141.

FIEHN W (1978). The effect of experimental uremia on potassium-activated phosphatase from erythrocyte and cardiac membranes. Clin Chim Acta 84: 149-152.

FIEHN W, SEILER D & HEIMBERG K-W (1976). Transport ATPases of cardiac sarcolemma in experimental uremia. Clin Chim Acta 73: 93-96.

FINEAN JB & MICHELL RH (1981). Isolation, composition and general structure of membranes. In: FINEAN JB & MICHELL RH, editors. Membrane structure, Series: New Comprehensive Biochemistry, Volume 1, Elsevier/North-Holland Biomedical Press, New York, 1981, 1-36.

FINE LG, BADIE-DEFOOLY B, LOWE AG, HAMZEH A, WELLS J & SALEMOGHADDAM S (1985). Stimulation of Na^+/H^+ antiport is an early event in hyperthrophy of renal proximal tubule cells. Proc Natl Acad Sci USA 82: 1736-1740.

FINE LG, HOLLEY RW, NASRI H & BADIE-DEFOOLY B (1985a). BSC-1 growth inhibitor transforms a mitogenic stimulus into hyperthrophic stimulus for renal proximal tubular cells: relationship to Na^+/H^+ antiporter activity. Proc Natl Acad Sci USA 82: 6163-6166.

FINE LG, NORMAN JT, KUJUBU DA & KNECHT A (1992). Renal hyperthrophy. In: SELDIN DW & GIEBISCH G, editors, The kidney: Physiology and pathophysiology, Second edition, Raven Press, New York, USA: 3113-3133.

FISCHER M, LEVINE PH, LEAF A (1989). N-3 Fatty acids and cellular aspects of atherosclerosis. Arch Int Med 149: 1726-1728.

FISHBEIN WN (1986). Lactate transporter defect: A new disease of muscle. Science 234: 1254-1256.

FISHBEIN WN, FOELLMER JW, DAVIS JI, FISHBEIN TM & ARMBRUSTMACHE R (1988a). Clinical assay of the human erythrocyte lactate transporter. I: Principles, procedure and variation. Biochem Med Metab Biol 39: 338-350.

FISHBEIN WN, DAVIS JI, FOELLMER JW & CASEY MR (1988b). Clinical assay of the human erythrocyte lactate transporter.

II: Analysis and display of normal human data. Biochem Med Metab Biol 39: 351-359.

FOSTER DW (1989). Insulin resistance- secret killer. N Engl J Med 370: 733-734.

FRANCHI A, CRAGOE E & POYSSÉGUR J (1986). Isolation and properties of fibroblasts mutants overexpressing an altered Na/H antiporter. J Biol Chem 261: 14614-14622.

FRASER CL, SARNACKI P & ARIEFF AI (1985a). Abnormal sodium transport in synaptosomes from brain of uraemic rats. J Clin Invest 75: 2014-2023.

FRASER CL, SARNACKI P & ARIEFF AI (1985b). Calcium transport abnormality in uremic rat brain synaptosomes. J Clin Invest 76: 1789-1795.

FRELIN C, VIGNE P, BARBRY P & LAZDUNSKI M (1987). Molecular properties of amiloride action and of its Na transporting targets. Kidney Int 32: 785-793.

FRIEDMAN E, SHERMAN KA, FERRIS SH, REISBERG B, BARTUS RT & SCHNECK MK (1981) Clinical response to choline plus piracetam in senile demential: relation to red-cell choline levels. N Engl J Med 304: 1490-1491.

FRÖLICH J, SCHÖLMERICH J, HOPPE-SEYLER G, MAIER KP, TALKE H, SCHOLLMEYER P & GEROK W (1974). The effect of acute uraemia on gluconeogenesis in isolated perfused rat livers. Eur J Clin Invest 4: 453-458.

FRÖLICH J, HOPPE-SEYLER G, SCHOLLMEYER P, MAIER KP & GEROK W (1977). Possible sites of interaction of acute renal failure with amino acid utilization for gluconeogenesis in isolated perfused rat liver. Eur J Clin Invest 7: 261-268.

FROMMER JP & KURTZMAN NA (1984). Water and electrolyte abnormalities in chronic renal failure. IN: "The systemic consequences of renal failure". EDs: EKNOYAN G & KNOCHEL JP, Grune & Stratton Inc, Orlando, FL, USA: 405-420.

FUNDER J, TOSTESON DC & WIETH JO (1978). Effects of bicarbonate on lithium transport in human red cells. J Gen Physiol 71: 721-746.

GALTON DJ & THOMPSON GR, editors (1990). Lipids and cardiovascular disease. Br Med Bull 46 (4): 865-1087.

GANZ MB, BOYARSKY G, STERZEL B & BORON W (1989). Arginine vasopressin enhances pHi regulation in the presence of HCO_3^- by stimulating three acid-base transport systems. Nature 337: 648-651.

GARAY RP, DAGHER G, PERNOLLET M-G, DEVYNCK M-A & MEYER P (1980). Inherited defect in a Na,K-cotransport system in erythrocytes from hypertensive patients. Nature 284: 281-283.

GARBER AJ (1978a). Skeletal muscle protein and aminoacid metabolism in experimental chronic uremia in the rat. J Clin Invest 62: 623-632.

GARBER AJ (1978). The regulation of skeletal muscle alanine and glutamine formation and release in experimental chronic uremia in the rat. J Clin Invest 62: 633-641.

GARBER AM, SOX HC & LITTENBERG B (1989). Screening asymptomatic adults for cardiac risk factors: the serum cholesterol level. Ann Int Med 110: 622-639.

GARDINER JE & PATON WDM (1972). The control of the plasma choline concentration in the cat. J Physiol 227: 71-86.

GENNIS RB (1989). Biomembranes. Molecular structure and function. Springer-Verlag, New York, USA.

GIARDINI O, TACCONE-GALLUCCI M, LUBRANO R, RICCIARDI-TENORE G, BANDINO D, SILVI I, RUBERTO U & CASCIANO CU (1984). Evidence of red cell membrane peroxidation in haemodialysis patients. Nephron 36: 235-237.

GIBBONS GF (1986). Hyperlipaedaemia of diabetes. Clin Sci 71: 477-486.

GIER JD, NOORDAM PC, van ECHTELD CAJ, MANDERSLOOT JG, BIJLVELD C, VERKLEIJ J, CULLIS PR & de KRUIFF B (1980). The barrier function of membrane lipids. In: LASSEN UV, USSING HH & WIETH JO, Membrane transport in erythrocytes, Alfred Besson Symposium 14, Munsksgaard, Copenhagen, 75-90.

GLEN AIM, YATES CM, SIMPSON J, CHRISTIE JE, SHERING A, WHALLEY LJ & JELLINEK EH (1981). Choline uptake in patients with Alzheimer's pre-senile dementia. Psychological Medicine 11: 469-476.

GOLDSTEIN JL & BROWN MS (1983). Familial hypercholesterolaemia. In: STANBURY JB, WYNGAARDEN JB, FREDERICKSON DS, GOLDSTEIN JL, BROWN MS, editors. Metabolic basis of inherited disease, McGraw-Hill, New York, 672-712.

GOLDSMITH DJA & HILTON PJ (1991). The effect of bicarbonate on the regulation of intracellular pH by human leucocytes (abstract). Clin Sci 80 (S24): 33p.

GOLDSMITH DJA & HILTON PJ (1992). Relationship between intracellular proton buffering capacity and intracellular pH. KIdney Int 41: 43-49.

GOLDSMITH DJA, POSTON L, CRAGOE EJ & HILTON PJ (1989). 5-(N,N-hexamethylene)amiloride is an effective inhibitor of Na/H exchange in human leucocytes (abstract). Clin Sci 80 (S24): 32

GOLDSMITH DJA, POSTON L, WATSON M, MORRIS J, HILTON PJ & CRAGOE EJ (1990). Effect of autologous serum on human leucocyte Na/H exchange and intracellular pH. Clin Sci 79: 357-364.

GOLDSMITH DJA, TRIBE RM, POSTON L, CAPPUCCIO FP, MARKANDU ND, MACGREGOR GA & HILTON PJ (1991). Leucocyte intracellular pH and Na-H exchange activity in essential hypertension: an in vitro study under physiological conditions. J Hypertension 9: 645-653.

GOLDSMITH DJA, TRIBE RM & HILTON PJ (1991a). The use of human leucocytes in vitro to study the effect of bicarbonate administration on intracellular pH, and pCO_2 (abstract). Clin Sci 80 (S24): 32p.

GONICK HC, KRAMER HJ, PAUL W & LU E (1977). Circulating inhibitor of the sodium-potassium activated adenosine triphosphatase after expansion of extracellular fluid volume in rats. Clin Sci 53: 329-334.

GOULD GW (1992). Tissue-specific expression of glucose transporters in normal and diseased states. J Physiol (In the press).

GRAVES SW, BROWN B & VALDES R (1983). An edogenous digitalis-like substance in patients with renal impairement. Ann Int Med 99: 604-608.

GRAVES SW & WILLIAMS GH (1987). Endogenous digitalis-like natriuretic factors. Ann Rev Med 38: 433-444.

GREEN JW (1949). The relative rate of penetration of the lower saturated monocarboxylic acids into mammalian erythrocytes. J Cell Comp Physiol 33: 247-266.

GREEN J & MUALLEM S (1989). A common mechanism for activation of the Na^+/H^+ exchanger by different types of stimuli. FASEB J 3: 2408-2414.

GREENWALD BS, EDASERY J, MOHS RC, SHAH N, TRIGOS GG & DAVIS KL. Red blood cell choline. I: Choline in Alzheimer's sisease. Biol. Psychiatry 20: 367-374, 1985.

GREGG VA & REITHMEIER RAF (1983). Effect of cholesterol on phosphate uptakee by human red blood cells. FEBS Lett 157: 159-164.

GRINSTEIN, editor (1988). Na^+/H^+ Exchanger. CRC Press, Boca Raton, Fl, USA.

GRINSTEIN S & DIXON SJ (1989). Ion transport, membrane potential, and cytoplasmin pH in lymphocytes: changes during activation. Physiol Rev 69: 417-541.

GRINSTEIN S & FURUYA W (1986). Characterization of the amiloride-sensitive Na^+/H^+

antiport of human neutrophils. Am J Physiol 250C: C283-C291.

GRINSTEIN S & ROTHSTEIN A (1986). Mechanisms of regulation of the Na^+/H^+ exchanger. J Membrane Biol 90: 1-12.

GRINSTEIN S, COHEN S & ROTHSTEIN OD (1984). Cytoplasmic pH regulation in thymic lymphocytes by amiloride sensitive Na^+/H^+ antiport. J Gen Physiol 83: 341-369.

GRINSTEIN S, GOETZ JD, FURUYA W, ROTHSTEIN A & GEFALD EW (1984a). Amiloride -sensitive Na/H exchange in platelets and leucocytes. Detection by electronic cell sizing. Am J Physiol 247: C293-C298.

GRINSTEIN S, GOETZ JD, COHEN S & ROTHSTEIN A (1985). Osmotic and phorbol ester-induced activation of Na/H role of protein phosphorylation in lymphocyte volume regulation. J Cell Biol 101: 269-276.

GRINSTEIN S, FURUYA W & BIGGAR WD (1986). Cytoplasmic pH regulation in normal and abnormal neutrophils. J Biol Chem 261-512.

GRINSTEIN S, FURUYA W, NASMITH PE & ROTSTEIN OD (1988a). Na^+/H^+ exchange and the regulation of intracellular pH in polymorphonuclear leucocytes. Comp Biochem Physiol[A] 90A: 543-549.

GRINSTEIN S, FURUYA W & ROTSTEIN OD (1988b). Intracellular pH regulation in neutrophils: properties and distribution of the Na^+/H^+ antiporter. In: GUNN RB & PARKER JC, editors. Cell Physiology of the Blood, The Rockefeller University Press, New York, 303-314.

GRINSTEIN S, ROTIN D & ROTHSTEIN A (1989a). Na^+/H^+ exchange growth-induced cytosolic pH changes. Role in cellular proliferation. Biochim Biophys Acta 988: 73-97.

GRINSTEIN S, COHEN S, GOETZ-SMITH JD & DIXON SJ (1989b). Detection of Na/H antiporter in lymphocytes. Methods in Enzymology 173: 777-790.

GRUNDY SM (1988). HMG CoA reductase for the treatment of hypercholesterolemia.

N Engl J Med 319: 24-33.

GRUNZE M, FORST B & DEUTICKE B (1980). Dual effect of membrane cholesterol on simple and mediated tranport processes in human erythrocytes. Biochim Biophys Acta 600: 860-869.

GUISADO R, ARIEFF AC, MASSRY S (1977). Muscle water and electrolytes in uremia and the effects of hemodialysis. J Lab Clin Med 89: 322-331.

GUNN RB (1985). Bumetanide inhibition of anion exchange in human red blood cells (abstract). Biophysical J 47: 326a.

HAAG-WEBER M, HABLE M, FIEDLER G, BLUM I, SCHOLLMEYER P, KREUβER W & HÖRL WH (1991). Alterations of polymorphonuclear leukocyte glycogen metabolism and glucose uptake in dialysis patients. Am J Kidney Dis 17: 562-568.

HAAS M, SCHOOLER J & TOSTESON DC (1975). Coupling of lithium to sodium transport in human red cells. Nature 258: 425-427.

HADDDY FG, PAMNANI MB, CLOUGH DL (1979). Humoral factors and the sodium-potassium pump in volume expanded hypertension. Life Sci 24: 2105-2118.

HAGA T & NODA H (1973). Choline uptake systems of rat brain synaptosomes. Biochim Biophys Acta 291: 564-575.

HAGERMAN JS & GOULD RG (1951). The in vitro interchange of cholesterol between plasma and red cells. Proc Soc Exp Biol Med 78: 329-332.

HAJEM S, MOREAU T, HANNAERT P, LELLOUCH J, ORSSAUD G, HUEL G, CLAUDE JR & GARAY RP (1990). Erythrocyte cation transport systems and plasma lipids in a general male population. J Hypertension 8: 891-896.

HALESTRAP AP (1975). The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. Biochem J 148: 85-96.

HALESTRAP AP (1976). Transport of pyruvate and lactate into human erythrocyte. Evidence for the involvement of the chloride carrier and a chloride-independent carrier. Biochem J 156: 193-207.

HALESTRAP AP & DENTON RM (1974). Specific inhibition of pyruvate transport in rat liver mitochondria and human red blood cell by α-cyano-4-hydroxycinnamate. Biochem J 138: 313-316.

HALESTRAP AP & POOLE RC (1989). The transport of pyruvate and lactate across mitochondrial and plasma membranes. IN: "Anion transport protein of the red blood cell membrane", Eds: HAMASAKI N & JENNINGS ML, Elsevier Science Publishers BV, 73-86.

HALESTRAP AP, POOLE RC & CRANMER SL (1990). Mechanisms and regulation of lactate, pyruvate and ketone body transport across the plasma membrane of mammalian cells and their metabolic consequences. Biochem Soc Trans 18: 1132-1135.

HALL AC & ELLORY JC (1986). Evidence for the presence of volume-sensitive KCl transport in young human red cells. Biochim Biophys Acta 858: 317-320.

HALKIN A, BENJAMIN N, DOKTOR HS, TODD SD, VIBERTI GC & RITTER JM (1991). Vascular responsiveness and cation exchange in insulin-dependent diabetes. Clin Sci 81: 223-232.

HANNAERT PA & GARAY RP (1986). A kinetic analysis of Na-Li Countertransport in human red blood cells. J Gen Physiol 87: 353-368.

HARAN JS & SHPORER M (1977). Proton magnetic resonance study of cholesterol transfer between egg yolk lecithin vesicles. Biochim Biophys Acta 465: 11-18.

HARGREAVES AD, LOGAN RL, THOMSON M, ELTON RA, OLIVER MF & RIEMERSA RA (1991). Total cholesterol, low density lipoprotein cholesterol, and high density lipoprotein cholesterol and coronary heart disease in Scotland. Br Med J 303: 678-681.

HARRIS RC, SEIFTER JL & BRENNER BM (1984). Adaptation of Na/H exchange in renal microvillus membrane vesicles. J Clin Invest 74: 1979-1987.

HARRIS RC, BRENNER BM & SEIFTER JL (1986). Sodium-hydrogen exchange and glucose transport in renal microvillus membrane vesicles from rats with diabetes mellitus. J Clin Invest 77: 724-733.

HASSTEDT SJ, WU LL, ASH O, KUIDA H & WILLIAMS R (1988). Hypertension and sodium-lithium countetransport in Utah pedigrees: evidence for major-locus inheritance. Am J Hum Genet 43: 14-22.

HAUBRICH DR, WAY PF, CLODY DE & WEDILKING PW (1975). Increase in rat brain acetylcholine induced by choline and deanol. Life Sci 17:975-980.

HENDRY BM & ELLORY JC (1988).Molecular sites for diuretic action. TIPS 9: 416-421.

HENÉ RJ, BOER P, KOOMANS HA & MESS EJD (1985). Sodium potassium ATPase activity in human rectal mucosa with and without renal insuficiency. Am J Kidney Dis 5: 177-181.

HIGGINS CF & HYDE SC (1991). Channeling our thoughts. Nature 352: 194-195.

HILTON PJ (1986). Cellular transport in essential hypertension. N Engl J Med 314: 222-229.

HOFFMANN EK & SIMONSEN LO (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol Rev 69: 315-381.

HÖBER R (1935). The permeability of the red blood corpuscles to organic anions. J Cell Comp Physiol 7: 367-391.

HÖFER M & HOGGETT JG (1981). Transport across biological membranes. Pitman, Marshfield, Massachusetts, USA.

HOLMAN GD (1992). Role of glucose transporters in insulin action and insulin

resistence. J Physiol (In the press).

HOOG M, NG LL & DAVIES JE (1991). Chloride influx in human leucocytes: a triple-isotope technique for the assessment of chloride transporters. Clin Sci 81: 405-412.

HRUSKA KA, KLAHR S & HAMMERMAN MR (1982). Decrease luminal membrane transport of phosphate in chronic renal failure. Am J Physiol 242: F17-F22.

HSIA SL, PEREZ GO, MENDEZ AJ, SCHIFFMAN J, FLETCHER S & STOUDEMIRE JB (1985). Defect in cholesterol transport in patients receiving maintanance hemodialysis. J Lab Clin Med 106: 53-61.

HULLEY SB (1988). Risk factors for coronary heart disease: selected recent epidemiological advances. Drugs 36 (S3): 1-4.

HUOT SJ, PAMNANI MB, CLOUGH DL, BUGGY J, BRYANT HJ, HARDER DR & HADDY FJ (1983). Sodium-potassium pump activity in reduced-mass hypertension. Hypertension 5: I94-I100.

HUNT GE, BEILHARZ GR, STORLIEN LH, JOHNSON GSF & KUCHEL PW (1984). Plasma and erythrocyte choline concentrations in rats following chronic treatment with lithium or choline. Life Sci 34: 1853-1859.

HUNT SC, WILLIAMS RR, SMITH JB & ASH KO (1986). Associations of three erythrocyte cation transport systems with plasma lipids in Utah subjects. Hypertension 8: 30-36.

HUNT SC, WU LL, HOPKINS PN ET AL (1989). Apolipoproteins, low density lipoprotein fraction and insulin. Associations with familial dyslipidaemic hypertension. Arteriosclerosis 29: 335-344.

HUNT SC, STEPHENSON SH, HOPKINS PN, HASSTEDT SJ & WILLIAMS RR (1991). A prospective study of sodium-lithium countertransport and hypertension in Utah. Hypertension 17: 1-7.

IBSEN KK, JENSEN H, WIETH JO & FUNDER J (1982). Essential hypertension: sodium-lithium countertransport in erythrocyte from patients and from children having one hyprtensive parent. Hypertension 4: 703-709.

IGARASHI P, REILLY RF, HILDEBRANDT F, BIEMESDERFER D, REBOUCAS NA, SLAYMAN CW & ARONSON PS (1991). Molecular biology of renal Na-H exchangers. Kidney Int 40 (S33): S84-S89.

IRVINE ROH & DOW J (1966). Intracellular pH and electrolyte content of voluntary muscle in renal acidosis. Clin Sci 31: 317-324.

IZUMO H, IZUMO S, DELUISE M & FLIER JS (1984). Erythrocyte Na,K pump in uremia. J Clin Invest 74: 581-588.

JACKSON PA & MORGAN DB (1982). The relation between the membrane cholesterol content and anion exchange in erythrocytes of patients with cholestasis. Biochim Biophys Acta 693: 99-104.

JACOBSON MP, RODRIGUEZ HJ, HOGAN WC & KLAHR S (1980). Mechanism of activation of renal Na-K-ATPase in the rat: effects of reduction of renal mass. Am J Physiol 239: F281-F288.

JAIN MK (1988). Introduction to biological membranes. Wiley & Sons, New York, USA.

JENNINGS ML & ADAMS-LACKEY M (1982). A rabbit erythrocyte membrane protein associated with L-lactate transport. J Biol Chem 257: 12866-12871.

JENSSEN JS, MATHIESEN ER, NORGAARD K, HOMMEL E, BORCH-JONHSEN K, FUNDER J, BRAHM J, PARVING HM & DECKERT T (1990). Increased blood pressure and sodium-lithium countertransport are not inherited in diabetic nephropathy. Diabetologia 33: 619-624.

JESSOP S & EALES L (1977). Erythrocyte electrolyte content and sodium efflux in

chronic renal failure. Nephron 18: 82-87.

JOHNSON JH, BELT JA, DUBINSKY P, ZIMMIAK A & RACKER E (1980). Inhibition of lactate transport in Ehrlich ascites tumor cells and human erythrocytes by a synthetic anhydride of lactic acid. Biochemistry 19: 3836-3840.

JONES SL, TREVISAN R, TARIQ T, SEMPLICINI A, MATTOCH M, WALKER JD, NOSADI R & VIBERTI GC (1990). Increased sodium-lithium countertransport activity in insulin-dependent diabetic patients with microalbuminuria. Hypertension 15: 570-575.

JOPE RS, JENDEN DJ, EHRLICH BF & DIAMOND JM (1978). Choline accumulates in erythrocytes during lithium therapy. N Engl J Med 299: 833-834.

JOPE RS, JENDEN DJ, EHRLICH BE, DIAMOND JM & GOSENFELD LF (1980). Erythrocyte choline concentrations are elevated in manic patients. Proc Natl Acad Sci USA 77: 6144-6146.

JORGENSON KE & SHEIKH MI (1984). Renal transport of monocarboxylic acids. Biochem J 223: 803-807.

JUEL C, BANGSBO J, GRAHAM T & SALTIN B (1990). Lactate and potassium fluxes from human skeletal muscle during and after intensive, dynamic knee extensor exercise. Acta Physiol Scand 140: 147-159.

JUHAN-VAGUE I, DRISS F, ROUL C ET AL (1984). Abnormalities of erythrocyte membrane lipids in insulin-dependent diabetes are improved by short term strict control of diabetics. Clin Hemorrheology 4: 455-459.

KAJI D & KAHN T (1987). Na-K pump in chronic renal failure. Am J Physiol 252: F785-F793.

KAHN CR (1978). Insulin resistance, insulin insensivity, and insulin unresponsiveness: a necessary distinction. Metabolism 27 (S2): 1893-1902.

KAHN AM, CRAGOE EJ, ALLEN JC, HALLIGAN RD & SHELAT H (1990). Na/H and Na-dependent Cl-HCO_3^- exchange control pHi in vascular smooth muscle. Am J Physiol 259: C134-C143.

KAHN AM, CRAGOE EJ, ALLEN JC, SEIDEL CL & SHELAT H (1991). Effects of pHi on Na/H and Na-dependent, and Na-independent Cl-HCO_3^- exchangers in vascular smooth muscle. Am J Physiol 261: C837-C844.

KANAANI J & GINSBURG H (1991). Transport of lactate in Plasmodium falciparum-infected human erythrocytes. J Cell Physiol 149: 469-476.

KANOF PD, GREENWALD BS, MOHS RC & DAVIS KL (1985). Red blood cell choline. II: Kinetics in Alzheimer's disease. Biol Psychiatry 20: 375-383.

KASTENDIECK E & MOLL W (1977). The placental tranfer of lactate and bicarbonate in the guinea-pig. Pflugers Arch 370: 165-171.

KATZ AI, TOBACK GF & LINDHEIMER MD (1976). Independence of onset of compensatory kidney growth from changes in renal function. Am J Physiol 230: 1067-10711

KEECH AC (1990). Randomised trial of cholesterol lowering with simvastatin in patients at increased risk of coronary heart disease (abstract). Symposium on lipoprotein metabolism and lipid lowering agents: F-022.

KELLY RA & SMITH TW (1989). The search for the endogenous digitalis: an alternative hypothesis. Am J Physiol 256: C937-C950.

KELLY RA, O'HARA DS, CANESSA ML, MITCH WE & SMITH TW (1985). Characterization of digitalis-like factors in human plasma. J Biol Chem 260: 11396-11405.

KELLY RA, O'HARA DS, MITCH WE, STEINMAN TI, GOLDSZER RC, SOLOMON HS & SMITH TW (1986a). Endogenous digitalis-like factors in hypertension and chronic renal insufficiency. Kidney Int 30: 723-729.

KELLY RA, O'HARA DS, MITCH WE & SMITH TW (1986b). Identification of NaK-ATPase inhibitors in human plasma as nonesterified fatty acids and lysophospholipids. J Biol Chem 261: 11704-11711.

KELLY RA, CANESSA ML, STEINMAN TI, MITCH WE (1989). Hemodialysis and red cell cation transport in uremia: role of membrane free fatty acids. Kidney Int 35: 595-603.

KELLY RA, ENGLAND BK, DRUML W & MITCH WE (1990). Specificity of abnormal cation transport in uremia: differences in myocardium from skeletal muscle (Abstract). Kidney Int 37: 510.

KIER AB, SWEET WD, COWLEN MS & SCHROEDER F (1986). Regulation of transbilayer distribuition of a fluorescent sterol in tumor cell plasma membranes. Biochim Biophys Acta 861: 287-301.

KIKERI D, ZEIDEL ML, BALLERMANN BJ, BRENNER BM & HERBERT SC (1990). pH regulation and response to AVP in A10 cells differ markedly in the presence and absence of CO_2-HCO_3^-. Am J Physiol 259: C471-C483.

KIRK K, WONG HY, ELFORD BC, NEWBOLD CI & ELLORY JC (1991). Enhanced choline and Rb^+ transport in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. Biochem J 278:521-525.

KIRK K, ELFORD BC, ELLORY JC & NEWBOLD CI (1992). A transport pathway responsible for the increased permeability of malaria-infected erythrocytes shows characteristics of a Cl^- channel. J Physiol (In the press).

KLINGENBERG M (1989). Survey of carrier methodology: strategy for identification, isolation, and characterization of transport system. Methods in enzymology 171: 12-23.

KLIP A (1988). Action of insulin on Na/H exchange. IN: GRINSTEIN S, editor. Na^+/H^+ exchanger, CRC Press, Inc., Boca Raton, FL, USA, 285-303.

KNOCHEL JP (1984). The biochemistry of renal failure. IN: EKNOYAN G & KNOCHEL JP, editors. The systemic consequences of renal failure. Grune & Stratton, Orlando, FL, USA, 1-14.

KOCH A, WEBSTER B & LOWELL S (1981). Cellular uptake of L-lactate in mouse diaphragm. Biophys J 36: 775-796.

KÖHNE W, HAEST CWM & DEUTICKE B (1981). Mediated transport of anions in band 3/phospholipid vesicles. Biochim Biophys Acta 664: 108-120.

KOMIDORI K, KAMADA T, YAMASHITA T, HARADA R, OTSUJI Y, HASHIMOTO S, CHUMAN Y & OTSUJI S (1985). Erythrocyte membrane fluidity is decreased in uraemic hemodialyzed patients. Nephron 40: 185-188.

KRAMER HJ, GOSPODINOV D & KRÜCK F (1976). Functional and metabolic studies on red blood cell sodium transport in chronic uremia. Nephron 16: 344-358.

KRAMER HJ, PENNIG J, KLINGMÙLLER D, KIPNOWSKI J, GLÄNZER K & DÙSING R (1985). Digoxin-like immunoreacting substance(s) in the serum of patients with chronic renal failure. Nephron 40: 297-302.

KROLEWSKI AS, WARRAM JH, CHRISTLIEB AR, BUSICK EJ, KAHN CR (1985). The changing natural hystory of diabetic nephropathy in type I diabetes. Am J Med 78: 785-794.

KROLEWSKI AS, CANESSA M, WARRAM JH, LAFFEL LMB, CHRISTLIEB AR, KROWLER WB, RAND LI (1988). Predisposition to hypertension and suceptibility to renal disease in insulin-dependent diabetes mellitus. N Engl J Med 318: 140-145.

KROLEWSKI AS, CANESSA M, WARRAM JH, LAFFEL LMB, CHRISTLIEB AR, KROWLER WB, RAND LI (1988a). Genetic factors and suceptibility to diabetic nephropathy (letter). N Engl J Med 319: 181-182.

KRUPKA RM & DEVÉS R (1980a). The choline transport system of erythrocytes.

Distribuition of the free carrier in the membrane. Biochim Biophys Acta 600: 228-232.

KRUPKA RM & DEVÉS R (1980b). The eletrostatic contribuition to binding in the choline transport system of erythrocytes. J Biol Chem 255: 8546-8549.

KRUPKA RM & DEVÉS R (1988). The choline carrier of erythrocytes: location of the NEM-reactive thiol group in the inner gated channel. J Membrane Biol 101: 43-47.

KUHAR MJ & ZARBIN MA (1978). Synaptosomal transport: a chloride dependence for choline, GABA, glycine and several other compounds. J Neurochem 31: 251-256.

KUTCHAI H, GEDDIS LM & MARTIN MS (1978). Mediated transport and metabolism in rat aorta. Biochim Biophys Acta 541: 312-320.

KRZESINSKI JM & RORIVE G (1983). Sodium-lithium countertranport in red cells (Letter). N Engl J Med 309: 987-988.

LACY WW (1970). Uptake of individual amino acids by perfused rat liver: effect of acute uraemia. Am J Physiol 219: 649-653.

LAFFEL L, WARRAN JH & KROLEWSKY AS (1991). Letter to the editor. Diabetologia 34: 452-453.

L'ALLEIMAN G, FRANCHI A, CRAGOE E & POYSSÉGUR J (1984). Blockade of the Na/H antiport abolishes growth factor-induced DNA synthesis in fibroblasts. J Biol Chem 259: 4313.

LAMERS JMJ (1975). Some characteristics of monocarboxylic acid transfer across the cell membrane of epithelial cells from rat small intestine. Biochm Biophys Acta 413: 265-276.

LANCET editorial (1991). Welcome to ouabain- a new steroid hormone. Lancet 338: 543-544.

LANDOUX A, KRAWICE I, CRAGOE EJ, ABITA JP & FRELIN C (1987). Properties of the Na-dependent Cl/HCO_3^- exchange system in U937 human leukemic cells. Eur J Biochen

170: 43-49.

LANGE Y & D'ALESSANDRO JS (1977). Characterization of mechanisms for transfer of cholesterol between human erythrocytes and plasma. Biochemistry 16: 4339-4343.

LANGE Y, CUTLER HB & STECK TL (1980). The effect of cholesterol and other intercalated amphipaths on the contour and stability of the isolated red cell membrane. J Biol Chem 255: 9331-9337.

LANGE Y, DOLDE J & STECK TL (1981). The rate of transmembrane movement of cholesterol in the human erythrocyte. J Biol Chem 256: 5321-5323.

LAURENZI M & TREVISAN M (1989). Sodium-lithium countertransport and blood pressure: The Gubbio Population Study. Hypertension 13: 408-415.

LEE JA, LEE HA & SADLER PJ (1991). Uraemia: is urea more important than we think? Lancet 338: 1438-1440.

LEEKS DR & HALESTRAP AP (1978). Chloride-independent transport of pyruvate and lactate across the erythrocyte membrane. Biochem Soc Trans 6: 1363-1366.

LEEKS DR & HALESTRAP AP (1979). Pyruvate and lactate transport across the cell membrane of the erythrocyte and other cells. In: QUAGLIARIELLO E et al eds, Function and molecular aspects of biomembrane transport, Elsevier/North-Holland Biomedical Press: 427-430.

LEVIN GE, COLLISON P & BARON DN (1976). The intracellular pH of human leucocytes in response to acid-base changes in vitro. Clin Sci 50: 293-299, 1976.

LEVY R, PARAN E, KEYNAN A & LIVNE A (1983). Essential hypertension: improved differentiation by the temperature dependence of Li efflux in erythrocytes. Hypertension 5: 821-827.

LEVY R & LIVNE A (1984). The erythrocyte membrane in hypertension. Characterization of the temperature dependence of lithium efflux. Biochim Biophys Acta 769: 41-

48.

LEVY R, BIALE Y, LATZER S, HEVRONI D, PARAN E, INSLER V & LIVNE A (1986a). Thermodynamic properties of erythrocyte Li efflux may facilitate detection of essential hypertension in pregnant women. J Cardiovasc Pharmacol 8(S5): S71-S75.

LEVY R, HEVRONI D, CABANTCHIK ZI & LIVNE A (1986). Li_i-Na_o countertransport and Li leak in erythrocytes are differentially affected by membrane enrichment with cholesteryl hemisuccinate. Bioch Biophys Acta 854: 325-328.

LIENHARD GE, SLOT JW, JAMES DE & MUECKLER MM (1992). How cells absorb glucose. Scientific Am, Jan 92: 86-91.

LIEB WR (1982). A kinetic approach to transport studies. IN: ELLORY JC & YOUNG JD, editors. Red cell membranes - A methodological approach. Biological Techniques Series, Academic Press, London, 135-164.

LIFTON RP, SARDET C, POYSSÉGUR J & LALOUEL J-M (1991). Cloning of the human genomic amiloride-sensitivee Na/H ntiporter gene, identification of genetic polymorphisms and localization on the genetic map of chromosome 1p. Genomics 7: 131-135.

LIFTON RP, HUNT SC, WILLIAMS, POYSSÉGUR J & LALOUEL J-M (1991a). Exclusion of the Na-H antiporter as a candidate gene in human hypertension. Hypertension 17: 8-14.

LIM VS, HENRIQUEZ C, SEO H, REFETOFF S & MARTINO E (1980). Thyroid function in uremic rat model. Evidence suggesting tissue hypothyroidism. J Clin Invest 66: 946-954.

LINDNER A, CHARRA B, SHERRARD DJ & SCHRIBNER BH (1974). Accelerated atherosclerosis in prolonged maintenance hemodialysis. N Engl J Med 290: 697-701.

LIVNE A, BALFE JW, VEITCH R, MARQUEZ-JULIO A, GRINSTEIN S, ROTHSTEIN

A (1987). Increased platelet Na^+/H^+ exchange rate in essential hypertension. Application of a novel test. Lancet ii: 533-536.

LUCIO FJ, HENDRY BM & ELLORY JC (1991). The effects of cholesterol depletion on the sodium pump in human red cells. Experimental Physiology 76: 437-443.

LUDENS JH, CLARCK MA, DUCHARME DW, HARRIS DW, LUTSKE BS, MANDEL F, MATTHEWS WR, SUTTER DM & HAMLYN JM (1991). Purification of an endogenous digitalis like factor from human plasma for structural analysis. Hypertension 17: 923-929.

LULY P (1989). Membrane lipid changes in the abnormal red cell. In: RAESS BU & TUNNICLIFF G, editors. The red cell membrane: a model for solute transport, Humana Press, Clifton, New Jersey, 399-421.

LUPO MA, CEFALU WT & PARDRIDGE WM (1990). Kinetics of lactate transport into rat liver in vivo. Metabolism 39: 374-377.

MACPHAIL S, THOMAS TH, WILKINSON R, DAVISON JM & DUNLOP W (1990). A serial study of erythrocyte sodium content and sodium pump kinetics in pregnancy. Clin Sci 79: 631-638.

MADIAS NE & KRAUT JA (1989). Uremic acidosis. In: SELDIN DW & GIEBISCH G, editors. The regulation of acid-base balance. Raven Press Ltd., New York, 285-317.

MADSHUS IH (1988). Regulation of intracellular pH in eukaryotic cells. Biochem J 250: 1-8.

MADUELL F, FRENANDEZ J & DÍEZ J (1990). Alteration of the $Cl^-/NaCo_3^-$ anion exchanger in erythrocytes of uraemic patients. Nephrol Dial Transplant 5: 1018-1022.

MAGUIRE PA & SHERMAN IW (1990). Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells. Molecular and Biochemical Parsitology 38: 105-112.

MAHER JF (1989). Replacement of renal function by dialysis. Kluwer Academic Publishers, Dordrecht, Holland.

MAHER VMG & THOMPSON GR (1990). HMG CoA reductase inhibitors as lipid lowering agents: five years experience with lovastatin and apppraisal of simvastatin and pravastatin. Quart J Med 274: 165-175.

MAHLBERG FH & ROTHBLAT GH (1992). Cellular cholesterol efflux. Role of cell membrane kinetic pools and interactions with apolipoproteins AI, AII and Cs. J Biol Chem 267: 4541-4550.

MAHNESMITH RL & AROSON PS (1985). The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circulation Research 56: 773-788.

MAIN J, THOMAS & WILKINSON R (1990). Sodium content and transsport of white cells and red blood cells in undialysed uraemic and continuous ambulatory peritoneal dialysis patients. Nephrol Dial Transplant 5: 369-375.

MAK RHK (1989). Insulin secretion in uraemia: effect of parathyroid hormone and vitamin D metabolites. Kidney Int 36 (S27): S227-S230.

MALLINGER AG, KOPP U & HANIN I (1984). Erythrocyte choline transport in drug-free and lithium-treated individuals. J Psych Res 18: 107-117.

MALOFF BL, McCALEB ML & LOOCKWOOD DH (1983). Cellular basis of insulin resistence in chronic uremia. Am J Physiol 245: E178-E184.

MANGILI R, BENDING JJ, SCOTT G, LI LK, GUPTA A, VIBERTI GC (1988). Increased sodium-lithium countertransport activity in red cells of patients with insulin-dependent diabetes and nephropathy. N Engl J Med 318: 146-150.

MANGILI R, ZERBINI G, GARBETTA F, SIRONI A & MASCHIO AD (1990). Red

blood cells sodium/lithium countertransport and kidney volume in short-term type I diabetes mellitus (abstract). Diabetes 39 (S1): 91A.

MARONI BJ, KARAPANOS G & MITCH WE (1986a). System A amino acid transport in incubated muscle: effects of insulin and acute uremia. Am J Physiol 251: F74-F80.

MARONI BJ, KARAPANOS G & MITCH WE (1986b). System ASC and sodium-independent neutral amino acid transport in muscle of uremic rats. Am J Physiol 251: F81-F86.

MARSH D (1985). ESR spin label studies of lipid-protein interactions. In: WATTS A, DE PONT JJHHM, editors. Progress in protein lipid interactions, Vol 1, Amsterdam Elsevier, 143-172.

MARTIN K (1968). Concentrative accumulation of choline by human erythrocytes. J Gen Physiol 51: 497-516.

MARTIN K (1971). Some properties of an SH group essential for choline transport in human erythrocytes. J Physiol 213: 647-667.

MARTIN K (1972). Extracellular cations and the movement of choline across the erythrocyte membrane. J Physiol 224: 207-230.

MARTIN K (1977). Choline transport in red cells. In: ELLORY JC & LEW VL, editors. Membrane transport in red cells. Academic Press, London, UK, 101-113.

MARTIN MJ, HULLEY SB, BROWNER WS, KULLER LH & WENTWORTH D (1986). Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361 662 men. Lancet: 934-936.

MASON MJ & THOMAS RC (1985). Evidence for facilitated difussion across frog skeletal muscle membranes. J Physiol (London) 361: 23P.

MASON MJ, SMITH JD, GARCIA-SOTO JJ & GRINSTEIN S (1989). Internal pH-sensitive site couples Cl-HCO_3^- exchange to Na-H antiport in lymphocytes. Am J Physiol 256:

C428-C433.

MASUDA M, MURAKAMI T, EGAWA H & MURATA K (1989). Decrease fluidity of polymorphonuclear leukocyte memnbrane in streptocitocin-induced diabetic rats. Diabetes 39: 466-470.

MATHEWS WR, DUCHARME DW, HAMLYN JM, HARRIS DW, MANDEL F, CLARK MA & LUNDES JH (1991). Mass spectral characterization of an endogenous digitalis like factor from human plasma. Hypertension 17: 930-935.

MATO JM (1991). Phospholipid organization in cellular membranes. In: MATO JM, Phospholipid metabolism in cellular signaling, CRC Press, Chapter 3: 19-32.

MATTHEWS C, HEIMBERG K-W, RITZ E, AGOSTINI B, FRITZSCHE J & HASSELBACH W (1977). Effect of 1,25-dihydroxycholecalciferol on impaired calcium transport by tha sarcoplasmatic reticulum in experimental uremia. Kidney Int 11: 227-235.

MAZZANTI L, RABINI RA, POLENTA M, LOMBARDELLO M, TOCHCINI, TESTA I & BERTOLI E (1990). A study on simvastatin effects on the erythrocyte membrane (abstract). International symposium on lipoprotein metabolism and lipid lowering agents: F-063.

MAY RC, CLARK AS, GOHEER MA & MITCH WE (1985). Specific defects in insulin-mediated muscle metabolism in acute uremia. Kidney Int 28: 490-497.

MAY RC, KELLY RA & MITCH WE (1986). Metabolic acidosis stimulates protein degradation in rat muscle by a glucocorticoid-dependent mechanism. J Clin Invest 77: 614-621.

MAY RC, KELLY RA, MITCH WE & KARAPANOS G (1987). Mechanisms for defects in muscle protein metabolism in rats with chronic uremia. Influence of metabolic acidosis. J Clin Invest 79: 1099-1103.

MAY RC, KELLY RA & MITCH WE (1991): Pathophysiology of uremia. IN: BRENNER BM & RECTOR FC, editors, The Kidney, 4th edition, WB Saunders Co., Philadelphia,

PA, USA: 1997-2018.

MCCALEB ML, MEVORACH R, FREEMAN RB, IZZO MS & LOCKWOOD DH (1984). Induction of insulin resistance in normal adipose tissue by uremic human serum. Kidney Int 25: 416-421.

MCDONALD AM, DYER AR, LIU K, STAMLER R, GOSH FC, GRIMM R, BERMAN R & STAMLER J (1988). Sodium,lithium-countertransport and blood pressure control by nutritional intervention in 'mild' hypertension. J Hypertension 6: 283-291.

MCDONALD AM, LIAO YL, TREVISAN M (1990). Sodium-lithium countertransport and systolic blood pressure response to exercise. J Hypertension 8: 129-137.

MENDELS J & FRAZER A (1973). Intracellular lithium concentration and clinical response: towards a membrane theory of depression. J Psychiatry Res 10: 9-18.

MENDELS J & FRAZER A (1974). Alterations in cell membrane activity in depression. Am J Psychiatry 131: 1240-1246.

MENGUAL R, LEBLANC G & SUDAKA P (1983). The mechanisms of Na-L-lactate cotransport by brush-border membrane vesicles from horse kidney. J Biol Chem 258: 15071-15078.

MENGUAL R & SUDAKA P (1983). The mechanisms of Na-L-lactate cotransport by brush-border membrane vesicles from horse kidney: analysis of rapid equilibrium kinetic in abscence of membrane potential. J Membrane Biol 71: 163-171.

MERRIN PK, RENTON S, FISCHER C, HENDERSON A, RICHMOND W, NICHOLAIDES A & ELKELES RS (1992). Serum lipids, macrovascular disease and arterial wall changes in type 1 diabetes (abstract). Clin Sci 82 (S26): 6p.

MICHALAK J, KADZIOLKA A, PRUSZKOWSKA R, LEDWOZYW & MADEJCZYK A (1988). Compensatory mechanisms in erythrocyte lipids in patients with atherosclerosis. Lipids

23: 476-480.

MILLER BL, JENDEN DJ, CUMMINGS JL, READ S, RICE K & BESON DF (1986). Abnormal erythrocyte choline and influx in Alzheimer's disease. Life Sci 38: 485-490.

MILLER NE (1984). Current concepts of the role of HDL in reverse cholesterol transport. In: MILLER NE & MILLER GJ, editors. Clinical and metabolic aspects of high-density lipoproteins. Elsevier Science Publishers, 187-215.

MILLER RT, POLLOCK AS (1987). Modification of the internal pH sensitivity of the Na^+/H^+ antiporter by parathyroid hormone in a cultured renal cell line. J Biol Chem 262: 9115-9120, 1987.

MIILUPALO S, SAARINEN R & MARMINIEMI J (1985). Red-cell sodium-potassium pump and sodium-lithium countertransport in human obesity. Re-evaluation of the methods and association in a Finnish population. Int J Obesity 9: 313-321.

MINKOFF L, GAERTNER G, DARAB M, MERCIER C & LEVIN ML (1972). Inhibition of brain sodium-potassium ATPase in uremic rats. J Lab Clin Med 80: 71-78.

MITCH WE & WILCOX CS (1982). Disorders of body fluid, sodium and potassium in chronic renal failure. Am J Med 72: 536-550.

MOLL W, GIRARD H & GROSS G (1980). Facilitated diffusion of lactic acid in the guinea-pig placenta. Plufugers Arch : 229-239.

MONDON CE, DOLKAS CB, REAVEN GM, ALTO P & FIELD M (1978). The site of insulin resistance in acute uremia. Diabetes 27: 571-576.

MONGEAU J-G, MAURAN P, POIROT G & DAVIGGNON A (1990). Longitudinal study of various transport abnormalities as an index of severety in children and adolescents suffering from essential hypertension. J Hypertension 8: 657-662.

MONSON JP, SMITH JA, COHEN RD & ILES RA (1982). Evidence for a lactate

transporter in the plasma membrane of the rat erythrocyte. Clin Sci 62: 411-420.

MONTI JP, GALLICE P, BAAS M, MURISASCO A & CREVAT A (1987). Modification of intra-erythrocytic homeostasis in uremic patients as studied with ^{31}P nuclear magnetic resonance. Clin Chem 33: 76-80.

MONTI JP, GALLICE P, BAZ M, MURISASCO A, CREVAT A & ELSEN R (1987). Intraerythrocytic pH variations during hemodialysis: A ^{31}P NMR study. Kidney Int 35: 871-874, 1989.

MONTROSE MH & MURER H (1988). Properties of Na^+/H^+ exchange. IN: GRINSTEIN S, editor. Na^+/H^+ exchange, CRC Press, Inc., Boca Raton, FL, USA, 57-75.

MOOLENAAR WH, TSIEN RY, van DER SAAG PT & de LATT SW (1983). Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature 304: 645-648.

MORGAN JM & MORGAN RE (1964). Study of the effect of uremic metabolites on erythrocyte glycolysis. Metabolism 13: 629-635.

MORGAN DB, STEWARD AD & DAVIDSON (1986). Relations between erythrocyte lithium efflux, blood pressure and family hystories of hypertension and cardiovascular disease: studies in a factory workforce and hypertension clinic. Journal of Hypertension 4: 609-615.

MORGAN K, SPURLOCK G, COLLINS PA & MIR MA (1989). Interaction of inhibitin with the human erythrocyte $Na(Li)_i/Na_o$ exchanger. Biochim Biophys Acta 979: 53-61.

MUJAIS SK & KURTZMAN N (1986). Regulation of renal Na-K-ATPase in the rat: effect of uninephrectomy. Am J Physiol 251: F506-F512.

MULEC H, JOHNSON S-A & BJORCK S (1990). Relation between serum cholesterol and diabetic nephropathy (abstract). Lancet 335: 1537-1538.

MURER H, HOPFER U & KINNE R (1976). Sodium/proton antiporter in brush-border

membrane vesicles isolated from rat small intestine and kidney. Biochem J 154: 597-604.

NEYSES L, LOCHER R, STIMPEL M, STRAULI R & VITTER (1985). Steroscopic modulation of calcium channel in human erythrocyte by cholesterol and its oxided derivatives. Biochem J 227: 105-112.

NG LL & BOMFORD J (1989). Altered stoichiometry of the human leucocyte Na^+/H^+ antiport with decreasing pH. Biochem J 259: 311-314.

NG LL & DUDLEY C (1989). Intracellular pH clamping of human leucocytes: a technique for determination of cellular buffering power and Na^+/H^+ antiport characteristics. Clin Sci 77: 417-423.

NG LL & HARKER M (1988). Sodium influx in human leucocytes: a novel technique for assessment of sodium-proton antiport activity. Clin Sci 75: 179-184.

NG LL, HARKER M, ABEL ED (1988). Mechanisms of leucocyte sodium influx in essential hypertension. Clin Sci 75: 521-526.

NG LL, DUDLEY C, BOMFORD J , FENNELL D & HAWLEY D (1989). Leucocyte Na^+/H^+ antiport kinetics in human hypertension (abstract). Clin SCi 76 (S20): 55p-56p.

NG LL, DUDLEY C, BOMFORD J , FENNELL D & HAWLEY D (1989). Leucocyte intracellular pH and Na^+/H^+ antiport activity in human hypertension. J Hypertens 7: 471-475.

NG LL, SIMMONS D, FRIGHI V, GARRIDO MC, BOMFORD J, FRAYN KN & HOCKADAY TD (1989). Leucocyte pH and Na^+/H^+ antiport activity in type 1 (Insulin-dependent) diabetes with nephropathy (abstract). Diabetologia 32: 522A.

NG LL, SIMMONS D, FRIGHI V, GARRIDO MC & BOMFORD J (1990a). Effect of protein kinase C modulators on the leucocyte Na^+/H^+ antiport in type I (Insulin-dependent) diabetic subjects with albuminuria. Diabetologia 33: 278-284.

NG LL, SIMMONS D, FRIGHI V, GARRIDO MC, BOMFORD J, HOCKADAY TD

(1990b). Leucocyte Na^+/H^+ antiport activity in type 1 (Insulin-dependent) diabetic patients with nephropathy. Diabetologia 33: 371-377.

NG LL & DAVIES JE (1991). HMG CoA reductase inhibitors affect Na^+/H^+ antiport activity in human lymphoblasts. Am J Physiol 261: C780-C786.

NG LL & DAVIES JE (1992). Lipids and cellular Na^+/H^+ antiport activity in diabetic nephropathy. Kidney Int 41: 872-876.

NIEMAN LK, DAVIS FB, DAVIS PJ, CUNNINGHAM EE, GUTMAN S, BLAS SD & SCHOENL M (1983). Effect of end-stage renal disease on responsiveness to calmodulin and thyroid hormone of calcium-ATPase in human red blood cells. Kidney Int 24 (S16): S167-S170.

NORD EP, HAFEZI A, KAUNITZ JD, TRIZNA W & FINE LG (1985). pH gradient-dependent incresed Na/H antiport capacity of the rabbit remanent kidney. Am J Physiol 249: F90-F98.

NORLING LL, GOLDRING D, HERNANDEZ A, ROBSON AM & LANDT M (1986). Sodium-lithium countertransport in erythrocytes of children and adolescents with hypertension. Dev Pharmacol Ther 9: 231-240.

NORTH P & FLEISCHER S (1983). Alteration of synaptic membrane cholesterol/phospholipid ratio using lipid tranfer protein. J Biol Chem 258: 1242-1253.

NORUM KR, BERG T, HELGERUD P & DREVON CA (1983). Transport of cholesterol. Physiol Rev 63: 1343-1419.

NOSADI R, VIBERTI GC, DORIA A, FIORETTO P, TREVISAN R, AVOGARO A, SEMPLICINI A, DONADON V, ZANETTE G & LUSIANI (1989). Increased Na/H countertransport activity is associated with cardiac hypertrophy and insulin resistance in hypertensive Type 1 (insulin-dependent) diabetic patients (abstract). Diabetologia 32: 523A.

NOSADI R, SEMPLICINI A, FIORETTO P, LUSIANI L, TREVISAN R, DONADON

V, ZANETTE G, NICOLOSI GL, DALL'AGLIO, ZANUTTINI D & VIBERTI GC (1991). Sodium-lithium countertransport and cardiorenal abnormalities in essential hypertension. Hypertension 18: 191-198.

OKAMOTO H, KAWAGUCHI H, MINAMI M, SAITO & YASUDA H (1989). Lipid alterations in renal membrane of stroke-prone spontaneously hypertensive rats. Hypertension 13: 456-462.

OKERLUND LS & GILLIE RJ (1988). Measurements of pH and Na^+ by nuclear magnetic resonance, IN: GRINSTEIN S, editor, Na^+/H^+ exchanger, CRC Press, Inc., Boca Raton, FL, USA, 21-43.

O'MEARE NMG, DEVERY RAM, OWENS D, COLLINS PB, JOHNSON AH & TOMKIN GH (1990). Cholesterol metabolism in alloxan-induced diabetic rabbits. Diabetes 39: 626-633.

ORLOWSKY J et al (1992). J Biol Chem in the press.

OTA K, SANAKA T, AGISHI T & NAKAJIMA O (1980). Influence of uraemic middle molecules on blood cells. Int J Art Organs: 113-115.

OWEN NE (1986). Effect of catecholamines on Na/H exchange in vascular smooth muscle cells. J Cell Biol 103: 2053-2060.

PANDEY GN, OSTROW DG, HAAS M, DORUS E, CASPER, DAVIS JM & TOSTESON DC (1977). Abnormal lithium and sodium transporter in erythrocytes of manic patient and some members of his family. Proc Nat Acad Sci 74: 3607-36011

PANDEY GN, SARKARDI B, HAAS M, GUNN RB, DAVIS JM & TOSTESON DC (1978). Lithium transport pathways in human red blood cells. J Gen Physiology 72: 233-247.

PARKER JC (1980). Hypertension and the red cell. N Engl J Med 302: 804-805.

PARRISH A, ZIKIRA M, TOMACELLI C & KENNEY R (1990). Carbohydrate

utilization in exercising muscle in subtotally nephrectomized rabbits. Nephron 55: 187-192.

PARRISH AE (1981). The effect of minimal exercise on blood lactate in azotemic subjects. Clin Nephrol 16: 35-39.

PATRICK J & JONES NF (1974). Cell sodium, potassium and water in uraemia and the effects of regular dialysis as studied in the leucocyte. Clin Sci 46: 583-590.

PATRICK J, JONES NF, BRADFORD B & GAUNT J (1972). Leucocyte potassium uraemia: comparisons with erythrocyte potassium and total exchangeable potassium. Clin Sci 43: 669-678.

PENPARGKUL S, BHAN AK & SCHEUER J (1976). Studies of subcellular control factors in hearts of uremic rats. J Lab Clin Med 88: 563-570.

PEUCHANT E, SALLES C, VALLOT C, WONE C & JENSEN R (1988). Increase of erythrocyte resistance to hemolysis and modification of membrane lipids induced by hemodialysis. Clin Chim Acta 178: 271-282.

POLLOCK AS, WARNOCK DG & STREWLER GJ (1986). Parathyroid hormone inhibition of Na^+-H^+ antiporter activity in a cultured cell line. Am J Physiol 250: F217-F225, 1986.

POOLE RC, HALESTRAP AP, PRICE SJ & LEVI AJ (1989). The kinetics of trasnport of lactate and pyruvate into isolated cardiac myocytes from guinea pig. Kinetic evidence for the presence of a carrier distinct from that in erythrocytes and hepatocytes. Biochem J 264: 409-418.

POOLE RC, HALESTRAP AP & JENNINGS ML (1990). The erythrocyte lactate transporter. Book of abstracts of The Dundee Meeting of The European Red Cell Club held in July 1990.

POSTNOV YV & ORLOV SN (1985). Ion transport across plasma membrane in primary hypertension. Physiol Rev 65: 904-945.

POSTNOV YV (1990). An approach to the explanation of cell membrane alteration in

primary hypertension. Hypertension 15: 332-337.

POUYSSÉGUR J, FRANCHI A, L'ALLEMAIN G, MAGNALDO I, PARIS S & SARDET C (1987). Genetic approach to structure, function, and regulation of the Na^+/H^+ antiporter. Kidney Int 32 (S23): s144-s149.

POUYSSÉGUR J, FRANCHI A, LANGARDE A & SARDET C (1987). Molecular genetics of the mammalian Na^+/H^+ antiporter. IN: GRINSTEIN S, editor. Na^+/H^+ exchange, ED:CRC Press, Inc., Boca Raton, FL, USA, 337-347.

PREISIG PA & ALPERN RJ (1991). Increased Na/H antiporter and Na/3HCO_3 symporter activities in chronic hyperfiltration. A model of cell hyperthrophy. J Gen Physiol 97: 195-217.

PUTNAM RW (1988). Basic principles of pH regulation. IN: GRINSTEIN S, Na^+/H^+ exchange, CRC Press, Inc., Boca Raton, FL, USA, 139-153.

QUARELLO F, BOERO R, GUARENA C, ROSATI C, GIRAUDO G, GIACCHINO F & PICCOLI (1985). Acute effects of hemodialysis on erythrocytes sodium fluxes in uremic patients. Nephron 41: 22-25.

REAVEN GM (1988). Insulin resistence in human disease. Diabetes 37: 1595-1608.

REDGRAVE J, CANESSA M,GLEASON R, HOLLENBERG NK & WILLIAMS (1989). Red cell sodium-lithium countertransport in non-modulating essential hypertension. Hypertension 13: 721-725.

REINERTSEN KV, TONNESSEN TI, JACOBSEN J, SANDVIG K & OLSNES S (1988). Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. J Biol Chem 263: 11117-11125.

RELMAN AS (1972). Metabolic consequences of acid-base disorders. Kidney Int 1: 347-359.

RELMAN AS (1978). Lactic acidosis. Contemporary issues in Nephrol 2: 65-100.

RENNICK B (1974). Choline and the organic transport system. In: WESSON LG& FANELLI GM, editors, Recents advances in renal physiology and pharmacology, Medical and techincal publishing Co., Lancaster, England: 81-97.

RENNICK B, ACARA M, HYSERT P & MOOKERJEE B (1976). Choline loss during hemodialysis: homeostatic control of plasma choline concentrations. Kidney Int 10: 329-335.

REUSS L & PETERSON KV (1985). Cyclic AMP inhibits Na/H exchange in apical membrane of Necturus gallbladder. J Gen Physiol 85: 409.

RINGOIR S, VANHOLDER R & MASSRY SG, Eds (1986). Uremic toxins. Series: Advances in experimental medicine and biology, Volume 223, Plenum press, New York.

RINK TJ, TSIEN RY & POZZAN T (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. J Cell Biol 95: 189-196.

RIORDAN JR, ROMMENS JM, KEREM B-S, ALON N, ROZMAHEL R, GRZELCZAK Z, ZIELENSKI J, LOK S, PLAVSIC, CHOU J-L, DRUMM MC, IANNUZZI MC, COLLINS FS & TSUI LC (1989). Identification of the cystic fibrosis gene cloning and characterization of complementary DNA. Science 245: 1066-1073.

ROONEY MW, LANGE Y & KAUFFMANN JW 1984). Acyl chain organization and protein secondary structure in cholesterol-modified erythrocyte membranes. J Biol Chem 259: 8281-8285.

ROOS A & BORON WF (1981). Intracellular pH. Physiological Reviews 61: 296-434.

ROTHE KF, HARZMANN R & SCHORER (1984). Influences of experimental uremia on the intra- and extracellular acid-base status of the rat. Uro Int 39: 332-335.

RUIZ P, GOMEZ F & SCHREIBER AD (1990). Impaired function of macrophage Fc_γ receptors in end-stage renal disease. N Engl J Med 322: 717-722.

RUSSELL JM (1988). Methods for the study of Na^+/H^+ exchange: ion-selective electrodes,

IN: GRINSTEIN S, editor, Na^+/H^+ exchanger, CRC Press, Inc., Boca Raton, FL, USA, 45-54.

RUTHERFORD PA, THOMAS TH & WILKINSON R (1990). Increased erythrocyte sodium-lithium countertransport in essential hypertension is due to an increase affinity for extracellular sodium. Clin Sci 79: 365-369.

RUTHERFORD PA, THOMAS TH & WILKINSON R (1992). Erythrocyte sodium-lithium countertransport: clinically useful pathophysiologically instructive or just phenomenology? Clin Sci 82: 341-352.

RUTHERFORD PA, THOMAS TH, CARR S, TAYLOR R & WILKINSON R (1992a). Changes in erythrocyte sodium-lithium countertransport kinetics in diabetic nephropathy. Clin Sci 82: 301-307.

RUTHERFORD PA, THOMAS TH, CARR S, TAYLOR R & WILKINSON R (1992b). Kinetics of sodium-lithium countertransport in patients with uncomplicated type I diabetes. Clin Sci 82: 291-299.

SACKS FM, STAMPFER MJ, MONOZ A, McMANUS K, CANESSA M & KASS EH (1987). Effect of linoleic and oleic acids on blood pressure, blood viscosity, and erythrocyte cation transport. J Am Coll Nutr 6: 179-185.

SACKS G & FLEISCHER S (1989). Transport machinery: an overview. Methods in enzymology 171: 3-12.

SACKTOR B & KINSELLA J (1986). Hormonal regulation of Na-H exchange activity. Curr Top Membr Transp 26: 223-244.

SACKTOR B & KINSELLA J (1988). Regulation of Na/H exchange activity by adaptative mechanisms. IN: GRINSTEIN S, Na^+/H^+ exchange, CRC Press, Inc., Boca Raton, FL, USA, 307-334.

SAGE SO, JOBSON TM & RINK TJ (1990). Agonist-evoked changes in cytosolic pH

and calcium concentration in human platelets: studies in physiological bicarbonate. J Physiol 420: 31-45.

SALEH AM & BATLLE DC (1991). Basic mechanisms of intracellular pH homeostasis in lymphocytes. Seminars in Nephrology 1: 3-15.

SALEHMOGHADDAM S, BRADLEY T, MIKHAIL N, BADIE-DEZFOOLY B, NORD EP, TRIZNA W, KHEYFETS R & FINE LG (1985). Hyperthrophy of basolateral Na-K pump activity in the proximal tubule of the remant kidney. Lab Invest 53: 443-452.

SALIHAGIC A, MACKOVIC M, BANFIC H & SABOLIC I (1988). Short-term and long-term stimulation of Na-H exchange in cortical brush-border membranes during compensatory growth of the rat kidney. Pflügers Arch 413: 190-196.

SAMUELSON O (1988). Experiences from hypertension trials: impact of other risk factors. Drugs 36 (S3): 9-20.

SARDET C, FRANCHI A & POUYSSÉGUR J (1989). Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. Cell 56: 271-280.

SARDET C, COUNILLON L, FRANCHI A & POUYSSÉGUR J (1990). Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. Science 247: 723-726.

SARDET C, WAKABAYASHI S, COUNILLON L, FAFOURNOUX P, PAGES G & POUYSSÉGUR J (1992). Structure-function of the Na^+/H^+ antiporter: molecular mechanisms of its activation. J Physiol (in the press).

SARKARDI B, ALIFIMOFF JK, GUNN RB & TOSTESON DC (1978). Kinetics and stoichiometry of Na-dependent Li transport in human red blood cells. J Gen Physiology 72: 249-265.

SAVIG M, RUDOY J, ROTSTEIN A, FISHER N & BEN-ARI J (1991). Exercise

tolerance of end-stage renal disease patients. Nephron 57: 424-427.

SCHON DA, SILVA P & HAYSLETT JP (1974). Mechanism of potassium excretion in renal insufficiency. Am J Physiol 227: 1323-1330.

SCHOTS A, VANHOLDER R, RINGOIR S & CRAMERS C (1986). Retation patterns. In: RINGOIR S, VANHOLDER R & MASSRY SG, editors. Uremic toxins. Series: Advances in experimental medicine and biology, Volume 223, Plenum press, New York, 19-26.

SEIFTER JL & HARRIS RC (1986). Adaptation of Na-H exchange in the proximal tubule: studies in microvillus membrane vesicles. Curr Top Membr Transp 26: 245-261.

SELBY JV, FITZSIMMONS SC, NEWMAN JM, KATZ PP, SEPE S & SHOWSTACK J (1990). The natural history and epidemiology of diabetic nephropathy. Implications for prevention and control. JAMA 263: 1954-1960.

SEMPLICINI A, SPALVINS A & CANESSA M (1989). Kinetics and stoichiometry of the human red cell Na/H exchanger. J Membrane Biol 107: 219-228.

SEMPLICINI A, MOZZATO MG, SAHA B, NOSADI R, FIORETTO P, TRVISAN R, PESSINA AC, CREPALDI G, PALU CD (1989). Na/H and Li/Na exchange in red blood cells of patients with insulin-dependent diabetes mellitus (IDDM). Am J Hypertension 2: 174-177.

SEMPLICINI A, LUSIANI M, MARZOLA M, CEOLOTTO G, MOZZATO MG, ZANETTE G, DONADON V, STEFANINI MG, ZANUTTINI & PESSINA AC (1992). Erythrocyte Li/Na and Na/H exchange, cardiac anatomy and function in insulin-dependent diabetics. Eur J Clin Invest 22: 254-259.

SHINITZKY M, BOROCHOV H & WILBRANDT W (1980). Lipid fluidity as a physiological regulator of membrane transport and enzyme activities. In: LASSEN UV, USSING HH & WIETH JO, Membrane transport in erythrocytes, Alfred Besson Symposium 14, Munsksgaard, Copenhagen, 91-107.

SHINITZKY M & INBAR M (1974). Difference in microviscosity induced by different cholesterol levels in the surface membrane lipid bilayer of normal lymphocytes and malignant lymphoma cells. J Mol Biol 85: 603-615.

SIMCHOWITZ L (1988). Properties of the principal anion-exchange mechanism in human neutrophils. In: GUNN RB & PARKER JC, editors. Cell Physiology of the Blood, The Rockefeller University Press, New York, 193-314.

SIMCHOWITZ L & DAVIS AO (1990). Intracellular pH recovery from alkalinization. Characterization of chloride and bicarbonate transport by the anion exchange system in human neutrophils. J Gen Physiol 96: 1037-1059.

SIMCHOWITZ L & ROOS A (1985). Regulation of intracellular pH in human neutrophils. J Gen Physiol 85: 443-470.

SIMCHOWITZ L & WEER P (1986). Chloride movements in human neutrophils. Diffusion, exchange and active transport. J Gen Physiol 88: 167-194.

SIMCHOWITZ L, RATZLAFF R & WEER P (1986). Anion/Anion exchange in human neutrophils. J Gen Physiol 88: 195-217.

SIMCHOWITZ L, TEXTOR JA & VOGT SK (1991). Use of tributylin to probe contribution of CL-HCO_3^- exchange to regulation of steady-state pHi in human neutrophils. Am J Physiol 261: C906-C915.

SIMENHOFF ML (1975). Metabolism and toxicity of aliphatic amines. Kidney Int 7: S314-S317.

SIMENHOFF ML, ASATOOR AM, MILNE MD & ZILVA JF (1963). Retention of aliphatic amines in uraemia. Clin Sci 25: 65-77.

SIMENHOFF ML, SAUKKONEN JJ, BURKE JF, WESSON LG & SCHAEDLER RW (1976). Amine metabolism and small bowel in uraemia. Lancet: 818-821.

SIMENHOFF ML, GINN HE & TESCHAN PE (1977). Toxicity of aliphatic amines in uremia. Trans Am Soc Artif Intern Organs 23: 560-561.

SIMENHOFF ML, SAUKKONEN JJ, BURKE JF, SCHAEDLER RW, VOGEL WH, BOVEE K & LASKER N (1978). Importance of aliphatic amines in uremia. Kidney Int 13: S16-S19.

SIMON G (1989). Different cation transport inhibitor in malignant renal hypertension and in uraemia. Clin Sci 76: 329-33.

SINGER SJ & NICOLSON GL (1972). The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

SLATER EE & MACDONALD JS (1988). Mechanism of action and biological profile of HMG CoA reductase inhibitors: a new therapeutic alternative. Drugs 36 (S3): 72-82.

SMITH JB, ASH KO, GREGORY MC, SPROWELL WL, HENTSCHEL WM & WILLIAMS (1984). Hemodialysis does not affect erythrocyte sodium-lithium countertransport. Clin Chim Acta 143: 275-279.

SMITH EKM & WELT LG (1970). The red blood cell as a model for the study of uraemic toxins. Arch Int Med 126: 827-831.

SMITH JB, PRICE AL, WILLIAMS RR, HENTSCHEL, SPROWELL W, HUNT SC & ASH KO (1982). A reproducible sodium-lithium countertransport assay: the outcome of changing key laboratory parameters. Clin Chim Acta 122: 327-335.

SMITH JB, ASH KO, HUNT SC ET AL (1984). Three red cell sodium transport system in hypertensive and normotensive Utah adults. Hypertension 6: 159-166.

SPECTOR AA & YOREK MA (1985). Membrane lipid composition and cellular function. Journal of lipid research 26: 1015-1035.

SPENCER TL & LEHNINGER AL (1976). L-Lactate transport in Ehrlich ascites tumor

cells. Biochem J 154: 405-414.

STEELE JA, POZNANSKY MJ, DOUGLAS CE & BRODWICK MJ (1981). Lipid vesicle alterations of membrane cholesterol levels: effect of Na and K channels currents in squid axon. J Membr Biol 63: 191-198.

STEIN WD (1986). Transport and diffusion across cell membrane. Academic Press INC, San Diego, CA, USA.

STEIN WD (1989). Kinetics of transport: Analyzing, testing, and characterizing models using kinetics approaches. Methods in enzymology 171: 23-62.

STEIN WD (1990). Channels, Carriers, and Pumps. An introduction to membrane transport. Academic Press INC, San Diego, CA, USA.

STEINER G (1988). Diabetes and atherosclerosis: metabolic links. Drugs 36 (3): 22-26.

STRAZZULLO P & CANESSA M (1990). Kinetics of the human lymphocyte Na/H exchanger. Clin Sci 79: 531-536.

STRINGER W, CASABURI R & WASSERMAN K (1992). Acid-base regulation during exercise and recovery in humans. J Appl Physiol 72: 954-961.

STUBBS CD (1983). Structure and dynamics of membrane lipids. Essays Biochm 19: 1-39.

STUBBS CD & SMITH AD (1984). The modification of mammalian membrane fluidity and function. Biochim. Biophys. Acta 779: 89-137.

SUZUKI Y, FAUSTO A, HRUSKA KA & AVIOLI LV (1987). Stimulation of phosphatidylcholine biosynthesis in diabetic hyperthrophic kidneys. Endocrinology 120: 595-601.

SWALES JD (1990). Functional disturbance of the cell membrane in hypertension. J Hypertension 8 (S7): S203-S211.

SWALLOW CJ, GRINSTEIN S & ROTSTEIN OD (1990). Regulation and functional

significance of cytoplasmic pH in phagocytic leucocytes. Current topics in membranes and transport 35: 227-247.

SWAMINATHAN R (1984). Red blood cell sodium transport and phosphate release in uremia (Letter). Nephron 37: 142.

SWAMINATHAN R, CLEGG G, CUMBERBATCH M, ZAREIAN Z & McKENNA F (1982). Erythrocyte sodium transport in chronic renal failure. Clin Sci 62: 489-494.

SWEADNER KJ & GOLDIN SM (1980). Active transport of sodium and potassium ions. N Engl J Med 302: 777-783.

SYME PD, ARNOLDA L, GREEN Y, ARONSON JK, GRAHAME-SMITH DG & RADDA GK (1990). Evidence for increased in vivo Na-H antiporter activity and an altered skeletal muscle contractile response in spontaneously hypertensive rat. J Hypertension 8: 1027-1036.

SYME PD, ARONSON JK, THOMPSON GH, WILLIAMS EM, GREEN Y & RADDA GK (1991). Na/H and HCO_3^-/Cl exchange in the control of intracellular pH in vivo in the spontaneously hypertensive rat. Clin Sci 81: 743-750.

TALBOT N, HARVEY CM & HENDRY BM (1992). Characterization of human erythrocyte choline transport in chronic renal failure. J Physiol 446: 355p.

TAMURA M, HARRIS TM, HIGASHIMORI K, SWEETMAN BJ, BLAIR IA & INAGAMI T (1987). Lysophosphatidylcholine containing polyunsaturated fatty acids were found as Na,K-ATPase inhibitors in acutely-expanded hog. Biochemistry 26: 2797-2806.

THOMAS RC (1989). Bicarbonate and pHi response (News and Views). Nature 337: 601.

TIZIANELLO A, DEFERRARI G, GURRERI G & ACQUARONE M (1977). Effect of metabolic alkalosis, metabolic acidosis and uraemia on whole body intracellular pH in man. Clin Sci 52: 125-135.

TIZIANELLO A, DEFERRARI G, GARIBOTTO G, ROBAUDO C, SAFFIOTI S, GURRERI G & PAOLETTI E (1987). Abnormalities in amino acid metabolism in patients with chronic renal failure. Contr Nephrol 55: 1-10.

TOBACK FG (1984). Phosphatidylcholine metabolism during renal growth and regeneration. Am J Physiol 246: F249-FF259.

TOOKE JE (1989). Microcirculation and diabetes. Br Med Bull 45: 206-223.

TOSTESON DC (1981). Cation countertransport and cotransport in human red cells. Federation Proceeedings 40 (5): 1429-1433.

TOSTESON DC, ADRAGNA N, BIZE I, SOLOMON H & CANESSA M (1981). Membranes, ions and hypertension. Clin Sci 61: 5s-10s.

TREVISAN M, OSTROW D, COOPER R, LIU K, SPARKS S & STAMLER J (1981a). Methodological assessment of assays for red cell sodium concentration and sodium dependent-lithium efflux. Clin Chim Acta 116: 319-329.

TREVISAN M, COOPER R, OSTROW D, MILLER W, SPARKS MS, LEONAS Y, ALLEN A, STEINHAUER & STAMLER J (1981). Dietary sodium, erythrocyte sodium concentration, sodium-stimulated lithium efflux and blood pressure. Cli Sci 61: 39s-32s.

TREVISAN M, OSTROW D, COOPER R, LIU K, SPARKS S, OKONEK A, STEVENS E, MARQUADT J & STAMLER J (1983). Abnormal red blood cell ion transport and hypertension. The people's gas company study. Hypertension 5: 363-367.

TREVISAN M, DE SANTO N, LAURENZI M, DI MURO M, DE CHIARA F, LATTE M, FRANZESE A, IACONE R, CAPODICASA G & GIORDANO C (1986). Intracellular ion metabolism in erythrocyte and uraemia: the effect of different dialysis treatments. Clin Sci 71: 545-552.

TREVISAN R, LI LK, WALKER JD & VIBERTI GC (1989). Overactivity of Na^+/H^+

antiport and enhanced cell growth in fibroblasts of type 1 (Insulin-dependent) diabetic patients with nephropathy (abstract). Diabetologia 32: 549A.

TREVISAN R, JONES S, TARIQ T, SEMPLICINI A, MATTOCK M & VIBERTI G (1990). Increased sodium-lithium countertransport activity (Na/Li CT) in insulin dependent diabetics (IDDs) with microalbuminuria (abstract). Diabetes 39 (S1): 72A.

TREVISAN R, NOSADINI R, FIORETTO P, SEMPLICINI A, DONADON V, DORIA A, NICOLOSI G, ZANUTTINI D, CIPOLLINA MR, LUSIANI L, AVOGARO A, CREPALDI G & VIBERTI G (1992). Clustering of risk factors in hyperetensive insulin-dependent diabetics with high sodium-lithium countertransport. Kidney Int 41: 855-861.

TROSPER TL & PHILIPSON KD (1987). Lactate transport by cardiac sarcolemmal vesicles. Am J Physiol 252: C483-C489.

TROSPER TL & PHILIPSON KD (1989). Functional characteristics of the cardiac sarcolemmal monocarboxylate transporter. J Membrane Biol 112: 15-23.

TSIEN RY (1989). Fluorescent indicators of ion concentrations. Methods in Cell Biology 30: 127-156.

TUCKER EM & YOUNG JD (1982). Reticulocyte maturation in culture. IN: "Red cell membranes- A methodological approach" Eds:ELLORY JC & YOUNG JD, Biological Techniques Series, Academic Press, London, 31-66.

TUNSTALL-PEDOE H & SMITH WCS (1990). Cholesterol as a risk factor in coronary heart disease. Br Med Bulletin 46: 1075-1087.

TURNER ST & MICHELS VV (1991). Sodium-lithium countertransport and hypertension in Rochester, Minnesota. Hypertension 18: 183-190.

TURNER ST, JOHNSON M, BOERWINCKLE E, RICHELSON E, TASWELL HF & SING CF (1985). Sodium-lithium countertransport and blood pressure in healthy blood donors.

Hypertension 7: 955-962.

TURNER ST, BOERWINKLE E, JOHNSON M, RICHELSON E & SING CF (1987). Sodium-lithium countertransport in ambulatory hypertensive and normotensive patients. Hypertension 9: 24-34.

UNEY JB, MARCHBANKS RM & MARSH A (1985). The effect of lithium on choline transport in human erythrocytes. J Neurology, Neurosurgery ans Psychiatry 48: 229-233.

VANHOLDER R, SCHOOTS A & RINGOIR S (1989). Uremic toxixcity. In: MAHER JF, editor. Replacement of renal function by dialysis, Kluwer Academic Publishers, Dordrecht, Holland, 4-19.

VAUGHAN-JONES RD (1988). pH slective microelectrodes: construction and use in investigation of transmembrane sodium-hydrogen exchange. IN: GRINSTEIN S, editor: Na^+/H^+ exchanger, CRC Press, Inc., Boca Raton, FL, USA, 3-19.

VELEZ RL & HENRICH WL (1984). The vasculature. In: EKNOYAN G & KNOCHEL JP, editors. The systemic consequences of renal failure., Grune & Stratton, Orlando, FL, USA, 93-112.

VERKLEIJ AJ, ZWAAL RFA, ROELOFSEN B, COMFURIUS P, KASTELIJN D & VAN DEENEN LLM (1975). The assymetric distribuition of phospholipids in the human red cell membrane. Biochim Biophys Acta 323: 178-193.

VERKMAN AS & FRASER CL (1986). Water and nonelectrolyte permeability in brain synaptosomes isolated from normal and uremic rats. Am J Physiol 250: R306-R312.

VERMES I, STEINMETZ ET, ZEYEN LJJM, VAN DER VEEN EA (1987). Rheological properties of white cells are changes in diabetic patients with microvascular complications. Diabetologia 30: 434-436.

VIBERTI GC, HILL RD, JARRETT RD, MAHMUD U & KEEN H (1982).

Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet i: 1430-1432.

VILJOEN M, OLIVEIRA AA & MILNE FJ (1991). Physical properties of the red blood cells in chronic renal failure. Nephron 59; 271-278.

VILLAMIL MF, RETTORI V & KLEEMAN CR (1968). Sodium transport by bloood cells in uraemia. J Lab Clin Med 72: 308-317.

VYAS S & O'REGAN S (1985). Reconstituition of carrier-mediated choline transport in proteiliposomes prepared from presynaptic membranes of *Torpedo* electric organ, and its internal and external ionic requirements. J Membr Biol 85: 111-119.

WALKER JD, TARIQ T & VIBERTI GC (1989). Sodium-lithium countertransport activity in parents of type 1 (insulin-dependent) diabetic patients with nephropathy. Diabetologia 32: 555A.

WALKER JD, TARIQ T & VIBERTI GC (1990). Sodium-lithium countertransport activity in red cells of patients with insulin dependet diabetes and nephropathy and their parents. Br Med J 301: 635-638.

WALKER JF (1988). HMG CoA reductase inhibitors. Current clinical experience. Drugs 36 (S3): 83-86

WALTER U & BECHT E (1983). Red blood cell sodium transport and phosphate release in uremia. Nephron 34: 35-41.

WARNOCK DG (1988). Uremic acidosis. Kidney Int 34: 278-287.

WASSERMAN K & CASABURI R (1991). Acid-base regulation during exercise in humans. In: WHIPP BJ & WASSERMAN K & CASABURI R, editors. Exercise. Pulmonary physiology and patolophysiology, 405-448.

WATT PW, MACLENNAN PA, HUNDAL HS, KURET CM & RENNIE MJ (1988).

L(+)-Lactate tranbsport in perfused rat skeletal muscle: kinetic characteristics and sensitivity to pH and transport inhibitors. Biochim Biophys Acta 944: 213-222.

WATTS GF, LEWIS B, BRUNT JNH, LEWIS ES, COLTART DJ, SMITH LDR, MANN JI & SWAN AV (1992). Effects on coronary artery disease of lipid lowering diet, or diet plus cholestyramine, in the St Thomas' atherosclerosis regression study (STARS). Lancet 339: 563-569.

WEDER AB & SCHORCK NJ (1989). Mixture analysis of erythrocyte lithium-sodium countertransport and blood pressure. Hypertension 13: 145-150.

WEDER AB, TORRETTI BA & JULIUS S (1984). Racial differences in erythrocyte cation transport. Hypertension 6: 115-123.

WEDER AB, FITZPATRICK MA, TORRETTI BA, HINDERLITER AL, EGAN BM & JULIUS S (1986). Red cell sodium-lithium countertransport as a genetic marker in essential hypertension. J Hypertension 4 (S6): 373-375.

WEDER AB, FITZPATRICK MA, TORRETI BA, HINDERLITER AL, EGAN BM & JULIUS S (1987). Red blood cell Li-Na countertransport, Na-K cotransport, and the hemodynamics of hypertension. Hypertension 9: 459-466.

WEDER AB, SCHORK NJ, KRAUSE L & JULIUS S (1991). Red cell lithium-sodium countertransport in the Tecumseh blood pressure study. Hypertension 17: 652-660.

WEDER AB, SCHORK NJ & JULIUS S (1991a). Linkage of MN locus and erythrocyte lithium-sodium countertransport in Tecumseh, Michingan. Hypertension 17: 652-660.

WEDER AB, SERR C, TORRETI BA, BASSETT DR & ZWEIFLER (1991). Effects of lovastatin treatment on red blood cell and platelet cation transport. Hypertension 17: 203-209.

WEINMAN EJ, DUBINSKY W & SHENOLIKAR S (1989). Regulation of the Na^+/H^+ exchanger by protein phosphorylation. Kidney Int 36: 519-525.

WELT LG, SACHS JR & McMANUS TJ (1964). An ion transport defect from uremic

patients. Trans Assoc Amer Phys 77: 169-181.

WELT LG, SMITH EKM, DUNN MJ, CZERWINSKI A, PROCTOR H, COLE C, BALFE JW & GILTELMAN HJ (1967). Membrane transport defect: The sick cell. Trans Assoc Amer Phys 80: 217-226.

WIETH JO & BRAHM J (1985). Cellular anion transport. In: SELDIN DW & GIEBISCH G, editors. The Kidney: physiology and patophysiology. Raven Press, New York, 49-89.

WILEY JS & COOPER RA (1975). Inhibition of cation cotransport by cholesterol enrichement of human red cell membranes. Bioch Biophys Acta 413: 425-431.

WILLIAMS RR, HUNT SC, KUIDA H, SMITH JB & ASH KO (1983). Sodium-lithium countertransportm in erythrocytes from hypertensive prone families in Utah. Am J Epidemiol 118: 338-344.

WILLIAMS RR, HUNT SC, DADONE MM, ET AL (1982). Preliminary analysis of sodium-lithium countertransport and blood pressure in Utah pedigrees. Procedings of the symposium to the Ninth Scientific Meeting of the International Society of Hypertension, Acapulco, Mexico, Feb 17-20 1982. In: Villarreal H, Sambhi MP, eds. Topics in pathophysiology of hypertension. Boston: Martinus Nijhoff, 1984: 112-135.

WILLIAMS RR, HASSTEDT SJ, HUNT SC, WU LL, & ASH KO (1987). Genetic studies of cation tests and hypertension. Hypertension 10: 137-141.

WILLIAMS RR, HUNT SC, HOPKINS PN, ET AL (1988). Familial dyslipidaemic hypertension: evidence from 58 Utah families. J Am Med Assoc 259: 3579-3586.

WOLOWYCK MW (1982). Cell separation techniques. IN: "Red cell membranes- A methodological approach" Eds:ELLORY JC & YOUNG JD, Biological Techniques Series, Academic Press, London, 1-11.

WOOD DA, BUTLER SD, RIEMERSMA RA (1984). Adipocyte tissue and platelet fatty

acid and coronary artery disease in Scottish men. Lancet ii: 117-122.

WOODS JWW, FALK RJ, PITTMAN AW, KLEMMER PJ, WATSON BS & NAMBOODIRI K (1982). Increased red-cell sodium-lithium countertranport in normotensive sons of hypertensive parents. N Engl J Med 306: 593-595.

WOODS JWW, PARKER JC & WATSON BS (1983a). Pertubation of sodium-lithium countertranport in red cells. N Engl J Med 308: 1258-1261.

WOODS JWW, PARKER JC & WATSON BS (1983b). Sodium-lithium countertranport in red cells (Letter). N Engl J Med 309: 989.

WOOLF N (1988). Interaction of lipoprotein with the artery wall. Drugs 36 (S3): 45-50.

WOOLF N (1990). Pathology of atherosclerosis. Br Med Bulletin 46: 986-1004.

WORLEY RJ, HENTSCHEL WM, CORMIER C, NUTTING S, PEAD G, ZELONKOV K, SMITH JB, ASH KO & WILLIAMS RR (1982). Increased sodium-lithium countertransport in erythrocytes of pregnant women. N Engl J Med 307: 412-416.

WRIGHT EM, TURK E & HAGER KM (1992). Familial glucose-galactose malabsorption. J Physiol (In the press).

YAMORI Y, NARA Y, SHIMIZU S, MANO M & NABIKA T (1989). Common cellular mechanisms in the development of hypertension and atherosclerosis. In: MEYER P & MARCHE P, editors. Blood cells and arteries in hypertension and atherosclerosis, Raven Press, New York, 233-245.

YAP L, ARRAZOLA A, SURIA F & DIEZ J (1989). Is there increased cardiovascular risk in essential hypertensive patients with abnormal kinetics of red cell sodium-lithium countertransport. J Hypertension 7: 667-673.

YEAGLE PL (1985). Cholesterol and the cell membrane. Biochim. Biophys. Acta 822: 267-287.

YEAGLE P (1987). The membranes of cells. Academic Press, Orlando, FL.

YEAGLE PL (1989). Lipid regulation of cell membrane structure and function. FASEB J 3: 1833-1842.

YOUNG JD & ELLORY JC (1982). Flux measurements. In: "Red cell membranes- A methodological approach" Eds:ELLORY JC & YOUNG JD, Biological Techniques Series, Academic Press, London, 119-134.

YULI I, WILBRANDT W SHINITZKY M (1981). Glucose transport through cell membranes of modified lipid fluidity. Biochemistry 20: 4250-4256.

ZANNAD F, KESSLER M, ROYER RJ, & ROBERT J (1985). Effect of hemodialysis of red blood cell Na-K ATPase activity in terminal renal failure. Nephron 40: 127-128.

ZANNAD F, ROYER RJ, KESSLER M, HURIET B & ROBERT J (1982). Cation transport in erythrocyte of patients with renal failure. Nephron 32: 347-350.

ZEISEL SH, COSTA K-A, FRANKLIN PD, ALEXANDER EA, LAMONT JT, SHEARD NF & BEISER A. Choline an essential nutrient for humans. FASEB J 5: 2093-2098, 1991.

PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS

NG LL, POLI DE FIGUEIREDO CE, GARRIDO MC, BOMFORD J, ELLORY JC & HENDRY BM (1990). Leucocyte intracellular pH and Na/H antiporter aactivity in patients with chronic renal failure (abstract). *Clin Sci* 79 (S22): 9p.

POLI DE FIGUEIREDO CE, NG LL, DAVIES JE, CAZANA FJL, HENDRY BM & ELLORY JC (1990). Effect of cell cholesterol on Na/H antiporter activity in human lymphoblasts (abstract). *J Physiol* 432: 26p.

POLI DE FIGUEIREDO CE, NG LL, GARRIDO MC, DAVIES JE, ELLORY JC & HENDRY BM (1990). Leukocyte intracellular pH and Na/H antiporter in endstage diabetic nephropathy (abstract). *Nephrol Dial Transplant* 5: 667.

ELLORY JC, HALL AC, ODY SA, POLI DE FIGUEIREDO CE, CHALDER S & STUART J (1991). KCl cotransport in HbAA and HbSS red cells: activation by intracellular acidity and disappearance during maturation. In: MAGNANI M & DE FLORA A, Red cell aging. Advances in experimental medicine and biology, volume 307. Plenun Press, New York, 47-57.

POLI DE FIGUEIREDO CE, NG LL, GARRIDO MC, DAVIES JE, ELLORY JC & HENDRY BM (1991). Leukocyte intracellular pH and Na/H antiporter in uraemia and type I diabetes mellitus. *Nephrol Dial Transplant* 6: 615-620.

POLI DE FIGUEIREDO CE, NG LL, DAVIS JE, CAZANA FJL, ELLORY JC & HENDRY BM (1991). Modulation of Na/H antiporter activity in human lymphoblasts by altered membrane cholesterol. *Am J Physiol* 261: C1138-C1142.

POLI DE FIGUEIREDO CE, ELLORY JC & HENDRY BM (1992). Erythrocyte choline uptake after renal transplantation. *Lancet* 339: 146-148.

POLI DE FIGUEIREDO CE, ELLORY JC & HENDRY BM (1992). A longitudinal study of human erythrocyte choline transport after renal transplantation (abstract). *J Physiol* 446: 403p.

KIRK K, POLI DE FIGUEIREDO CE, ELFORD BC & ELLORY JC (1992). Effect of cell age on erythrocyte choline transport: implications for the incresed permeability of malaria-infected erythrocytes. *Biochemical J* 283: 617-624.

POLI DE FIGUEIREDO CE, NG LL, GARRIDO MC, DAVIES JE, ELLORY JC & HENDRY BM (1992). Leukocyte intracellular pH in uraemia and diabetes (letter reply). *Nephrol Dial Transplant* 7: (In the press).

POLI DE FIGUEIREDO CE, ELLORY JC & HENDRY BM (1992). Erythrocyte lactate transport in patients with chronic renal failure (abstract). *J Physiol* (In the press).