MEMBRANE TRANSPORT ABNORMALITIES

IN

PATIENTS WITH RENAL FAILURE

A thesis presented in part fulfilment of the

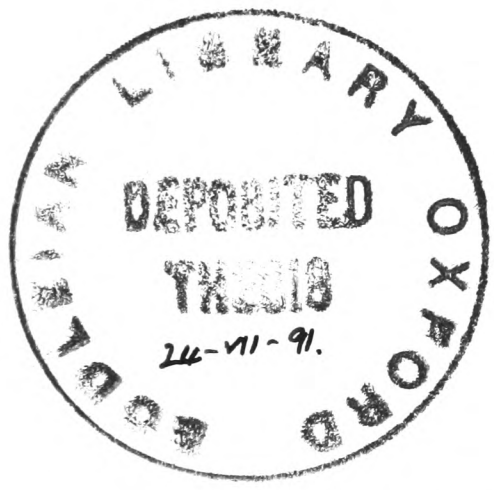
requirements for the degree

of

Doctor of Philosophy

of the

University of Oxford



by

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Michaelmas Term

1990

Jesus College

Oxford

ABSTRACT

The possibility that changes in membrane transport systems may contribute to the pathophysiology of the uraemic syndrome has not been extensively studied.

This thesis presents a study of eight erythrocyte membrane transport systems, namely the Na/K pump, the amino acid systems y^+, ASC, gly, L and T, the nucleoside and choline transporters.

The results indicate that, compared to normal controls, K^+ flux through the Na/K pump was reduced in chronic renal failure patients (CRF), on haemodialysis (HD), and on continuous ambulatory peritoneal dialysis (CAPD), but was normal in functional transplant (FT) patients' erythrocytes. The number of Na/K pumps per erythrocyte was decreased in CRF and CAPD but showed no differences between HD, FT and Normal controls. The mean turnover rate per pump site was reduced in patients on HD, whereas other groups were not significantly different from controls.

Cross-incubation experiments suggest that the lowered pump flux seen in the HD group was due to plasma factors since reversibility of the defect was achieved when those cells were incubated in normal plasma. The defect was completely reversed with a successful transplant.

Erythrocytes from haemodialysis patients exhibited an increased uptake of L-lysine through the y^+ system. The uptake of L-serine was decreased and the affinity of the ASC system for L-serine was increased in these patients compared with controls. The glycine transporter showed a significant increase in affinity for glycine. The flux of L-leucine and L-tryptophan showed no differences from control cells.

Erythrocyte membrane transport of uridine was similar in normal control cells and in those obtained from uraemic patients.

Choline influx rates were significantly increased and affinity of the transporter for choline reduced in dialysis patients' erythrocytes. Renal transplant and CRF patients showed variable influx rates which gave a significant negative correlation with creatinine clearance.

These results show that there are selective abnormalities in some membrane transport system of the erythrocyte in patients with renal failure. The mechanism and possible significance of these changes are discussed.

This thesis is dedicated to my parents

Fernando and Lorena

and to my wife

Ivete

ACKNOWLEDGEMENTS

I would like to thank all those who contributed and helped me, in one way or another, towards the completion of this thesis.

My most sincere thanks go to my parents, whose moral and financial support made it possible for me to come to Oxford, and without whom I could not have had the pleasure and privilege of coming to Oxford.

I am deeply grateful to my wife, Ivete, for her love, understanding, encouragement and support during this entire period, not always without difficulties.

I would like to thank Dr. Martin Portner in Brazil, for having given me the idea of Oxford as a place were I could broaden my knowledge and for all his encouragement and support on the stages of applying and coming to Oxford. I would also like to thank Dr. Leonel Lerner, in Brazil, for his invaluable help in preparing the necessary documents to register my former Medical School in England.

I will be always grateful to my supervisor Dr. Clive Ellory, this brilliant scientist who very kindly allowed me to work in his laboratory in the Physiology Department. He always gave me constant encouragement and skilled supervision, and his sense of humour made it a pleasure to work for him. I would consider myself very gifted if I could keep with me a tiny part of Clives's insight into scientific thinking.

I am also deeply grateful to Dr. Bruce Hendry for all his help when I first arrived in Oxford, full of enthusiasm and good will, but with no place or project to work with. He kindly introduced me to Dr. Ellory and has always been a fund of friendship, encouragement, enthusiasm, constructive ideas and optimism, even in the darkest hours. His help has been invaluable to me during the course of my studies.

I am grateful to Prof. J.G.G. Ledingham who kindly answered my first letters and gave me the opportunity of coming to Oxford.

I am profoundly grateful to Dr. D.O. Oliver, this fine nephrologist always concerned with the day to day care of his patients, who taught me how to assess and manage renal failure. He, together with Dr. C.G. Winearls and Prof. P.J. Morris gave me the golden opportunity of working and being trained at the Renal Unit, Churchill Hospital, Oxford, and allowed me to study their patients. I am very grateful to them all.

I am very grateful to Dr. G. Rushworth, my academic tutor, for all his kind attention and help during the entire period in Oxford.

I would like to thank all of the Ellory laboratory, for their help in the day-to-day problems that arose during the different stages of the thesis. In particular, I would like to thank Dr. A.C. Hall, Dr. C.M. Harvey and Mr. S. Pittman.

I am sincerely indebted to C.N.P.q. (Brazil) for having granted me a scholarship and support for of this project.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background.

This thesis presents studies of membrane transport systems in patients with renal failure and in patients with kidney transplants. The initial idea arose from the fact that some of the signs and symptoms present in uraemia suggest that a membrane transport defect may be involved in its pathogenesis. In addition, internal cell composition is known to be abnormal in uraemia and it is possible that membrane transport abnormalities may contribute to the widespread cellular dysfunction of uraemia.

This is an area in which there has been some work on a specific transport system, but a general approach to transport has not been taken before.

In fact, a defect of the sodium pump was described more than 20 years ago by Welt, studying red cells from patients with severe untreated uraemia. He found that 25% of these patients had significantly high intracellular levels of sodium, associated with a decreased Na,K-ATPase activity

(Welt, Sachs and McManus, 1964). The abnormalities could be corrected by several weeks of haemodialysis but there was no correlation between the degree of inhibition of the pump, and biochemical parameters such as plasma creatinine or urea levels. Since then, different groups have also reported an increased red cell intracellular sodium concentration in patients with renal failure (Kinsey, Smith and Welt, 1970), inhibition of the red cell membrane Na,K-ATPase (Villamil, Rettori and Kleeman, 1968; Cole, 1973; Kramer, Gospodinov and Kruck, 1976), and induction of the defect by incubating normal cells in uraemic plasma (Cole, Balfe and Welt, 1968). These studies support the view that a circulating toxin rather than an intrinsic defect in the red cell membrane, is responsible.

Decreased Na,K-ATPase activity has also been demonstrated in neurones (Fraser, Sarnacki and Arieff, 1985), brain homogenate from uraemic rats (Minkoff, Gaertner, Darab et al, 1972), leucocytes (Edmondson, Hilton, Jones et al, 1975), skeletal muscle (Cotton, Woodward, Carter and Knochel, 1979), jejunum and midgut (Kramer, Baker and Kruck, 1974) and in adipocytes (Druml, Kelly, May and Mitch, 1988). These changes also appear to occur in vivo (Boon, Aronson, Hallis et al, 1984).

Many reports support the existence of a circulating endogenous digoxin-like substance responsible for Na/K pump inhibition in uraemia (Graves, Brown and Valdes, 1983; Kelly, O 'Hara, Mitch et al, 1986b; Graves and Williams, 1987;

Walker, Bialy, Walker et al, 1987; Bosch, Hernando, Casado and Lopez-Nova, 1989). However, an intrinsic defect in the cell membrane or metabolism may also occur, and the relative contributions of these two factors have not been completely defined (Walter and Brecht, 1983; Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984; Diez, Virto, Yap et al, 1986; Kaji and Kahn, 1987). It has not been clear whether the decreased ouabain-sensitive Na/K pump activity in uraemia is due to a reduced number of pumps per erythrocyte or to a decreased pump turnover, and different views on this have been reported (Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984; Kaji and Kahn, 1987). The fact that most ion-flux and ouabain-binding experiments have been performed with erythrocytes suspended in artificial media rather than in plasma, where effects arising from a plasma factor may have been altered or lost, makes it difficult to interpret such results clearly (Welt, Sachs and McManus, 1964; Welt, Smith, Dunn et al, 1967; Walter and Brecht, 1983; Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984; Diez, Virto, Yap et al, 1986; Zannad, Royer, Kessler et al, 1982; Corry, Tuck, Brickman et al, 1986).

The experiments reported in this thesis were designed to measure both erythrocyte Na/K pump ion fluxes and specific ouabain-binding to normal red cells and red cells obtained from uraemic patients incubated in their own or autologous plasma. The aim was to obtain information about membrane

transport function under physiological conditions. With these results it was possible to assess the effect of dialysis and transplantation on the number of functional Na/K pumping sites per cell and to calculate the ion turnover rate per site. Cross-incubation experiments of cells obtained from uraemic patients in normal plasma and vice-versa were performed to test the reversibility of the effects observed and these results will be presented in Chapter 3 together with data on the NaKCl cotransport system. This system represents another route for potassium transport and was easy to study, at the same time, in the experimental protocol used for the sodium pump experiments, to see if it was also affected in uraemia.

Having studied the sodium pump in uraemia, another question arose: was the defect unique to the pump or would other transport systems be affected?

For example, plasma and intracellular amino acid concentrations are found to be abnormal in uraemia (Young and Parsons, 1970; Kopple and Swendseid, 1975; Alvestrand, Furst and Bergstrom, 1983; Tizianello, Deferrari, Garibotto et al, 1987a,b). In plasma, an increase in conjugated amino acid concentrations and both increases and decreases of individual free amino acid levels have been reported (Frimper, Thompson and Luckey, 1961; Burzynski, 1969). The most important changes are higher concentrations of several non-essential amino acids and lower concentrations of essential amino acids (Bergstrom and Furst, 1983). Low intracellular lysine

(Alvestrand, Furst and Bergstrom, 1983) valine, tyrosine and histidine (Bergstrom, Furst, Noree and Vinnars, 1972, 1975) and high concentration of aspartic acid, citrulline, ornithine and arginine have been reported in muscle (Furst, Alvestrand and Bergstrom, 1980).

The reasons for these abnormalities are not clear but nutritional imbalance and effects of uraemic toxins in amino acid transport systems could be implicated. The second possibility has not been extensively studied, although changes in amino acid uptake by system A into rat skeletal muscle have been reported in experimental uraemia (Maroni, Karapanos and Mitch, 1986). Inter-organ transport of amino acids is also altered in renal failure (Tizianello, Deferrari, Garibotto et al, 1987a,b; Deferrari, Garibotto, Robaudo et al, 1987).

It was logical therefore that the next step in investigating membrane transport in uraemia would be to look at amino acid transport systems. The experiments to be described in Chapter 4 are investigations in patients on haemodialysis of the five amino acid transport systems present in the human red cell. Each system is defined by L-lysine, L-serine, glycine, L-leucine and L-tryptophan as paradigm substrates. The aim was to test the hypothesis that abnormal internal amino acid composition reported in uraemia could be at least in part due to changes in the amino acid transport systems affected by the uraemic environment.

From these amino acid results it was clear that transport

abnormalities in uraemia were not restricted to the Na/K pump, but that other systems also showed marked changes. So what next? The red cell has other well-characterised transport systems that could have been studied but in particular two other systems caught the attention. They were the nucleoside and the choline transport systems.

Red cells obtained from uraemic patients have been reported to contain increased amounts of purine and pyrimidine nucleosides, but the reasons for this are not clear (Angle, Swanson, Stohs and Markins, 1985) and could well be due to changes in its transport. Experiments on nucleoside transport are presented in Chapter 4.

In the case of choline, its transport has been reported to be altered in Alzheimer's disease (Miller, Jenden, Cummings et al, 1986; Butterfield, Nicholas and Markesbery, 1985). Also, in patients on lithium therapy, a pronounced irreversible inhibition of the transport of choline in human red cells has been shown; when lithium is stopped the transport recovers slowly over a period of three months (Lingsch and Martin, 1976; Mallinger, Kopp and Hanin, 1984; Uney, Marchbanks and Marsh, 1985). Patients with renal failure can suffer from a variety of neurological problems including poor memory, and cognitive difficulties and a membrane transport defect could play a role in its pathogenesis.

A study of choline transport in patients with renal failure both predialysis and on dialysis (both haemodialysis

and continuous ambulatory peritoneal dialysis) and with a renal transplant will be presented on Chapter 5.

It is the intention of this thesis to demonstrate some effects that renal impairment may have on the behaviour of selected transport processes. The defects are not exclusive to the Na/K pump but occur in other systems as well. It is however, not a generalized membrane defect since some systems appeared to behave normally. Also interesting is the fact that all systems are not affected equally but there are big differences in selectivity.

Before the results are presented, a brief word of introduction will be given about the uraemic syndrome and the potential uraemic toxins. Because in this thesis a number of membrane transport systems are dealt with, a short review of the cell membrane (its structure and dynamics), transport mechanisms, its kinetics, and the role exerted by ions in transporting some substances will also be mentioned. Special emphasis will be given to each individual transport system studied in this thesis (Na/K Pump, NaKCl cotransport, Amino Acid, Nucleoside and Choline Transport) as this is thought to be necessary for a better understanding of their particular characteristics.

1.2. Uraemia.

A complete discussion about the uraemic syndrome is

beyond the scope of this thesis but a brief introduction to the subject will be given here.

The original meaning of uraemia was "urine in the blood" and carries with it the idea that a number of substances normally excreted in the urine are retained in the blood of patients with renal failure and cause toxicity (Knochel and Seldin, 1981). This would fit well with the clinical aspects of patients with severe renal impairment since uraemia resembles poisoning by an ingested toxin (Ringoir and Schoots, 1988). The syndrome encompasses a host of clinical symptoms and almost every system in the body is affected. Table 1.1. illustrates some important clinical aspects of the uraemic syndrome.

At the present time, uraemia could be defined as symptomatic renal failure together with the complications and metabolic events associated with its treatment (Mujais, Sabatini and Kurtzman, 1988). Signs and symptoms of uraemia in patients with severe renal impairment are usually not correlated with gross water or electrolyte abnormalities and therefore the idea that substances accumulated in uraemia may be responsible for the syndrome has evolved.

1.3. Uraemic Toxins.

It has been known for a long time that a rise in blood urea would follow removal of the kidneys. This finding led to

Table 1.1. Some Clinical Aspects of Uraemia:

Neurological

Malaise, Drowsiness, Insomnia, Irritability, Neuropathy, Flapping tremor, Cramps, Hiccup, Shortened attention span, Headache, Erratic memory, Stupor, Coma

Gastrointestinal

Anorexia, Nausea, Vomiting

Cardiovascular

Pericarditis, Hypertension

Haematological

Anaemia, Bleeding tendency

Endocrine

Growth retardation, Hypogonadism, Glucose intolerance, Hyperlipidaemia

Bones

Osteodystrophy, Osteomalacia

Others

Immune-response depression

the start of the chemical theory of the pathogenesis of the uraemic syndrome. The idea that substances retained in the blood when kidney function deteriorates could account for the pathogenesis of the signs and symptoms described above, was substantiated by the fact that a dramatic clinical improvement followed dialysis treatment. Since then an enormous amount of research has developed, aiming to identify the "uraemic toxin(s)" and a number of different solutes have been found to be present in abnormal quantities in the uraemic plasma.

To be considered an uraemic toxin a substance must: a) be biochemically identifiable, b) show high serum levels in renal failure, c) have a clear relationship between its levels and signs and symptoms in uraemic patients, d) give clinical improvement after lowering its level, e) provide experimental evidence of toxicity which closely resembles that seen in patients (Bergstrom and Furst, 1978).

Many <u>in vitro</u> experiments demonstrated that plasma, serum, ultrafiltrate, and dialysate from uraemic patients could affect several biological processes including erythropoiesis (Bozzini, Devoto and Tomio, 1966; Fisher, Hatch, Roh el al, 1968; Lamperi, 1974; Wallner, 1978; Savdie, Gibson and Crawford, 1980), fibroblast growth (Rachmilewitz, 1941), lymphocyte transformation (Kasakura and Lowenstein, 1967; Newberry and Sanford, 1971; Navarro, Grossetete, DeFrasne et al, 1985), fibrinolysis (Bennett and Ogston, 1970), and platelet functions (Horowitz, Cohen, Marinez and

Papayoanou, 1967; Bazilinski, Shaykh, Dunea et al, 1985).

Glucose utilisation and glycolysis inhibition (Morgan and Morgan, 1964; Dzurik and Krajci-Lazary, 1967; DeFronzo, Alvestrand, Smith et al, 1981; DeFronzo, Smith and Alvestrand, 1983; Kaufmann and Caro, 1983; Alvestrand, Mujacic, Wajngot and Effendic, 1989), enhanced or inhibited gluconeogenesis (Renner and Heintz, 1965; Dzurik, Niederland and Cernacek, 1969; Horl, Riegel and Schollmeyer, 1986) have also been demonstrated together with inhibition of protein synthesis (Delaporte, Gross and Anagoustopoulos, 1980; Mitch, 1988), and abnormal lipid metabolism (Boyer and Scheig, 1970; Heuck and Ritz, 1980; Chan, Varghese and Moorhead, 1981; Norbec and Carson, 1981; Nestel, Fidge and Ran, 1982).

Transport systems affected by uraemia include calcium transport in mitochondria (Russell and Avioli, 1974), uric acid transport in liver slices (White and Nachev, 1975), hippuric acid derivatives in kidney and liver tissue (White, 1966), amino acids in muscle (Cernacek, Spustova and Dzurik, 1982) and folate in Ehrlich ascites tumour cell (Jennette and Goldman, 1975).

There are few <u>in vivo</u> studies and they are based on either administering a potential toxin, uraemic plasma, serum and most recently fractions of uraemic ultrafiltrate to experimental animals, modifying patients' dialysis programme in order to prevent the elimination of a particular substance or comparing the plasma profiles of uraemic solutes between

patients who are clinically well and those severely symptomatic, using specific body function tests (Solders, Persson and Wilczeck, 1986).

Several reviews have been published on this subject, e.g. (Bergstrom and Furst, 1978; Contreras, Later, Navarro et al, 1982; Schoots, Mikkers, Cramers et al, 1984). Table 1.2. shows some of the most important potential "uraemic toxins". It is of course a simplification since there are probably more compounds still to be discovered.

The concept of a single "uraemic toxin" is probably an oversimplification, (Ringoir and Schoots, 1988) and it is most likely that many substances taken together are responsible for the abnormalities demonstrated in uraemia. Single toxic effects can be potentiated when several compounds are present simultaneously in the environment in abnormal concentrations. In fact, although some signs or symptoms can be demonstrated when a specific compound is tested, in more than 150 years of research, in no circumstance has been possible to reproduce the entire syndrome by administering a single toxin.

1.4. The Cell Membrane

The cell is the functional unit of all living organisms. Each cell is bounded by a cell membrane. This membrane is selectively permeable containing proteins specialised in the transportation of substances in and out of the cell, in order

Table 1.2. Potential Uraemic Toxins.

Urea (a)	Guanidines (b)
"Middle-molecules" (1)(c)	Phenols (d)
PTH (e)	Myoinositol (f)
B_2-Microglobulin (g)	Hippuric acid (h)
Free fatty acids (i)	Vanadium (j)
Polyamines (k)	Amino acids (l)

For references see: [1]a.Rajogopalan, Fridovich and Handler, 1960; Balestri et al, 1972. b.Davidoff, 1974; Ingerowski, Ingerowski and Dunn, 1972; Shainkin, Giatt and Berlyne, 1975; Giovannetti et al, 1968. c.Bergstrom, Furst and Zimmerman, 1979; Menyhart and Grof, 1981; Contreras et al, 1982; Keshaviah and Kjellstrand, 1983. d.Wardle and Wilkinson, 1976. e.Slatopolsky, Martin and Hruska, 1980; Massry and Goldstein, 1978, 1979; Massry, 1986. f.Blumberg, Esslen and Burgi, 1978. g.Ypersele de Strihou et al, 1988. h.Boumendil-Podevin, Podevin and Richet, 1975. i. Tamura et al, 1985; Kelly et al, 1989. j.Grantham, 1980. k.Radtke et al, 1981. l.Bergstrom and Furst, 1983.

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[1](c)molecular weight 800-2000.

to maintain an adequate environment suitable for cellular function. Some properties of the membrane will depend on the different specialised functions of the cell (Hendry, 1981).

1.4.1. **Membrane Lipids.**

The membrane is a bilayer of amphipathic lipids (see fig 1.1). They are mainly phospholipids and glycolipids in a variable proportion according to the type of membrane, and constitute fifty per cent of the red cell membrane by weight. In the human erythrocyte, phospholipids represent 61%, cholesterol 22% and glycolipid 11% of the total lipid content by weight. However, they coexist in a molar ratio of phospholipid:cholesterol close to 1:1 making cholesterol a major component of the red cell membrane (Carruthers and Melchior, 1983; Yeagle, 1985). The polar group on cholesterol is a simple hydroxyl group and the remainder of the rigid sheet molecule is buried deeply in the bilayer, probably distributed equally on both sides of the membrane. The permeability of a model phospholipid bilayer is reduced by cholesterol and the high cholesterol content of red cells completely removes the lipid temperature-phase transition phenomenon. The cholesterol of red cell membranes appears to be freely exchangeable and complete exchange between inner and outer layers is achieved within twenty four hours. Cholesterol can be exchanged with plasma cholesterol with a half life of

Figure 1.1. The three-dimensional representation of the fluid mosaic membrane model. A fluid-like phospholipid bilayer with proteins (solid bodies) embedded to varying degrees in the membrane. (reproduced from Singer and Nicolson, 1972).

two hours and transbilayer movement of cholesterol occurs with a half time of less than one hour at 37°C (Cooper, Arner, Wiley and Shattil, 1975). The major lipid constituents of the human red cell membrane are shown in Table 1.3. and the distribution of lipids in the red cell membrane is illustrated in figure 1.2. Under physiological conditions (35 - 37°C in humans) membrane lipids are in a fluid state.

1.4.2. Protein-Lipid Interaction.

The interaction between lipids and proteins is central to the understanding of membrane function. Membrane lipids must affect protein function significantly, in view of interaction between protein and lipids in the membrane (Kleinfeld, 1987). A vast literature on the effects of lipid composition on membrane protein-mediated transport mechanisms exists, e.g. (Carruthers and Melchior, 1986; Deuticke and Haest, 1987; Yeagle, 1989). The effect of cholesterol on the Na,K-ATPase varies according to concentration: high cholesterol levels inhibit the pump, while low levels stimulate it (Giraud, Claret, Bruckdorfer and Chailley, 1981; Yeagle, 1983; Yeagle, 1985). The apparent immunity of the activity of the calcium pump to membrane cholesterol has been explained by exclusion of cholesterol from the region of the pump (Warren, Houslay, Metcalfe and Birdsall, 1975). It is unclear how membrane protein function can be modulated by the

Table 1.3. Lipid composition of the human erythrocyte membrane.

From Christensen (1975)

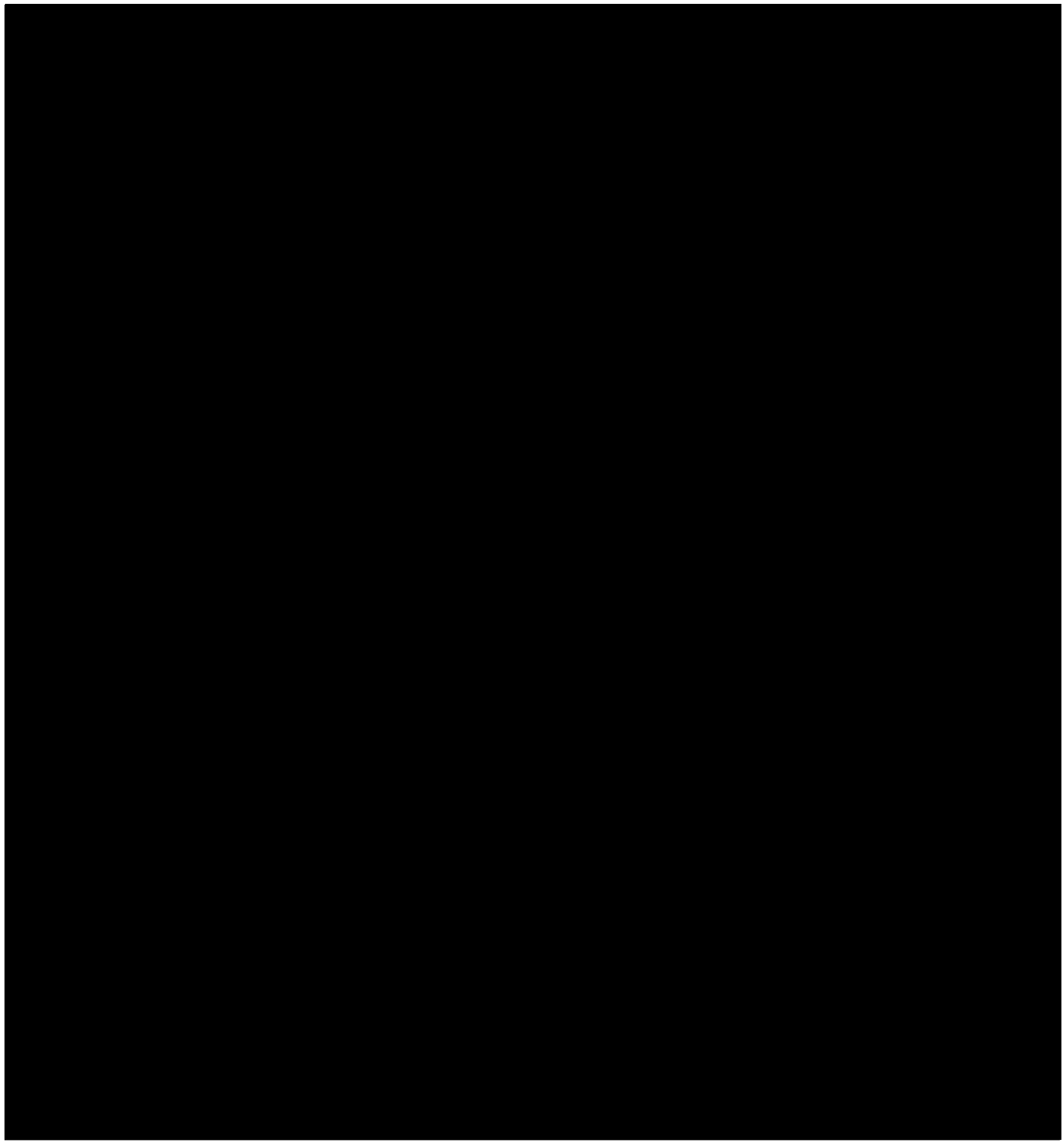

Figure 1.2. The asymmetrical distribution of phospholipids in the membrane of human erythrocytes. (reproduced from Gennis, 1989).

surrounding lipids. Interactions may be either of low or high specificity taking place at a particular binding site, probably at the polar-apolar membrane interface, or they may be non-specific with lipid acting simply as a solvation shell necessary to maintain the protein in a conformational and motionally intact state for activity (Watts, 1989).

1.4.3. **Proteins.**

Membrane proteins can be extrinsic or intrinsic. Transmembrane proteins have polar amino acid sequences located at both sides of the membrane interacting with phospholipid head groups. In the erythrocyte, glycophorin, membrane channels, Na/K-ATPase, Band 3 (the major transmembrane transport protein in the erythrocyte which accounts for 25-30% of the total membrane protein), amino acid transporters, nucleoside transporters and various receptors belong to this group of proteins.

1.5. Concepts of Mediated Transport.

Processes other than simple diffusion are involved in membrane transport; for amino acids, for example, interaction with a number of specialised membrane proteins or carriers, facilitate their transport across the membrane. Membrane carriers are able to reorientate and undergo conformational

changes during transmembrane movement of their substrate. Such carriers exist in at least two conformational states and alternately expose their binding sites to each side of the membrane.

The conventional carrier model is illustrated in figure 1.3, where the four sequential reversible steps can be seen.

The process starts with the binding of the substrate on the cis side (step 1, C_1S); then translocation of the substrate and carrier occurs (step 2, C_2); this is followed by release of the substrate at the trans side (step 3, C_2); the last step is the relocation of the transporter to its original state (step 4, C_1). Only steps 2 and 4 are vectorial. For most transporters the reorientation can happen with the carrier loaded (exchange) or empty. The carrier performance can be studied at defined extreme experimental conditions, and this can be of relevance when analyzing the kinetics of the transporter. The trans-stimulation phenomenon, for example, helps to distinguish between carriers and channels (Lieb, 1982). In secondary active transport a ternary complex is involved: between the substrate, the carrier and the co-ion, and there is some evidence that this operates in Na^+-dependent amino acid transport systems (Eddy, 1987).

Figure 1.3. The simple carrier model. C represents forms of the carrier; CS forms of the carrier-substrate complex. Rate constants are indicated next to reaction steps. Modified after Lowe and Walmsley, 1986.

1.6. The Role of Ions.

The presence of ion transport mechanisms provides the cells with a route to maintain a steady-state volume and allows for homeostatic adjustments to be made to changes in the environment. Ion movements between the cell and the external environment depend intimately on the membrane. The ionic gradient is used to transfer solutes across the membrane and to generate the membrane potential among other functions. For example, the active transport of Na^+ *via* the Na/K pump generates and maintains an inwardly-directed electrochemical gradient of Na^+ and an outwardly-directed chemical K^+ gradient in almost every cell. The transport of amino acids by secondary active transport is driven by the Na^+ gradient. In Na^+-independent systems, it is possible that the uptake of amino acid, is driven by the efflux of other amino acids (heteroexchange). Such systems are not dependent upon Na^+ or K^+ gradients or immediate supplies of metabolic energy. Ions can, in addition, affect the carrier-substrate affinity and also increase the translocation velocity (Crane, 1977).

1.7. Kinetic Analysis.

Kinetic analysis of membrane transport has been much helped by the fact that many transport systems behave as

enzymes, displaying the same dependence of rate on substrate concentration. The Michaelis-Menten type of analysis is therefore appropriate in estimating K_m and V_{max} for a particular transport system, and has allowed the discovery and characterization of many transport routes in red cells (Ellory, 1987). The use of kinetic parameters obtained at different pre-established conditions allows the characteristics of the ratios of these parameters to be tested for the operation of a simple carrier (Stein, 1986).

Several transport systems have been studied by this method: the L system for leucine transport (Hoare, 1972a,b; Rosenberg, 1981a); the T system for tryptophan (Rosenberg, 1981b), the nucleoside transporter, (Plagemann and Wohlhueter, 1984; Jarvis, Hammond, Paterson and Clanachan, 1983) and choline transport (Deves and Krupka, 1980).

When the number of carriers in a cell is known and good kinetic data are available, it is possible to determine the turnover rate of the carrier; this will be used when analyzing the K^+ influx results in Chapter 3. However this is not possible, for example, for the amino acid transporter since the total number of transporters per cell is unknown due to the lack of a specific high affinity ligand.

1.8. The Sodium-Potassium Pump.

Living cells are known to have a high intracellular K^+

and low Na^+. These gradients of Na^+ and K^+ across cell membranes reflect the existence of a specific transport system responsible for their maintenance, the Na/K pump. Glynn demonstrated a link between the metabolically dependent uptake of K^+ and efflux of Na^+, a link already suggested by the complementary nature of the movements of potassium and sodium during incubation after cold-storage (Glynn, 1956). A year later, the ratio of Na^+ efflux to K^+ influx was demonstrated to be 1.5 (Post and Jolly, 1957). The 3Na/2K stoichiometry is the basis for the pump being electrogenic, moving net charge outward (Hoffman, Kaplan and Callahan, 1979).

The Na/K pump is inhibited by cardiac glycosides. The molecular features necessary for inhibition, the external site of action, the parallel inhibition of transport and ATPase activity, and the protective effect of external K^+ have been characterized (Glynn, 1957; Glynn, 1964; Hoffman, 1966; Ruoho and Kyte, 1974; Chipperfield, 1985).

Skou linked the Na,K-ATPase to the transport of Na^+ and K^+ (Skou, 1957). The Na,K-ATPase was studied further in red-cell ghosts (Post, Merritt, Kinsolving and Albright, 1960; Dunham and Glynn, 1961). The red cell ghost model (Hoffman, Tosteson and Whittam, 1960) demonstrated that the energy for active transport of Na^+ came from ATP, and that other triphosphates could not replace it (Hoffman, 1960; Hoffman, 1962; Sen and Post, 1964; Garraham and Glynn, 1967). ATP interacts with and phosphorylates the α subunit on its inward-

facing aspect (Uesugi, Dulak, Dixon et al, 1971). Finally, purified Na,K-ATPase was reconstituted into proteoliposomes and shown to catalyze ATP-dependent Na/K exchange (Hilden, Rhee and Hokin, 1974).

In studying the Na/K pump, $^{86}Rb^{+}$ quantitatively substitutes for K^{+} in this system (Beauge and Lew, 1977).

1.8.1. **Mechanism.**

The basic mechanism of sodium pump function is as follows: Intracellular sodium and ATP combine with the enzyme; this is followed by phosphorylation, release of ADP, followed by a conformational change leading to the release of sodium in the extracellular space. Then the enzyme binds potassium, the phosphate residue is released into the cell by hydrolysis, leading to a new conformational change and releasing potassium into the intracellular space (Post, Hegyvary and Kume, 1972; Fahn, Koval and Albers, 1966; Tanaguchi and Post, 1975). The net result is that three Na^{+} ions are transported out of and two K^{+} into the cells for each molecule of ATP that is consumed.

Proof that the E_1P form of the enzyme contains three occluded sodium ions came recently (Glynn, Hara and Richards, 1984) together with confirmation of two potassium ions per phosphorylation site for the E_2P conformation (Beauge and Glynn, 1979).

When the concentration gradients of sodium and potassium are enormous, the energy obtained from the downhill movement of ions could make the system run backwards, synthesizing ATP (Garrahan and Glynn, 1967; Glynn and Lew, 1970; Lew, Glynn and Ellory, 1970).

There is no complete explanation for the specific mechanism of ion pumping by the Na,K-ATPase. For example, it is impossible to say anything about the identity or position of the sites that bind ions at the surface of the membrane or participate in their occlusion (Glynn, 1988); furthermore, the way ions are transported from the surface to the interior and vice-versa can only be speculated about by analogy with better-understood enzymes.

Steady-state kinetic experiments support a ping-pong mechanism for sodium and potassium translocation (Sachs, 1986) where sodium ions are first bound and then released early in the reaction cycle, before the addition of two potassium ions (Forbush, 1984). The transport is electrogenic; each cycle of the pump is accompanied by the outward movement of a positive charge (Hoffman, Kaplan and Callahan, 1979).

The number of pumps varies according to the type of cell and species but human red cells contain an average of 300 pumps, each capable of "pumping" about 150 potassium ions.site^{-1}.sec^{-1}. (Hoffman, 1969; Joiner and Lauf, 1978).

Several reviews of the subject can be found (Glynn and Karlish, 1975; Cavieres, 1977; Sweadner and Stanley, 1980;

Jorgensen and Andersen, 1988; Glynn, 1988; Sachs, 1989; Blostein, 1989; Baxter-Lowe and Hokin, 1989).

1.9. NaKCl Cotransport.

In 1960, Tosteson and Hoffman believed that the regulation of the ions inside the cell was just an interaction between pump and leak (Tosteson and Hoffman, 1960). We now know that those leak pathways are not electrodiffusion but in fact represent transport through other systems. In the context of the red cell, the most important of these systems is the NaKCl cotransporter. This system represents an electroneutral 1Na1K2Cl transporter, inhibited by loop diuretics (Ellory and Stewart, 1982) through competition with Cl^- (Dunham, Stewart and Ellory, 1980; Chipperfield, 1980; Lauf, McManus, Haas et al, 1987; Hendry and Ellory, 1988) that can perform net and exchange fluxes. As with the Na/K pump (Beauge and Lew, 1977), $^{86}Rb^+$ quantitatively substitutes for K^+ in studying this system (Geck, Pietrzyk, Burckhardt et al, 1980) and the activity of the transporter does not change with cell age (Hall and Ellory, 1986). An extensive literature exists about the subject (Dunham, Stewart, and Ellory, 1980; Chipperfield, 1980; Dagher, Brugnara and Canessa, 1985; Hoffman, 1986; Chipperfield, 1986; O'Grady et al, 1988; Stein, 1989; Stewart and Ellory, 1989).

In human red cells, the system has no clear role

identified so far; It is however of fundamental importance in the thick ascending limb of the loop of Henle.

1.10. Amino Acid Transport in Red Cells.

1.10.1. Background.

It was known as early as 1913, that the membrane of red cell was permeable to amino acids, and that red cells could contain amino acids at higher concentrations than the surrounding tissue fluid. Subsequent studies showed that the distribution of amino acids in the intra or extracellular space could be influenced by the presence of other amino acids (Christensen, Streicher and Elbinger, 1948). The detailed study of these effects, and the application of traditional enzyme kinetics, made it possible to start defining distinct transport systems for different amino acids (Oxender and Christensen, 1963) and Winter and Christensen in 1964, studying neutral amino acid transport took the first major step in the understanding of these transport systems. It was confirmed that transport systems could accumulate amino acids against a concentration gradient and the kinetic properties and stereoselectivity could not be explained by passive permeability. They described two saturable uptake systems: one for all neutral amino acids and a second one, of lower capacity, for alanine and glycine. Christensen described seven

different transport systems for amino acids in different cell types but only five of them are present in human red cells (Christensen, 1984).

Various cells have different amino acid requirements depending on several factors; differentiation and growth, protein synthesis, neurotransmission, and glutathione (GSH) biosynthesis are examples of those factors. The ways in which these factors may alter amino acid transport are complex and have been reviewed recently (Saier, Daniels, Boerner and Lin, 1988).

Several reasons have been proposed for why red cells possess amino acid transport systems. They include: 1. Functional relics of reticulocytes (Antonioli and Christensen, 1969; Tucker and Young, 1980; Johnstone, Adam, Hammond et al, 1987; Johnstone and Teng, 1989). 2. Glutathione biosynthesis (Young and Ellory, 1977; Bannai and Tateishi, 1986). 3. Accidental carriage on other transport systems (Ellory, Jones and Young, 1981b; Young, Jones and Ellory, 1981). 4. Interorgan amino acid nutrition (Elwyn, 1966; Elwyn, Launder, Parikh and Wise, 1972; Phang, Yeg and Hagedorn, 1980; Christensen, 1982; Christensen, 1990). 5. Volume Regulation (Pierce, 1981; Hoffmann and Lambert, 1983; Hoffmann, 1987; Hall, Bianchini and Ellory, 1989). There are several reviews of the subject (Young and Ellory, 1977; Ellory, 1987; Tunnicliff, 1989).

In the human red cell there are five, well characterized

and kinetically analyzed, discrete amino acid transport systems, which have been studied in this thesis in the context of uraemia and are the y^+, ASC, gly, L and T systems. They will be reviewed briefly below.

1.10.2. The y^+ system.

First described in Ehrlich tumour cells, the y^+ system was identified in human red cells (Gardner and Levy, 1972) as a saturable, Na^+-independent route for cationic amino acids and was characterised further and found to be present at an increased level in reticulocytes (Christensen and Antonioli, 1969; Antonioli and Christensen, 1969). Its properties were characterised later in human red cells (Young and Ellory, 1979; Young, Jones and Ellory, 1980).

L-lysine is transported across the erythrocyte membrane by the y^+ system which is highly stereoselective (D-isomers are not transported) and has a high affinity for its preferred substrates, lysine, ornithine and arginine (Ellory, 1987). The system is sodium-independent and in humans the total flux of L-lysine is divided into a high affinity, saturable component via this transporter and a second, non-specific, non-saturable component represented in this thesis as the linear component. The system can operate in an exchange mode for these preferrred substrates and this is probably the dominant mode

<u>in vivo</u> (Ellory, Fervenza, Harvey and Hendry, 1988). The y^+ system provides a relatively well-characterised high affinity transporter as a suitable paradigm for the study of erythrocyte amino acid transport in renal failure.

1.10.3. **The ASC system.**

The ASC system has its name derived from its major subtrates: alanine, serine and cysteine. This widespread system is Na^+-dependent and is a secondary active transporter, i.e. does not require ATP. It has significant affinity for alanine and serine and is the principal route for entry of cysteine in mature erythrocytes at physiological concentrations (Young, Wolowyk, Jones and Ellory, 1979; Young, Jones and Ellory, 1980; Rosenberg, 1982). L-cysteine is essential for glutathione (GSH) synthesis and thus protects the cell against oxidative damage. In human red cells the system appears to operate in an exchange as well as unidirectional flux mode (Barker and Ellory, 1990).

The system appears to be insensitive to hormonal control, adaptive regulation and transformation but is subject to trans-stimulation (Saier, Daniels, Boerner and Lin, 1988). It has been reported that the system can be inhibited by harmaline through competition with Na^+ at its binding site (Young, Mason and Fincham, 1988).

1.10.4. The gly system.

First described in avian erythrocytes (Vidaver and Shepherd, 1968) the gly system has been also studied in human erythrocytes and reticulocytes. Intracellular glycine plays an important role in the synthesis of erythrocyte glutathione (Ellory, Jones and Young, 1981a,b).

Glycine transport in the red cell can occur through Band 3, L, T and y^+ systems (Young, Jones and Ellory, 1980, 1981). However, a specific pathway for glycine also exist in human red cells: the gly system (Ellory, Jones and Young, 1981a,b; Al-Saleh and Wheeler, 1982). The gly system has a very limited substrate specificity, carrying only glycine, sarcosine and proline, and it is unique in showing an absolute requirement for both Na^+ and Cl^- ions and in being substantially inhibited by N-ethylmaleimide and N-methylated amino acids but not by loop diuretics (Ellory, Jones and Young, 1981; Al-Saleh and Wheeler, 1982). The stoichiometry of the transport is thought to be two Na^+, one Cl^-, and one glycine (Eddy, 1987) although it may be more complex. The gly system, although significant under physiological conditions, does not play the dominant role in glycine transport and a significant contribution is made by Band 3, Na^+-dependent ASC and Na^+-independent L systems and a Na^+-independent residual flux. In fact at an extracellular concentration of 0.2mM glycine, the glycine

influx was 42, 16,15,11 and 16% by Gly, band 3, L, ASC and residual systems, respectively (Ellory, Jones and Young, 1981).

Glycine transport through the gly system has been reported in synaptosomes (Kuhar and Zarbin, 1978) and kidney (Christensen, 1975) suggesting the presence of the system in different cells. In contrast to the ASC system, depletion of intracellular amino acids has a negligible effect on Cl^- dependent glycine flux under the same experimental conditions (Barker and Ellory, 1990) and so the system does not appear to perform exchange fluxes but depends on the Na^+ gradient to operate.

1.10.5. The L system.

This system was described by Winter and Christensen (1964) and confirmed by other studies (Hoare, 1972a,b; Young and Ellory, 1977; Young, Jones and Ellory, 1980; Rosenberg, 1981a).

The L system represents the dominant amino acid transport system in the red cell, both in terms of abundance and capacity. It is very efficient in transporting L-isomers of neutral amino acids, in particular, leucine and phenylalanine. Due to its high transport capacity even low affinity substrates may have a significant flux through it, e.g.

alanine and cysteine, and it might represent the major route of histidine uptake by human erythrocytes (Liou and Ellory, 1990). It operates in either exchange or zero-trans modes (Ellory, 1987). It is Na^+-independent (Winter and Christensen, 1964) and is the best characterised transport system (Hoare, 1972a,b; Rosenberg, 1981a; Walmsley and Lowe, 1987).

1.10.6. **The T system.**

More specialized than system L, the T system shows a significant affinity only for aromatic amino acids, namely tryptophan, tyrosine and phenylalanine (Rosenberg, 1979; Young and Ellory, 1979; Rosenberg, Young and Ellory, 1980). This Na^+-independent system although representing the major route for tryptophan transport at physiological concentrations still accounts for only 70% of the total tryptophan transport, the remaining been carried by the low affinity system L. However, system L contributions become important progressively at higher concentrations (Rosenberg, 1981b). Tryptophan is important for serotonin synthesis in the brain (Rosenberg, 1979; Rosenberg, Young and Ellory, 1980; Rosenberg, 1981b).

D-tryptophan, phloretin and SH-group reagents inhibit L-tryptophan influx, although it appears that D-tryptophan is not transported into the cell but rather inhibits the transport through binding to the carrier (Lopez-Burrillo,

Garcia-Sancho and Herros, 1985).

1.11. Nucleoside Transport.

Human red cells are known to have a selective, high-capacity nucleoside transport system which has been well-characterised in its kinetics at temperatures between 4°C and 25°C (Plagemann and Wohlhueter, 1980; Jarvis, McBride and Young, 1982; Cabantchick, 1989).

The transport of nucleosides in human red cells is a system of nonconcentrative facilitated diffusion with a broad substrate specificity for purine and pyrimidine nucleosides (Oliver and Paterson, 1971). The kinetics of the system have been studied in detail using pyrimidine nucleosides since they are free of intracellular metabolic conversion (Paterson, Kolassa and Cass, 1981). The transporter behaves according to the simple carrier model (Lieb and Stein, 1974; Cabantchik and Ginsburg, 1977; Cabantchick, 1989) and is directionally symmetric (Jarvis, Hammond, Paterson and Clanachan, 1983; Plagemann and Wohlhueter, 1984).

p-nitrobenzylthioinosine (NBMPR) is a potent and specific inhibitor of nucleoside transport at nanomolar concentrations. The inhibition is competitive and both binding and inhibition of the transporter are very fast (Cabantchik and Ginsburg, 1977) but its precise site of action is not clear. It can

either bind to the transporter or exert its action on an allosteric site preferentially at the external membrane surface (Jarvis, McBride and Young, 1982).

With the help of NBMPR, a functional transport protein of approximately 55 kDa was identified by photoaffinity labelling (Young, Hammond, Paterson and Clanachan, 1983) by isolation and purification (Jarvis and Young, 1984) and by reconstitution procedures (Tse, Belt, Jarvis et al, 1985). More recently, the transporter has been purified by immunoaffinity chromatography (Kwong, Davies, Tse et al, 1988). The first indication that the human red cell nucleoside transporter, as well as the glucose transporter, comes from a polypeptide in band 4.5 was obtained using reversible high-affinity NBMPR binding activity as an assay for the nucleoside carrier protein (Jarvis and Young, 1981) and has been further studied (Wu, Jarvis and Young, 1983). However, the glucose and nucleoside carrier differ from each other in their proteolytic profiles and exofacial susceptibility to trypsin (Janmohamed, Young and Jarvis, 1985).

The behaviour of the system at 37°C has not been studied extensively because of difficulties associated with very high transport rates for nucleosides and the consequent need for membrane flux measurements to be performed with very short incubations. This thesis presents the first study of uridine transport in human erythrocytes at 37°C performed using a brief flux incubation stopped with cold NBMPR.

Uridine was chosen as the substrate to study nucleoside transport in this thesis because it has been shown not to be cleaved or phosphorylated in human red cells (Oliver and Paterson, 1971) and uridine transport fulfills all the kinetic criteria of a simple carrier mechanism (Lieb and Stein, 1974).

1.12. Choline Transport.

Apart from being vital in excitable tissues as a precursor of acetylcholine, choline is also needed for membrane phospholipid synthesis. It is therefore logical to expect a specific transport system facilitating the entry of choline into cells of various tissues, e.g. neurones and erythrocytes (Martin, 1968).

Choline flux in red cells is sodium-independent (Martin, 1972) and can be divided into two components: a simple diffusion and a saturable component. When extracellular choline concentration is 100 μM or higher, the flux through the saturable component is a negligible function of the total flux (Askari, 1966). The transporter seems to be, like the amino acid transporters, a vestige left during cell maturation (Askari, 1966). In the red cell, choline is neither metabolized nor incorporated into phospholipids (Askari, 1966; Martin, 1968). This makes the red cell an ideal model to study choline transport. On the other hand, cells that do metabolise choline, e.g. neurones, express a "high-affinity" sodium-

dependent choline transport system (K_m = 1-4 μM) in addition to the low affinity system already discussed (Haga and Noda, 1973); this system is associated with an efficient conversion of choline to acetylcholine, is distributed in the brain in parallel to the distribution of choline acetyltransferase (Kuhar and Murrin, 1978) and has also been reconstituted in proteoliposomes (Vyas and O'Regan, 1985). It has been suggested that the "low affinity" system may in fact be more important to choline homeostasis in vivo (Carroll and Goldberg, 1975).

At micromolar extracellular choline concentrations the erythrocyte influx of choline follows Michaelis Menten kinetics, with a K_m between 20 and 30 μM and a V_{max} of the order of 60 μmol.l cells^{-1}.h^{-1} (Askari, 1966; Martin, 1968; Martin, 1972). The system does not appear to need metabolic energy to operate since it is not affected by low ATP levels; it is also capable of moving choline against an electrochemical gradient. This ability however is dependent on the composition of the external medium, and fluxes are subject to trans-stimulation, the carrier-substrate complex been more mobile than the free carrier (Deves and Krupka, 1979). The activity of the transporter has also been found to be pH dependent with influx being higher at lower pH, but not influenced by membrane potential (Deves, Reyes and Krupka, 1986).

High affinity competitive and reversible inhibition of

choline transport is achieved with hemicholinium HC-3 (Askari, 1966; Martin, 1969). Noncompetitive and irreversible inhibitors are represented by N-ethylmaleimide (NEM) and the more specific compound,cysteamine, which produces an almost complete inhibition. Studies using the last two compounds suggest that the transporter exists in two conformations, with the inward-facing form being at least 200 times more reactive towards NEM than the other (Edwards, 1973; Deves and Krupka, 1981). This cannot be explained by an unequal carrier distribution (Krupka and Deves, 1980).

Regarding the choline transporter there are still some unresolved questions as, for example, the kinetic model of the carrier. It was not the intention of this thesis to clarify the kinetics or the kinetic model of choline transport. It is agreed that choline transport is complex since it shows both trans-stimulation by different monovalent cations but also trans-inhibition depending on the exact conditions. The aim of the present work was to answer a simple question: is choline transport under the same physiological experimental conditions, different in red cells from haemodialysis patients compared with those obtained from normal controls?

1.13. The Red Cell Membrane as a Model.

Given the absence of internal organelles, many red cell

functions must be directly related to the properties of the membrane. These particular specialisations therefore impose limitations on structure and so we cannot say that it is a "typical" membrane. It is however a true lipid bilayer membrane that has been very well characterized with respect to its cholesterol and phospholipid contents and dynamics (Schatzmann, 1989).

Although artificial lipid bilayers may have some advantages in some specific problems, the red cell membrane is invaluable as a model for study under physiological conditions. Many transport systems have been found and studied in red cell membranes and the high quality kinetic data have allowed many other systems to be explored in detail and extrapolated to other cells, e.g. Na/K-transport (e.g. Glynn, 1956; Gardos, 1958; Garay and Garrahan, 1973), Aminoacids (e.g. Rosenberg, 1981a,b) and choline transport (e.g. Jennings, 1985). Therefore, red cells have been well established as a model for studying membrane transport and the kinetic data obtained have generally proved valid when analyzing these transport systems in other tissues.

Additional advantages are: they are single cells, and when incubated immediately come into contact with the medium from which they can be quickly and easily separated; since the whole cell is a single compartment, it does not present the difficulties of multicompartment analysis of data obtained from studying more complex cells. Finally and perhaps even

more important is the fact that they are the most easily available human cells (Schatzmann, 1989) and this is particularly relevant in the clinical context.

1.14. Implications in the Use of Red Cells.

The red cell is an easily obtained model where membrane transport properties can be studied in a variety of conditions without damaging the membrane. However, the term "normal human erythrocyte" has been called "an abstraction of convenience" (Pennell, 1964) since a blood sample contains a population of cells at different stages of maturation. Many functions have been demonstrated to change with red cell maturation (Marks, 1964; Johnstone and Teng, 1989), including transporters (Tucker and Young, 1980). Nevertheless, providing the subjects are neither very young nor very old, the population distribution is constant between individuals (Harris, 1963; Dacie and Lewis, 1975).

The mature human red cell has a relatively simple metabolism since it does not need to synthesise protein or lipid, or replicate its genetic material. It must however, keep its shape, its internal "milieu" and structural integrity. There are also advantages in studying transport in a model where the transported substance is not rapidly metabolised.

It has been the objective of these pages to introduce

the subject of this thesis and to substantiate the red cell as a model for the studies to be presented in the chapters that will follow.

CHAPTER 2

METHODS

This Chapter will describe the methodology used to perform the experiments to be presented in the chapters to follow. A general description of the group of patients studied will be given first; this will be followed by the protocols used to perform the fluxes in the different studies. After that, the composition of the solutions used and a list of the chemicals will be given. Finally, the theoretical basis for the experiments will be presented.

2.1. Groups Studied.

Blood samples were either taken from normal volunteers both from the University Laboratory of Physiology and the Oxford Renal Unit, or from uraemic and transplant patients at the Renal Unit, Churchill Hospital, Oxford. Each patient gave their consent and this work has been approved by the Oxford Regional Ethical Committee. None of the patients (except when mentioned) had received blood transfusion in the 3 months before blood sampling. All patients on renal replacement

therapy [haemodialysis or continuous ambulatory peritoneal dialysis (CAPD)] had been on treatment for at least 3 months previous to the study. For haemodialysis patients, blood samples were usually taken in the morning immediately prior to dialysis; pre-dialysis, CAPD and transplant (with exception of those in the early post-operative period) patients were recruited from out patient clinics at the time they came for routine follow-up.

Blood was sampled using vacutainers with lithium heparin; usually 15 - 20ml of blood was drawn and used within 3 hours. Diabetic subjects, patients with hyperthyroidism, hepatic disease, taking cardiac glycosides, and those on recombinant human erythropoietin treatment were excluded. Use of prednisolone was confined to the transplant patients.

2.1.1. Potassium Fluxes and Ouabain Binding.

Eleven patients with chronic renal failure who had never been dialysed were studied (CRF group). These patients all had plasma creatinine concentrations greater than 0.5 mM (57 mg/l). Thirteen patients who had been on haemodialysis for at least 3 months were employed (HD group) and thirteen patients on continuous ambulatory peritoneal dialysis for at least 3 months (CAPD group). Fifteen patients with renal transplants were studied between 1 week and 10 years after transplantation (mean 24 months); all had plasma creatinine concentrations of

less than 0.25 mM (28 mg/l) (FT group). In this group all patients were taking prednisolone (5-20 mg daily); 8 patients were also on both cyclosporin A and azathioprine, 4 were on azathioprine and 3 on cyclosporin A. Twenty four normal volunteers (N group) were also studied.

Clinical and laboratory details of the patients employed are displayed in Table 2.1. This table represents the group of patients used for ouabain binding and rubidium ion flux experiments. For the amino acid, nucleoside and choline flux experiments, very similar groups were used, although they would not always represent experiments on the same patient.

2.1.2. For Amino Acid Experiments.

For L-lysine fluxes ten patients on maintenance haemodialysis and three patients with chronic renal failure who had not yet received dialysis were employed. The non-dialysed patients had creatinine clearances of less than 5ml/min. Twelve healthy volunteers were employed as controls. For L-serine, glycine, L-leucine and L-tryptophan, fluxes were studied only in haemodialysis patients. For L-serine and glycine nine patients were employed and nine healthy volunteers served as controls. For l-leucine, eight patients were employed together with eight healthy volunteers as controls. Finally, for L-tryptophan ten patients and nine normal controls were employed. Clinical and laboratory

Table 2.1. Details of the 5 groups studied in the Na/K pump experiments

	Normal	CRF	HD	CAPD	FT
Number	24	11	13	13	15
Age, years					
Mean	37	42	46	41	41
Range	24-54	17-63	26-69	23-59	24-64
Urea, mM (g/l)					
Mean	4.6 (0.28)	34 (2.0)	24 (1.4)	21 (1.3)	8.9 (0.53)
Range	3.3-6.6	19-47	15-35	16-33	3.4-14
Creatinine, uM (mg/l)					
Mean	117 (13.2)	769 (87)	1036 (117)	852 (96)	151 (17)
Range	100-138	505-943	706-1389	467-1301	93-245
K, mM					
Mean	4.1	4.8	5.2	4.5	3.9
Range	3.6-4.8	4.0-5.6	4.1-6.0	3.8-5.5	3.0-5.1
Na, mM					
Mean	138	138	138	136	140
Range	133-142	132-143	129-146	130-140	132-144
MCHC, g/dl					
Mean	33.6	33.1	33.2	33.0	33.8
Range	32-35	31-34	31-34	32-35	32-36
MCV, fl					
Mean	92	95	92	91	96
Range	85-99	89-100	83-100	80-97	87-103

The values for urea, creatinine, K and Na are plasma concentrations
MCHC = Mean cell haemoglobin concentration; MCV = mean cell volume.

details of the patients employed are displayed in Table 2.2.

2.1.3. **For Nucleoside Experiments.**

Eight patients on haemodialysis and eight normal subjects were employed. Clinical and laboratory details of the patients employed are displayed in Table 2.3.

2.1.3. **For Choline Experiments.**

Erythrocyte choline transport was studied in 24 patients with chronic renal failure not on dialysis, 9 patients on haemodialysis, 6 patients on continuous ambulatory peritoneal dialysis (CAPD), 31 patients with renal transplants and in 9 normal controls. Clinical and laboratory details of the patients employed are displayed in Table 2.4.

2.2. <u>The Na/K pump.</u>

2.2.1. **Preparation of the erythrocytes.**

For the rubidium flux measurements, experiments were performed in whole blood. For controls, and transplant patients, the blood was diluted with autologous plasma to reduce the haematocrit to 0.2 in order to be equivalent to the uraemic groups. For ouabain-binding studies, blood samples

Table 2.2. Details of the 5 groups studied in the Amino Acid experiments

	Normal	HD Lysine	HD Serine	HD Glycine	HD Leucine	HD Tryptophan
Number	19	13	12	10	9	10
Age, years						
Mean	39	40	45	44	45	51
Range	18-68	17-67	24-69	18-64	24-64	24-72
Urea, mM (g/l)						
Mean	4.2	25.5	24.4	23	26.5	25.7
Range	3.3-6.1	22-41	19-37	15.2-26	13.7-42.7	12.4-35.4
Creatinine, uM (mg/l)						
Mean	108	872	902	798	859	857
Range	96-116	600-1162	580-1254	690-945	594-1352	668-1150
K, mM						
Mean	3.7	5.1	5.3	5.2	4.9	5.1
Range	3.5-4.3	3.9-6.0	5-6.2	4.3-5.9	3.5-5.9	4-6.4
Haemoglobin g/dl						
Mean	15.4	9.0	9.8	9.2	8.5	8.8
Range	13.2-16	7.8-12.5	6-11.5	7.1-10.7	6.4-12	7.4-10.2
MCHC, g/dl						
Mean	33.7	33.1	33.0	32.3	33.1	33
Range	33-38	30-34	32-34.1	31.3-33	32.1-34.6	32-33.9
MCV, fl						
Mean	89	92	93	93	91.1	94.6
Range	82-99	84-95	84-102	88-99	85.5-97.1	88.1-104

The values for urea, creatinine and K are plasma concentrations
MCHC = Mean cell haemoglobin concentration; MCV = Mean cell volume.

Table 2.3. Details of the 2 groups studied in the Nucleoside experiments

	Normal	Haemodialysis
Number	8	8
Age, years		
Mean	39	44
Range	24-62	22-69
Urea, mM (g/l)		
Mean	4.5	24
Range	3.3-6.1	18.9-30
Creatinine, uM (mg/l)		
Mean	110	813
Range	88-121	607-1183
K, mM		
Mean	4.0	5.9
Range	3.5-4.5	4.4-6.2
Haemoglobin g/dl		
Mean	15.3	8.2
Range	13-16.1	5.0-10.2
MCHC, g/dl		
Mean	34	33.1
Range	32-35.5	32.3-34.5
MCV, fl		
Mean	88	91.9
Range	82-96	87.3-94.6

The values for urea, creatinine and K are plasma concentrations
MCHC = Mean cell haemoglobin concentration; MCV = Mean cell volume.

Table 2.4. Details of the 5 groups studied in the Choline experiments

	Normal	CRF	HD	CAPD	FT
Number	11	24	9	6	31
Age, years					
Mean	38	50	56	55	46
Range	24-56	17-70	43-68	21-78	20-67
Urea, mM (g/l)					
Mean	4.5	14.7	29.4	24.4	14.6
Range	3.4-6.3	4.0-36.9	15.5-44.6	20.0-33.2	4.1-35.5
Creatinine, uM (mg/l)					
Mean	115	282	859	971	226
Range	98-125	85-919	573-1203	531-1359	88-846
K, mM					
Mean	3.9	4.2	5.7	5.2	4.4
Range	3.6-4.3	3.0-5.5	5.1-6.4	3.9-6.1	3.4-5.5
Haemoglobin g/dl					
Mean	14.5	11.1	8.0	8.2	11.9
Range	13.4-15.8	6.1-14.3	6.3-10.8	6.5-9.4	6.4-18.0
MCHC, g/dl					
Mean	33.6	32.6	33.0	32.4	33.0
Range	32-35	32.1-34.2	32.4-33.4	30.6-33.3	31.6-34.6
MCV, fl					
Mean	90	88.5	95.6	91.6	94.3
Range	85-96	84.5-95.6	93-99.8	84.8-98.4	82.6-102

The values for urea, creatinine and K are plasma concentrations
MCHC = Mean cell haemoglobin concentration; MCV = Mean cell volume.
Mean creatinine clearance in CRF = 46.3 (5-100).

were centrifuged initially and the plasma was separated and saved; the buffy coat was discarded. A sample of the harvested plasma was used for potassium measurements. The erythrocytes were then rapidly centrifuged and washed (2000g; 5min) three times in ice-cold saline (see below for composition). Cells were then resuspended in their native plasma (or, for cross incubation experiments, in autologous plasma) to produce an haematocrit of approximately 0.2, and then incubated at 37°C for 2 hours. This suspension of washed erythrocytes was then used for ouabain binding studies and for the cross-incubation ^{86}Rb flux experiments. All flux and ouabain binding experiments were done in 1.5 ml Eppendorf tubes and the cell suspensions were spun down using Eppendorf microcentrifuges (model 5415). The haematocrits in this suspension were in the range 0.1 to 0.2.

2.2.2. K^+ Influx.

Fluxes were measured by using radioactive tracer methods (Ellory, 1982), using $^{86}Rb^+$, as a congener for potassium, (Garay and Garrahan, 1979).

The following procedure was followed both for the standard flux measurements and for fluxes measured after a 2 hour incubation in autologous or heterologous plasma. Duplicate tubes containing 1 ml blood were incubated at 37°C for 5 min with 0.1 mM ouabain, 0.1 mM ouabain and 0.1 mM

bumetamide, or neither. Tubes were then transferred to ice for 3 minutes. Tracer amounts of ^{86}Rb were added to each tube and mixed, and a 5 minute incubation for flux measurement at 37°C was performed. Fluxes were stopped by transferring the samples back to ice so that the cells were allowed to cool for 3 minutes before washing procedures were started; the cooling curves are symmetric and equilibrate within two minutes (see fig 2.1).

After the tubes had been cooled for 3 minutes, they were centrifuged rapidly and 10 μl samples of the supernatant were taken for counting of ^{86}Rb. The erythrocytes were then quickly centrifuged-washed 3 times in cold $MgCl_2$, 12 000 g for 10-15s. Cell lysis was achieved by addition of 0.5 ml 0.1% Triton X-100 to the pellet, and the protein was precipitated by addition of 1 ml 5% trichloroacetic acid and pelleted by centrifugation (12000 g, 5 min). The supernatant was transferred to scintillation vials containing 2 ml of distilled water, and intracellular ^{86}Rb was counted by Cerenkov radiation in a ß-scintillation spectrophotometer (Packard Tricarb 2000CA). The ratio of intracellular and extracellular ^{86}Rb counts per minute (c.p.m.) together with the haematocrit (Hct) and measured plasma K^+ concentration $[K^+]$, was used to calculate the total K^+ influx rate and its ouabain-sensitive and bumetamide-sensitive components.

Flux rates were calculated using the formula:

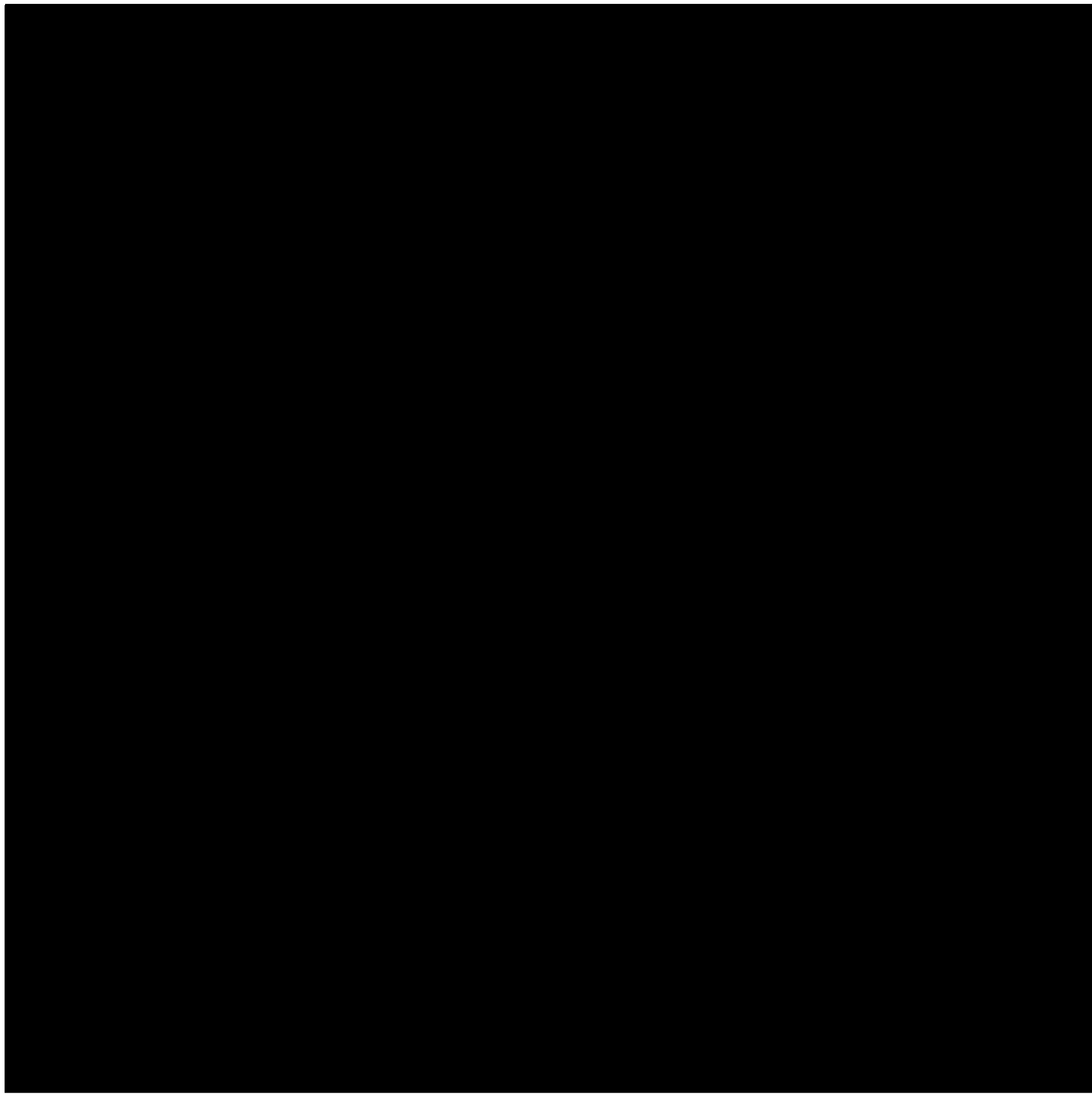

Figure 2.1. Time course of temperature changes inside an Eppendorf 1.5 ml polypropylene tube, on transfer from an ice-bath to a 37°C water bath and back again. Temperature equilibration is within two minutes. (reproduced from Young and Ellory, 1982).

$$\frac{(\text{c.p.m sample}) \times [K^+] \times (\text{vol.supernat.}) \times 12}{(\text{c.p.m supernat.}) \times (\text{Hct}) \times (\text{vol.sample})} = K^+ \text{ influx rate}$$

K^+ influx rate has units of mmol.l cells^{-1}.h^{-1}.

cpm sample= counts per minute in the cell sample counted.

$[K^+]$ = potassium concentration in the plasma.

vol. supernat. = volume of the supernatant counted.

cpm supernat. = counts per minute in the sample of supernatant.

Hct = Haematocrit of the cell suspension.

vol. sample = volume of cells in the sample counted.

The multiple of 12 converts the 5 minutes flux result into a rate per hour.

^{86}Rb flux measurements were performed in plasma and therefore extracellular potassium concentration was not standardised. Separate experiments were performed in saline to confirm that the stimulation of Na/K pump activity by external K^+ was the same in the control as in the uraemic groups. Results were consistent with the existence of 2 identical external K^+ binding sites, each with an apparent dissociation constant of 0.5 mM (Garay and Garrahan, 1973) (see figure 3.7). The measurements made of ouabain-sensitive K^+ influx could be corrected to a standard external K^+ of 4 mM.

These corrections were small (<8%). The uncorrected potassium fluxes are reported in Chapter 3, and the effects of applying these corrections are considered in the Discussion.

2.2.3. **Measurement of ^{3}H-ouabain Binding.**

Tubes containing a 1 ml aliquot of the experimental washed erythrocyte suspension in plasma were incubated at 37°C for 5 minutes with or without 0.1 mM unlabelled ouabain. ^{3}H-labelled ouabain was then added to each tube to a final concentration of 300 nM. The suspensions were incubated at 37°C for 1 hour and tubes were gently agitated at regular intervals. The erythrocytes were then washed by 3 rapid centrifugations in ice-cold $MgCl_2$. The bound ^{3}H-ouabain was solubilised by sequential addition of 0.1% Triton-100 and 5% trichloroacetic acid, and counted by ß scintillation in Packard Pico-fluor 40 scintillation fluid. The specific ouabain binding was obtained by subtraction of the ^{3}H-ouabain bound in the presence of excess cold ouabain. Appropriate standards of ^{3}H-ouabain were counted so that the experimental counts could be converted into moles of ouabain bound per sample tube. The measured haematocrit of the cell suspension and an assumed mean cell volume of 100 fl were then used to convert the result into a value of moles of ouabain bound per cell. This result was multiplied by Avogadro's number (6×10^{23}) to produce a final result for the specific binding of

ouabain as the number of molecules per cell. Experiments were also performed to confirm that 1 hour incubation with 275 nM ouabain was sufficient for equilibrium to be achieved, and to confirm that all the specific ouabain binding sites were occupied at this concentration.

2.2.4. **Reversibility Experiments.**

Reversibility experiments were performed in blood taken from patients on haemodialysis immediately prior to a haemodialysis session. At the same time, 100ml of ABO and Rh compatible blood was taken from normal controls. The uraemic cells were rapidly centrifuged, the buffy coat was discarded together with part of the uraemic plasma. An aliquot of cells was then centrifuged-washed 3 times in cold standard saline (as above) and resuspended in its uraemic plasma to give a haematocrit of about 0.1-0.15. It was then incubated at 37°C for 1 hour and ^{86}Rb fluxes were performed, the result of this flux being considered the value for time 0. The other aliquot of the cells separated previously was resuspended in normal plasma at a similar haematocrit and incubated at 37°C for 4 hours. At every hour samples of the cell suspension were withdrawn and ^{86}Rb fluxes were performed. At the same time, cells were centrifuged rapidly and the plasma was replaced by fresh normal plasma. Therefore ^{86}Rb fluxes were obtained at time 0,1,2,3 and 4 hours incubation in normal plasma.

The same protocol was followed for the incubation of normal cells in uraemic plasma.

2.3. Amino Acid Transport.

Amino acid influx rates were measured using established methods (Young and Ellory, 1982; Harvey and Ellory, 1989).

Initially a report of how red cells were prepared, together with a general description of the amino acid fluxes procedure will be given. Although this will be based on the amino acid transport experiments, in principle, the same method was used for nucleoside and choline transport experiments. A detailed explanation will be given every time a modification of this general protocol had to be made due to particular considerations of the substrate under investigation.

For amino acid fluxes, red cells were initially prepared as follows: after a centrifugation (2000g for 5 minutes) the plasma and the buffy coat were removed by aspiration; then cells were rapidly washed 3 times in saline and resuspended accordingly in the required medium aiming for a haematocrit of 0.08-0.16. Care was taken that the cells were kept cold (4°C) during the washes to minimise the effect of removing intracellular substrates, e.g. amino acids, since for transport systems that operate in an exchange mode this could affect the results. To avoid changes in the extracellular

substrate concentration during the influx the final haematocrit in all amino acid incubations was 0.04-0.08.

Flux measurements were made by incubating the cells at 37°C in standard saline solution, unless stated otherwise, with tracer amounts of radioactive amino acid present at a chosen concentration, according to the amino acid studied. After incubation, cells were separated quickly from the medium, and washed 3 times. Subsequent lysis and counting of intracellular radioactivity, allows an estimation of the total amount of amino acid taken up. This method allowed the movement of amino acids across red cell to be followed. The errors due to counting of extracellular label after 3 washes were negligible and so no extracellular space marker was required (Young and Ellory, 1982). Duplicate tubes were employed for each amino acid concentration, and the agreement between duplicates was excellent.

For serine experiments, fluxes were also performed in sodium-free media [NMDG replacement (NMDG = N-Methyl-D-Glucamine)] and for glycine in a chloride-free solution (Sodium Methylsulphate replacement). Similarly, for certain choline fluxes, half of the cell suspension was incubated in a sodium-free solution.

For L-lysine, L-serine and glycine fluxes, experiments were started and stopped on ice; after incubation the cells were left to cool down for two minutes in ice and then were washed free of extracellular label in ice-cold $MgCl_2$ medium

(see below) using the rapid wash technique (Young, Ellory and Tucker, 1976).

For L-leucine and L-tryptophan, flux measurements were started by transferring the pre-incubated cell suspension (at 37°C) to an Eppendorf tube containing saline with labelled L-leucine/L-tryptophan, and shaking vigorously. Fluxes were stopped by transferring 200 μl of the incubated cell suspension to the top of an ice-cold Eppendorf tube containing 500 μl of $MgCl_2$ solution plus 250μM phloretin on the top of 600 μl of n-buthyl-phthalate and immediately spinning down the cells. The supernatant was aspirated and the tube wall was dried with cotton wool. This oil separation method is fully described by Harvey and Ellory (1989).

Time course experiments showed the flux to be linear up to 7 minutes for lysine and serine, 15 minutes for glycine, 2.5 minutes for leucine and 3 minutes for tryptophan. Therefore, incubation times were chosen as follows: for lysine and serine, 5 minutes; for glycine, 15 minutes, for leucine, 2 minutes and for tryptophan, 2.5 minutes.

The amino acid concentration range also varied. For L-lysine, L-serine and glycine the cells were suspended in saline (standard saline, Na^+-free, or Cl^--free) containing amino acids at concentrations of 0.02-0.5 mmol/l, 0.02-0.5 mmol/l and 0.01-1 mmol/l, respectively. Duplicate 1.0 ml aliquots of cell suspension were used for each amino acid concentration. For L-leucine and L-tryptophan, the

concentrations were 1-50 mmol/l and 0.25-25 mmol/l respectively. A 200 μl aliquot of cell suspension was used for each measurement.

The routine washing procedure will not deplete the cell completely of its intracellular amino acid content. The fact that the transporter may perform exchange fluxes of different amino acids or be trans-stimulated by intracellular amino acids, makes it important to study transport properties in zero-trans conditions, where these factors can be removed. Therefore, zero-trans experiments were performed using the same group of patients, to compare fluxes under the two conditions. Zero-trans conditions were established by incubation of erythrocytes in saline at a low haematocrit (< 0.025) for 4-6 hours. These results showed a significant change between the initial and the zero-trans results for lysine and serine, a very small change for glycine, but no change at all for L-leucine and L-tryptophan fluxes.

The cells were then lysed by addition of 0.5ml of 0.1% Triton-X100, and 1.0 ml of 5% trichloroacetic acid was added to precipitate the protein. The ^{14}C content of the erythrocytes was measured by liquid scintillation counting in Pico-Fluor 40 scintillation fluid in a Packard Tri-carb 2000 CA Liquid Scintillation Analyser against similarly quenched blanks. With this information, the initial influx rate of amino acid per litre of erythrocytes was calculated. Results are usually expressed in terms of mmol.l cells^{-1}.h^{-1}.

2.4. Nucleoside transport.

Nucleoside ^{14}C uridine fluxes across the red blood cell membrane were performed preparing the cells the same way as described for the amino acid experiments with modification of the 'stopping solution'. This was necessary in view of the fact that nucleoside fluxes are very fast at 37°C (Jarvis, Hammond, Paterson and Clanachan, 1983; Cabantchik, 1989).

In brief, 100 μl of the erythrocyte suspension was mixed with the 100 μl of (^{14}C) uridine (concentration range of 0.1 to 2 mM; both at 37°C) by repeated injection from and suction into a pipette tip, and then 100 μl were extracted and put into the stopping flux tube containing 500μl of ice-cold stop solution [107 mM $MgCl_2$, 10 mM MOPS and 40μM Nitrobenzylthioinosine at pH 7.4] overlying 600 μl of dibutyl phthalate oil, and immediately centrifuged (10000g; 15s); the supernatant aqueous phase and oil were removed by suction, the tube inverted and its walls were dried using cotton dental rolls. The time between adding the cells to the (^{14}C) uridine solution and addition to the stop solution was recorded with the help of an assistant with a watch accurate to 0.05 seconds, and this time was used to calculate the flux. Quadruplicate measurements were performed for each concentration of uridine (1 - 50μM). All measurements were performed with incubations of approximately 4 seconds. The

contamination of the oil-separated cells by extracellular label was estimated by performing control experiments in which 40μM NPMPR inhibitor was present throughout, by prior addition to the initial erythrocyte suspension. By this procedure two extra tubes were counted for each concentration and this number taken as the zero time value or radioactivity background. The corrected intracellular counts were obtained by subtraction of this component which normally represented less than 10% of the total.

The separated erythrocytes were lysed and the supernatant removed and counted the same way as for the amino acid fluxes.

Time course experiments were performed by measurement of uptake at variable incubation times from 2 seconds to 300 seconds. The fluxes were linear up to 5 seconds and justified the use of incubations of 2-5 seconds to estimate the initial rate of uridine influx. The experimental results are expressed as μmoles of uridine per litre of cells per second.

2.5. Choline Transport.

Choline purification was performed by dissolving choline chloride in hot ethanol. Recrystallization was by transferring the solution to an ice bath. Crystals were then separated on an Erlenmeyer funnel under suction, and the crystals were then washed with diethyl ether. They were then transferred to a dessicator and dried overnight under vacuum to eliminate the

ether. A solution of about 1 molar was made. The osmolarity was measured and the concentration adjusted accordingly.

Cells were rapidly washed 3 times with saline and then incubated in saline at aproximately 2% haematocrit at 37°C for 6 hours. Choline fluxes at 250 μM concentration were performed immediately after washing and at hourly intervals to determine time required to reach zero-trans. It was clear that 4 hours were enough for the cells to reach steady-state condition (assumed zero-trans condition) and therefore all experiments on this group were performed following 4 hours incubation for choline depletion.

Zero-trans fluxes were performed as follows: 200 μl of the cell suspension was mixed with the same amount of the labelled choline solution in Eppendorf tubes and incubated for 5 minutes at 37 °C. All experiments were started and stopped on ice. The final haematocrit was 6 - 8%, and the final concentration range from 1 to 50 μM.

In a separate series of experiments, immediate-use cell fluxes were performed as soon as the third wash had finished. The flux method was the same as for zero-trans but a final concentration of 250 μM was aimed for.

In addition, fluxes were also performed using the same cells both in the presence of standard saline solution and in the absence of Na^+, using NMDG as a substitute. These experiments were designed to confirm the Na^+-independence of the choline fluxes.

The separation and counting of the cell pellet was dealt with as previously described.

2.6. Haematocrit and electrolyte determinations.

Plasma and intracellular Na^+ and K^+ concentrations were determined by flame photometry (Instrumental Laboratory, model 743), using lithium as an internal standard. For intracellular ion measurements the cells were rapidly centrifuged and washed 4 times in ice-cold, sodium-free, isotonic $MgCl_2$ solution, and then lysed with deionized water. Extracellular sodium concentration was checked prior to lysis and from this the errors due to trapped plasma were calculated to be less than 2%. Haemoglobin was determined spectrophotometrically (Perkin-Elmer, lambda 5, UV/Vis spectrophotometer) in triplicates using Drabkin's reagent. 100 μl of the red cell suspension was added to 5 ml of Drabkin's reagent, and after mixing samples were allowed to rest for 1 hour before spectrophotometric determinations were performed. Results were normalised on the basis of O.D. at 540nm (packed cells) = 247. The same method was used to determine haemoglobin in all the other experiments: amino acid, nucleoside and choline fluxes.

Mean cell haemoglobin concentration and mean cell volume did not differ significantly between the groups studied.

2.7. Solutions.

Solution A: Normal saline: used for initial washing and incubation of cells in all experiments, except were specified.

NaCl	150mM	
KCl	5mM	
MOPS	10mM	
Glucose	5mM	pH: 7.4

MOPS = (3-[N-Morpholino]propanesulfonic acid)

Solution B: Washing solution:

$MgCl_2$	107mM	
MOPS	10mM	pH: 7.4

Solution C: Sodium free-medium:

NMDG (Cl)	140mM	
KCl	5mM	
MOPS	10mM	
Glucose	5mM	pH: 7.4

Solution D: Chloride free-medium:

CH_3SO_4Na	140mM	
CH_3SO_4K	5mM	
MOPS	10mM	
Glucose	5mM	pH: 7.4

Fine adjustments of osmolarity were made using sucrose. Care was also taken when amino acid changes in concentrations greater than 10 mM were made, since it may change the pH of the solution, and affect transporter affinity for its substrate. The osmolarity was measured using a Wescor 5100C Vapour Pressure Osmometer, calibrated with 100, 290 and 1000 mOsm/kg standards.

2.8. Chemicals and Isotopes.

The movement of substrates across the cell membrane was followed by using radioactive isotopes as tracers of total unidirectional flux, (Young and Ellory, 1982). This is based on the principle that labelled form of a substrate is not functionally different from the unlabelled form with respect to carrier systems, binding sites and cellular metabolism.

Specific activities were as follows:

^{3}H Ouabain: 42 mCi/mmol; ^{86} Rubidium: 628 mCi/mmol. ^{14}C L-lysine: 329 mCi/mmol; ^{14}C L-serine: 179 mCi/mmol, ^{14}C glycine:

113 mCi/mmol; ^{14}C tryptophan (side chain - 3 - ^{14}C): 54.8 mCi/mmol; ^{3}H leucine: 140 mCi/mmol; ^{14}C Uridine: 519 mCi/mmol; (methyl-^{14}C) Choline chloride: 55 mCi/mmol.

The amount of tracer used was in the region of 0.1 μCi of ^{14}C/ml, 1 μCi/ml when using ^{3}H, and 0.1 μCi/ml for ^{86}Rb.

All radioactive material was purchased from NEN Research Products, Stevenage, Herts or from Amersham International, Aylesbury, Bucks. Purity was greater then 97%.

All non-radioactive chemicals and reagents were of analytical grade and purchased from Sigma, BDH, or Hopkin & Williams. Eppendorf tubes were from Eppendorf Geratebau, Hamburg, FRG.

Scintillation vials were from Hughes & Hughes Ltd, Romford, Essex.

2.9. Experimental Design.

It will be discussed in the context on amino acid transport, but it is valid for the other transport systems studied in this Thesis.

2.9.1. Initial Rate.

The initial rate of amino acid entry was determined from time course curves; different transporters show very different rates (Ellory and Young, 1977; Young, Jones and Ellory, 1980).

The time course fits a simple hyperbola; as the transported substrate reaches significant concentrations on the trans side of the membrane the net forward rate changes, due to significant backflux. The rate of reaction at any given time is the slope of the tangent to the curve; the initial rate being found from the slope of the early time course when uptake is less than 10% of the final uptake. Time courses were carried out by serial incubations in triplicate by the washing and centrifugation method. Incubation times for subsequent experiments were chosen such that initial rate conditions operated.

2.9.2. Concentration Dependence.

Amino acid transport via specific transport system can be analysed using Michaelis-Menten type enzyme kinetics. The expression that relates the rate of transport (V) to the concentration of substrate [S] is then given by the Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot [S]}{[S] + K_m} \qquad (1)$$

Were V_{max} is the maximum rate of transport at very high values of [S], and K_m is the Michaelis-Menten apparent dissociation constant.

At low substrate concentrations, when [S] is much less than K_m, $V = [S]V_{max}/K_m$. In other words, the reaction rate is proportional to the substrate concentration. When [S] is much higher then K_m, $V = V_{max}$; the rate is then maximal and independent of substrate concentration.

Although amino acid transport is mediated by specific transport systems, a significant part of amino acid flux can be due to transport by multiple transport systems with overlapping specificities. There may also be a component due to diffusion. In general the transport by other components has a low affinity and this shows a linear dependence on substrate concentration. Therefore, an expression containing a second component of transport can be used and is represented by:

$$V = \frac{V_{max} \cdot [S]}{[S] + K_m} + K_d.[S] \qquad (2)$$

Where K_d represents the slope of a linear component and has units of $time^{-1}$.

The identification and extraction of the linear component of uptake can be done with the help of a computer program that will fit the results from amino acid flux experiments by nonlinear least squares regression into either equations (1 or 2) and estimates of K_m will be made and tried in repetitive cycles of calculations until it reaches a

stable value. The corresponding V_{max} value is then derived. The advantage of this approach is that is versatile and allows standard errors for K_m and V_{max} to be calculated. The method is a robust one and simulations have showed that it gives good estimates of K_m and V_{max} when correctly weighted. This approach was used by Rosenberg (Rosenberg, 1981a,b) in analyzing the leucine data of Hoare (Hoare, 1972a,b). There are many software packages available which are directly applicable for amino acid transport. In this thesis, the program used was "Enzfitter" (Sigma).

In using a computer program, it must be remembered that although these methods provide more accurate estimates of K_m and V_{max}, including non-linear weighting procedures, they can however produce obviously erroneous estimates, such as a negative K_d value (for review, see Atkins and Nimo, 1980). In order to avoid such inaccurate estimates, and to assess the best method for estimating all parameters, K_d was determined, when possible, by other independent experimental methods. For example system ASC fluxes were performed in parallel, in Na^+ and Na^+-free medium and the linear component determined directly in the Na^+-free medium. Similarly for system gly fluxes were performed in $Cl^-$and Cl^--free medium and the linear component determined accordingly.

If there is no linear component, the uptake can fitted by equation (1). In this case, the equation may be transformed into a linear form and a straight line can be fitted to the

data either visually or by means of an unweighted least-squares regression. The most common linear forms are the Lineweaver-Burk (1/V versus 1/[S]), Hanes ([S]/V versus [S]) and Eadie-Hofstee (V versus V/[S]) plots. For a review of this subject see Harvey and Ellory (1989).

2.10. Statistical Methods.

Linear functions, such as time courses and determinations of linear components of influx, were fitted by linear regression to the equation of a straight line: $ax + b = y$. This gives the slope (a) and the intercept b. The correlation coefficient (r) provides an indication of the fit of the data to the straight line; for a perfect fit $r = 1.0$.

Kinetic parameters were determined by non-linear least squares regression to fit data to either equation 2.1 or equation 2.2., using the commercial fitting program "Enzfitter" (Elsevier).

Student's t-tests were used to examine the differences between groups of results, paired or unpaired tests were employed as appropriate. The p values indicate the probability that a difference occurred by chance. These tests were performed with a commercial program "StatWorks". Differences which were significant at the $p < 0.05$ level by this method were also similarly significant when non-parametric statistical tests were substituted.

Correlations between parameters were analysed by Spearman correlation tests. These results are reported with the correlation coefficient (r) and the probability that the observed correlation occurred by chance (p).

The basis for using these methods and the equations describing them can be found in detail in the statistical text used (Armitage & Berry, 1987).

CHAPTER 3

THE Na/K PUMP

3.1. Introduction.

This chapter will present a study on the Na/K pump in erythrocytes obtained from patients with chronic renal failure before initiation of maintenance dialysis (CRF group), haemodialysis (HD group), continuous ambulatory peritoneal dialysis (CAPD group), patients with a functioning kidney transplant (FT group) and normal controls (N group). Clinical details of the patients in these groups have been described in Table I presented in chapter 2.

Experiments were designed to measure intracellular Na^+ and K^+ concentrations, ouabain binding and K^+ influx in red cells obtained from the above groups. In addition, studies involving cross-incubation of normal cells in uraemic plasma and red cells obtained from uraemic patients in normal plasma were performed to examine if any effect found could be reversed or be induced by these procedures.

The first set of results to be presented concerns measurements of intracellular Na^+ and K^+ concentration. They

will be followed by the ouabain binding, K^+ influx and reversibility experiments. Finally, a discussion concerning these results will be given. The methodology for these experiments has been described in chapter 2.

3.2. Intracellular Na^+ and K^+ concentrations

The measured intracellular Na^+ concentrations determined by flame photometry in the five studied groups are shown in figure 3.1. Each point is the mean of 2-4 determinations on one subject. The mean erythrocyte sodium concentration in each group in mmol/l cells (± SD) was as follows: CRF, 8.97 ± 2.17; HD, 6.68 ± 1.32; CAPD, 8.34 ± 2.12; FT, 6.48 ± 1.11; controls, 7.31 ± 1.94. The values for pre-dialysis (CRF) patients were significantly higher than controls ($p < 0.05$). The haemodialysis, CAPD, and transplant groups were not significantly different from controls (p values 0.30, 0.18, 0.14, respectively). The mean erythrocyte potassium concentrations in each group in mmol/l cells (± SD) were as follows: (CRF), 100 ± 10; (HD), 110 ± 8; CAPD, 110 ± 7; (FT), 110 ± 8; controls, 107 ± 6. These differences were not significant.

3.3. Ouabain Binding Experiments.

This group of experiments was designed to measure the

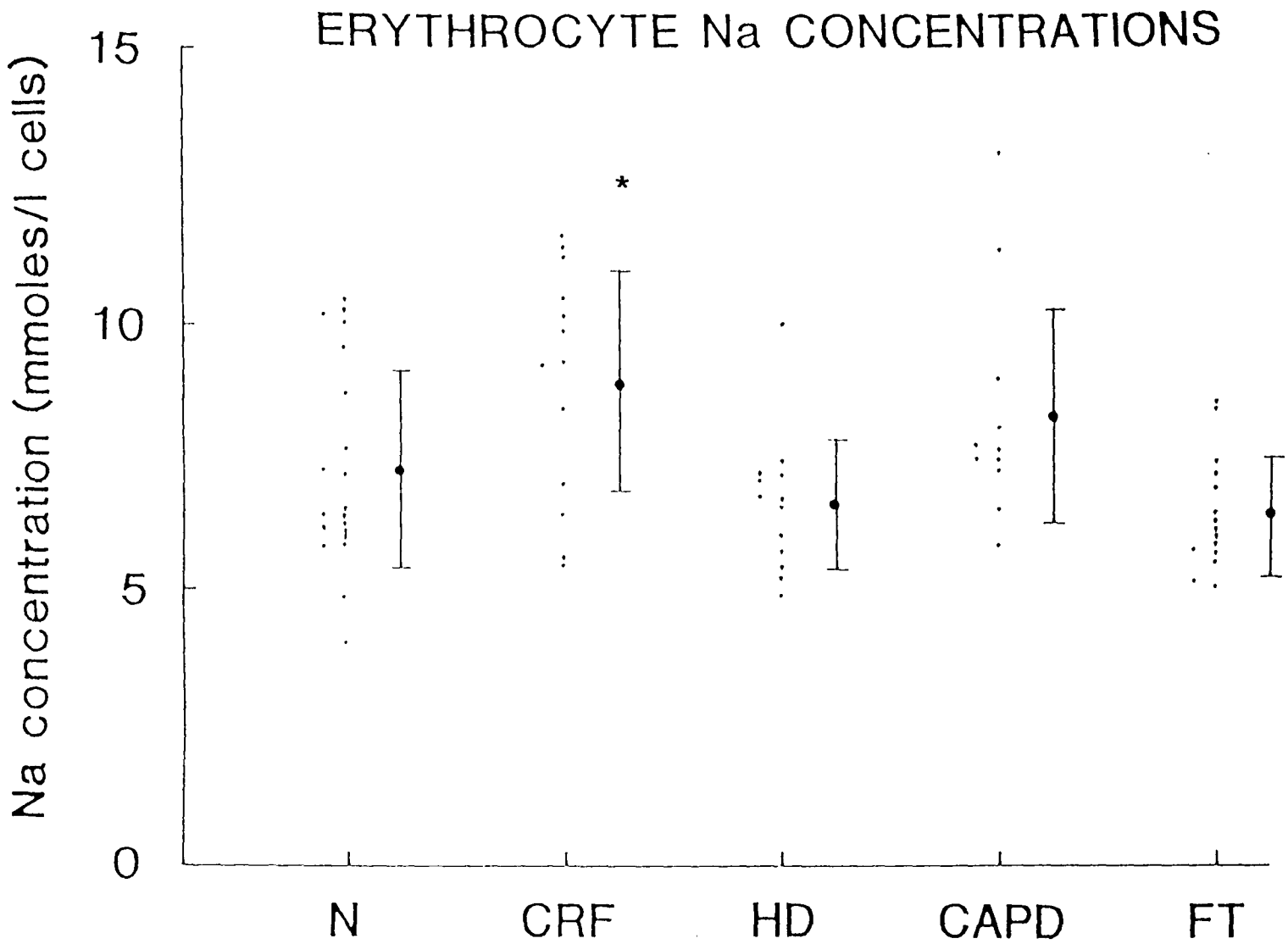

Figure 3.1. Measurements of erythrocyte sodium concentrations in the 5 subject groups. Each point is the mean of 2-4 estimations on a single subject. The mean and standard deviation for each group are displayed. The groups are: N= normal controls; CRF= non-dialysed chronic renal failure; HD= haemodialysis; CAPD= continuous ambulatory peritoneal dialysis; FT= functional transplants.

*Significant difference from the control group $p < 0.05$.

number of Na/K pumps in the red cell membrane. Ouabain binds irreversibly to the Na/K pump with a ratio 1 molecule of ouabain per pump (Jorgensen, 1974). Therefore, by measuring the number of specific ouabain molecules bound per cell it is possible to determine how many Na/K pumps there are.

Ouabain binding experiments were performed in cells suspended in their native plasma. Specific ouabain binding was calculated by subtracting counts obtained in cells previously incubated with cold ouabain from counts obtained in cells incubated directly in ^{3}H-ouabain. Experiments were performed to establish that a 1 hour incubation was sufficient for all specific binding to be achieved with 300 nM ouabain and this is illustrated in figure 3.2. A second group of experiments was also performed to measure K^{+} influx in relation to extracellular ouabain concentration. It has been shown that ouabain-sensitive K^{+} influx strictly correlates with the degree of ouabain binding (Joiner and Lauf, 1978). As can be seen in figure 3.3, ouabain-sensitive K^{+} influx was totally inhibited by incubating cells at 37°C for 1 hour at the above ouabain concentration.

Figure 3.4 shows the mean specific ouabain binding per erythrocyte in the 5 groups studied. Error bars are S.E.M. The results show a significant reduction in ouabain binding in the pre-dialysis (CRF) group (290 $\pm$ 16 molecules per cell) and in the CAPD group (321 $\pm$ 18 molecules per cell) compared to controls (366 $\pm$ 16 molecules per cell, ($p = 0.011$ and

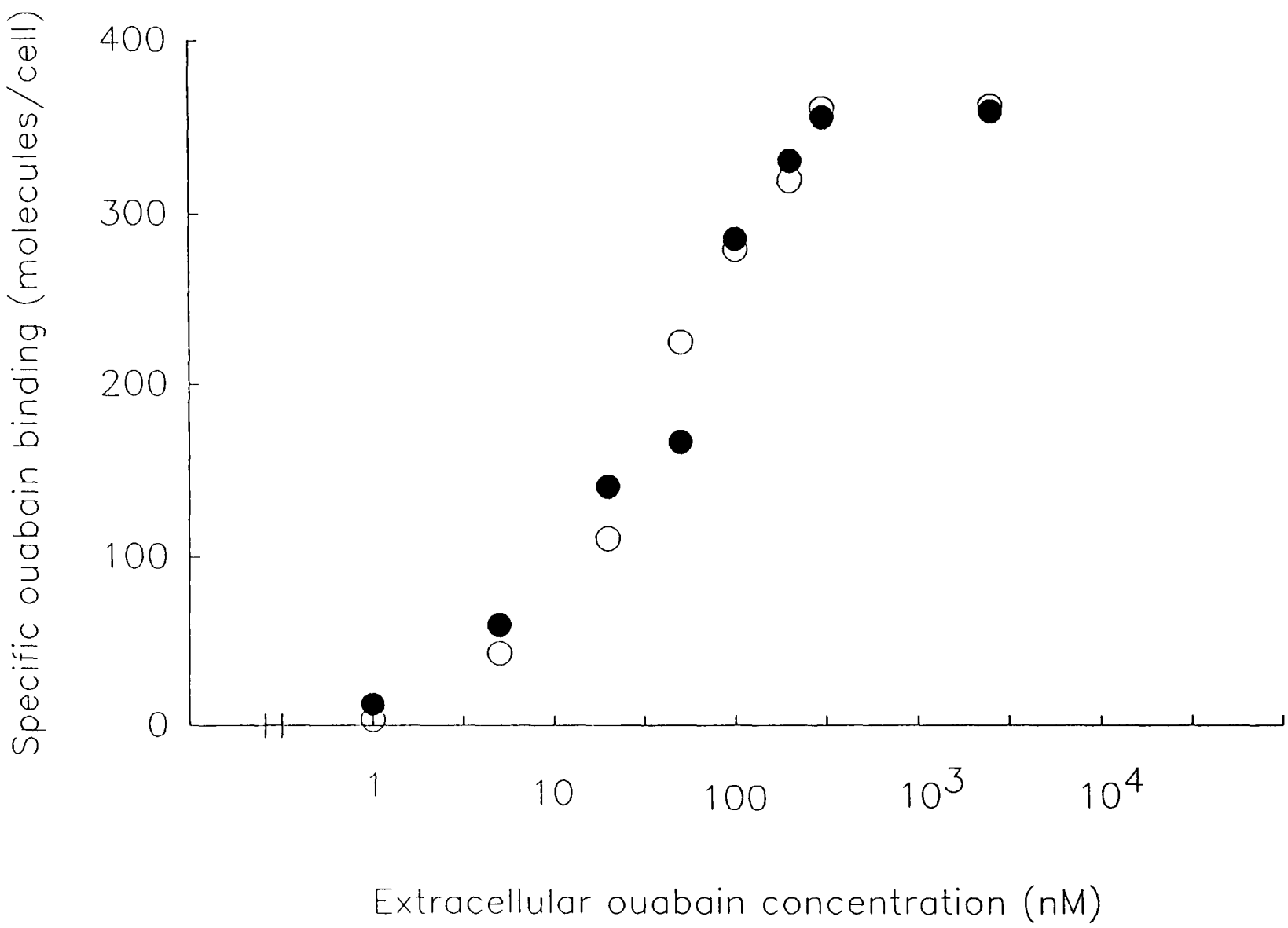

Figure 3.2. Specific ouabain binding in human erythrocytes as a function of extracellular ouabain concentration in cells incubated in plasma at 37°C for 1 hour. Normal control erythrocytes (open circles); erythrocytes obtained from haemodialysis patients (closed circles).

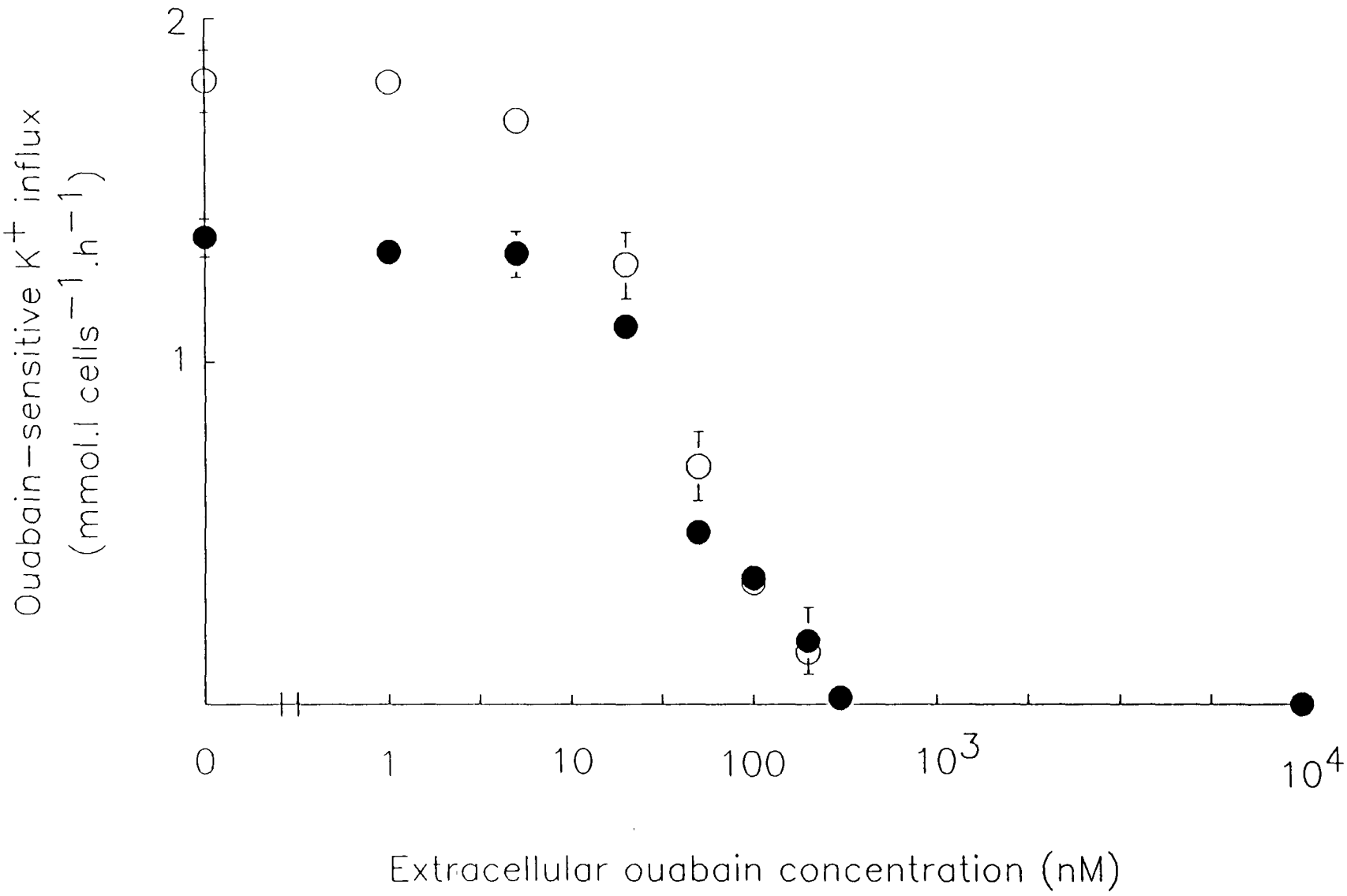

Figure 3.3. Ouabain-sensitive K^+ influx as a function of extracellular ouabain concentration in plasma. Open circles (normal control); closed circles (haemodialysis patient). Cells incubated for 1 hour at 37°C.

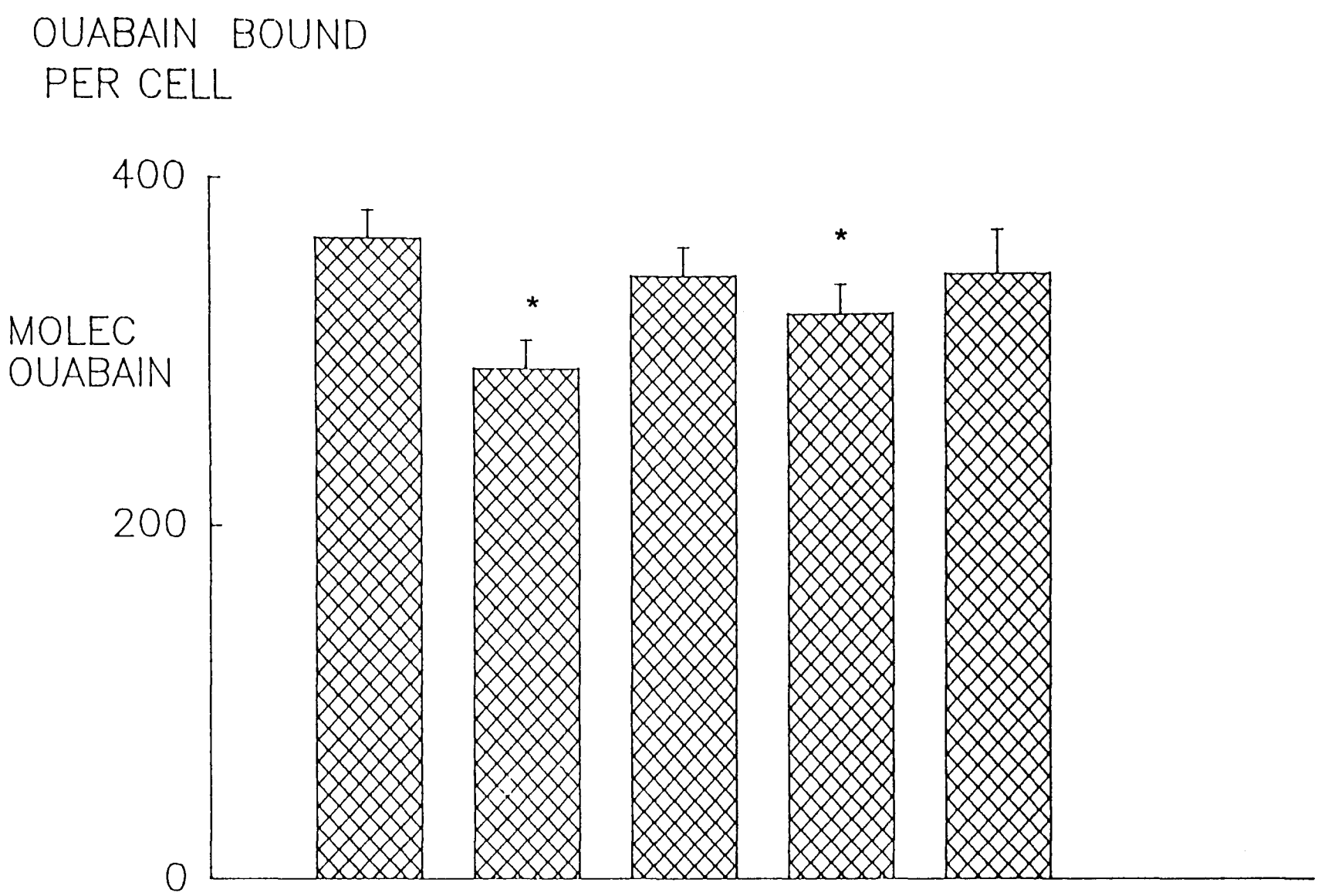

Figure 3.4. Mean specific ouabain binding (molecules per erythrocyte) in the 5 subject groups, as defined in the legend to figure 3.1. Error bars are S.E.M. *Significant difference from the control group $p < 0.05$.

0.021). The other groups were not significantly different from controls (HD = 344 ± 17, FT = 346 ± 26).

3.4. K^+ Influx Experiments.

These experiments were designed to measure ouabain sensitive and bumetanide-sensitive K^+ influx in whole blood. In this work ^{86}Rb has been used as a substitute for K^+ and it is assumed to be a valid congener both for the Na,K-ATPase and for the bumetanide-sensitive cotransporter. The leak value was the component of the flux not inhibited in cells incubated simultaneously in ouabain and bumetanide. Details of the methodology used to calculate these fluxes has been given in chapter 2.

The first group of results relates to K^+ influx rate in cells suspended in their native plasma. The mean (S.E.M) ouabain-sensitive K^+ influx values in normal controls, haemodialysis, pre-dialysis, CAPD and transplant groups were measured as 1.588 ± 0.03, 1.116 ± 0.04, 1.273 ± 0.09, 1.372 ± 0.03, 1.543 ± 0.07 mmol.l cells^{-1}.h^{-1} respectively. The mean (S.E.M) bumetanide-sensitive influx rate in normal controls was 0.214 ± 0.02; in haemodialysis 0.261 ± 0.07; in pre-dialysis 0.282 ± 0.05; in CAPD 0.322 ± 0.05 and in transplant patients 0.184 ± 0.02. The mean (S.E.M) residual K^+ influx for the same group of patients was 0.399 ± 0.02, 0.565 ± 0.07, 0.409 ± 0.05, 0.545 ± 0.06, and 0.430 ± 0.04 respectively.

These results are displayed for each of the 5 groups in figure 3.5.

The FT group was similar to the normal controls in all respects. A group of 5 transplant patients (FT) who were studied within 3 weeks of transplantation was also analysed separately and compared with the other 10 FT patients; these subgroups showed no significant differences. None of these patients had been given blood transfusion in the 3 months prior to being studied. The mean ouabain-sensitive K^+ influx was reduced by 21% in the CRF group and by 30% in the HD group compared to the controls ($p < 0.01$). The mean ouabain-sensitive influx in the CAPD group was 15% less than controls ($p < 0.02$). The mean bumetanide-sensitive component of K^+ influx was variable, and on average slightly raised in the uraemic groups, but only the 68% increase in the CAPD group with respect to controls was significant ($p < 0.05$). The mean residual K^+ influx was significantly increased in the HD and CAPD groups ($p < 0.05$).

These flux measurements were performed in plasma and therefore extracellular potassium concentration was not standardised, although it was measured in each experiment. Separate experiments were performed in standard saline to demonstrate the relationship between K^+ influx and extracellular potassium concentration (figure 3.6) and also to confirm that the stimulation of the Na/K pump activity by external K^+ was normal in the uraemic groups, and consistent

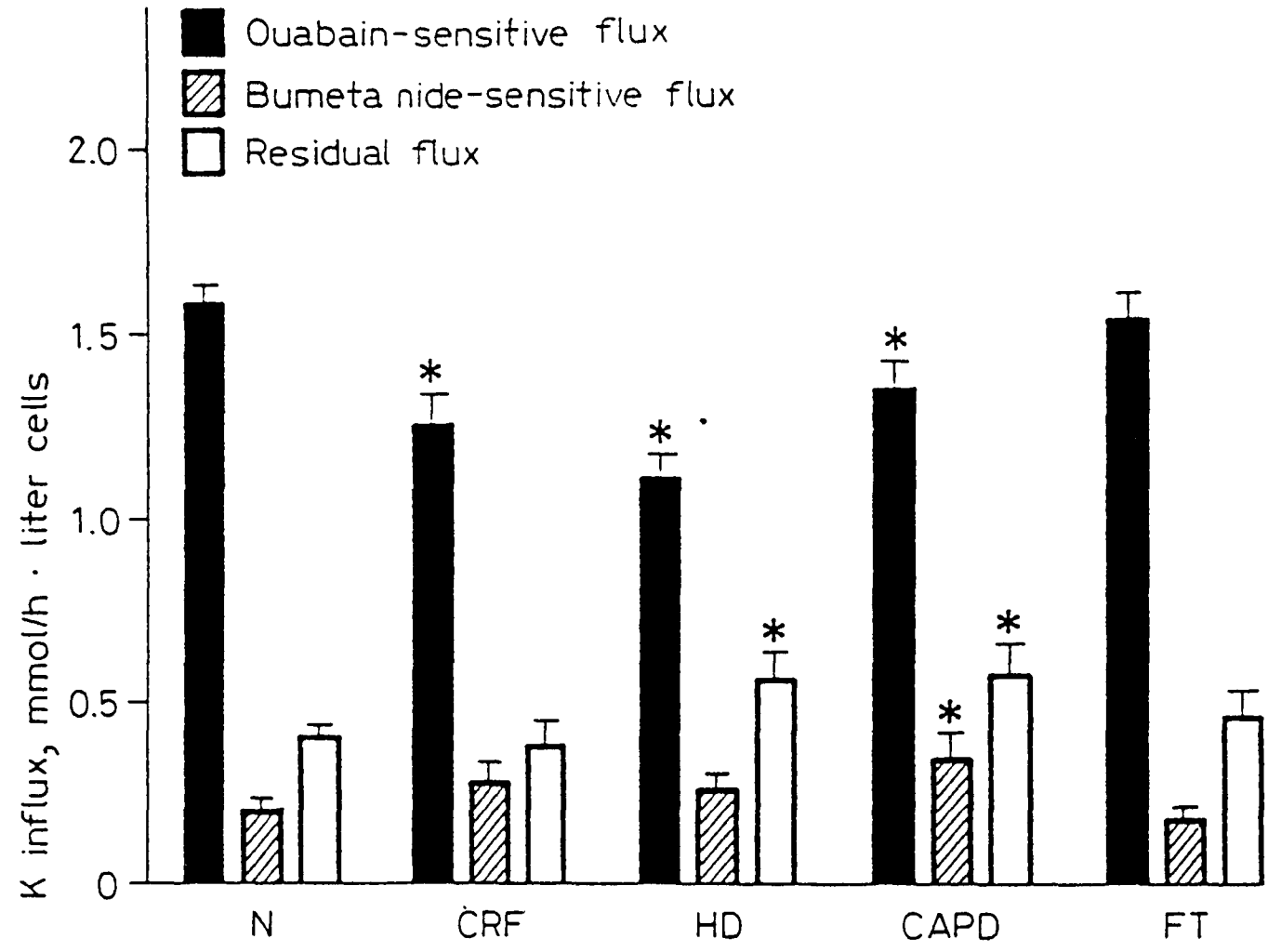

Figure 3.5. Estimates of the mean initial rate of erythrocyte K^+ influx (mmol.l cells^{-1}.h^{-1}) in the 5 groups. For each group the left-hand bar represents ouabain-sensitive flux, the middle bar bumetanide-sensitive flux and the right-hand bar the residual flux. Error bars are S.E.M. The groups are defined in the legend to figure 3.1. *Significant difference from the control group ($p< 0.05$).

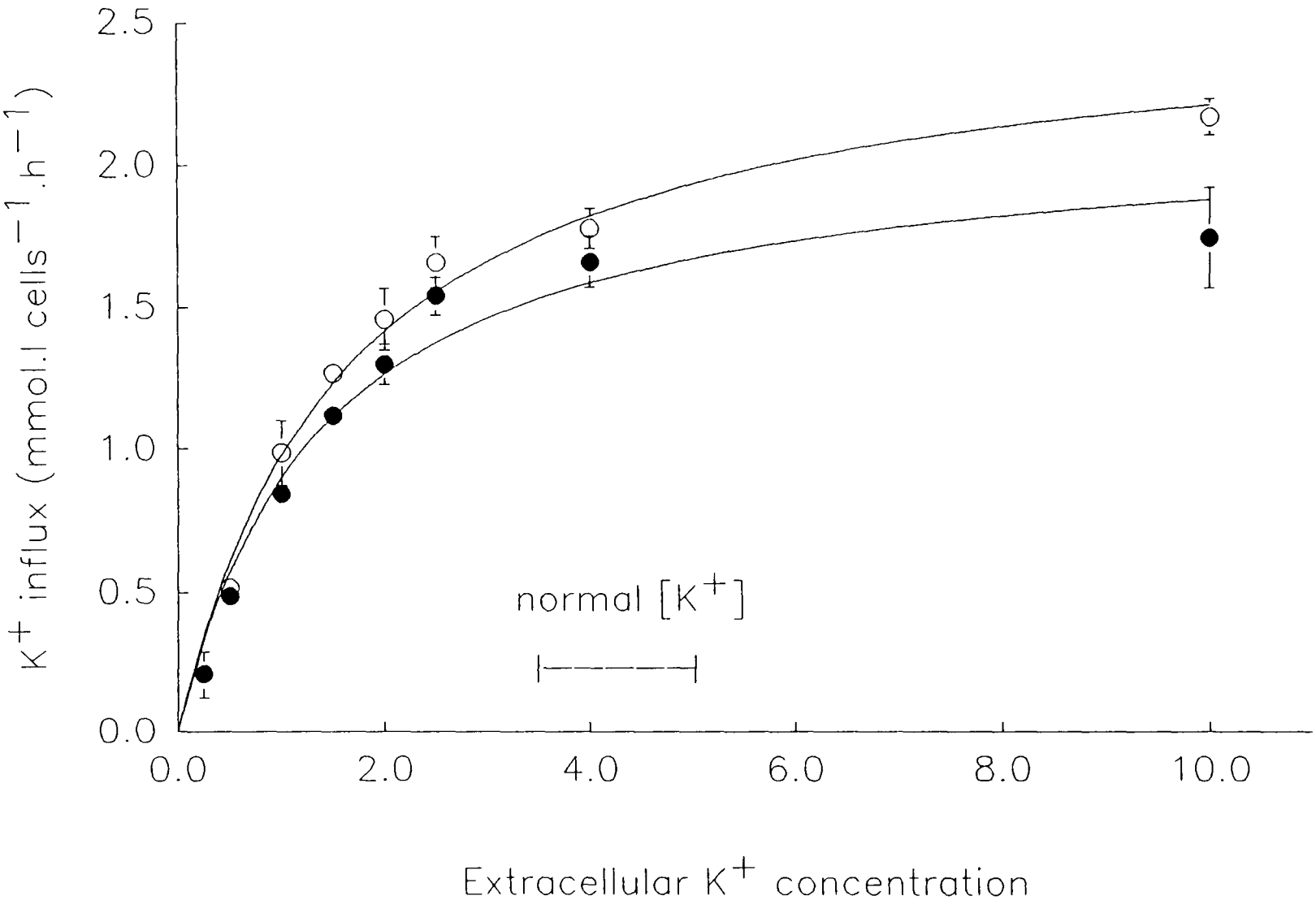

Figure 3.6. Active potassium influx as a function of extracellular potassium concentration. Open circles (normal controls); closed circles (haemodialysis patients).

with the existence of 2 identical external K^+ binding sites, each with an apparent dissociation constant of about 0.5 mM (see figure 3.7) (Garay and Garrahan, 1973). The measurements made of ouabain-sensitive K^+ influx could be corrected to a standard external K^+ of 4 mM. These corrections were small (< 8%), since as can be seen in figure 3.6, K^+ fluxes in physiological conditions are very near to the apparent V_{max}. The uncorrected potassium fluxes are reported here, and the effects of applying these corrections will be considered in the discussion.

3.5. Cross-Incubation Experiments.

Cross-incubation experiments were performed by placing erythrocytes from CRF and HD patients in normal plasma, and placing normal erythrocytes in CRF and HD plasma. After a 2 hour incubation at 37°C, K^+ influx and ouabain-binding measurements were made in the heterologous plasma. The results of these experiments are summarised in figure 3.8. Each measurement is expressed as a percentage of the value obtained for the same cells in their own plasma. Normal erythrocytes incubated in CRF plasma (N/CRF group) showed a 20% reduction in ouabain-sensitive K^+ influx and a 24% increase in bumetanide-sensitive K^+ influx. They also exhibited an 18% reduction in specific ouabain binding per cell. In other words the CRF plasma induced the type of abnormalities seen in the

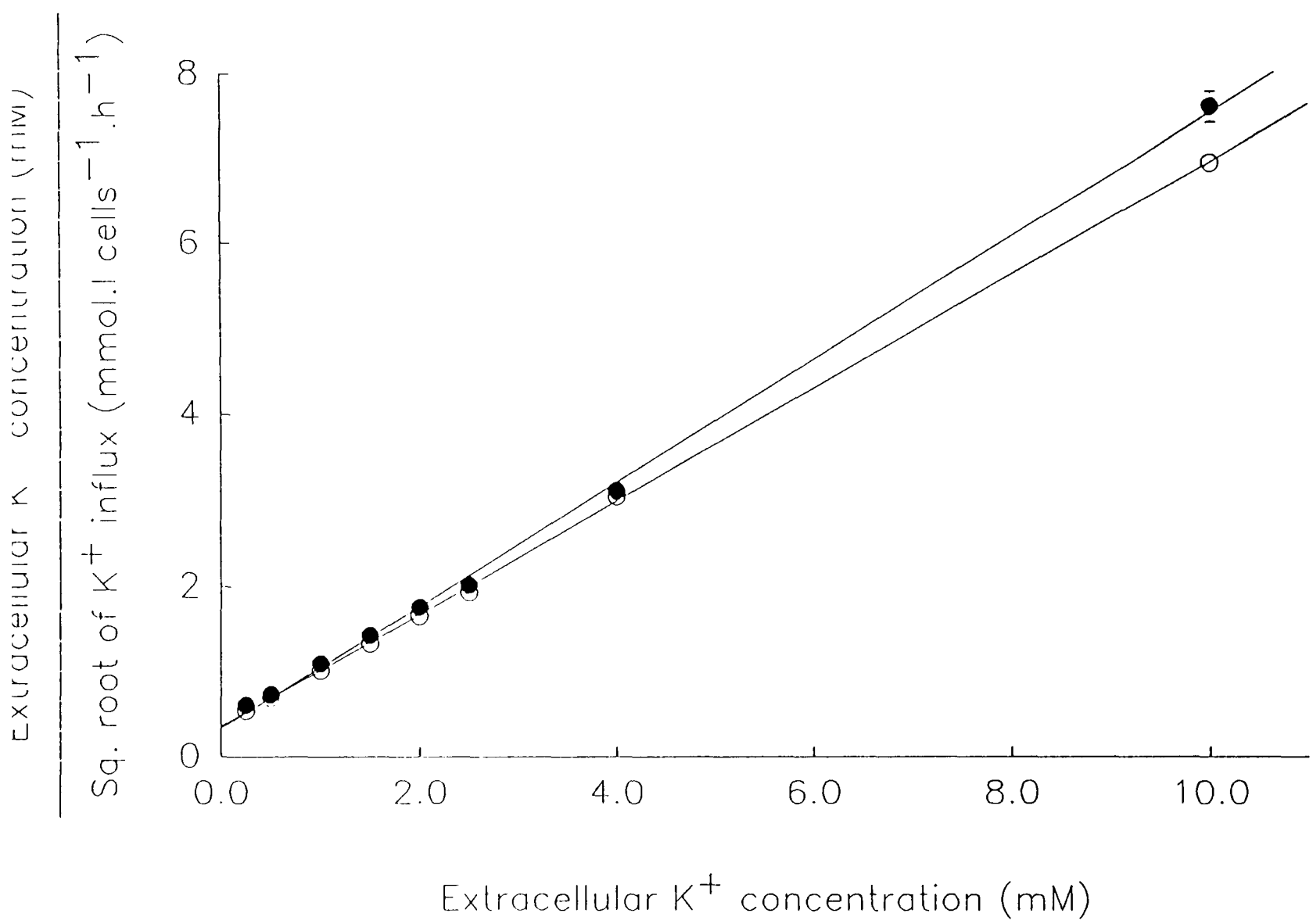

Figure 3.7. A test of the hypothesis that the sodium pump has 2 independent identical sites for potassium. The hypothesis is supported by the linear least-squares regression lines. The open circles are mean (± S.E.M) data from control erythrocytes while the closed circles are from haemodialysis patients' erythrocytes. The apparent dissociation constant for the external K^+ binding site can be estimated from the fits to be 0.48 mM in haemodialysis patients' cells and 0.51 mM in normal control cells (NS).

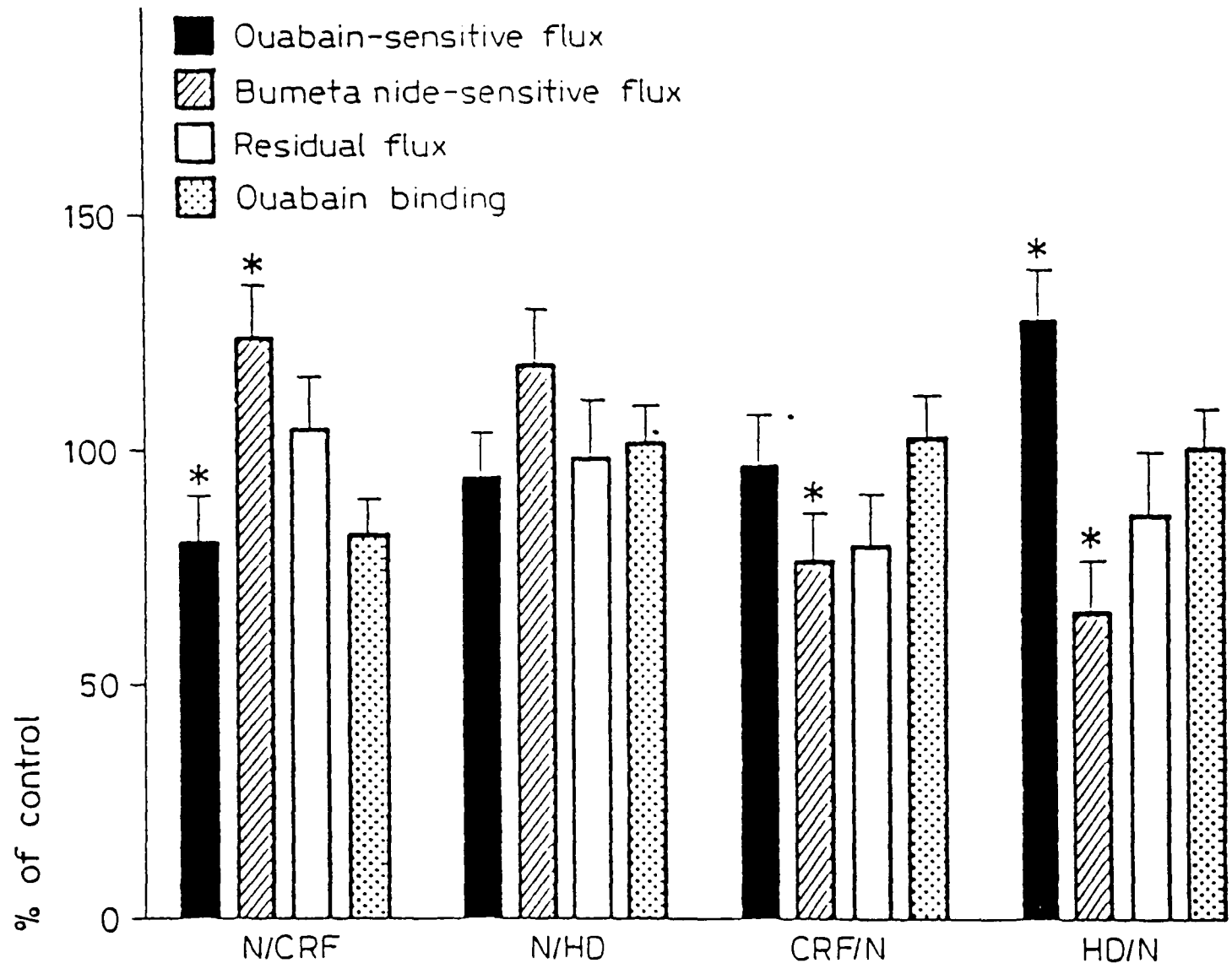

Figure 3.8. The results of cross-incubation experiments. The protocols were: N/CRF, normal erythrocytes incubated in CRF plasma; N/HD, normal erythrocytes in HD plasma; CRF/N, erythrocytes from CRF patients in normal plasma; HD/N, erythrocytes from HD patients in normal plasma. For each experiment the result has been expressed as a percentage of the result obtained for the same cells in their own plasma. The histograms show ouabain-sensitive K^+ influx, bumetanide-sensitive K^+ influx, residual K^+ influx, and specific ouabain binding per cell. Error bars are S.E.M. *Significant difference from 100% ($p < 0.05$).

CRF group (figure 3.4). Normal erythrocytes incubated in HD plasma (N/HD group) showed a small decrease in ouabain-sensitive K^+ influx, and a small increase in the bumetanide-sensitive K^+ influx. These effects were not significant. CRF erythrocytes incubated in normal plasma (CRF/N group) showed no change in ouabain-sensitive K^+ influx, and exhibited reductions in bumetanide-sensitive and residual K^+ influx. Incubation of HD patients' erythrocytes in normal plasma (HD/N group) produced a 28% increase in ouabain-sensitive K^+ ion flux and a 35% reduction in bumetanide-sensitive flux. There was no change in ouabain binding. The abnormalities seen in the HD group were therefore partially reversed by incubation in normal plasma.

3.6. Pump Turnover.

For each subject the ion-flux and ouabain-binding measurements were performed in parallel on the same sample of blood, and the results can be used to calculate the ouabain-sensitive K^+ influx per specific ouabain binding site, as an indication of the turnover rate of the Na/K pump. These turnover rates for the 5 groups studied and for the 4 cross-incubation protocols are displayed in figure 3.9. The mean (S.E.M) turnover rate of the Na/K pump for normal erythrocytes in native plasma was 79 ± 4.7 K^+ ions.sec^{-1}. The mean turnover was significantly reduced for erythrocytes from haemodialysis

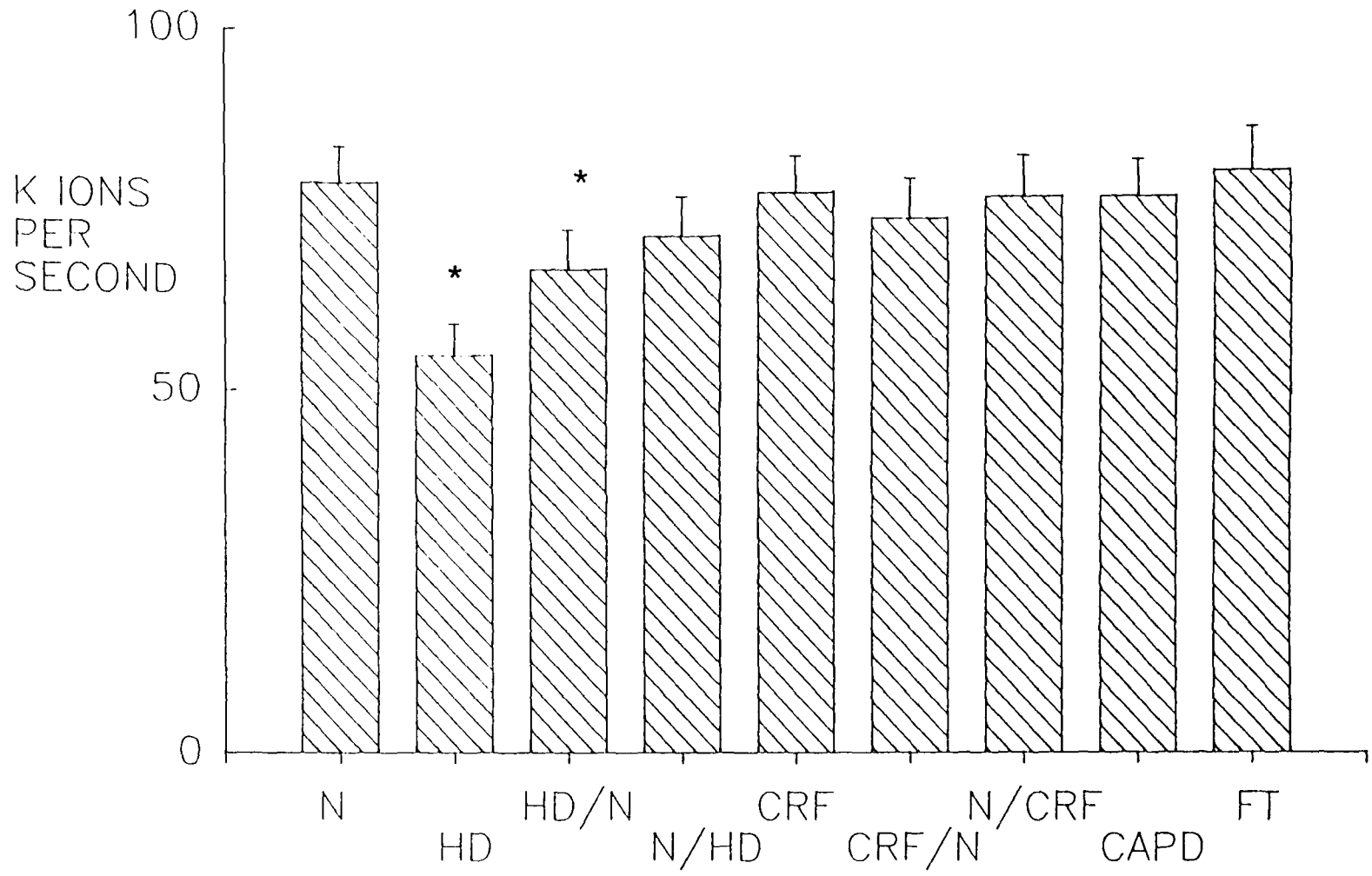

Figure 3.9. Mean Na/K pump turnover rates (K ions/s . ouabain binding site) for erythrocytes in their native plasma from the 5 subject groups, and in the 4 cross-incubation protocols. Error bars are S.E.M. *Significant difference from the control group ($p < 0.05$).

patients in their native plasma at 55 ± 5.2 K^+ ions.sec^{-1}, ($p < 0.01$), and showed significant reversal following incubation in normal plasma (HD/N group: 67 K^+ ions.sec^{-1}. $p < 0.05$). The mean turnover rate was normal in the CRF, CAPD and FT groups. Cross-incubation of CRF cells and normal plasma, and vice-versa, did not induce any abnormality in the turnover rate.

3.7. Reversibility Experiments.

The above experiments established that uraemic cells showed a significant degree of inhibition of ouabain-sensitive K^+ influx. They also showed that for haemodialysis patients' red cells, a significant part of this inhibition would recover after 2 hours incubation in normal plasma. It was therefore important to examine this in more detail in order to answer the following questions: What was the time-scale of recovery? Would there be a more significant improvement if cells were incubated for longer? If recovery occurs would this be a sustained effect?

Red cells obtained from 8 haemodialysis patients and 6 normal controls and prepared the same way as for the previous influx experiments, were suspended in their native plasma at 37°C in triplicate tubes in the presence or absence of 0.1 mM ouabain, and ouabain-sensitive ^{86}Rb influx rates were determined as above. These results were considered as influx rate at time zero. Uraemic cells were then washed 3 times in

standard saline, resuspended in normal plasma (ABO and Rh compatible) and incubated at 37°C. The same ^{86}Rb influx experiments were then repeated after cells had been incubated for 1, 2 and 4 hours in normal plasma.

In order to establish that alterations of the flux with time were not simply an artifact induced by placing cells in a foreign plasma, normal cells were also washed 3 times and resuspended in normal heterologous plasma, and influx measurements performed at the same times as for the uraemic cells in normal plasma.

Figure 3.10 presents the mean ouabain-sensitive K^+ influx in red cells from uraemic patients incubated in normal plasma (closed circles) and normal cells incubated in normal heterologous plasma (open circles) in relation to their flux at time 0. Uraemic cells showed a significant progressive recovery of the flux after 1 hour ($p < 0.03$), 2 hours ($p < 0.02$) and influx rates remained at this level at 4 hours incubation ($p < 0.01$). In normal cells, the K^+ influx rates were not significantly different from the initial values at any time during incubation in heterologous plasma. The mean influx rates (S.E.M) at different times is shown in Table 3.1. It must be emphasised that these experiments were performed 1 year apart from the K^+ influx experiments presented above. The 8 haemodialysis patients in the reversibility experiments represent part of the original haemodialysis group in whom K^+ influx was re-studied. As can be seen in Table 3.1, the mean

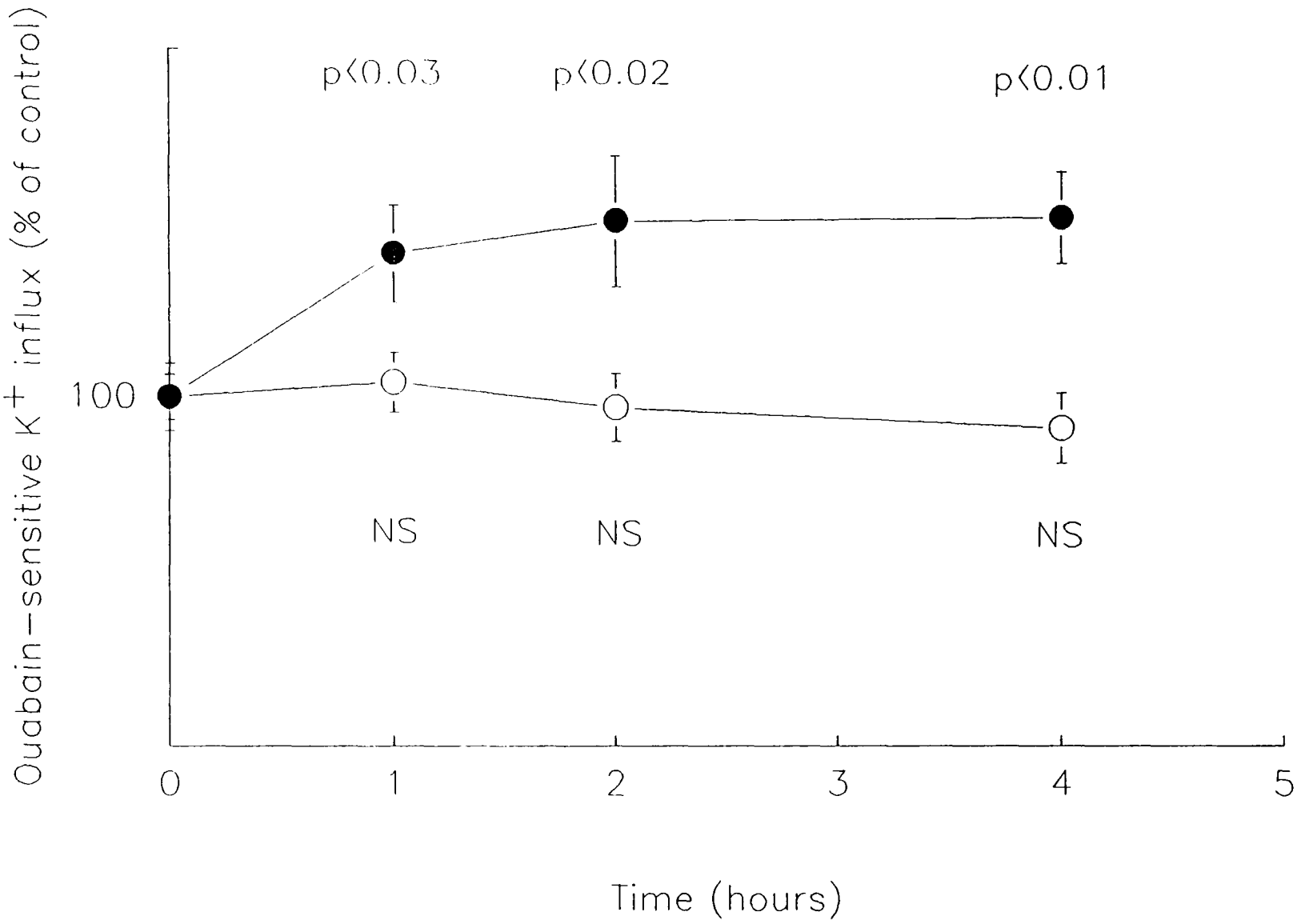

Figure 3.10. The results of the reversibility experiments. The figure shows the mean ouabain-sensitive K^+ influx in uraemic cells incubated in normal plasma (closed circles) and normal cells incubated in normal heterologous plasma (open circles) as a function of time (hours). For each experiment the result has been expressed as a percentage of the result obtained for the same cells in their own plasma at time 0. Error bars are S.E.M. Significant difference from 100% is presented in the figure.

Table 3.1. Reversibility flux experiments

	Time 0	Time 1h	Time 2h	Time 4h
Normal controls (n = 6)	1.410 (0.192)	1.432 (0.073)	1.375 (0.176)	1.218 (0.109)
Haemodialysis patients (n = 8)	1.125 (0.136)	1.350 (0.255)	1.496 (0.230)	1.398 (0.207)

Influx rates of potassium, obtained in red cells incubated in plasma at 37 C. Units = mmol/l cells/h (SD)

influx rates at time 0 were again significantly different from the results obtained for normal controls ($p < 0.01$) and testifies to the reproducibility of the findings presented above.

The ouabain-insensitive ^{86}Rb influx rates showed no changes on cross-incubation in heterologous plasma as illustrated in figure 3.11.

3.8. Discussion.

This chapter has shown that there are significant changes in red cells from uraemic patients. However, they are not uniform, since different groups of patients showed selective abnormalities. These results will now be discussed individually.

3.8.1. Normal Controls.

The number of specific ouabain binding sites in the membrane is equivalent to the number of Na/K pump sites (Jorgensen, 1974; Glynn and Karlish, 1975). In human red cells, this number has been variously reported as 259 ± 60 (Erdmann, 1982), 362 ± 69 (Wiley, Kraft and Cooper, 1979), 449 ± 11 (Kaji, Thakkar, Kahn and Torelli, 1981) and 450-500 sites per cell (Joiner and Lauf, 1978). The study presented above reports a value of 366 ± 16 pump sites per cell. The

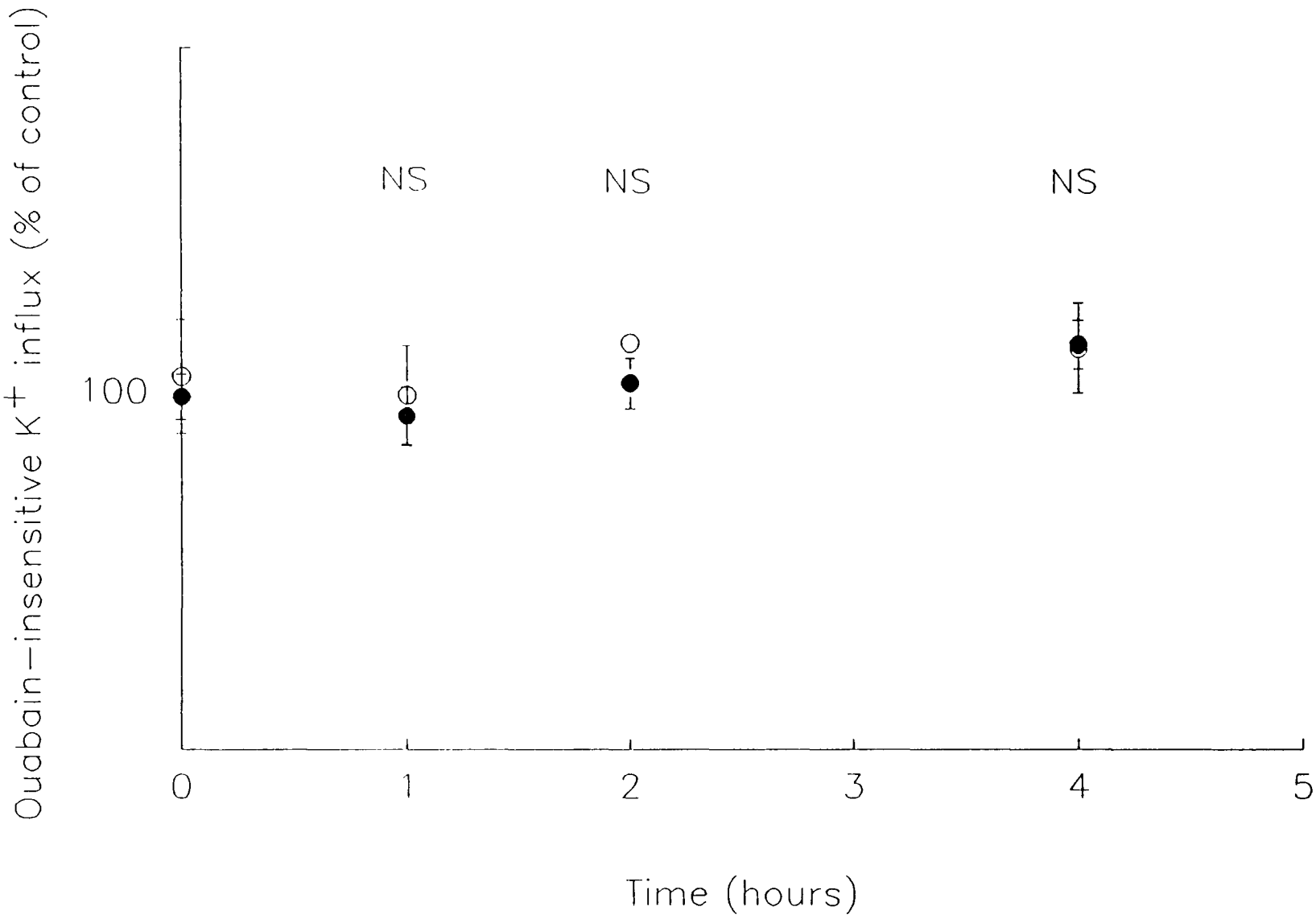

Figure 3.11. The results of the reversibility experiments. The figure shows the mean ouabain-insensitive ^{86}Rb influx in uraemic cells incubated in normal plasma (closed circles) and normal cells incubated in normal heterologous plasma (open circles) as a function of time (hours). For each experiment the result has been expressed as a percentage of the result obtained for the same cells in their own plasma at time 0. Error bars are S.E.M. No significant differences were observed.

values obtained for ouabain-sensitive ^{86}Rb influx in human erythrocytes at 37°C are also similar to those reported in the literature (Glynn, 1956; Glynn, 1957). The maximal Na/K pump turnover per site has been calculated as 170 K^+ ions/second (Erdmann and Hasse, 1975) and under physiological conditions as 80 K^+ ions/second (Solomon, 1952 cited by Erdmann, 1982). In normal controls, the results presented here showed the mean (± S.E.M) turnover rate to be 79 ± 4.7 K^+ ions/second (for review see Joiner and Lauf, 1978). Finally, the mean intracelullar sodium values for normal controls reported in this thesis are in line with the reported mean values of 5.6 ± 0.3 (Cole, 1973), 7.5 ± 0.2 (Cole, Steinberg and Guttmann, 1978), 7.54 ± 1.27 (Welt, Smith, Dunn et al, 1967) and 7.5 ± 0.7 mmol/l cells reported by Kramer, Gospodinov and Kruck (1976).

3.8.2. **Non-dialysed patients with chronic renal failure**

Mean erythrocyte Na^+ concentration was raised in these patients (CRF group) compared with controls, in agreement with previous reports (Cole, 1973; Kramer, Gospodinov and Kruck, 1976; Zannad, Royer, Kessler et al, 1982; Walter and Brecht, 1983, Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984). In animal models, intracellular sodium has been reported to be increased by 90% in adipocytes and by 50% in skeletal muscle from chronically uraemic rats (Druml, Kelly,

May and Mitch, 1988).

The CRF group exhibited a 21% reduction in ouabain-sensitive K^+ influx associated with a similar reduction in specific ouabain binding per erythrocyte. Accordingly, the calculated Na/K pump turnover number was not different from control values. The increased intracellular Na^+ and increased plasma K^+ in the CRF group compared to controls should activate pump fluxes over this range of concentrations. The extent of activation can be estimated if it is assumed that the affinities of the internal site for Na^+ and external site for K^+ are normal in the CRF cells. The mean stimulation can be estimated from the data of Garay and Garrahan (1973) to be 24%. Therefore, higher turnover rates would be expected in these CRF cells, and the present results can be interpreted as a modest decrease in turnover. The abnormalities of ouabain-binding are similar to those reported by Cheng, Kahn and Kaji (1984). The plasma of CRF patients induced equivalent effects in normal erythrocytes after a 2 hour incubation, as demonstrated by Kramer, Gospodinov and Kruck (1976). Plasma from patients in chronic renal failure has also been shown to inhibit the activity of isolated preparations of erythrocyte Na,K-ATPase (Cole, Balfe and Welt, 1968; Cole, 1973). These results favour the hypothesis that the abnormal membrane function seen in CRF is due to plasma factors. Zannad and collegues, (1982) were able to demonstrated a defect in the Na/K pump of erythrocytes of non-dialysed patients with acute

renal failure and their data support the idea of a plasma factor effect.

In a more recent report Stokes, Norris, Marwood et al, (1990) also found an inhibitory effect of uraemic plasma on Na,K-ATPase activity, measured in vitro by a linked-enzyme assay, and on ^{86}Rb uptake in guinea pig aortic strips.

The reduction in ouabain binding in CRF indicates that there is a reduction in the number of pump units undergoing cyclical phosphorylation-dephosphorylation (Lee and Fortes, 1984). Three explanations for this are possible: (a) a down regulation of the number of pumps inserted into the cell membrane during erythropoiesis, (b) the presence of an "endogenous digoxin" in CRF plasma which competes for the ouabain binding site, or (c) the existence of plasma factors in CRF which cause complete inhibition of a fraction of pumps, without competing with ouabain. The experiments reported here did not explore the process of ouabain binding in detail, and further information could be obtained by a study of the kinetics of ouabain binding in the presence of CRF plasma. The cross-incubation experiments suggest that plasma factors in CRF can significantly affect membrane transport systems following a 2 hour incubation, but little reversal occurs after 2 hours in normal plasma. The reasons underlying these phenomena are unknown. Recent reports indicate that the structural and compositional properties of the membrane can determine the catalytic properties of membrane transport

proteins (Carruthers and Melchior, 1988) and this possibility will be considered in more detail in chapter 7.

The ouabain-insensitive ion fluxes were not significantly different from controls in these experiments in CRF, and this is in agreement with most other reported studies (Kramer, Gospodinov and Kruck, 1976; Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984).

3.8.3. **Patients on Haemodialysis.**

This group of patients (HD group) produced different experimental results from those patients with CRF before initiation of maintenance dialysis. The mean erythrocyte Na^+ concentration was normal, a fact also reported by Quarello, Boero, Guareno et al, (1985).

The erythrocyte ouabain-sensitive potassium influx was reduced, but no changes were found in the ouabain binding properties of these cells. If the ouabain-sensitive K^+ fluxes are corrected for the variable plasma K^+ to a K^+ value of 4 mmol, the 30% decrease in HD compared to controls becomes a 33% decrease. The K^+ turnover rate per ouabain binding site was decreased. Two hours incubation in normal plasma partially reversed the inhibition and (as demonstrated in the reversibility experiments) the flux would then remain stable for at least 4 hours. However, there was no induction of the defect in normal red cells by incubation in HD patients'

plasma for 2 hours. Since intracellular sodium was normal in the HD patients whose plasma was used to incubate normal cells, this result is in agreement with Cole, Balfe and Welt (1968). They were able to induce the defect in normal cells only when plasma from patients with high intracellular sodium was used. The normal ouabain binding seen in HD patients is consistent with the observation of Cheng, Kahn and Kaji (1984) that ouabain binding increased towards normal over a period of 2 months in three CRF patients when they were started on maintenance haemodialysis. However, these authors also reported, using a larger group of patients, that the mean number of ouabain binding sites per erythrocyte was considerably reduced in HD patients, a finding which differs from the present results and from data of Izumo, Izumo, DeLuise and Flier, (1984) who found a normal erythrocyte ouabain-binding capacity.

The 30% reduction in ouabain-sensitive ion flux seen here in HD patients is consistent with most earlier reports (Zannad, Royer, Kessler et al, 1982; Walter and Brecht, 1983, Izumo, Izumo, DeLuise and Flier, 1984; Cheng, Kahn and Kaji, 1984; Quarello, Boero, Guareno et al, 1985) but some researchers report no pump defect at all in this group (Diez, Virto, Yap et al, 1986; Corry, Tuck, Brickman et al, 1986), while Bosch, Hernando, Casado et al, (1989) reported two groups of HD patients: group 1 (pump -) with decreased erythrocyte Na^+ pump activity, and group 2 (normal pump) with

normal erythrocyte Na^+ pump activity. One possible reason for these discrepancies is the fact that many studies have involved flux measurements in artificial media where removal of important factors present in uraemic plasma may cause the effect to be lost or altered. In the present experiments a significant reversal of the decrease in transport occurred on incubation in normal plasma. Furthermore, the available evidence indicates that the abnormal pump function in haemodialysis patients is partially reversed after each dialysis (Izumo, Izumo, DeLuise and Flier, 1984; Zannad, Royer, Kessler et al, 1985; Quarello, Boero, Guareno et al, 1985).

As with the CRF group, the most likely explanation for the altered Na/K pump function in HD patients is the presence of plasma factors. However these factors appear to have different properties from those in non-dialysed patients. The total number of functional pumps is unaltered but each turns over more slowly. The factors appear to undergo significant removal from the erythrocyte membrane over a 2 hour period, but do not seem to affect normal cells over the same time-course. The abnormalities of Na/K pump number seen in CRF are reversed by maintenance haemodialysis, perhaps due to removal of plasma factors, but the abnormalities of total pump function remain, arising perhaps from a different set of plasma constituents acting by a different mechanism. The presence of different factors in CRF and HD patients might

help to explain a puzzling feature of the data reported by Cheng, Kahn and Kaji (1984). Although 3 patients showed increased ouabain binding site density and decreased erythrocyte Na^+ after initiation of HD, on average the patients who had been on dialysis longest had the highest levels of erythrocyte Na^+. This might be consistent with a change in the origin of the membrane defect and accumulation of different toxins in long-term haemodialysis patients.

3.8.4. **Patients on CAPD.**

Erythrocyte Na/K pump function in patients on continuous ambulatory peritoneal dialysis was depressed, but the effects were smaller than those seen in CRF or HD patients. The difference between normals and CAPD patients was not altered significantly by applying corrections for the variation in extracellular K^+ concentration. However, erythrocyte Na^+ concentrations were slightly higher in CAPD than HD. This may be related to the increased ouabain-insensitive ion-fluxes seen in CAPD (figure 3.5). Cheng, Feinfeld, Briscoe et al, (1987) also found a significant increase in red cell sodium following 48 hours of intermittent peritoneal dialysis. However they did not have a clear explanation for this phenomenon.

Overall, it is not clear whether the erythrocyte membrane transport abnormalities of uraemia respond better to CAPD than

to HD, although CAPD has been suggested as more effective by Stokes, Norris, Marwood et al, (1990) who did not detect a significant inhibition of the Na,K-ATPase in the CAPD group of patients. The reduced Na/K pump flux in the CAPD patients' group was associated with a reduction in erythrocyte ouabain binding, and turnover of ions per site was normal. The abnormalities were therefore similar to those seen in the CRF group, but the effects seen in the CAPD patients were too small for firm conclusions to be drawn.

3.8.5. **Patients with Functional Transplants.**

It has been reported that erythrocyte membrane Na,K-ATPase activity is supranormal in renal transplant patients (Cole and Maletz, 1975), and that erythrocyte Na^+ content is normal or low (Cole, Steinberg and Guttmann, 1978; Diez, Virto, Yep et al, 1986). Ion flux studies suggest that Na/K pump function in transplanted patients is normal or increased (Cole, Steinberg and Guttmann, 1978; Zannad, Royer, Kessler et al, 1982). In the present experiments, erythrocytes from these patients had normal membrane transport, ouabain binding properties and intracellular sodium concentrations. The abnormalities associated with uraemia therefore appeared to be completely reversed by transplantation. The finding that the subgroup of patients studied within 3 weeks of transplantation had normal erythrocyte Na/K pump function is

consistent with the view that plasma factors, rather than intrinsic erythrocyte abnormalities are important in uraemia. However, the use of higher doses of corticosteroids in the early post-transplant period could have contributed to the improvement in membrane transport, although this would be an unlikely explanation in long standing transplant patients treated with low dose steroids (Cole, Steinberg and Guttmann, 1978). Other authors have found no significant effects of steroids on K^+ transport either *in vivo*, or *in vitro*. (Streeten and Solomon, 1953-4) and ouabain-sensitive K^+ influx was unchanged in erythrocytes from patients treated with steroids compared with normal controls (Kaji, Thakkar, Kahn and Torelli, 1981).

3.8.6. NaKCl Cotransport.

The results for K^+ influx throughout showed a tendency for the bumetanide-sensitive fluxes to be higher in patients in the CRF, HD and CAPD groups. However there was great variability and the increase was only significant in the CAPD patient group ($p < 0.05$). In general other authors have found these fluxes to be reduced in uraemic patients (Corry, Tuck, Brickman et al, 1986; Diez, Virto, Yep et al, 1986; Corry, Lee and Tuck, 1987) but this view is not unanimous (Trevisan, DeSanto, Laurenzi et al, 1986). These discrepancies may be the result of differences in methodology.

There have been reports of changes in the NaKCl cotransport system in essential hypertension but the literature is controversial. Some authors have reported a reduced Na^+ efflux in erythrocytes through this system (Garay and Meyer, 1979, Garay, Dagher, Pernollet et al, 1980) while other groups reported that the co-transport was in fact increased in essential hypertension (Adragna, Canessa, Solomon et al, 1982; Bin Talib, Chipperfield and Semple, 1984; Canessa, Spalvins, Adragna and Falkner, 1984). (for review see Chipperfield, 1986).

At this moment, the data presented in this thesis do not allow a definitive conclusion to be drawn.

3.8.7. **Residual Fluxes.**

The ouabain and bumetanide-insensitive ^{86}Rb influx (leak) was also variable. The mean value was increased in CAPD and HD patients ($p < 0.05$). This has been also noticed by another group (I. Bernhardt, unpublished observations). This is consistent with the presence of an increased background membrane ion permeability. It was accompanied by an increase in mean intracellular Na^+ in the CAPD group. The importance of these changes is difficult to assess.

3.9. Summary

The abnormalities of membrane transport associated with renal failure are complex, and remain poorly understood. The experiments presented in this thesis confirm that there are abnormalities in erythrocyte K^+ transport both in patients with renal failure not yet dialysed, and on dialysis. However, the groups studied did not behave the same way. Patients on haemodialysis exhibited reduced pump turnover, non-dialysed patients showed normal turnover with a reduced number of functional pumps. The abnormalities were corrected by a functional kidney transplant. A summary of these results is presented in Table 3.2.

The reasons for these abnormalities are not clear. The balance of evidence suggests that these effects are due to the presence of plasma factors. The chemical nature of these factors remains unclear, but some features of the effects seen (e.g. slow reversal) are consistent with the view that abnormal lipids are involved. These possibilities will be discussed in more detail in chapter 7.

Table 3.2. The Na/K pump experiments

	Number of Na/K pumps per erythrocyte	Ouabain-sensitive potassium influx	Mean turnover rate of the pump
Chronic renal failure	decreased	decreased	normal
Haemodialysis	normal	decreased	decreased
CAPD	decreased	decreased	normal
Functional transplants	normal	normal	normal

CAPD: continuous ambulatorial peritoneal dialysis.
For details, see text.

CHAPTER 4

AMINO ACID TRANSPORT

This chapter will present a study on red cell amino acid transport systems in normal controls and patients on haemodialysis. The five amino acid transport systems presently identified in human red cells are the y^+, ASC, gly, L and T systems and will be measured in this study by their substrates L-lysine, L-serine, glycine, L-leucine and L-tryptophan respectively. The results will be presented individually for each transport system studied and will be followed by a general discussion at the end of the chapter.

4.1. L-lysine.

The initial rate of lysine influx in immediately-washed human red cells at 37°C and an extracellular lysine concentration of 0.5 mM was established in normal control cells. It appears to be linear with time at least up to 10 minutes (figure 4.1) and this justifies the 5 minutes incubation time used in subsequent experiments.

Experiments measuring lysine influx as a function of

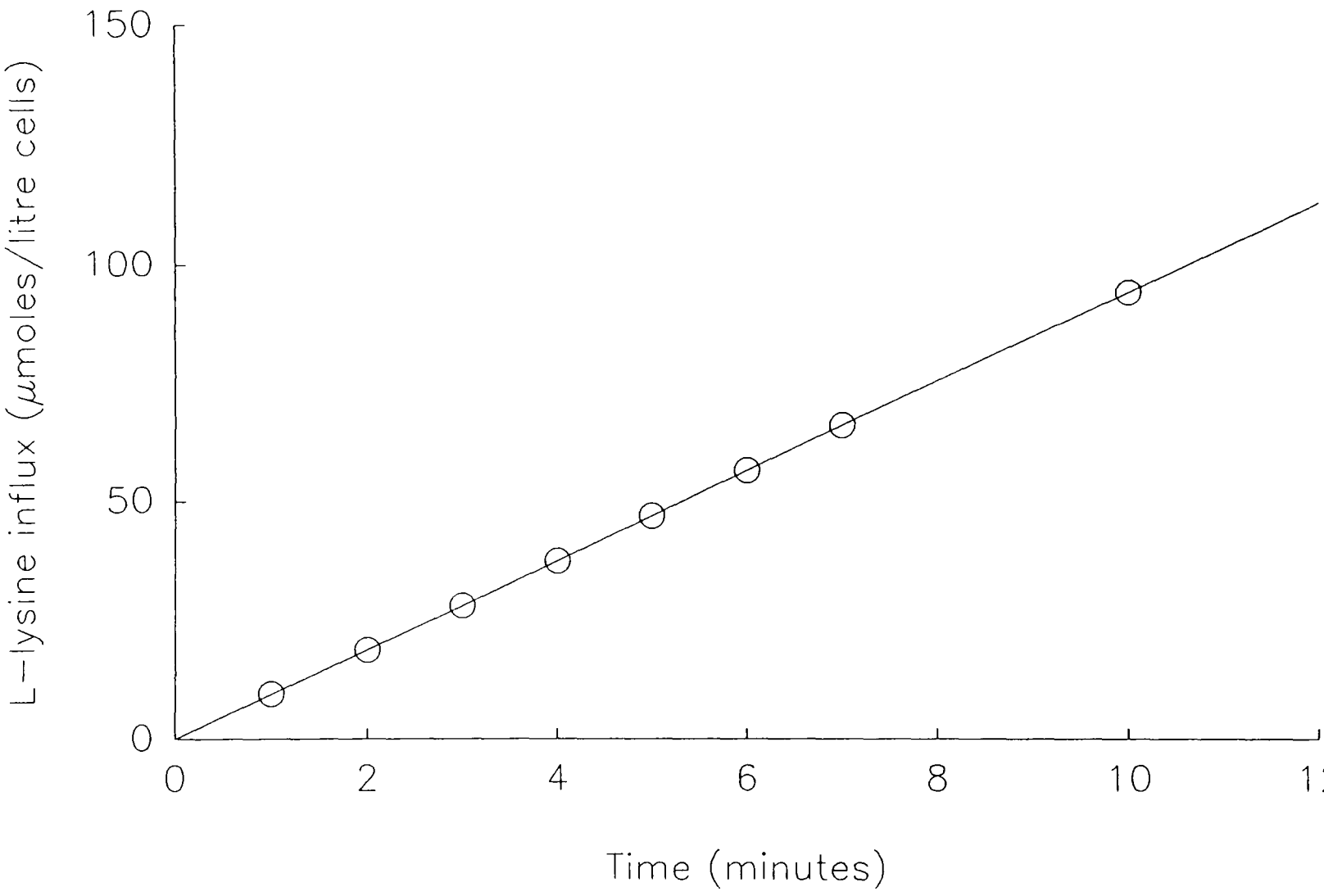

Figure 4.1. Time course of L-lysine influx in a normal control (extracellular lysine concentration 0.5 mM). Time points were fitted by linear regression.

extracellular lysine concentration in immediately washed cells were performed in red cells obtained from 10 patients on haemodialysis, 3 patients with chronic renal failure who were not on dialysis and 12 normal controls. Figure 4.2 presents the mean initial rates of L-lysine influx as a function of extracellular lysine concentration in patients on haemodialysis and normal controls. The relationship between flux rate and concentration can be fitted with a model in which there are two components of the flux. The first component is saturable at high concentrations and can be described by Michaelis-Menten kinetics, using the maximal flux at high lysine concentrations (V_{max}), and the concentration of lysine giving half-maximal flux (K_m). The second component is not saturable and is linearly related to the lysine concentration by an apparent diffusion constant, K_d. The total lysine flux (V) is the sum of these two components and is given by the relationship:

$$V = \frac{V_{max}.\ [S]}{[S] + K_m} + K_d.\ [S] \qquad (1)$$

where [S] is the extracellular lysine concentration. The plot in figure 4.2 was obtained by fitting the data to equation (1).

The results from each experiment were fitted by equation

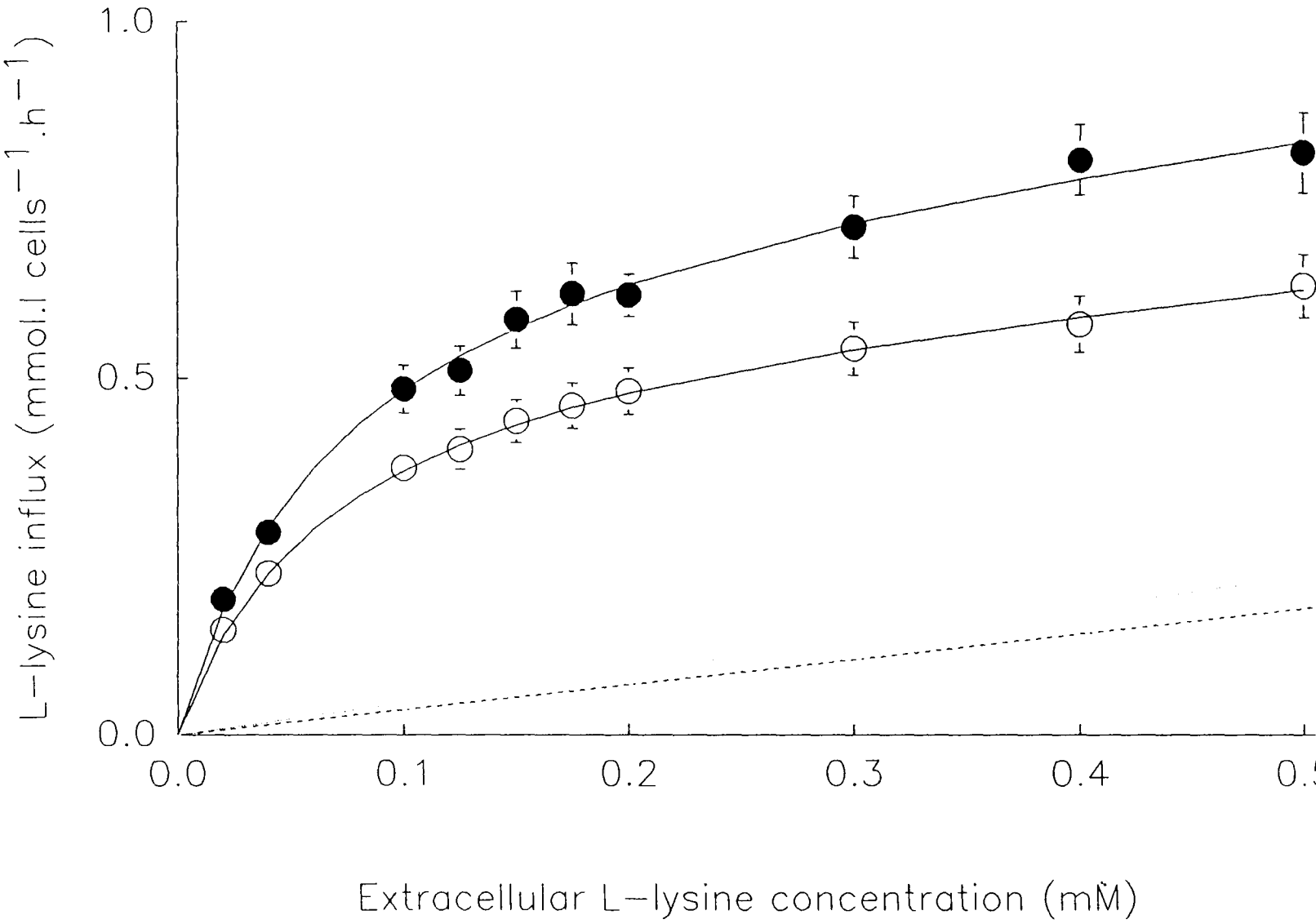

Figure 4.2. The mean initial rates of erythrocyte L-lysine influx in immediately washed cells as a function of extracellular lysine concentration in haemodialysis patients (closed circles) and normal controls (open circles). The error bars are S.E.M. The lines are Michaelis-Menten fits to the data with the values of V_{max} and K_m described in the text. Fitting for K_d in controls (dotted lines) and uraemics (dashed lines) are 0.178 h^{-1} and 0.224 h^{-1} respectively.

(1) using a least squares algorithm. In each case the coefficient of multiple correlation was greater than 0.99; the quality of fit shown in figure 4.2 was typical.

In erythrocytes from uraemic patients the saturable component had a mean V_{max} = 0.760 mmol.l cells^{-1}.h^{-1} (S.E.M. 0.07) and a mean K_m = 68.2 μmol/l (S.E.M. 5.7). The corresponding values in normal controls were 0.566 mmol.l cells^{-1}.h^{-1} (S.E.M. 0.033) and 70.5 μmol/l (S.E.M. 4.1), respectively (Table 4.1.). The mean apparent diffusion constant (K_d) for the linear component of influx was 0.224 h^{-1} (S.E.M. 0.039) in uraemic patients' cells and 0.178 h^{-1} (S.E.M 0.028) in normal controls. The 35% increase in mean V_{max} seen in uraemic red cells was statistically significant ($p < 0.02$). The mean K_m and mean slope of the linear component of flux (K_d) values were not significantly different, $p = 0.75$ and $p = 0.35$ respectively. The mean values obtained for the small subgroup ($n = 3$) of patients with renal failure who had never received dialysis were as follows: V_{max} = 0.730 mmol.l cells^{-1}.h^{-1} (S.E.M 0.128), K_m = 57.8 μmol/l (S.E.M. 9.8) and K_d = 0.283 h^{-1} (S.E.M. 0.020). These results were similar to those for haemodialysis patients.

The high lysine influx rate observed in patients with chronic renal failure could have been the result of flux trans-stimulation by increased intracellular levels of lysine in these cells. It was therefore important to investigate this further by performing flux experiments in lysine-depleted

cells, i.e. in zero-trans conditions, where this effect, if present, would be abolished.

Red cells were incubated at 37°C in standard saline for 6 hours to deplete intracellular amino acids and 5 minute influx experiments at V_{max} (0.5 mM extracellular lysine concentration) were performed at time 0, 1, 2, 4, and 6 hours. Figure 4.3 illustrates an experiment with normal control red cells. It can be seen that the influx rate declines and appears to reach a steady-state after 2 hours of incubation. To investigate further the possible trans-stimulatory effect of intracellular amino acid levels in studying lysine fluxes, control red cells under assumed zero-trans conditions were resuspended in standard saline containing non-radioactive L-lysine at 0.5 mM concentration to re-load the cells with L-lysine. This experiment is illustrated in figure 4.4. The arrow in the figure indicates the moment that cells were resuspended into the above solution. As can be seen, the lysine influx rate returns to the same level of influx obtained by immediate use of washed cells and supports the existence of a trans-stimulatory effect in lysine transport.

L-lysine influx experiments as a function of extracellular lysine concentration in zero-trans condition were performed in red cells obtained from 6 patients on haemodialysis and 6 normal controls. To ensure that cells were as near as possible under zero-trans condition, cells were used after 4 hours incubation in saline.

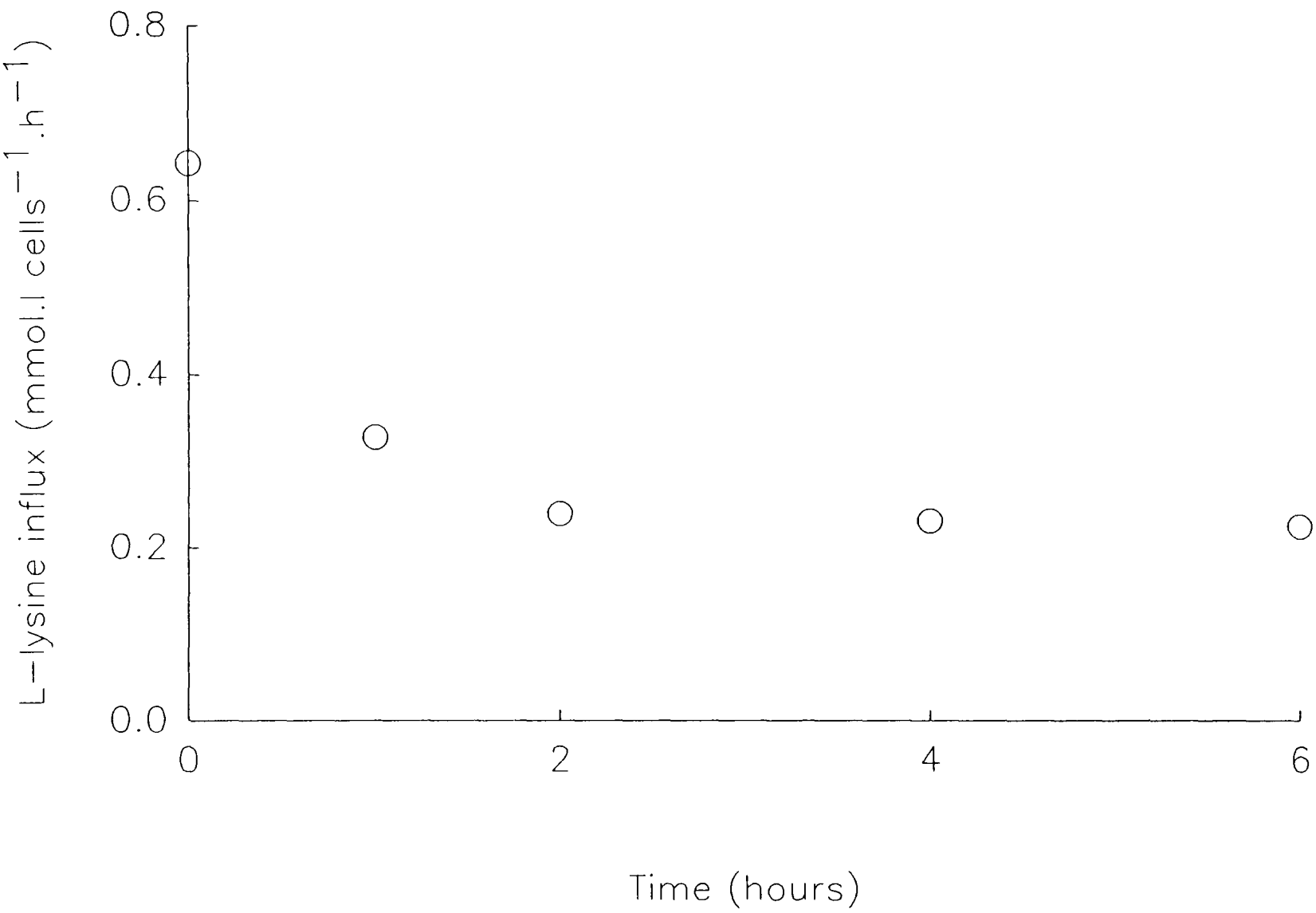

Figure 4.3. L-lysine influx rate in red cells of a normal control in standard saline (extracellular lysine concentration 0.5 mM). Fluxes were performed with immediately washed cells (time 0) and at every hour after the cells had been incubated at a haematocrit < 1% at 37°C in lysine-free saline. Influx rates reached steady-state condition after 2 hours.

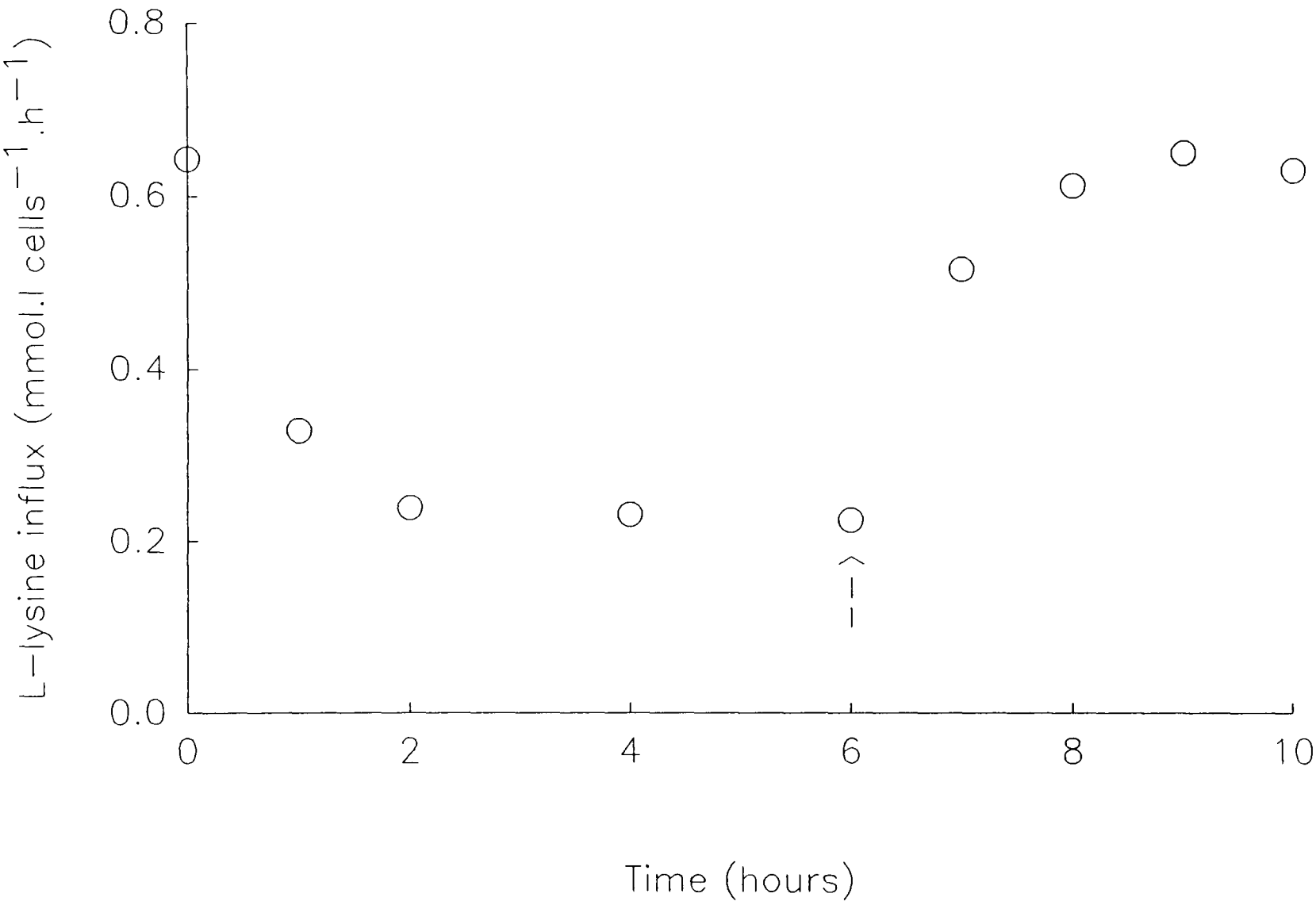

Figure 4.4. L-lysine influx rate at V_{max} (extracellular L-lysine at 0.5 mM) in immediately used red cells (time 0) and after these cells had been previously suspended at 37°C in standard saline at a haematocrit < 1%. Cells appeared to reach steady-state conditions after 2 hours incubation time. At the time indicated by the arrow, cells were resuspended in standard saline containing lysine at a concentration of 0.5 mM. Fluxes were measured at every hour subsequently. Lysine influx rate appears to return to values obtained at time 0.

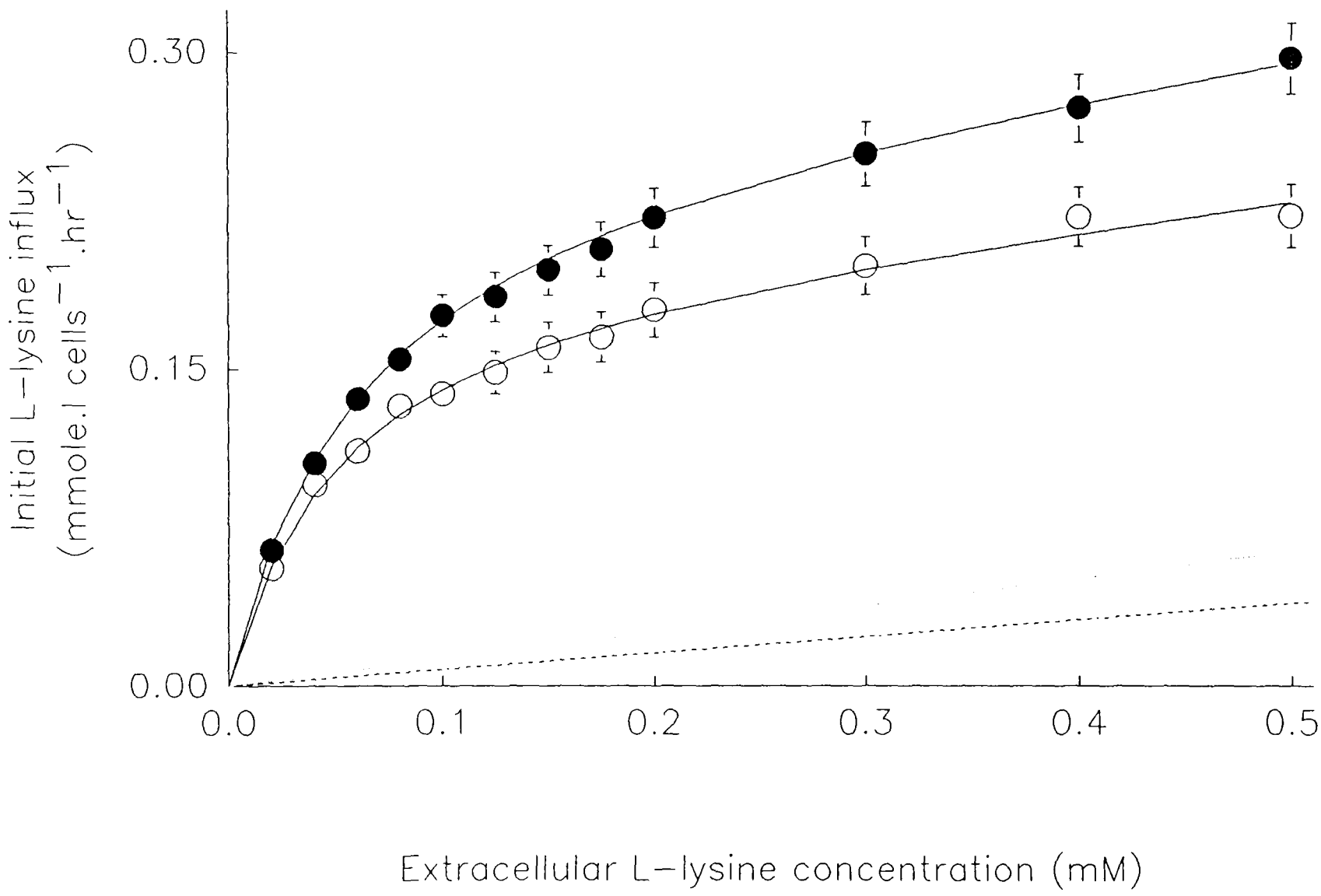

Figure 4.5. The mean initial rates of erythrocyte L-lysine influx in assumed zero-trans conditions as a function of extracellular lysine concentration in haemodialysis patients (closed circles) and normal controls (open circles). The error bars are S.E.M. The lines are Michaelis-Menten fits to the data with the values of V_{max} and K_m described in the text. Fitting for K_d in controls (dashed lines) and uraemics (dotted lines) are 0.08 h^{-1} and 0.13 h^{-1} respectively.

The uptake data under zero-trans conditions could be fitted with equation (1). The plot of this data is presented in figure 4.5. The mean values obtained in normal cells were as follows: V_{max} = 0.20 mmol.l cells^{-1}.h^{-1} of cells (S.E.M. 0.01), K_m = 56 μmol/l (S.E.M. 3), K_d = 0.08 h^{-1} (S.E.M. 0.02). These results in uraemic patients' cells were: V_{max} = 0.26 mmol.l cells^{-1}.h^{-1} (S.E.M. 0.05), K_m = 56 μmol/l (S.E.M. 3) and K_d = 0.13 h^{-1} (S.E.M. 0.02). There was therefore a significant ($p < 0.02$) 30% increase in V_{max} in uraemic patients' red cells compared with controls in cells under zero-trans conditions, in line with the results obtained for cells not depleted of amino acids.

There was no significant correlation within the uraemic group between the erythrocyte transport capacity for L-lysine (expressed as V_{max}) and the plasma creatinine, plasma urea, haemoglobin concentration or mean corpuscular volume.

4.2. L-serine.

The initial rate of influx of L-serine in immediately washed human red cells at 37°C with an extracellular serine concentration of 0.5 mM was established in normal controls. It appears to be linear with time at least up to 15 min incubation (figure 4.6) and justifies the 5 minutes incubation time used in the experiments.

L-serine transport via the ASC system is Na^+-dependent.

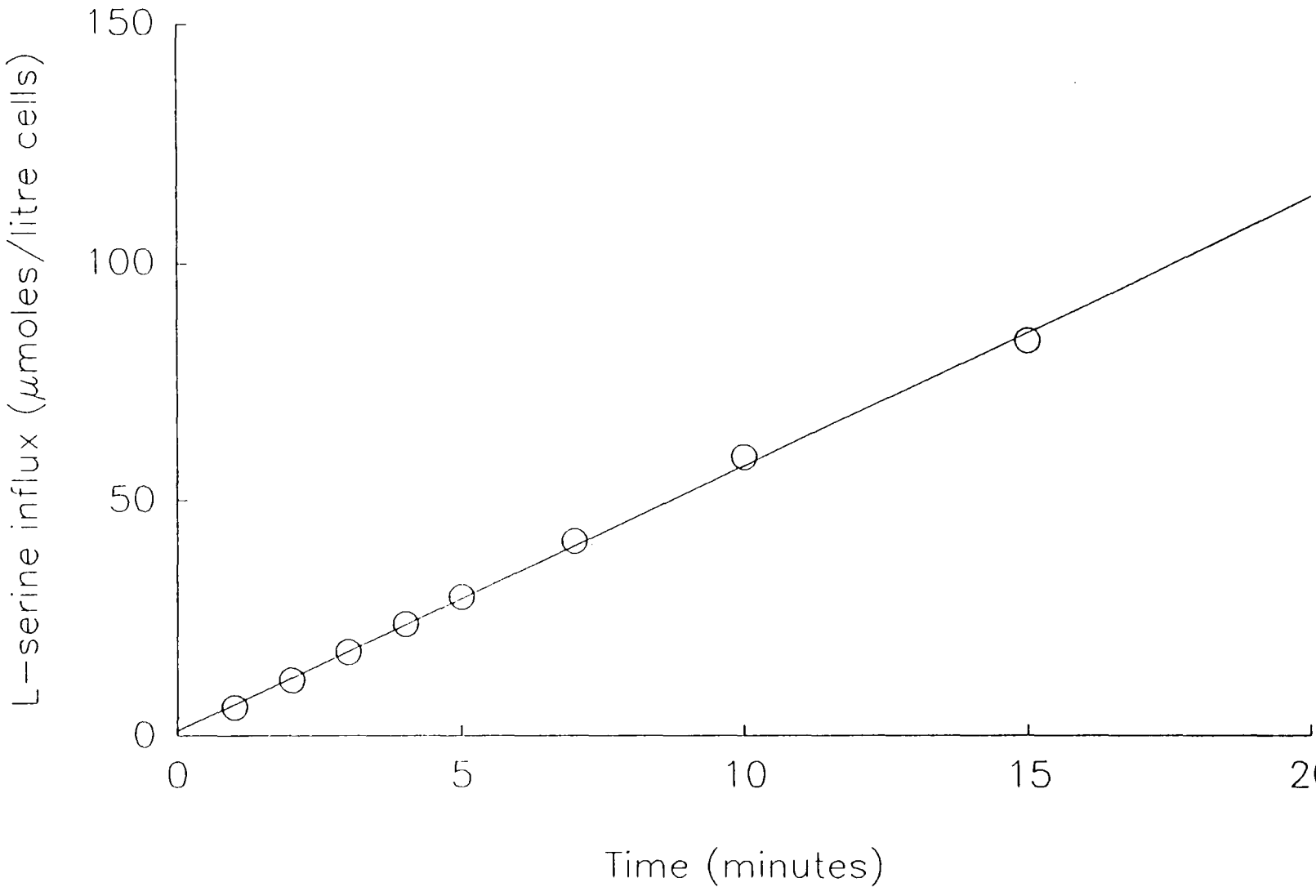

Figure 4.6. Time course of L-serine influx in standard saline at an extracellular serine concentration of 0.5 mM in a normal control. Points were fitted by a linear regression line.

In order to measure the Na-dependent component, influx experiments were performed in cells suspended in standard saline and in sodium-free (NMDG replacement) solution, each containing L-serine at concentrations of 0.02-0.5 mM. The sodium-dependent influx of L-serine could be obtained by subtraction of the data obtained in Na^+-free saline from the data obtained in standard saline. Figure 4.7 shows the mean of the Na^+-dependent and Na^+-independent L-serine influx for erythrocytes from 9 haemodialysis patients and from 9 normal controls. The Na^+-dependent flux data have been fitted to Michaelis-Menten kinetics, in which L-serine flux (V) is defined by a maximum flux (V_{max}) and by (K_m). In uraemic patients' erythrocytes the Na^+-dependent mean kinetic parameters were V_{max} = 233 μmol.l cells^{-1}.h^{-1} (S.E.M. 33), K_m = 105 μmol/l (S.E.M. 11) and K_d = 0.153 h^{-1} (S.E.M. 0.03). In normal erythrocytes these values were V_{max} = 352 μmol.l cells^{-1}.h^{-1} (S.E.M. 21), K_m = 160 (S.E.M. 12) and K_d = 0.206 h^{-1} (S.E.M. 0.05). Thus, there was a significant reduction in both V_{max} and K_m in uraemic erythrocytes compared with controls ($p < 0.02$).

The influx of L-serine via system ASC is known to be stimulated by the presence of intracellular (or trans) L-serine or other ASC substrates (Saier, Daniels, Boerner and Lin, 1988; Barker and Ellory, 1990). To confirm these reported observations a similar experiment to the one performed with lysine, was carried out for serine using cells under zero-

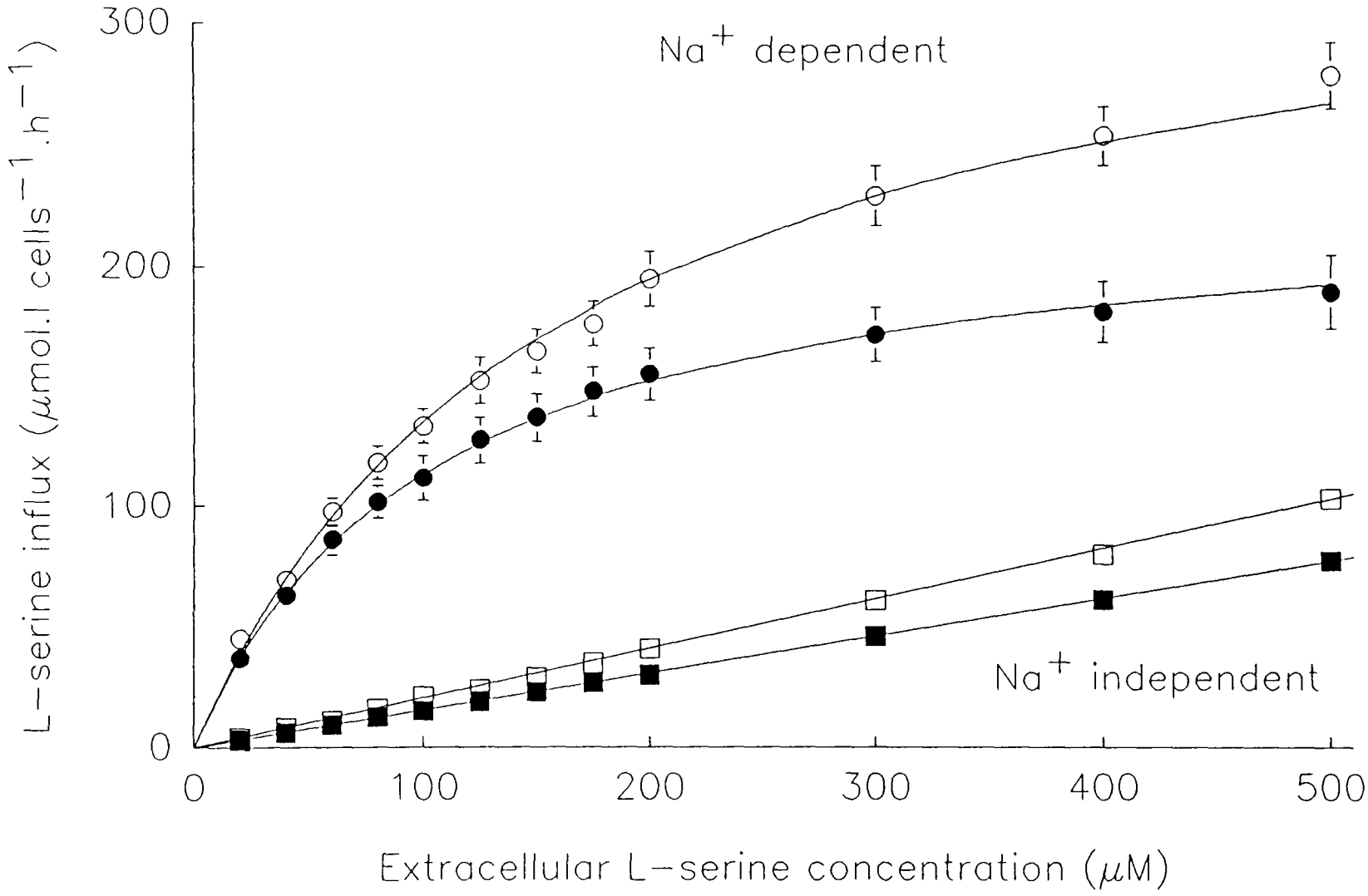

Figure 4.7. The mean Na^+-dependent initial rate of influx of L-serine as a function of extracellular L-serine concentration in normal controls (open circles) and in erythrocytes from haemodialysis patients (closed circles). The curved lines showed the fit of the data to equation 1 (parameters shown in table 4.1). The mean Na^+-independent influx of L-serine is shown at the bottom of the figure for normal controls (open squares) and cells from uraemic patients (closed squares). These results are fitted with linear regression lines and their slopes are reported as K_d values in table I. Error bars are S.E.M.

trans condition. Figure 4.8 shows the Na-dependent L-serine influx rate reaching a steady-state rate after 2 hours. The arrow shows the moment when cells are resuspended into standard saline containing L-serine at a concentration of 1 mM. The influx rate recovers although does not reach the initial influx rate of immediately used washed cells, at least after 2 hours.

Accordingly, the V_{max} for L-serine influx was measured under assumed zero-trans conditions. This was accomplished by incubation of the cells in saline at 37°C and at a haematocrit < 1% for 4 hours. Figure 4.9 shows the result of serine influx in these cells according to time. Influx appears to reach steady-state status after 2 hours. The Na^+-independent flux showed no changes with time.

Therefore, measurements in zero-trans conditions of the Na^+-dependent L-serine influx at V_{max} (extracellular L-serine concentration of 1.0 mM) were performed in 8 haemodialysis patients and 8 normal controls. The mean V_{max} for L-serine under zero-trans conditions was 0.153 mmol.l $cells^{-1}.h^{-1}$ (S.E.M. 0.009) in uraemic patients' erythrocytes; in control erythrocytes it was 0.233 mmol.l $cells^{-1}.h^{-1}$ (S.E.M. 0.006)($p< 0.03$) (figure 4.10). This 35% reduction in V_{max} was in line with the 34% reduction found when erythrocytes were used immediately (Table 4.2).

In sodium-free saline the rates of L-serine uptake were related linearly to extracellular L-serine concentration over

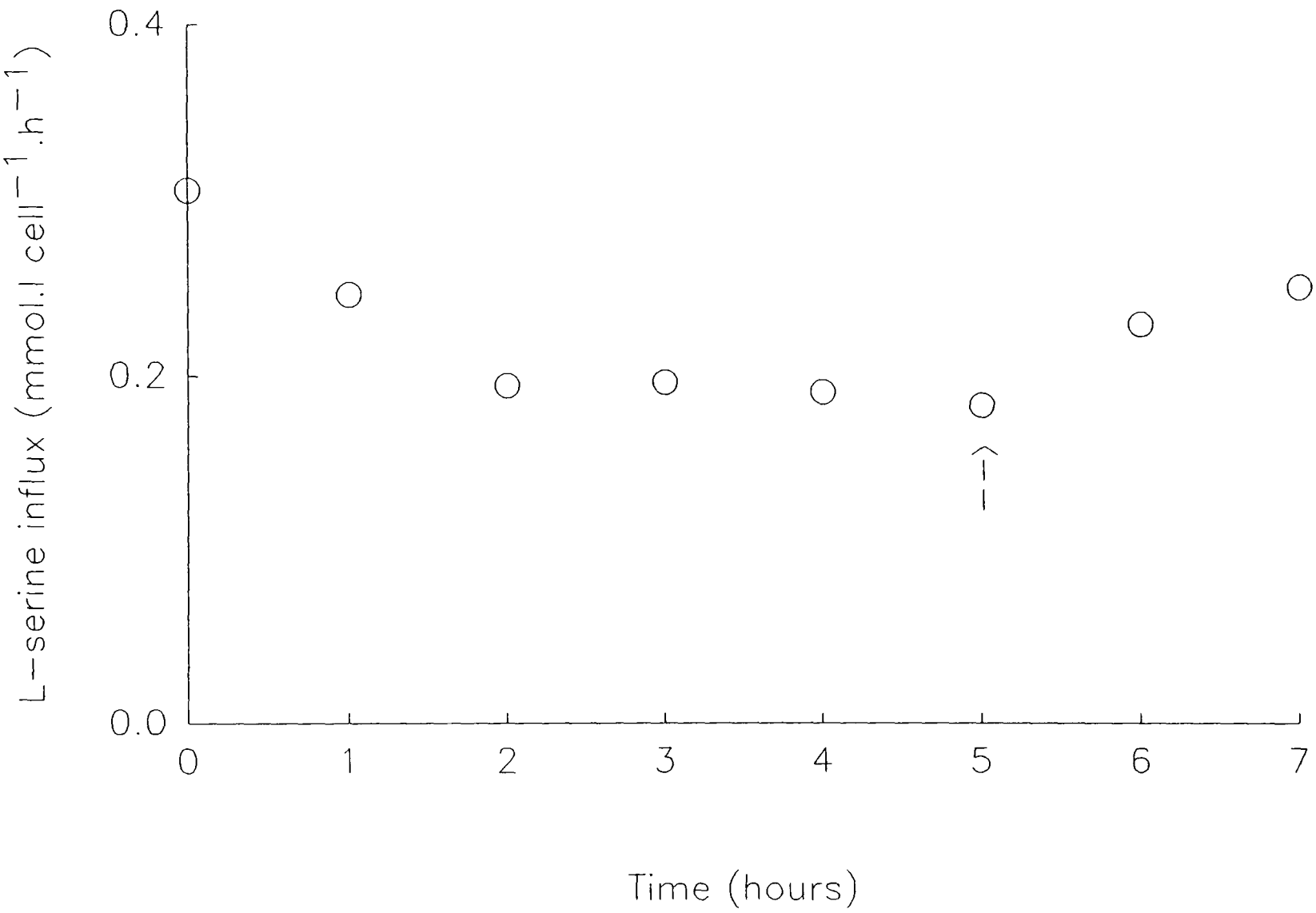

Figure 4.8. L-serine influx rate at V_{max} (extracellular L-serine at 0.5 mM) in immediate use red cells (time 0) and after these cells had been previously suspended at 37°C in standard saline at a haematocrit < 1%. Cells appeared to reach steady-state status after 2 hours. At the time indicated by the arrow, cells were resuspended in standard saline containing L-serine at a concentration of 1.0 mM. Fluxes were measured at every hour subsequently. Serine influx rate appears to partially recover up to 2 hours incubation.

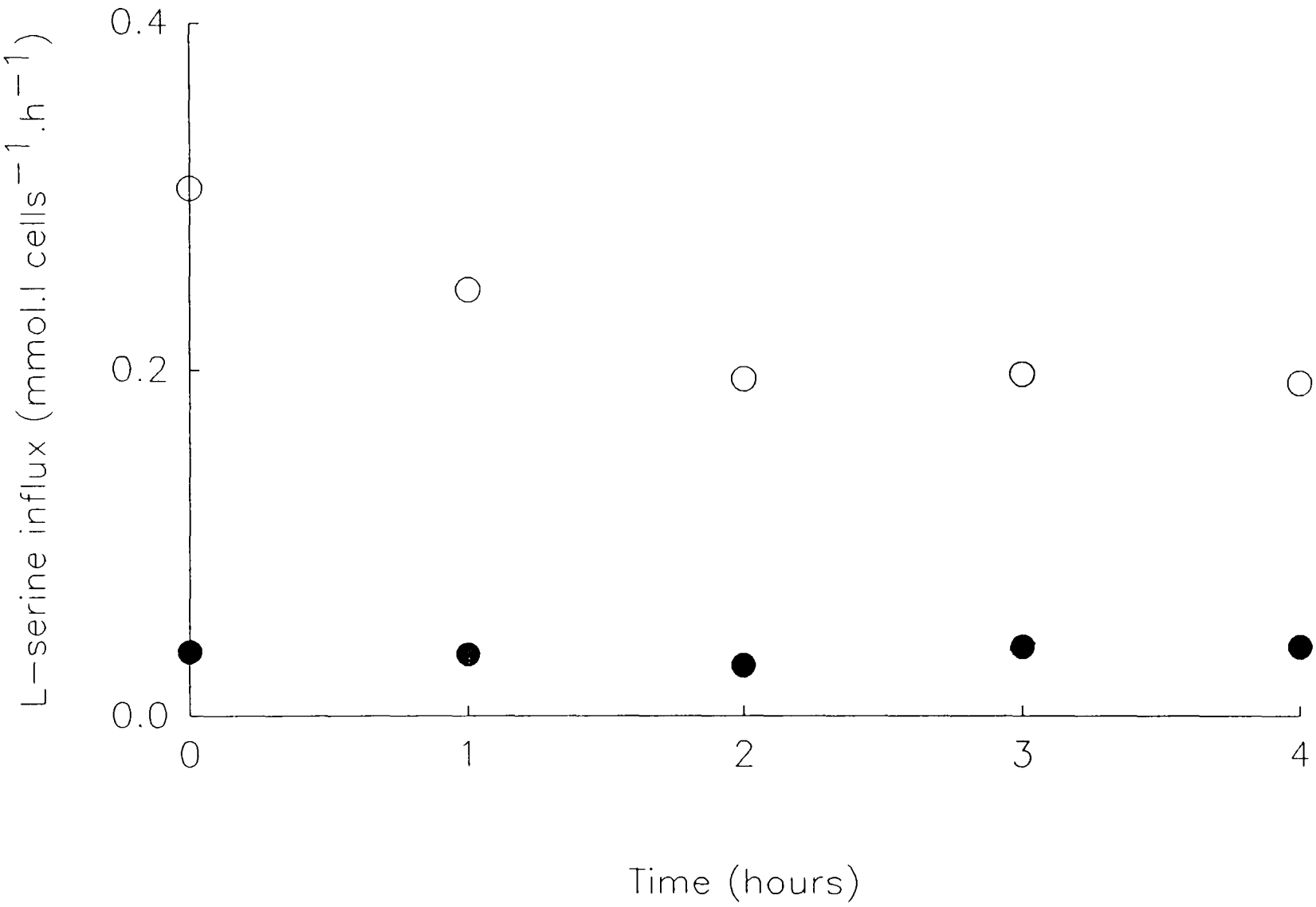

Figure 4.9. L-serine influx in standard saline (open circles) and in sodium-free saline (closed circles) in a normal control (extracellular serine concentration 0.5 mM). Fluxes were performed with immediately washed cells (time 0) and at every hour after the cells had been incubated in serine-free saline at a haematocrit < 1% at 37°C. Fluxes in saline appear to have achieved steady-state condition after 2 hours. The Na^+-independent flux appears not to change.

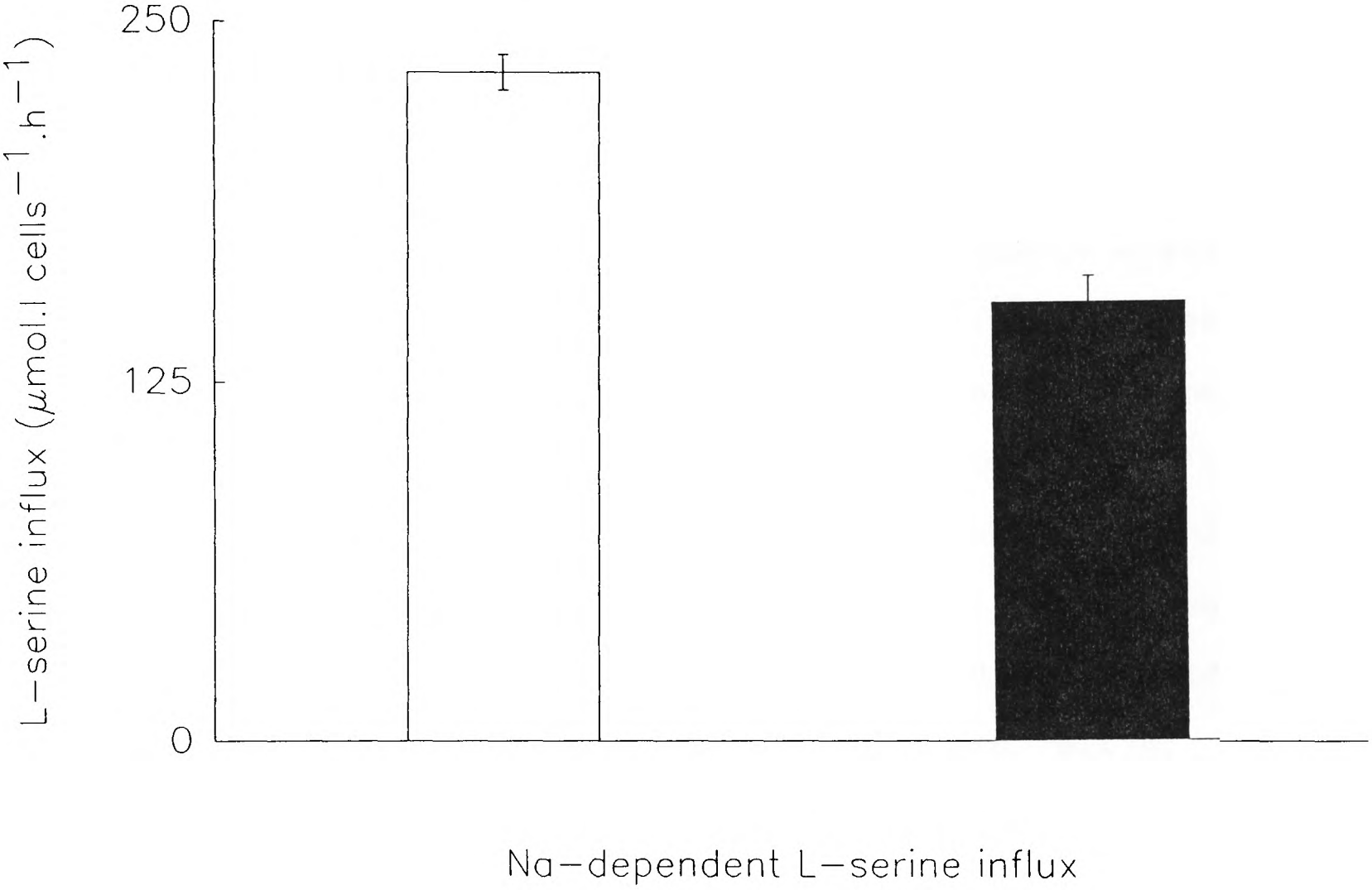

Figure 4.10. Mean Na^+-dependent influx of L-serine at V_{max} (extracellular L-serine concentration 1 mM) under assumed zero-trans condition in normal controls (open bar) and in haemodialysis patients' erythrocytes (closed bar). Error bars are S.E.M. ($p < 0.03$).

the range 0-0.5 mM serine and were non-saturable. The mean influx rates obtained in the absence of sodium are shown in figure 4.7. These results could be described by an apparent diffusion constant (K_d), the mean values for K_d are listed in Table 4.1. They did not differ significantly between HD and control cells.

4.3. Glycine.

Total glycine flux involves components through systems gly, ASC, L, Band 3 as well as true flux leak (Ellory, Jones and Young, 1981). Glycine flux through system gly is both sodium and chloride-dependent. In order to measure this, flux experiments were performed in standard saline and in chloride-free solutions. The chloride-dependent influx of glycine could be obtained by subtraction of the data obtained in chloride-free solution from the data obtained in standard saline.

Time course experiments at 37°C and at extracellular glycine concentration 0.5 mM, showed the initial glycine influx rate to be linear at least up to 20 minutes incubation time (figure 4.11).

Using a 15 minute incubation time, glycine influx experiments as a function of extracellular glycine concentrations of 0.01-0.5 mM were performed in red cells obtained from 9 haemodialysis patients and 9 normal controls.

The initial rates of glycine influx in chloride-free

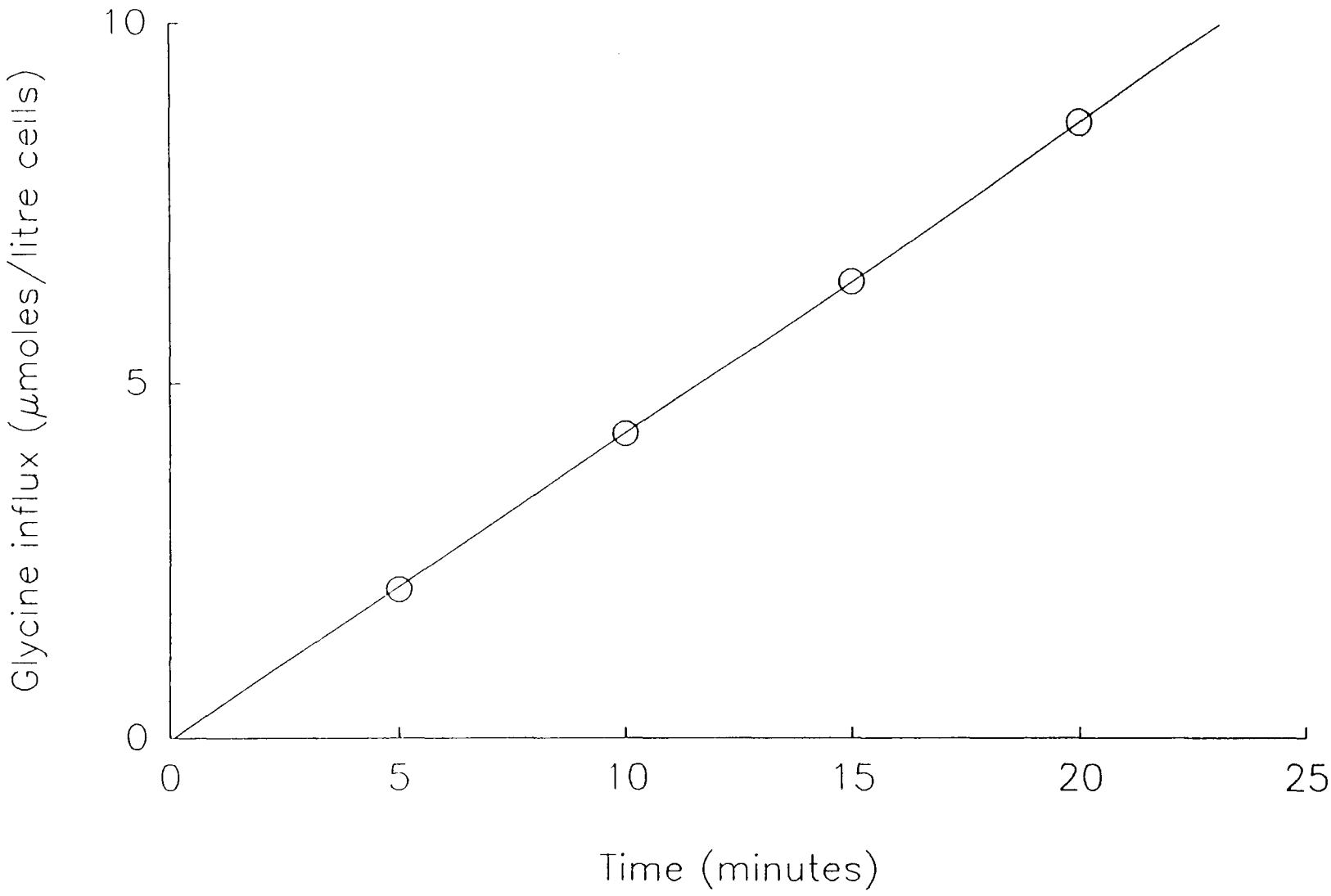

Figure 4.11. Time course of glycine influx at 37°C with immediate use of washed erythrocytes in a normal control (extracellular glycine concentration 0.5 mM). Time points were fitted to a linear regression.

saline were related linearly to glycine concentration and could be described conveniently by an apparent diffusion constant (K_d) although as pointed out before this flux is complex, involving components of ASC, L and Band 3 as well as the true flux leak. The mean value of this parameter was not altered significantly in haemodialysis patients' erythrocytes (Table 4.1.). The chloride-dependent component of glycine influx, obtained for each subject by subtraction of the influx obtained in chloride-free saline from the influx measured in standard saline, was saturable and could be fitted with Michaelis-Menten kinetics. Mean values for these parameters in uraemic patients' erythrocytes were V_{max} = 27.6 μmol.l cells^{-1}.h^{-1} (S.E.M. 2.9) and K_m = 26.3 (S.E.M. 2.3). In normal cells these parameters were V_{max} = 24.1 (S.E.M. 4.1) and K_m = 34.0 (S.E.M. 3.1) (Table 4.1.). There was a significantly decreased K_m for glycine in uraemic patients' erythrocytes ($p < 0.02$). This is illustrated in figure 4.12, which is a double reciprocal plot (Lineweaver-Burk plot) of the glycine influx data. The differences between the slopes of the two lines arises from a reduced K_m for glycine in the uraemic cells.

To establish if glycine flux rates were altered by depleting intracellular amino acid levels, red cells were preincubated in saline at an haematocrit < 1% . Influx measurements were performed hourly at an external glycine concentration 0.1 mM. Figure 4.13 illustrates the results of these experiments in a normal control. Influx rate appears to

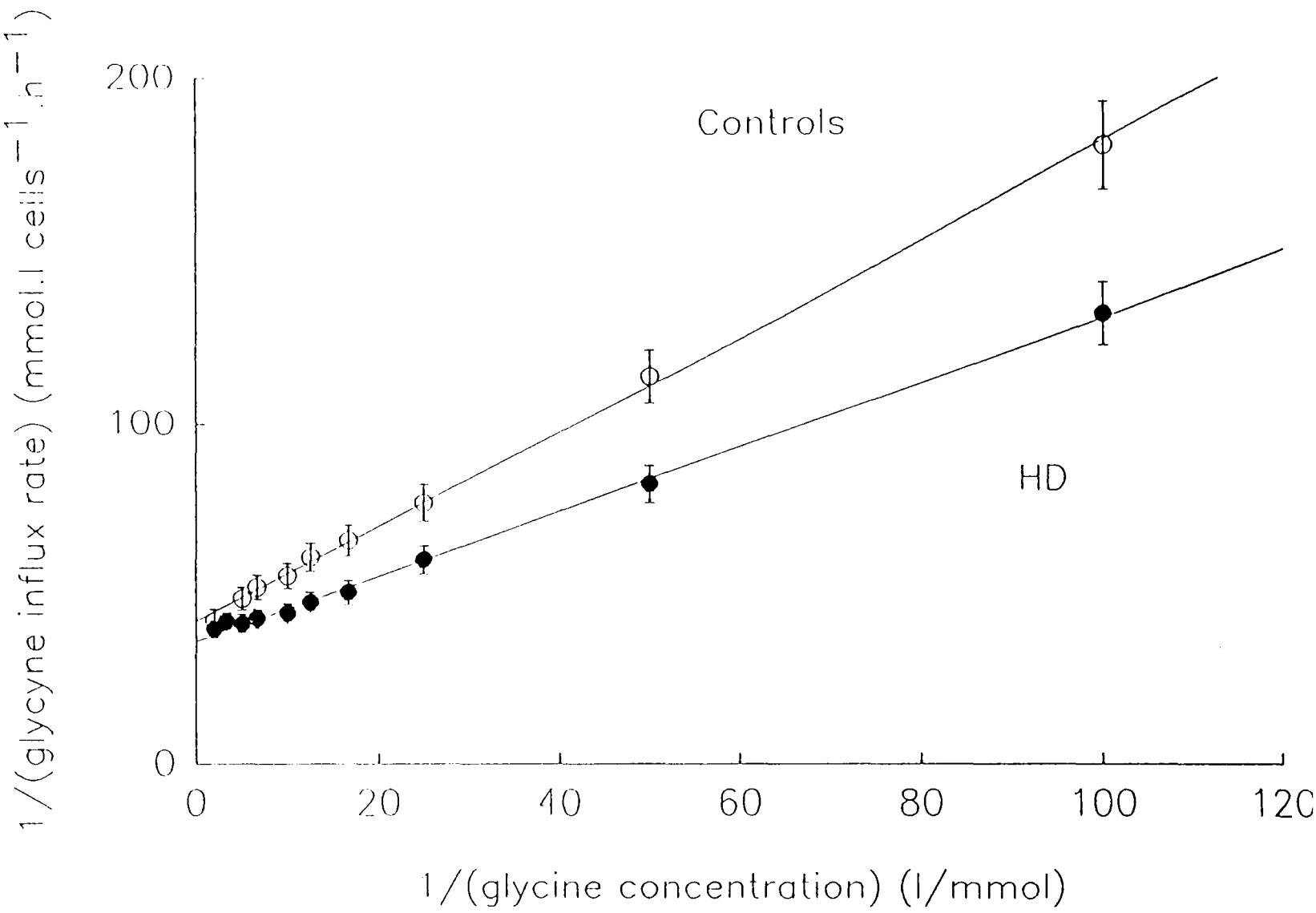

Figure 4.12. Double reciprocal plot of the mean initial rate of Cl^--dependent glycine influx as a function of extracellular glycine concentration in erythrocytes from haemodialysis patients (closed circles) and normal controls (open circles). The lines are drawn by a weighted least-squares linear regression. The fitted parameters are shown in Table I. Error bars are S.E.M. (n=9).

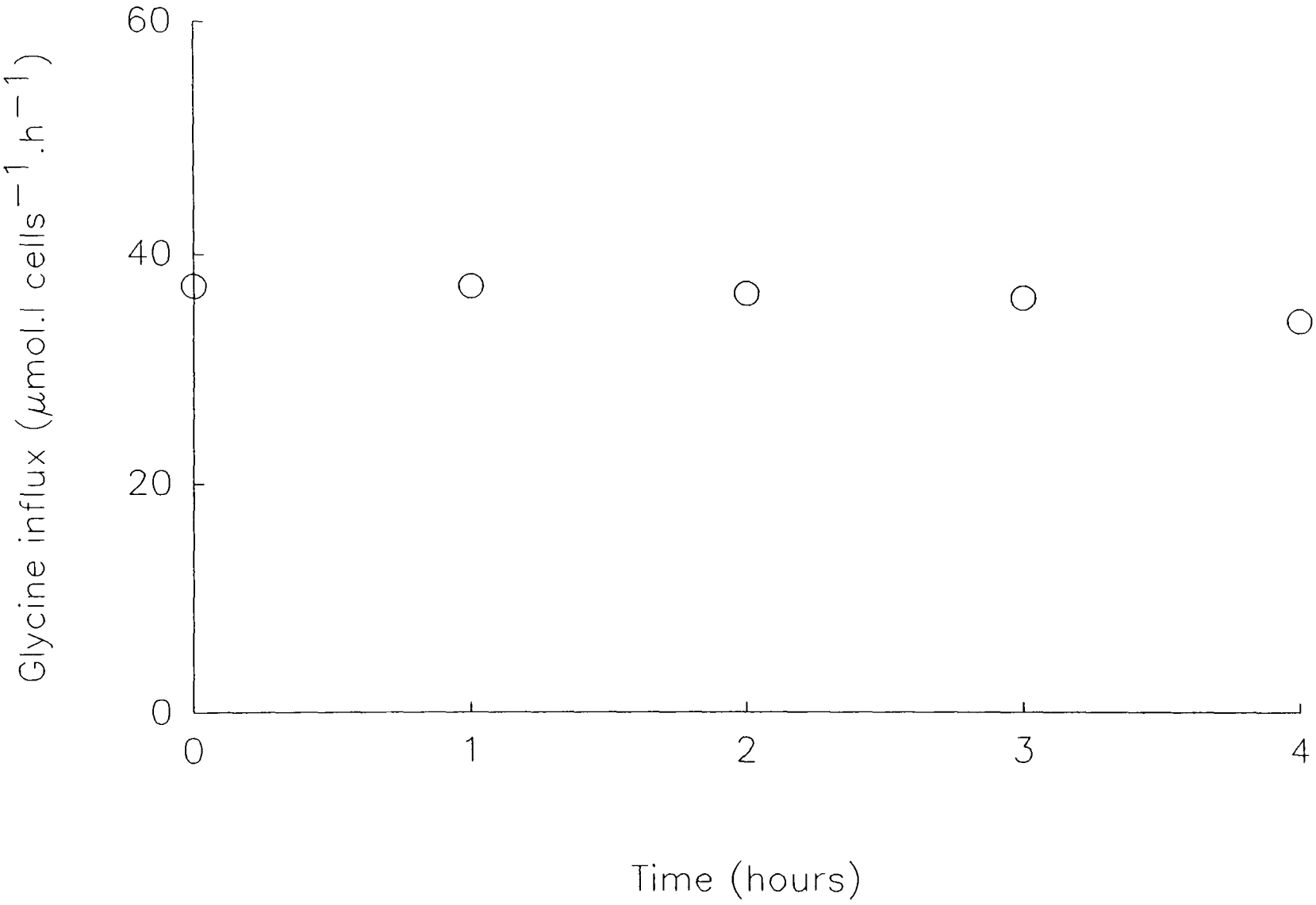

Figure 4.13. Glycine influx rate at V_{max} (extracellular glycine at 0.2 mM) in immediately used red cells (time 0) and at every hour after these cells had been previously incubated at 37°C in standard saline at a haematocrit <1%. Influx rate does not appear to change significantly with time.

be unaffected by incubation up to 4 hours.

4.4. L-leucine.

The next amino acid system to be studied was the L-system, represented by L-leucine as substrate.

Due to the high transport capacity of system L in human red cells, a shorter incubation time period was used in order to measure the initial rate of uptake of L-leucine at 37°C. (see chapter 2 for details of the flux measurement method used in these experiments).

Influx of L-leucine at an extracellular concentration 50 mM appeared to be linear up to 2 minutes incubation time and this experiment is illustrated in figure 4.14.

Experiments were also performed to establish if influx rates were different using immediately washed cells from cells pre-incubated at 37°C in standard saline. There were no differences in these results, probably reflecting the fact that cells are being depleted of leucine at the time of the initial washing procedure. Therefore, it was assumed that the cells used for these experiments were under zero-trans conditions.

The mean concentration-dependent influx rates for L-leucine in erythrocytes from 9 normal controls and 9 uraemic patients, obtained by immediate use of washed cells are illustrated in figure 4.15. The quality of fit shown was

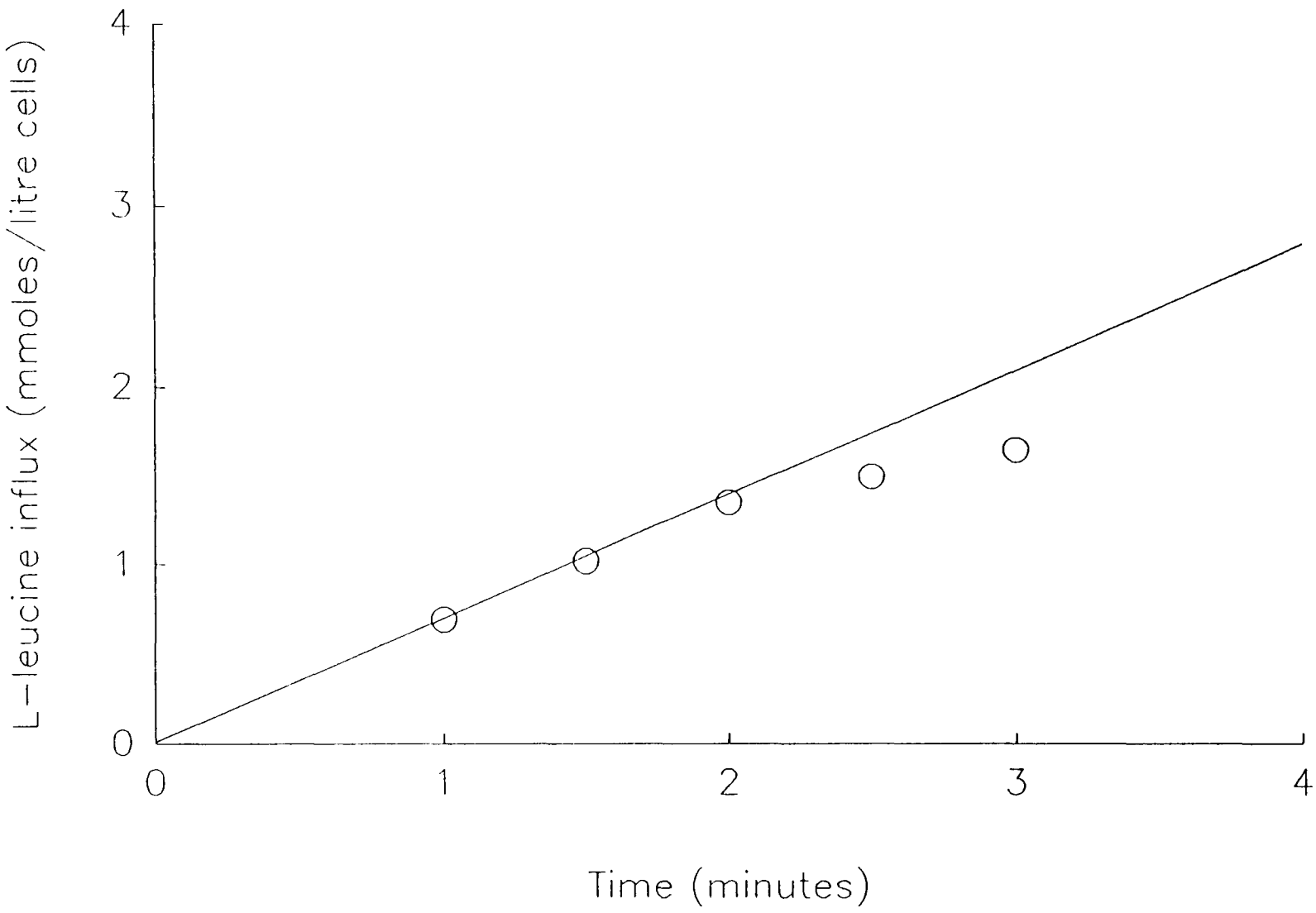

Figure 4.14. Time course of L-leucine influx in erythrocytes from a normal control at an extracellular leucine concentration of 50 mM. The first three points were fitted by linear regression.

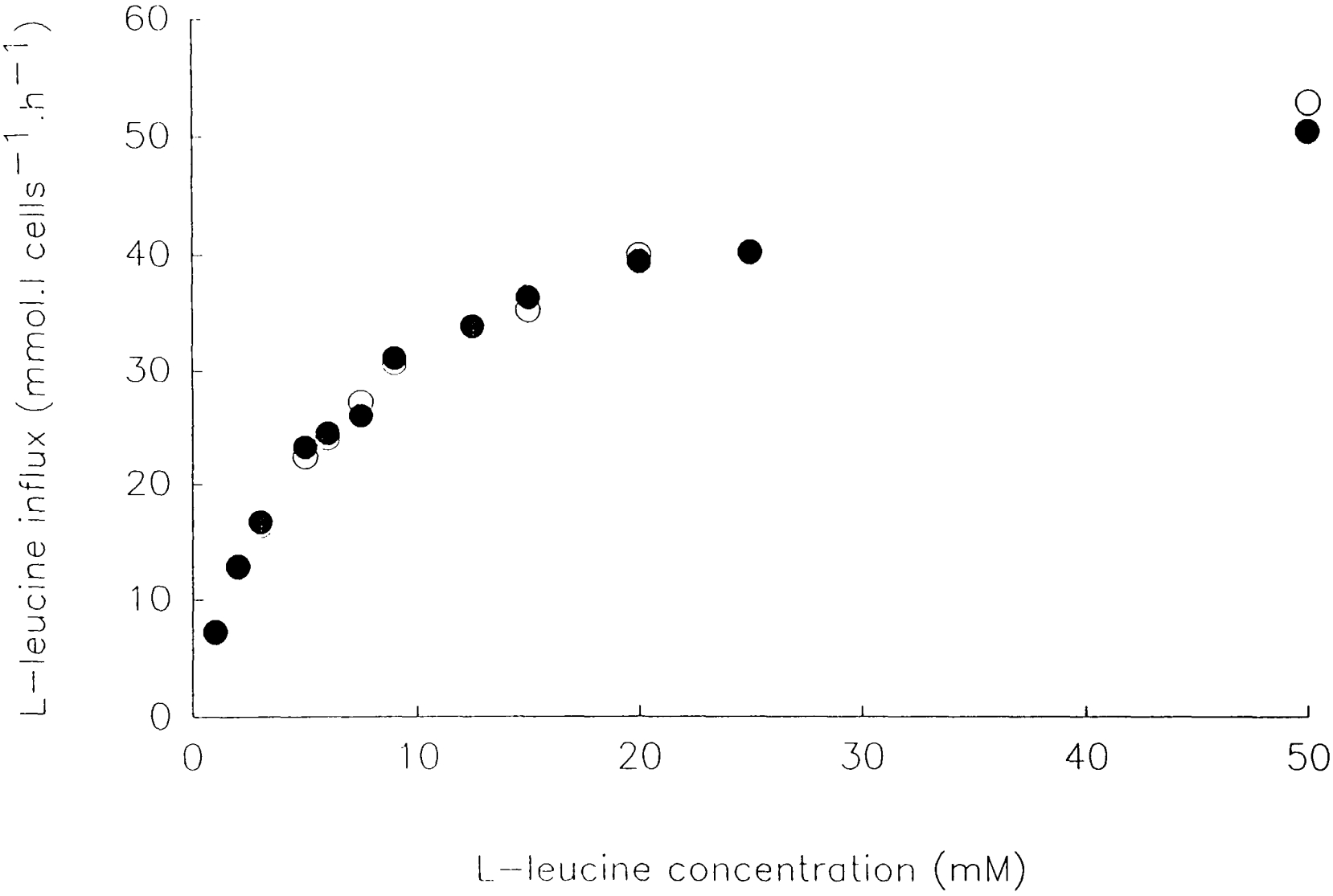

Figure 4.15. The mean initial rate of influx of L-leucine as a function of extracellular leucine concentration in erythrocytes from haemodialysis patients (closed circles) and from normal controls. Error bars are omitted for clarity but S.E.M was of the order of < 7% (n=9).

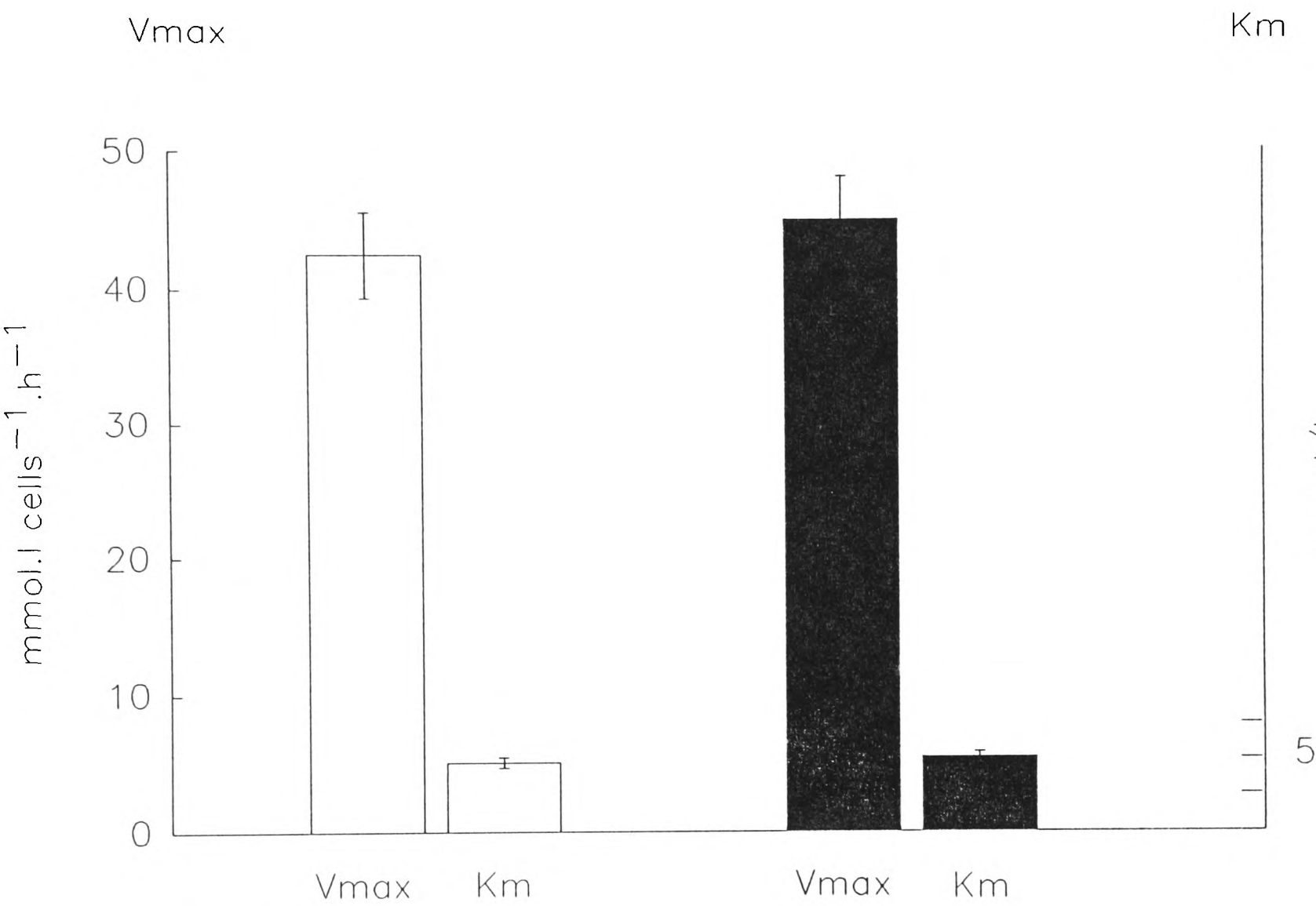

Figure 4.16. Mean V_{max} and K_m of L-leucine fluxes into erythrocytes from controls (open bars) and haemodialysis patients (closed bars) according to the values presented in table 4.1. Error bars are S.E.M. (n=9).

typical. The relationship between flux rate and concentration was consistent with a model in which there are two components of the flux, one obeying Michaelis-Menten kinetics, and a second non saturable component that is related linearly to the leucine concentration.

The results from each experiment were fitted by equation (1) using a least squares algorithm. In each case the coefficient of multiple correlation was greater than 0.95. The mean of the values obtained for V_{max} in erythrocytes from uraemic patients was 45.1 mmol.l cells^{-1}.h^{-1} (S.E.M. 3.15) while the mean V_{max} in normal erythrocytes was 42.5 mmol.l cells^{-1}.h^{-1} (S.E.M. 3.1). The difference between the two groups was not significant ($p = 0.865$). The mean K_m for uraemic patients' erythrocytes was 5.4 mmol/l (S.E.M. 0.43) and for normal cells it was 5.07 mmol/l (S.E.M. 0.4). The mean slope of the linear component of flux (K_d) was 0.185 h^{-1} (S.E.M. 0.04) in uraemic cells, and it was 0.274 h^{-1} (S.E.M. 0.05) in normal cells. These values were also not significantly different ($p = 0.843$ and $p = 0.562$ respectively) (figure 4.16).

4.5. L-tryptophan

The final amino acid transport system studied was the T system, represented in these experiments by L-tryptophan transport. This system also has a high transport capacity for

its substrates making it necessary to use the same methodology as for the L-leucine flux experiments.

Time course experiments measured the initial rate of influx of L-tryptophan at 37°C in normal control red cells, at an extracellular L-tryptophan concentration of 25 mM in standard saline. The influx rate appears to be linear up to 3 minutes as illustrated in figure 4.17.

Tryptophan influx rate measurements performed after the cells had been preincubated at 37°C for 3 hours in standard saline did not showed any difference with results obtained by use of immediately washed cells. As discussed above for leucine, this probably results from the fact that cells reach zero-trans condition during the routine washing procedure.

Measurements of L-tryptophan influx rate as a function of extracellular tryptophan concentration were performed in erythrocytes under assumed zero-trans conditions from 9 haemodialysis patients and 9 normal controls, using a 2.5 minute incubation time.

Analysis of the data was performed as above, again the relationship between flux rate and concentration fits the two component flux model, i.e. one obeying Michaelis-Menten kinetics, and the second, non-saturable component, linearly related to the tryptophan concentration.

The results from each experiment were fitted by equation (1) using a least squares algorithm. In each case the coefficient of multiple correlation was greater than 0.99.

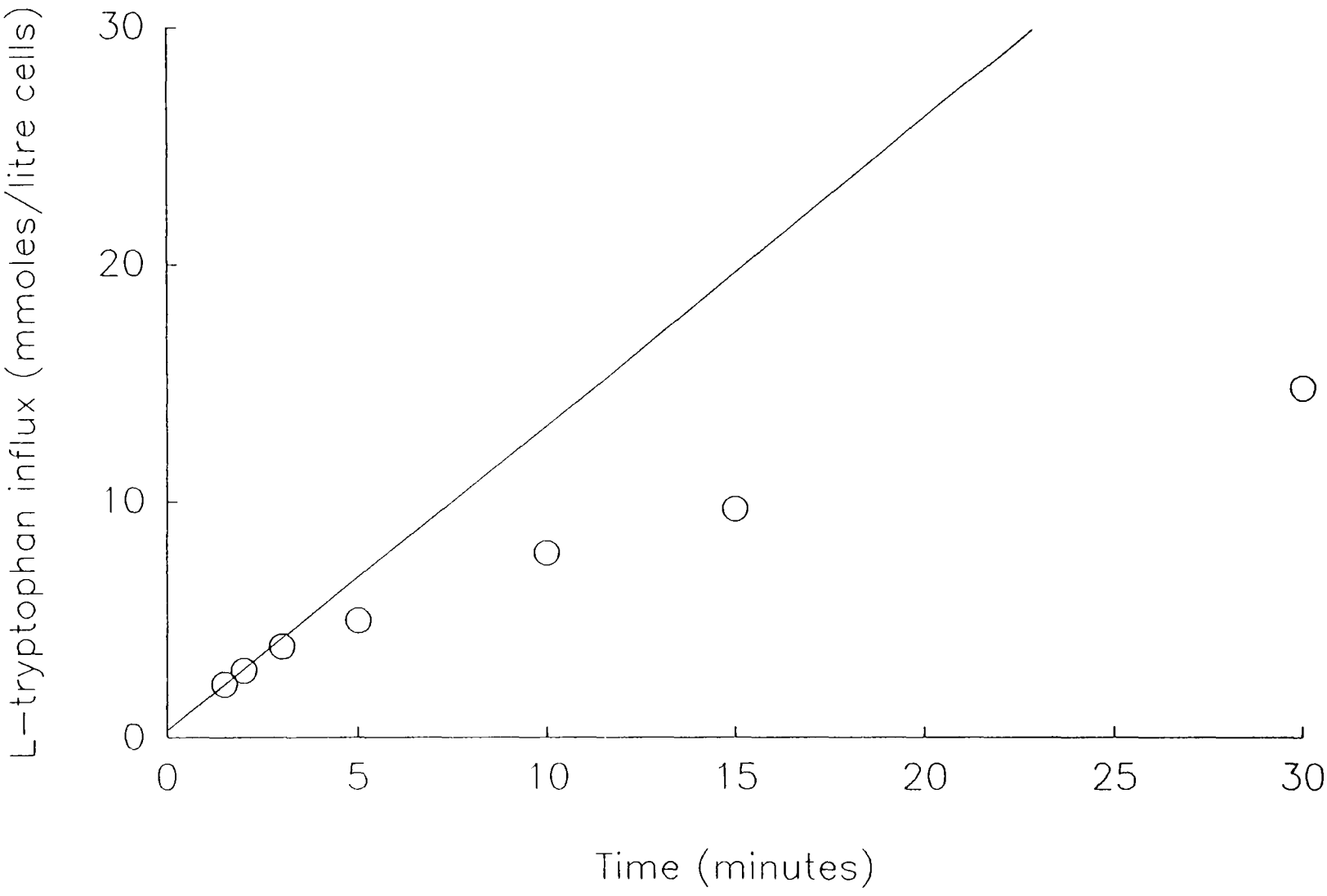

Figure 4.17. Time course experiment for L-tryptophan influx at 37°C in standard saline and at extracellular tryptophan concentration of 25 mM. The first 3 points were fitted by a linear regression line.

Statistical analysis showed no significant difference between the two groups. V_{max} in normal control erythrocytes was 4.08 mmol.l cells^{-1}.h^{-1} (S.E.M. 0.26) while the mean V_{max} in uraemic cells was 3.29 mmol.l cells^{-1}.h^{-1} (S.E.M. 0.3) ($p = 0.427$). The mean K_m for normal cells was 0.97 mmol/l (S.E.M. 0.12) and for uraemic patients' cells was 1.16 mmol/l (S.E.M. 0.16) ($p = 0.372$). Finally, the mean slope of the linear component of the flux (K_d) was 0.460 h^{-1} (S.E.M. 0.06) in normal cells and 0.540 h^{-1} (S.E.M. 0.05) in uraemic cells ($p = 0.723$). These results are illustrated in figure 4.18 and 4.19.

4.6. Discussion.

This chapter presents the first complete study in patients on haemodialysis of the five identified and kinetically characterised amino acid transport systems present in human red cells. These results will be discussed individually.

4.6.1. L-lysine.

L-lysine influx in human erythrocytes has been reported as having a saturable high-affinity component fulfilling simple Michaelis Menten kinetics with a V_{max} = 0.488 mmol.l cells^{-1}.h^{-1} and a K_m = 68 μmol/l (Gardner and Levy, 1972; Young, Jones and Ellory, 1980). The equivalent values for normal

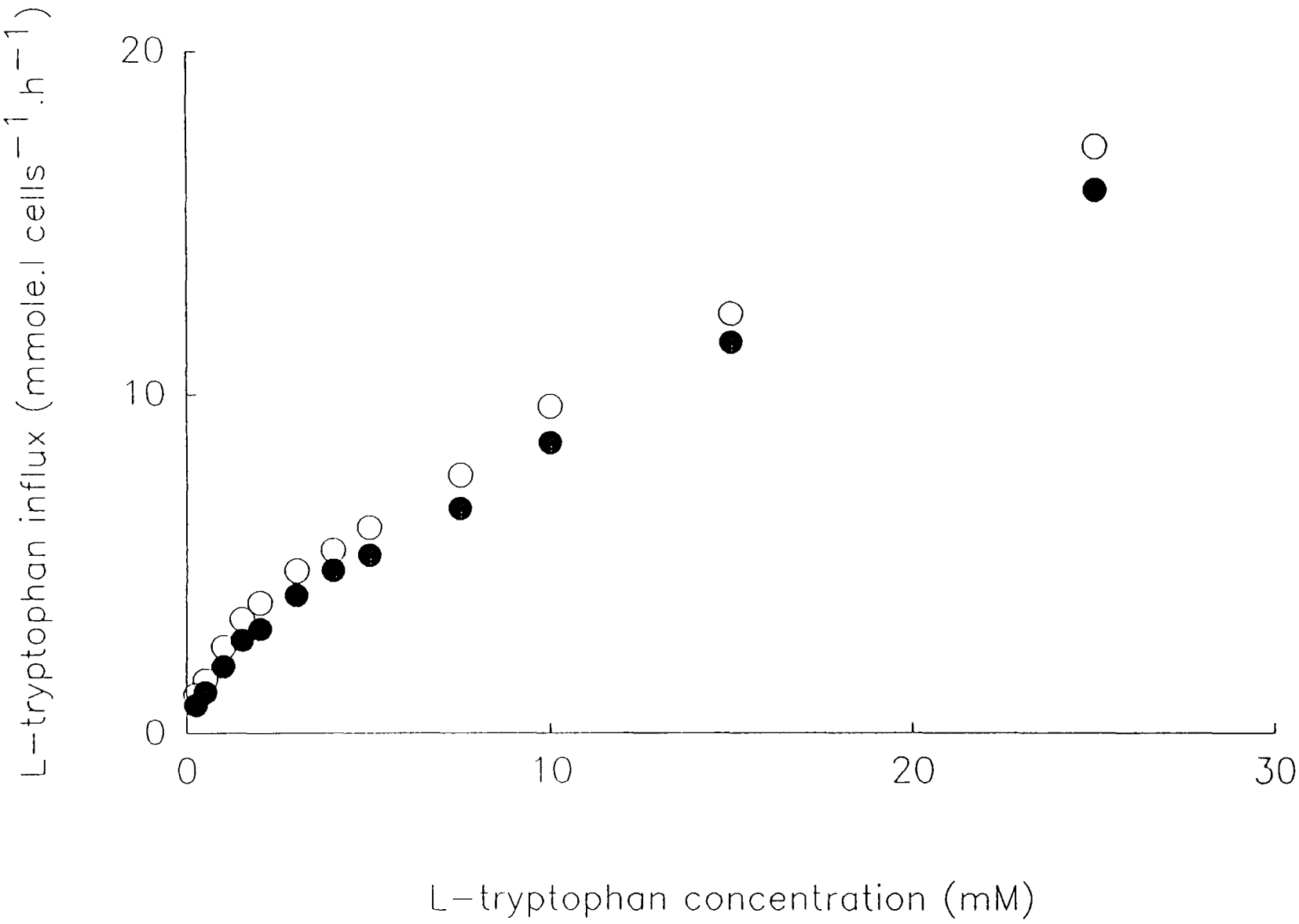

Figure 4.18. The mean initial rate of influx of L-tryptophan as a function of extracellular concentration in erythrocytes from haemodialysis patients (closed circles) and from normal controls (open circles). Error bars are omitted for clarity but S.E.M. were of the order of 7% (n=9).

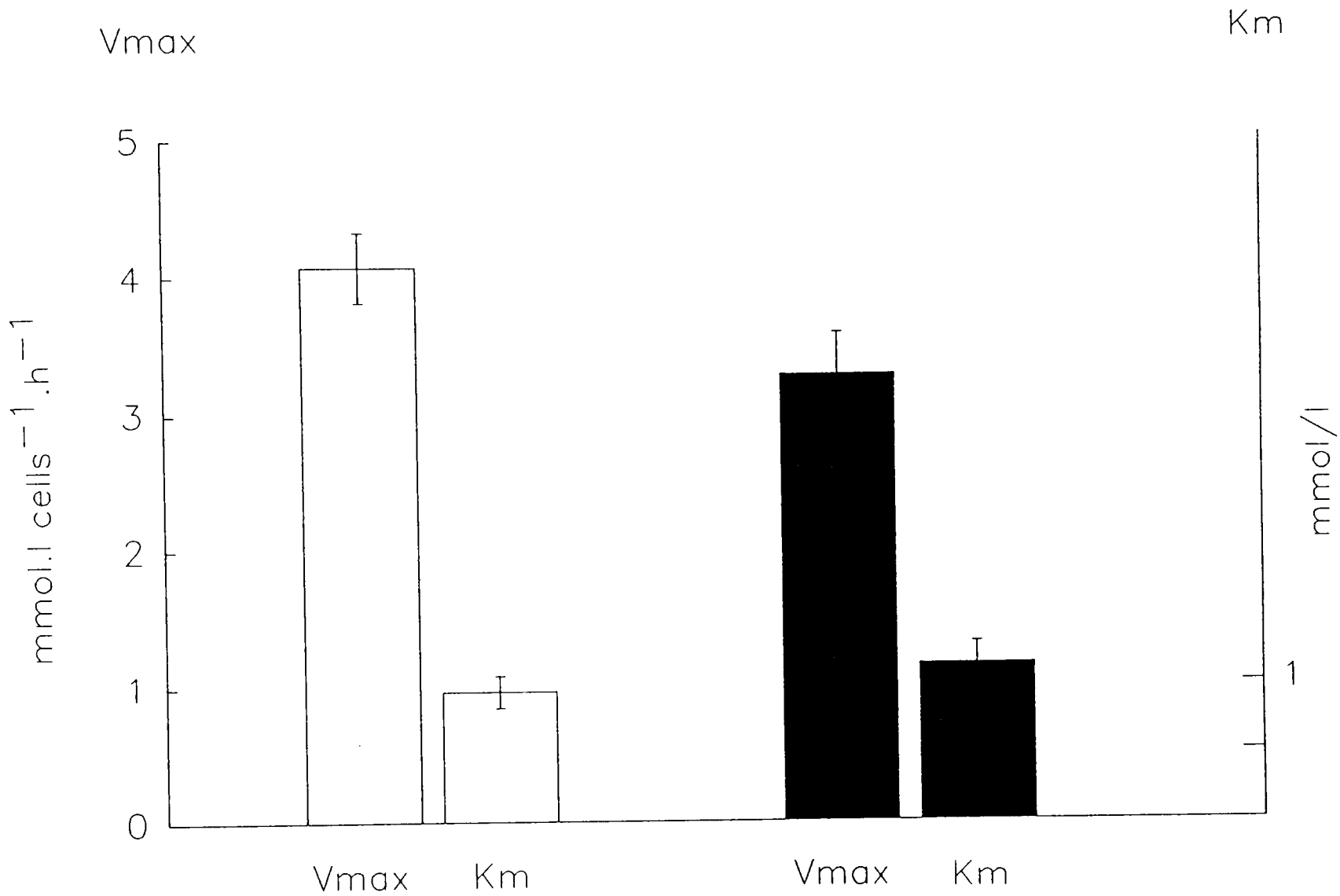

Figure 4.19. Mean V_{max} and K_m for L-tryptophan influx into erythrocytes from normal controls (open bars) and haemodialysis patients (closed bars) according to the values presented in table 4.1. There were no significant differences between the two groups. Error bars are S.E.M. (n=9).

controls in this thesis were not significantly different V_{max} = 0.566 mmol.l cells^{-1}.h^{-1} and K_m = 71 μmol/l.

The present experiments demonstrated an increased capacity for lysine transport in red cells from patients with chronic renal failure on haemodialysis. Flux through the saturable, high-affinity y^+ system was 35% greater than in controls, without any alteration in K_m. This increase in V_{max} was significant ($p < 0.02$). The mean non-saturable influx of lysine into uraemic cells was 26% greater than in controls, although this difference was not statistically significant, owing to the variability of K_d. Experiments performed to compare L-lysine uptake in haemodialysis patients with pre-dialysis chronic renal failure patients showed no significant differences in their K_m and V_{max}. The normal value of K_m in uraemia suggests that the same transport system as in normal cells is responsible for the saturable flux of lysine. The higher V_{max} could be explained by an increase in the number of membrane transporters per cell, by an increase in the turnover rate per transport site, or by a combination of these changes. At the present moment these possibilities cannot be distinguished since it is not possible to determine the number of lysine transporters in the membrane.

Amino acid flux into the erythrocyte may be stimulated by the availability of intracellular amino acids for exchange across the membrane (Gardner and Levy, 1972) and the results presented in figure 4.4 demonstrate that this is correct

regarding L-lysine. Therefore, the increased L-lysine flux observed in these experiments could have been due to increased intracellular amino acid levels in the erythrocytes from uraemic patients. However, this seems unlikely since the relative increase in V_{max} in uraemic cells compared with controls was seen even after intracellular amino acid depletion by preincubation. The conclusion derived from these experiments is that increased lysine influx results directly from altered membrane properties.

The origins of the non-saturable flux of lysine into erythrocytes are not understood. The observed apparent diffusion constants are too large to be explained by the permeability of the lipid bilayer (Klein, Moore and Smith, 1971; Young and Ellory, 1977; Harvey and Ellory, 1989). It seems likely that this component of flux represents "accidental" transport of lysine by membrane systems designed to transport other amino acids, for which lysine has a low affinity, (i.e. which exhibit K_m values for lysine at least an order of magnitude above the extracellular lysine concentrations studied). If this is correct, then the increase in K_d observed in these experiments in uraemia might indicate that erythrocyte amino acid transporters other than y^+ are also increased in number or turnover rate in renal failure. However, K_d showed considerable variability between subjects, and the difference between uraemic and normal groups in this study did not reach statistical significance. Furthermore, in

the other experiments described in this thesis there was no increase in V_{max} or changes in K_d and therefore lysine transport behaves uniquely in uraemia.

4.6.2. **L-serine.**

In humans, the Na^+-dependent, ASC system dominates the uptake of L-serine by the erythrocyte. The saturable, sodium-dependent component of L-serine influx represents transport through the ASC system, an amino acid transporter with L-alanine, L-serine, and L-cysteine as its preferred and eponymous substrates (Ellory, 1987). This system can operate in a net flux and exchange mode and exchange is the predominant mechanism in human red cells (Barker and Ellory, 1990). L-serine uptake into the erythrocyte is known to be stimulated by intracellular L-serine (Collarini and Oxender, 1987). This was also demonstrated in this chapter by the experiment presented in figure 4.8.

The sodium-dependent influx of L-serine system was significantly reduced in haemodialysis patients, through a decrease in V_{max}. The value of V_{max} = 353 μmol.l $cell^{-1}.h^{-1}$ for controls presented here is similar to the V_{max} = 299 μmol.l $cell^{-1}.h^{-1}$ reported by Al-Saleh and Wheeler (1982). There was also a decrease in K_m for L-serine in uraemic erythrocytes. The overall effect of the changes seen is that uraemic erythrocytes possess a reduced capacity for L-serine

transport, and this reduction is most marked at high concentrations of L-serine.

The reduction in erythrocyte L-serine uptake may reflect an intrinsic alteration in the membrane ASC system, or a reduction in the number of transport proteins, but could also arise through a reduction in the concentrations of ASC substrates inside the erythrocytes. This latest possibility is unlikely to be correct since similar reductions in V_{max} were seen in the zero-trans experiments suggesting that the transport abnormality seen in haemodialysis patients results from altered membrane properties.

4.6.3. **Glycine.**

Glycine transport in human erythrocytes can be resolved into five separate components of uptake (Ellory, Jones and Young, 1981). The saturable sodium and chloride-dependent transport of glycine into erythrocytes occurs through the gly transport system, which is selective for glycine and sarcosine only. The results presented in this chapter are related to influx rates via this high-affinity system.

The values of V_{max} = 24 μmol.l cells^{-1}.h^{-1} and K_m = 34 μmol/l for normal controls reported here are in line with reported values for V_{max} = 29 μmol.l cells^{-1}.h^{-1} (Ellory, Jones and Young, 1981) and 32 μmol.l cells^{-1}.h^{-1} (Al-Saleh and Wheeler, 1982). Similar values have also been reported by King

and Gunn (1989). This low rate of V_{max} reflects the fact that glycine is the neutral amino acid transported at the slowest rate in the human red cell (Ellory, Jones and Young, 1981). For K_m, Al-Saleh and Wheeler (1982) reported values of 60 μmol/l while Ellory, Jones and Young (1981) found this to be 25 μmol/l. In this thesis, normal controls had a K_m = 34 μmol/l.

In comparing the experimental results, V_{max} for this system was unaltered in haemodialysis patients' cells. However, uraemic cells showed an increased affinity for glycine since K_m was reduced by 23% compared to control cells. The origins of the altered K_m for the gly system in uraemic erythrocytes are not clear. It could be speculated that the change is likely to cause an increased rate of erythrocyte glycine uptake at physiological plasma concentrations.

4.6.4. **L-leucine.**

The uptake of L-leucine into erythrocytes occurs almost entirely through system L (Ellory, 1987; Rosenberg, 1981a).

The assumption that human red cells reach zero-trans condition for L-leucine fluxes during the washing procedure is in agreement with data reported by Rosenberg (1981a). This author also points out that at 40 mM external concentration, leucine influx rates at zero-trans were not different when compared with influx rates in cells pre-loaded with 80 mM L-

leucine.

Analysing leucine flux data obtained at 25°C, Hoare (1972a) and Rosenberg (1981a) concluded that a simple Michaelis-Menten relationship between flux and concentration would fit well with leucine kinetic parameters and that the goodness of the fit was not significantly improved when an additional linear component was added to the equation. In this thesis, the fitting of the leucine influx data was performed by using a three parameter equation (1). These data could also have been fitted by using a simple Michaelis Menten equation, as reported by Hoare (1972a) and Rosenberg (1981b) above. Hoare (1972a) pointed out that a linear component could only be demonstrated by using high concentrations of leucine (> 20 mM), although this would change either osmotic pressure or ionic strength (Rosenberg, 1981a). In fact, looking at figure 4.15, it appears that influx measurements at 50 mM leucine, fall away from the expected line for the concentration dependence curve. Nevertheless, in fitting the data presented in this chapter to equation (1) by using a least square algorithm with $1/V^2$ weighting, the computer programme used was able to give more weight to values obtained at low concentration than at high concentration and in each case the coefficient of multiple correlation was greater than 0.95. It is important to stress that the fit looked very good whatever the equation used, a fact already reported by Rosenberg (1979) and Young, Jones and Ellory (1980), and that no significant

difference was observed for leucine influx between normal controls and uraemic cells, regardless of the method used to fit the data.

The results of L-leucine flux measurements in normal controls and in uraemic erythrocytes indicate that this system functions normally in these cells, since no significant differences were observed regarding V_{max}, K_m, or K_d. The value of K_m = 5.07 mmol/l in controls is similar to reported values of 5 mmol/l (Tunnicliff, 1989) and 4.8 mmol/l (Young and Ellory, 1979). The reported values of V_{max} in the present experiments (42 mmol.l cells^{-1}.h^{-1}) were significantly lower than values in the literature: V_{max} = 68 mmol.l cells^{-1}.h^{-1} (Young and Ellory, 1979) and V_{max} = 119 mmol.l cells^{-1}.h^{-1} (Young, Jones and Ellory, 1980). The origins of these differences are not clear but may reflect differences in the methodology used.

4.6.5. **L-tryptophan.**

Transport of L-tryptophan in human red cells is mediated by a saturable, sodium-independent, high affinity and selective transport system (T) with a K_m = 1.3 mM and V_{max} = 7.2 mmol.l cells^{-1}.h^{-1} and a non-saturable route (K_d) representing transport via system L (K_d = 0.618 h^{-1}) (Young and Ellory, 1979; Rosenberg, 1979; Rosenberg, Young and Ellory, 1980). The K_m = 0.97 mmol/l and K_d = 0.460 h^{-1} for L-

tryptophan in normal controls in this thesis is similar to values above, although the values of V_{max} were lower than those reported.

The saturable influx of L-tryptophan via system T in uraemic erythrocytes was not significantly different from the controls. The unchanged mean value of K_d for L-tryptophan in uraemic cells (Table 4.1.) confirms the normal behaviour of system L.

4.7. Summary.

Table 4.3. summarises the findings on the different amino acid transport systems in relation to results obtained in normal controls.

There are abnormalities in the membrane amino acid transport in erythrocytes from haemodialysis patients. However, this is not a generalised effect. Rather, there are specific changes in selected amino acid transport systems. The reasons for these changes are not clear at the present time.

Table 4.1. Mean kinetic parameters for amino acid influx into erythrocytes from patients on haemodialysis and from controls.

Aminoacid	Vmax (SEM) (umol/l cells/h	Km (SEM) (umol/l)	Kd (SEM) (h)
L-lysine haemodialysis	762 (72) *	68.2 (5.7)	0.224 (0.039)
L-lysine controls	566 (33)	70.5 (4.1)	0.178 (0.028)
L-serine haemodialysis	233 (33) *	105 (11) *	0.153 (0.003)
L-serine controls	352 (21)	160 (12)	0.206 (0.05)
Glycine haemodialysis	27.6 (2.9)	26.3 (2.3) *	0.16 (0.01)
Glycine controls	24.1 (4.1)	34.0 (3.1)	0.15 (0.01)
L-leucine haemodialysis	45100 (3150)	5400 (430)	0.185 (0.04)
L-leucine controls	42500 (3100)	5070 (400)	0.274 (0.05)
L-tryptophan haemodialysis	3290 (300)	1160 (160)	0.540 (0.05)
L-tryptophan controls	4080 (260)	970 (120)	0.460 (0.06)

The value for Kd for L-serine represents the apparent diffusion constant for influx in Na-free saline. Kd for glycine was obtained in Cl-free saline. The values of Kd for L-lysine, L-leucine and L-tryptophan were obtained by computer fits of the fluxes measured in standard saline.
* indicates a significant difference from controls ($p<0.02$)

Table 4.2. Mean kinetic parameters for amino acid influx into erythrocytes from patients on haemodialysis and from controls under assumed zero-trans condition.

Aminoacid	Vmax (S.E.M) (umol/l cells/h	Km (S.E.M) (umol/l)	Kd (S.E.M) (h)
L-lysine haemodialysis	260 (50) *	56 (3)	0.13 (0.02)
L-lysine controls	200 (10)	56 (3)	0.08 (0.02)
L-serine haemodialysis	153 (9) *		
L-serine controls	233 (6)		

The values of Kd for L-lysine were obtained by computer fits of the fluxes measured in standard saline.

* indicates a significant difference from controls ($2P<0.03$)

Table 4.3. Amino acid transport systems in uraemia

System	Vmax	Km	Kd
y+	increased	unchanged	unchanged
ASC	decreased	decreased	unchanged
gly	unchanged	decreased	unchanged
L	unchanged	unchanged	unchanged
T	unchanged	unchanged	unchanged

CHAPTER 5

NUCLEOSIDE TRANSPORT

5.1. Nucleoside fluxes.

This chapter will present results of a study of nucleoside transport in normal controls and patients on haemodialysis. This transport system has been well studied in the temperature range 4-25°C. Carrier-mediated nucleoside transport is a function of temperature and measurement of transport rates at lower temperatures facilitates the experimental protocol. It has been the intention of this thesis to study transport systems in as near as possible physiological conditions and so for this reason the experiments were performed at 37°C. The data to be presented below have been collected with the use of an original methodology developed to measure nucleoside influx at this higher temperature.

The experiments were performed using uridine as substrate, since it has been shown not to be metabolized (Oliver and Paterson, 1971).

The linearity of the initial rate of uptake of uridine

at 37°C and at extracellular uridine concentration 1mM, was established by time course experiments. Figure 5.1 presents the time course of uridine uptake into normal human red cells fitted by linear regression, at an extracellular uridine concentration of 1 mM. As can be deduced from the figure, the flux is linear up to 7 seconds. Each point represents individual determinations. Extrapolation of the line back to zero time provides an estimate of the error involved in taking data at a single timepoint of 3.5 seconds as a measure of the initial rate of influx. This error was of the order of 10%.

To establish if the uridine flux rate is different in freshly isolated cells than in zero-trans conditions, uridine flux measurements at an extracellular uridine concentration of 1 mM, were performed immediately after washing the cells and after cells had been incubated at 37°C for 2 hours in standard saline, at a < 1% haematocrit. Figure 5.2 shows an example of a concentration-dependence curve for the initial rate of uridine influx in freshly isolated erythrocytes from normal controls (open circles) and under zero-trans condition (closed circles). No significant difference in influx rate was found using cells prepared by the two different methods.

Therefore, the rate of uridine influx was measured in 8 normal controls and 8 patients on haemodialysis at an extracellular uridine concentration of 0.05-1 mM, using immediately-washed cells.

The concentration-dependence curve of the initial rate

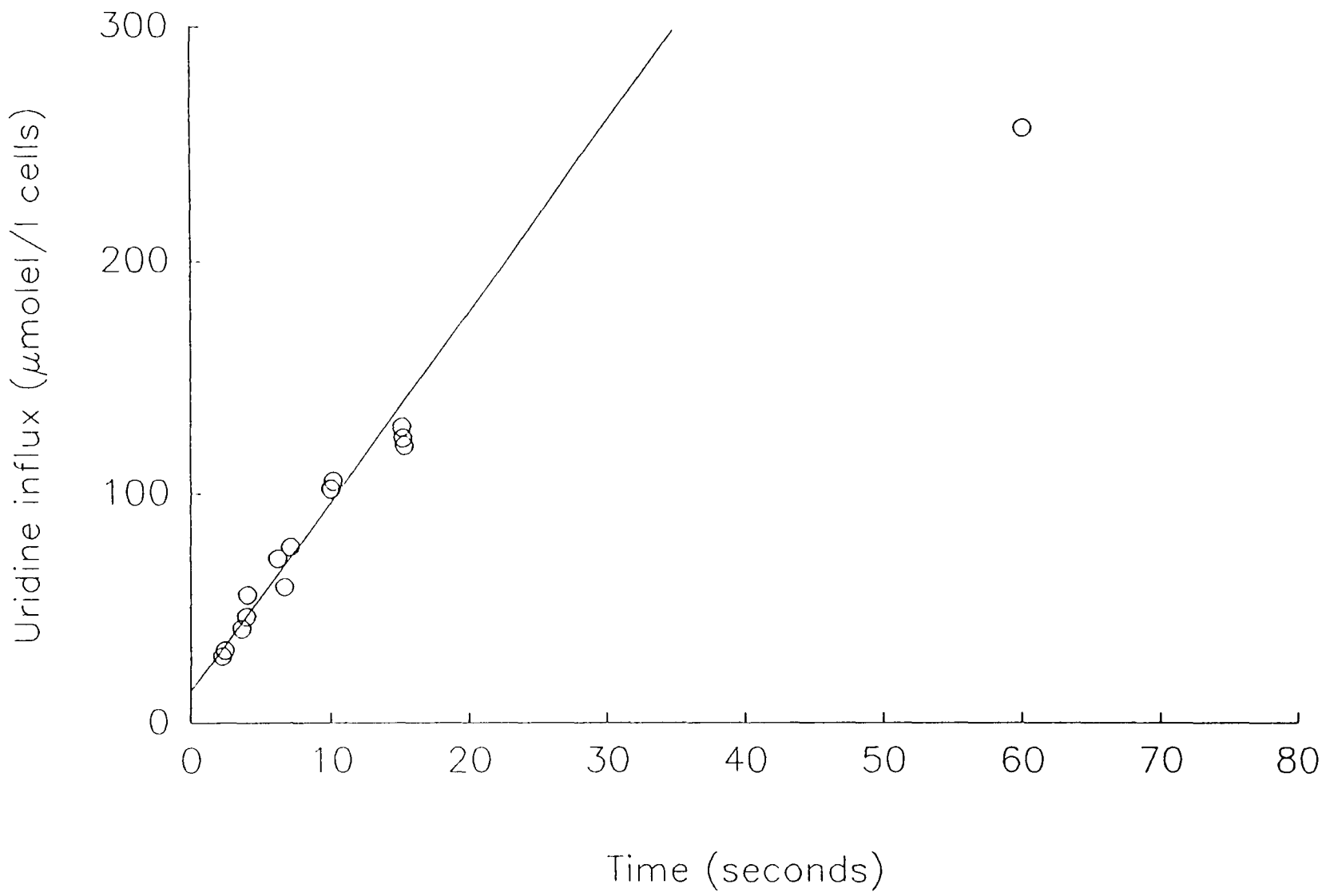

Figure 5.1. Time course for uridine uptake into human erythrocytes at 37°C. Each point represents a single measurement. Extracellular uridine concentration was 1 mM. The data for time < 7 seconds has been fitted by linear regression (r^2 = 0.972).

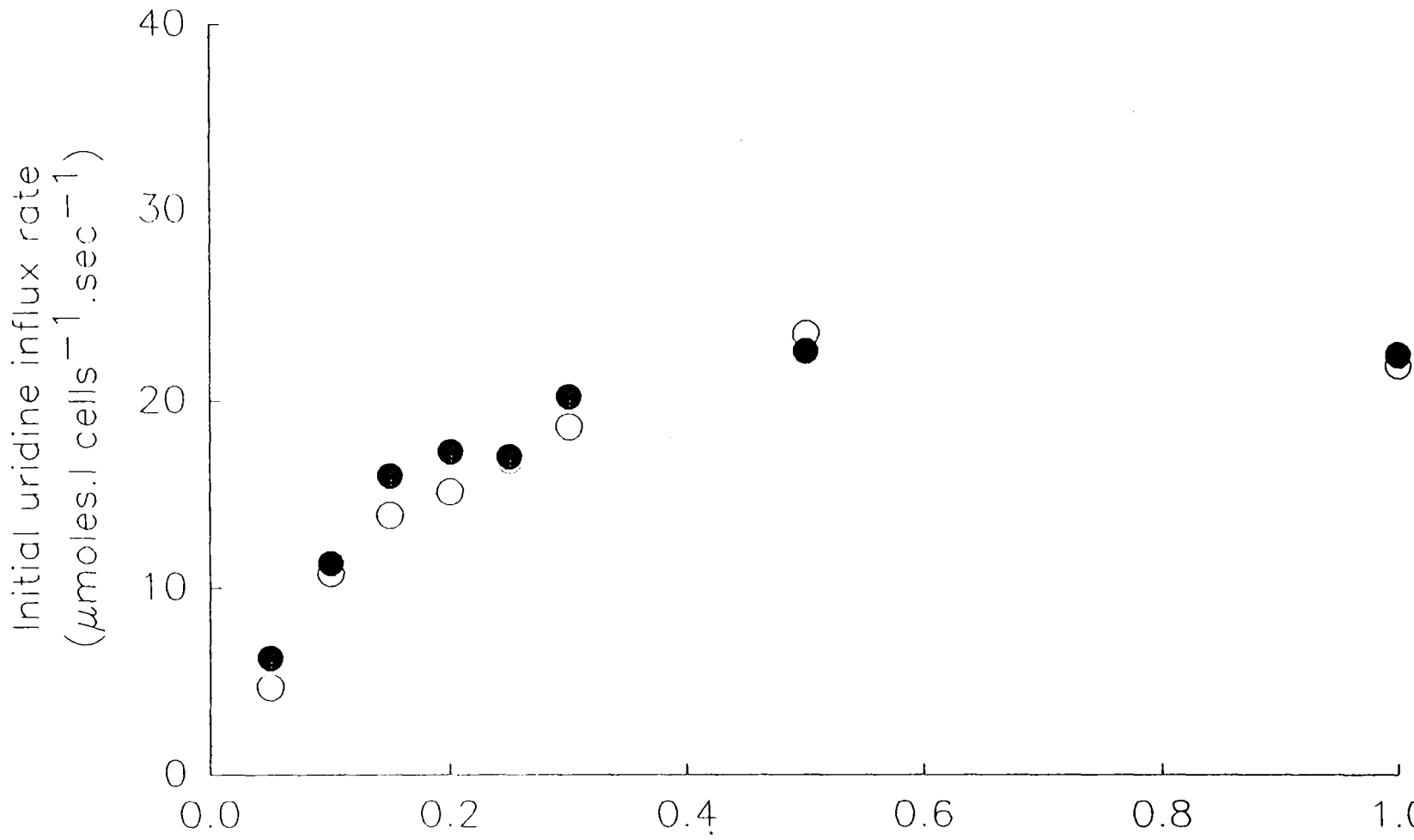

Figure 5.2. The initial rate of uridine uptake at 37°C into erythrocytes from a normal control as a function of extracellular uridine concentration in freshly isolated cells (open circles) and in cells previously incubated for 2 hours in standard saline at the same temperature (closed circles). For immediately used cells V_{max} = 27.2 μmol.l cells^{-1}.sec^{-1}, K_m = 152 μM. At assumed zero-trans condition V_{max} = 27.0 μmol.l cells^{-1}.sec^{-1} and K_m = 124 μM.

of uridine influx in a single experiment in a normal control is shown in figure 5.3. Results from each individual sample are shown to demonstrate the reproducibility of the measurements. The results were fitted by Michaelis-Menten kinetics as shown by the line drawn in the figure. A concentration-dependence curve showing the mean initial rate of influx in all 8 normal controls and 8 haemodialysis patients is shown in figure 5.4.

The calculated mean initial rate of uridine influx (V) for each uridine concentration [S] employed, can be presented as plots of [S]/V against [S] or V/[S] against V, to emphasize results obtained at different substrate concentrations and to test the Michaelis-Menten fitting of the measurements. If the fitting is correct, there will be no statistical significance between either plot. Figure 5.5 presents a plot of [S]/V against [S] (Hanes plot) for normal individuals (top figure) and a similar plot for haemodialysis patients (bottom figure). Both are good fits and the Michaelis-Menten parameters obtained are similar. The mean ($\pm$ S.E.M) V_{max} = 32.8 $\pm$ 2.4 μmoles.l cells^{-1}.sec^{-1}, and K_m = 190 $\pm$ 4.6 μM in normals were similar to uraemics: V_{max} = 30.6 $\pm$ 2.7 μmoles.l cells^{-1}.sec^{-1}, K_m = 185 $\pm$ 5 μM. Statistical significance analysis using student's t-test gave p values of 0.879 and 0.243 respectively and showed that there was no statistically significant difference between the V_{max} or K_m of the normal and uraemic patients' erythrocytes. Figure 5.6 are plots of V/[S] against

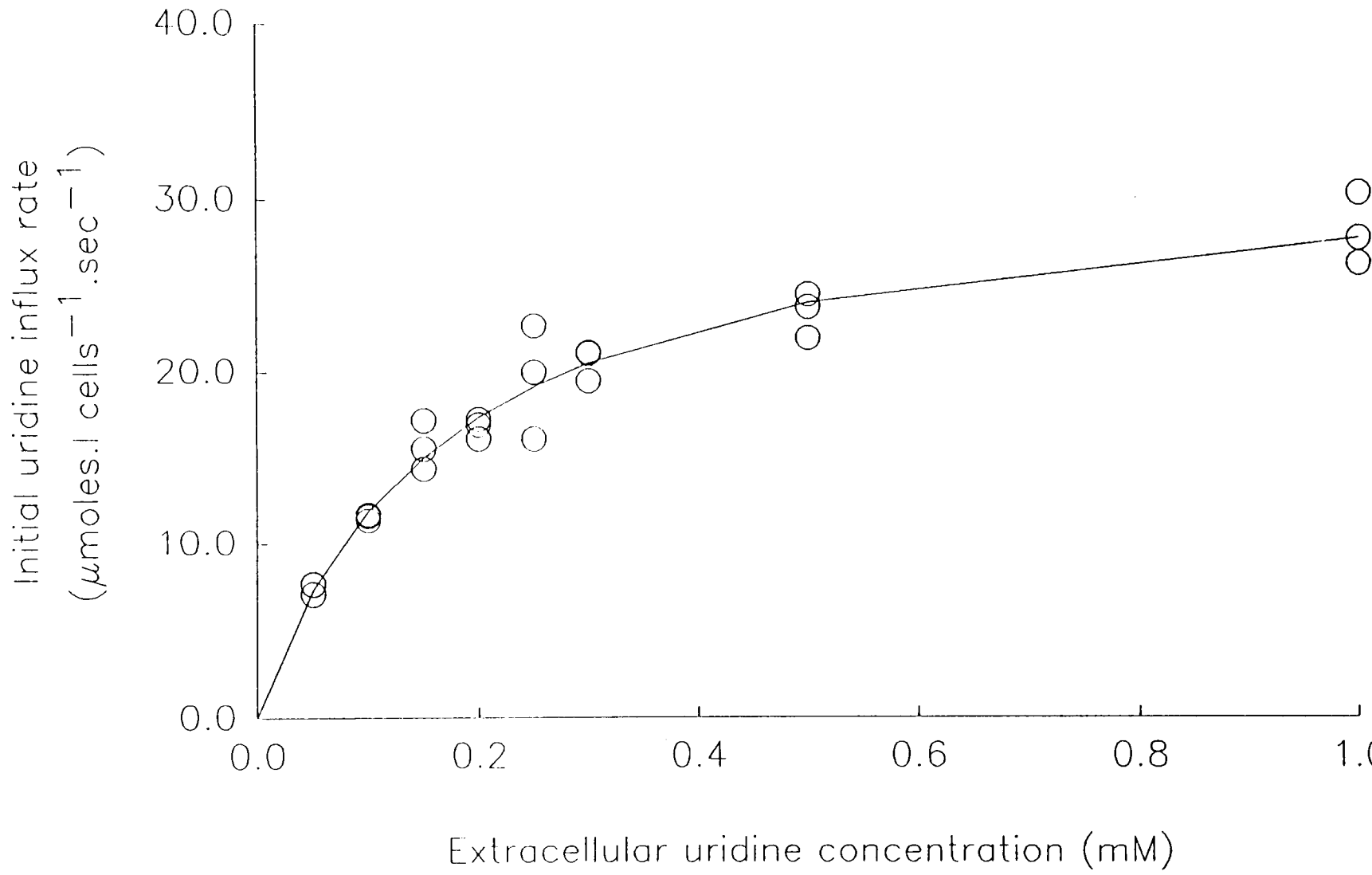

Figure 5.3. The initial rate of uridine uptake at 37°C into erythrocytes from one normal control as a function of extracellular uridine concentration. Each point represents a single measurement. The least-squares fit of the data to the Michaelis-Menten equation is shown by the line. Fitted parameters were V_{max} = 32.5 μmol.l cells^{-1}.sec^{-1}, K_m = 173μM.

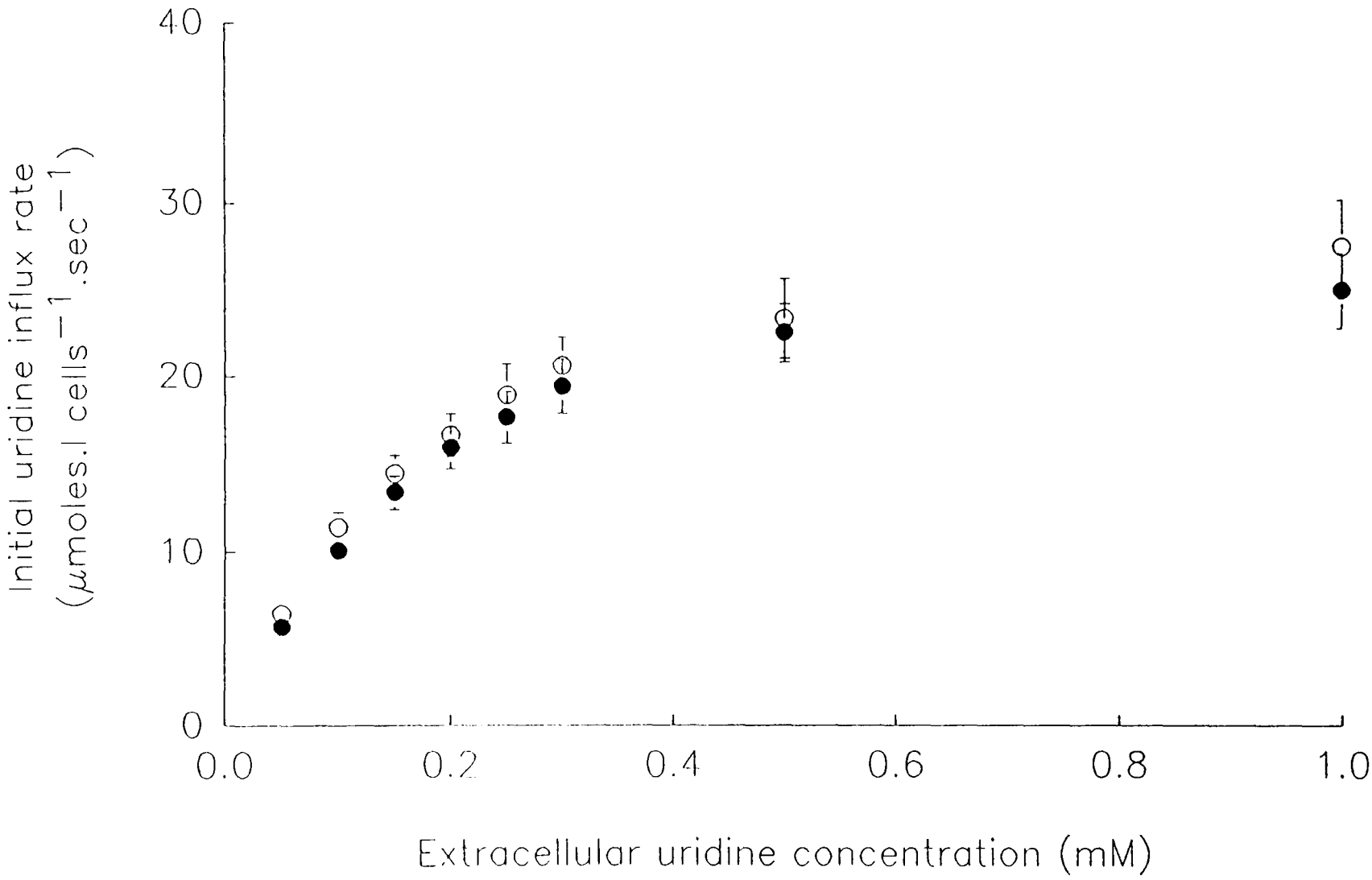

Figure 5.4. The initial rate of uridine uptake at 37°C into erythrocytes from normal controls (open circles) and haemodialysis patients (closed circles). Each point represents the mean of the total number of experiments (n= 8). Error bars are S.E.M. V_{max} = 32.8 μmol.l cells^{-1}.sec^{-1}, K_m= 190 μM in controls and V_{max} = 30.1 μmol.l cells^{-1}.sec^{-1}, K_m= 185 μM in cells obtained from haemodialysis patients.

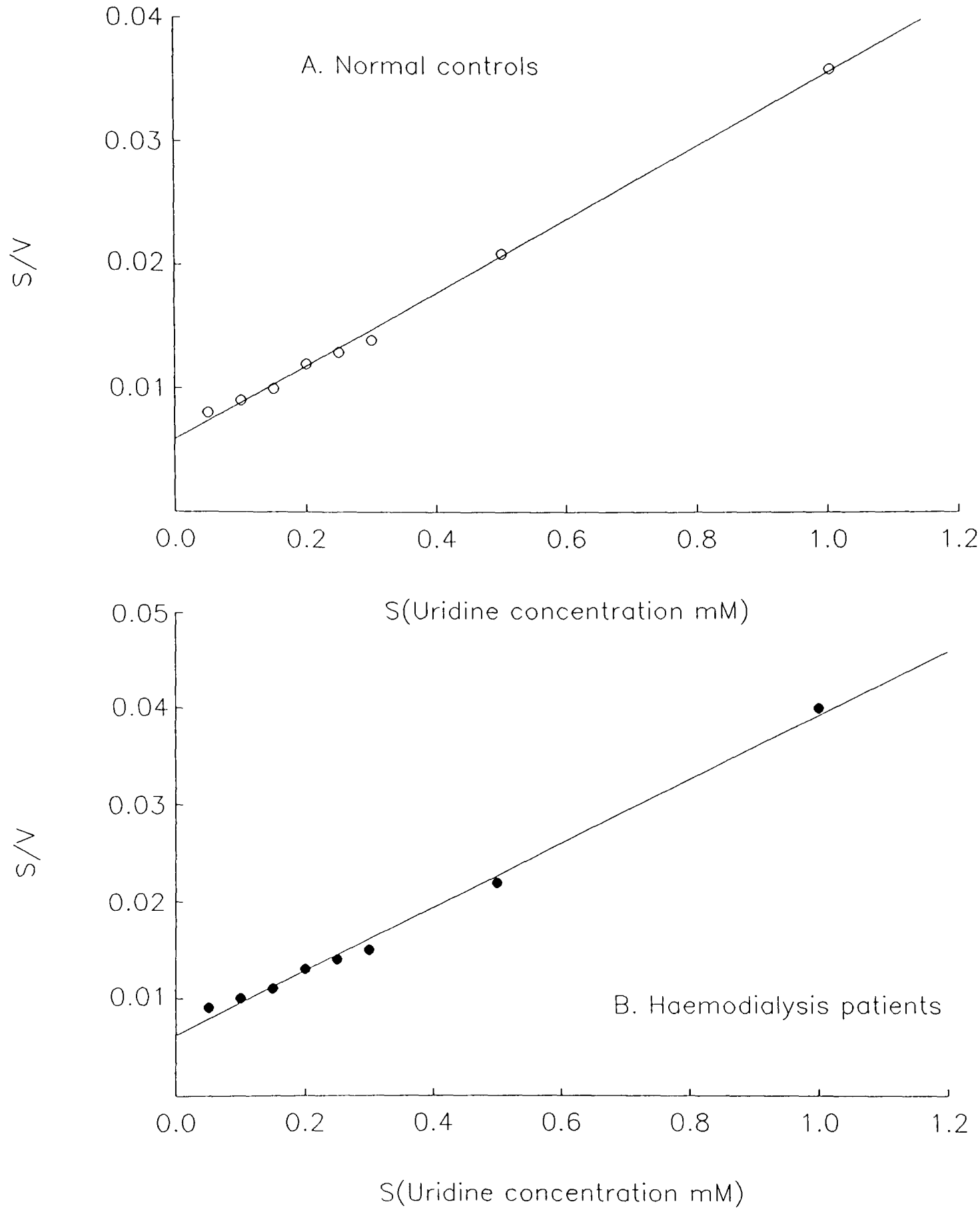

Figure 5.5. Plots of S/V against S for uridine uptake in erythrocytes obtained from eight normal controls (A) and eight haemodialysis patients (B). Error bars are omitted for clarity but the S.E.M. for measurements of V at each value of S were of the order of 5-8% of the mean. The data have been fitted with a least-square linear regression line. For controls V_{max} = 32.8 μM/sec, K_m = 190.4 μM; for haemodialysis patients V_{max} = 30.0 μM/sec, K_m = 185 μM. The values of r^2 for the upper figure was 0.999 and for the lower figure 0.986.

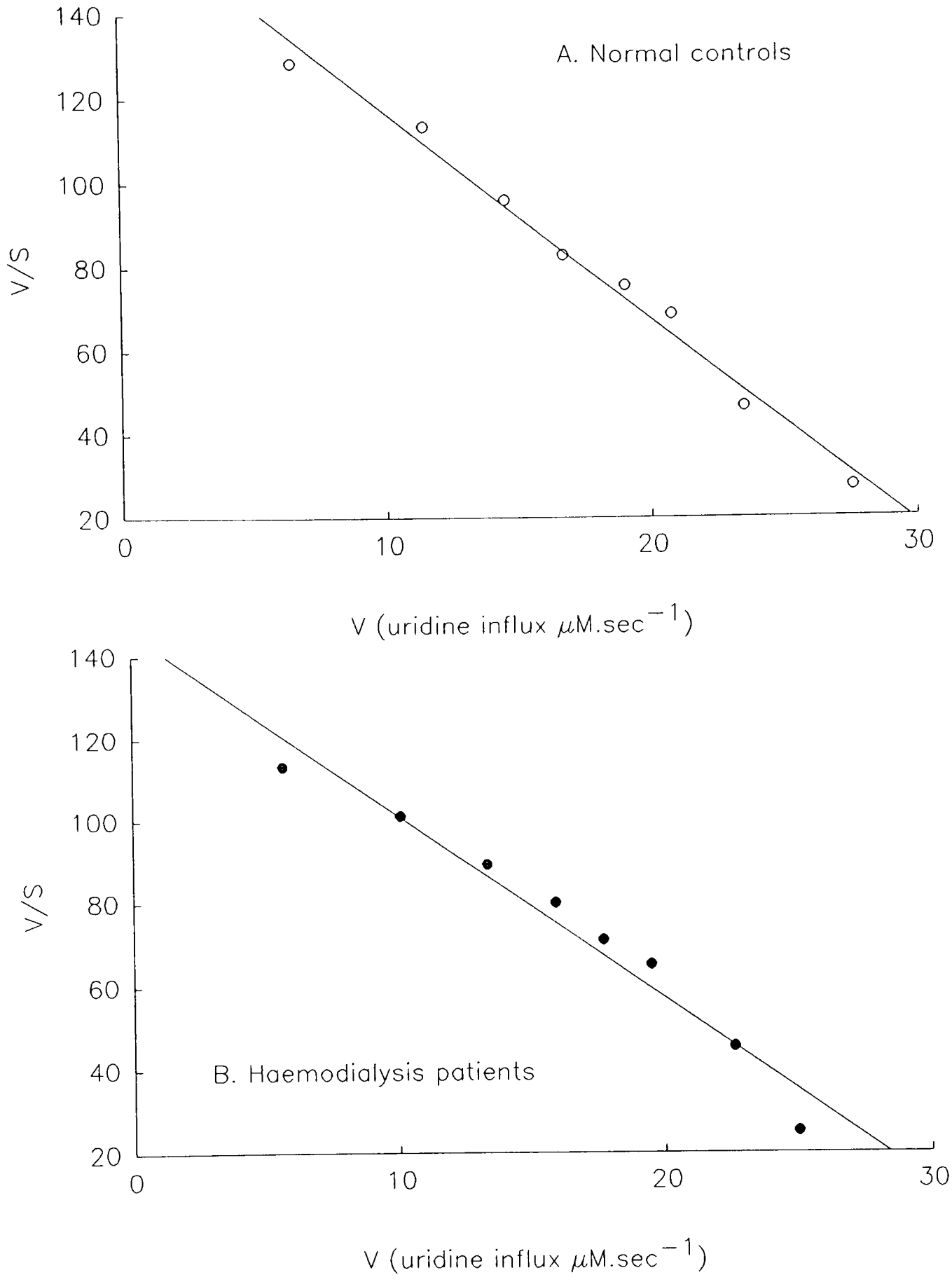

Figure 5.6. Represents plots of V/S against V in the same group. Normal controls (figure A), haemodialysis patients (figure B). Error bars have been omitted for clarity. The data have been fitted with least-squares linear regression. For controls V_{max} = 33.8 μM/sec, K_m = 203 μM; for haemodialysis patients V_{max} = 32.9 μM/sec, K_m = 226 μM. The r^2 values of the fits were 0.996 (A) and 0.961 (B).

V (Eadie-Hofstee plot) for normals and haemodialysis patients respectively. These plots provide a different weighting of the data but also show good linear fits and produce comparable values for V_{max} and K_m. This supports the use of the Michaelis-Menten equation to fit the data.

5.2. Discussion.

The major conclusions from this series of experiments is that erythrocyte uridine transport is normal in patients on haemodialysis.

The measurement of uridine influx at 37°C in human erythrocytes presents a number of difficulties because of the high capacity of the cell for uridine transport. Techniques for measurement of nucleoside transport include the use of sophisticated quenched-flow procedures (Paterson, Harley and Cass, 1985) and ATP-depleted adenosine deaminase-blocked red cells (Plagemann, Wohlhueter and Kraupp, 1985). This Thesis presents results obtained with the use of a simpler but nevertheless efficient technique.

The time course of uridine influx illustrated in figure 5.1 demonstrates that the methods used here provided a reproducible measurement of the initial rate of uptake. Also figure 5.3 shows each single measurement to be very reproducible. The systematic errors due to incorrect measurements of the true incubation time can be estimated as

< 10%. If a significant linear diffusion component was present, the results would not fit a simple Michaelis-Menten equation. Figures 5.4, 5.5 and 5.5 demonstrate that this is not the case. A further possible error arises from the accumulation of uridine inside the erythrocytes during the flux incubation and the loss of true zero-trans conditions. The accumulation of uridine in the 1 mM experiment at 3 seconds can be estimated to be <10% of the extracellular concentration and in all experiments the maximal accumulation was of the order of 25% of the external concentration at the end of the timed incubation period.

The high capacity of transport could explain the results shown in figure 5.2 where uridine fluxes are similar in cells used immediately after washing to cells suspended for 2 hours in standard saline, since red cells could become completely depleted of uridine during the initial washing procedure.

Fresh human erythrocytes exhibit a kinetically symmetrical zero-trans influx and efflux of uridine (Jarvis, Hammond, Paterson and Clanachan, 1983). The V_{max} for this process has been studied in detail in the temperature range 4-25°C. There is only one reported result for temperatures above this, excepting the work of this thesis (Plagemann and Wohlheuter 1984b). The present result of V_{max} = 32.8 μmoles.l cells^{-1}.sec^{-1} at 37°C is significantly different from the value of approximately 120 μmol.l cells^{-1}.sec^{-1} obtained at 35°C by Plagemann and Wohlheuter (1984b) using more indirect methods,

but the origins of this difference are not clear. It is unlikely that an underestimation of the flux by a factor of 4 by using a 3 second incubation time could account for it. The value of K_m for zero-trans influx at 37°C reported here is similar to the value of 141 μM measured at 35°C (Plagemann and Wohlhueter, 1984b). Uridine zero-trans influx into guinea pig erythrocytes shows a decrease in the temperature-dependence of V_{max} above 25°C with K_m values which are broadly similar to those found in fresh human erythrocytes (Jarvis and Martin, 1986). The uptake of uridine into Novikoff rat hepatoma cells and into 3T3 cells also shows a decrease in temperature-dependence above 25°C (Plagemann and Erbe, 1975; Stein and Rozengurt, 1975) but the interpretation of these results is controversial and effects due to metabolism of uridine may complicate these experiments (Plagemann and Wohlhueter, 1981). The data reported in this thesis support the view that nucleoside transport rates in human erythrocytes show a reduced temperature-dependence above 25°C.

Finally it is of interest to calculate the maximum turnover number of the human erythrocyte nucleoside transporter for the zero-trans influx of uridine at 37°C. NBMPR binding studies can be used to estimate that there are 10^4 nucleoside transporter units per erythrocyte (Jarvis et al, 1984). Assuming a mean cell volume of 90 fl, the V_{max} measured in these experiments of 32.8 μmoles.l cells^{-1}.sec^{-1} can be used to estimate a turnover number of 180 uridine

molecules per second for the nucleoside transport at 37°C. This compares with values of 100-200 per second for the maximal turnover of K^+ ions by the human erythrocyte sodium pump at 37°C (Chapter 3). Turnover numbers for the erythrocyte sugar transport system also appear to be in a similar range (Plagemann, Wohlhueter and Woffendin, 1988; Jarvis, 1989).

CHAPTER 6

CHOLINE TRANSPORT

This chapter will describe studies on erythrocyte choline fluxes in patients with chronic renal failure in pre-dialysis, haemodialysis, CAPD and kidney transplant patients.

Results will be presented in the order that the experiments were originally performed. Experiments were performed initially to establish the initial rate of uptake, zero-trans conditions and Na^+-dependence of the system studied. The experiments in zero trans condition in normal controls and in haemodialysis patients will follow. The experiments performed with immediate use of cells in controls, haemodialysis and CAPD patients will continue and finally, choline fluxes in transplant and pre-dialysis patients correlating their respective fluxes with their renal function will be presented.

6.1. Initial Rate of Uptake and Zero-Trans Conditions.

Time course experiments were performed to establish the initial rate of influx of choline. Figure 6.1 shows the

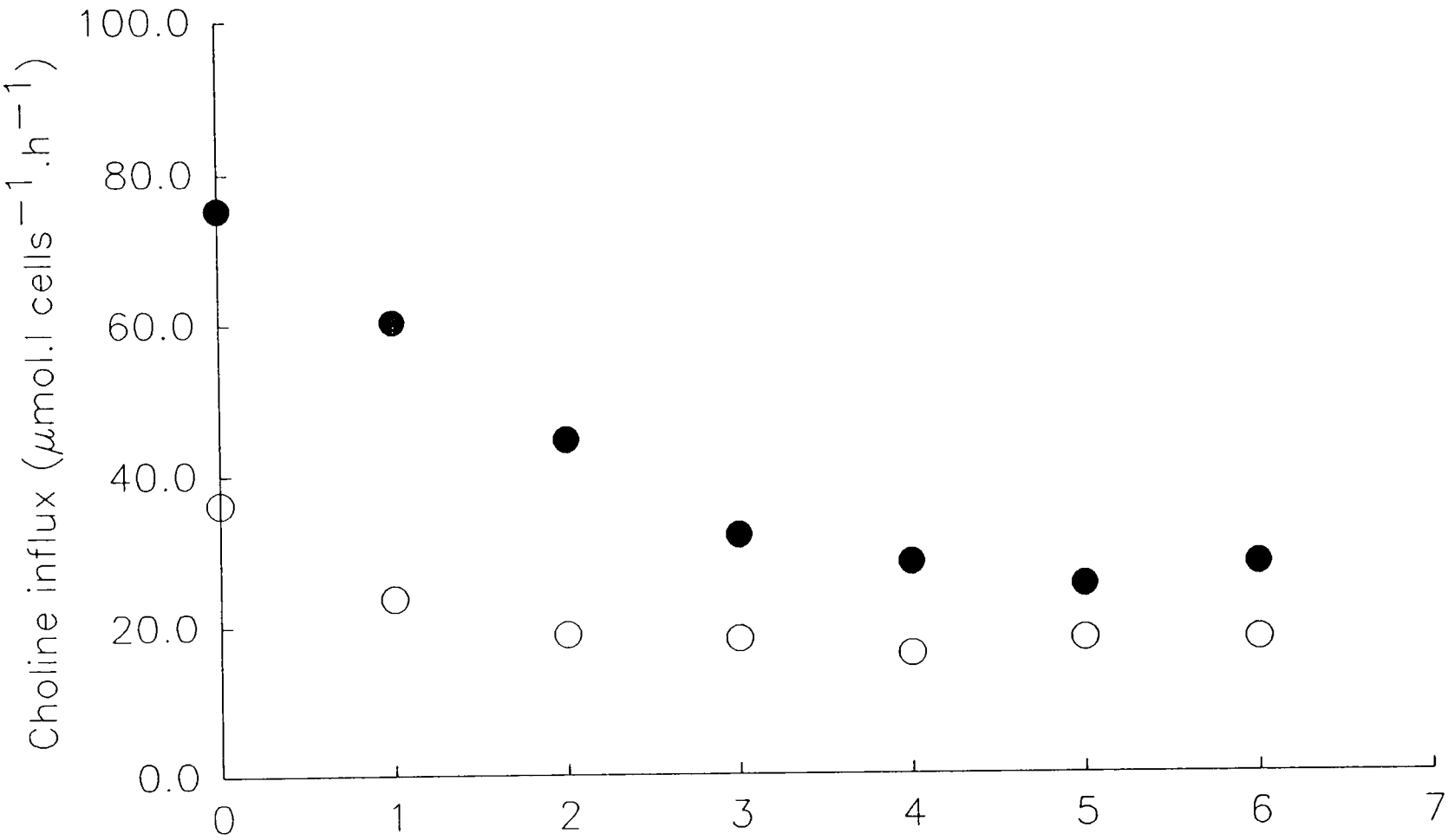

Figure 6.1. Choline fluxes in standard saline containing 250 μM choline (V_{max}). Fluxes were performed with immediately washed cells (time 0) and at every hour after the cells had been incubated in choline-free saline at a haematocrit < 1% at 37 °C. Open circles represent a normal control. Closed circles a haemodialysis patient. Cells appear to achieve steady-state condition after 4 hours.

results of a time course experiment in uraemic cells, at an extracellular choline concentration of 250 μM. Choline flux is linear up to 7 minutes and justifies the 5 minute incubation time used for the choline influx experiments.

Experiments performed at 250 μM extracellular choline concentration demonstrated that choline influx in human red cells, both of normal controls and haemodialysis patients, reached a low steady state value after 4 hours incubation in standard saline at an haematocrit < 1%. Figure 6.2 illustrates these results. From this figure it is apparent already that uraemic cells have a higher choline transport rate (V_{max}) than control cells.

6.2. Na^+-dependence.

Choline transport in human red cells, in contrast to neurones, has been shown to be Na^+-independent. The next group of experiments was directed to demonstrate that this was also correct for the cell population studied. Fig 6.3 illustrates the results of choline influx experiments in cells suspended in standard saline and NMDG replacement solution, both with immediate use and zero-trans condition cells. There was no difference between either cell group demonstrating that in control and uraemic red cells choline fluxes are indeed Na^+-independent.

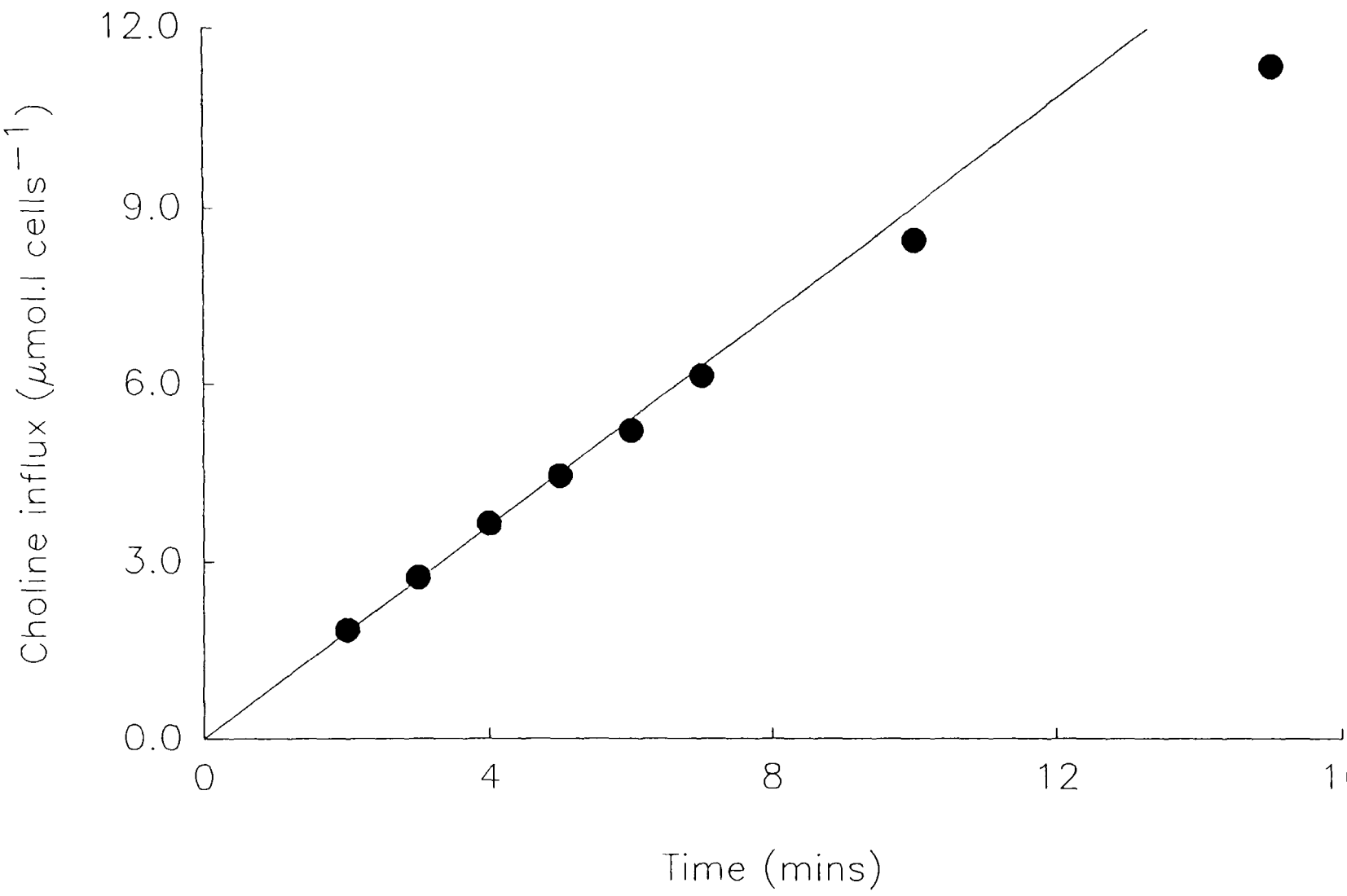

Figure 6.2. Time course of choline influx at an extracellular choline concentration of 250 μM, with immediate use of erythrocytes from a haemodialysis patient. The first four time points were fitted to a linear regression with a slope of 0.905 μmol.l cells^{-1}.min^{-1}.

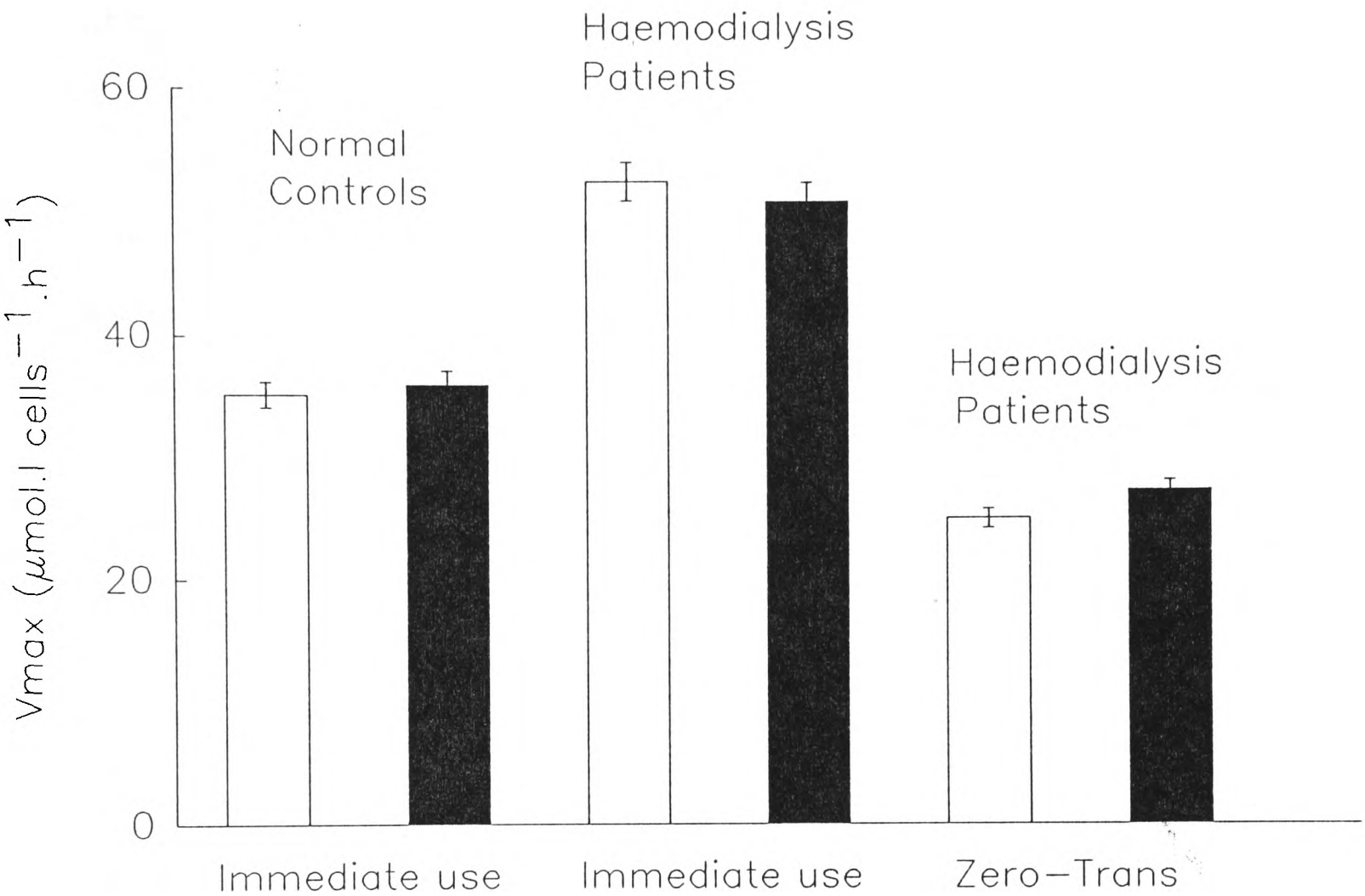

Figure 6.3. Choline fluxes at an extracellular choline concentration of 250 μM (V_{max}) in standard saline (blank boxes) and sodium-free solution (filled boxes) in immediate use and under assumed zero-trans condition erythrocytes obtained from normal controls and haemodialysis patients. Error bars are S.E.M. The fluxes are shown to be independent of Na^+.

6.3. Zero-Trans Condition Experiments.

Erythrocytes from normal controls and haemodialysis patients were studied in zero-trans conditions with choline influx measured as a function of extracellular choline concentration (1-50 μM).

The data can be fitted by Michaelis-Menten kinetics (Harvey and Ellory, 1989) to provide an estimate of the maximal rate of choline uptake in zero-trans conditions [V_{max} (ZT)], and of the apparent dissociation constant of the transporter [K_m (ZT)]. The fits are shown by the lines in Figure 6.4; The mean V_{max} (ZT) in HD patients was 38.4 μmol.l cells^{-1}.h^{-1}, compared with 14.2 μmol.l cells^{-1}.h^{-1} in controls. The value of K_m (ZT) was 19.4 μM in HD patients and 7.4 μM in controls. The differences between controls and HD patients were clearly significant ($p < 0.001$), with uraemic red cells showing an increased choline influx rate in comparison with normal cells.

6.4. Experiments on Immediately-Used Cells.

Once it was established that uraemic red cells had a higher choline transport rate at zero-trans conditions, it was important to determine if these changes were restricted to zero-trans conditions or would also be present when influx

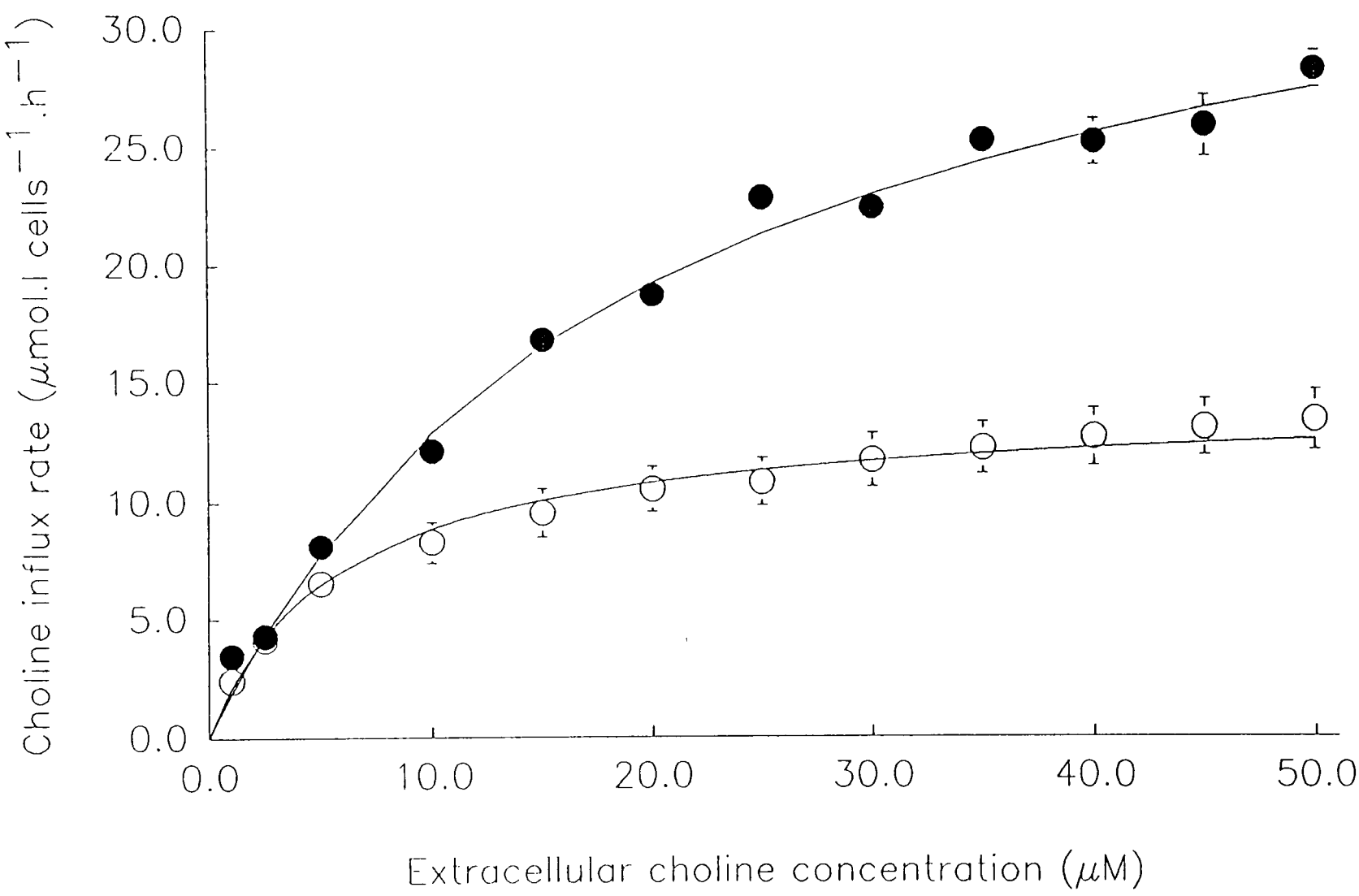

Figure 6.4. The mean initial rates of erythrocyte choline influx under assumed zero-trans condition as a function of extracellular choline concentration in haemodialysis patients' erythrocytes (closed circles) and controls (open circles). The error bars are S.E.M. The lines are Michaelis-Menten fits to the data with the values of V_{max} and K_m (ZT) described in the text.

experiments were performed with cells immediately after initial washing procedures.

Therefore, choline influx experiments at an extracellular choline concentration of 250 μM were performed using 5 minutes incubation time in normal controls, haemodialysis, CAPD, pre-dialysis and kidney transplant patients. These results estimate V_{max} for the choline transporter in erythrocytes studied immediately after initial washing. The results of these experiments are shown for each subject studied in Fig. 6.5. The mean V_{max} in normals was 30.5 (SD 4.9) μmol.l cells^{-1}.h^{-1} compared with 66.7 (SD 14.1) μmol.l cells^{-1}.h^{-1} in HD patients and 87.8 (SD 18.5) μmol.l cells^{-1}.h^{-1} in CAPD patients. There was no overlap between the individual results in normals (all < 40 μmol.l cells^{-1}.h^{-1}) and in dialysis patients (all > 50 μmol.l cells^{-1}.h^{-1}) and the HD and CAPD groups were significantly different from the controls ($p < 0.001$).

6.5. Correlating Influx Rate with Renal Function.

It was clear that the pre-dialysis and the transplant patient population studied had wide differences in their choline influx rate as seen in Figure 6.5. The values of V_{max} in the transplant patients range from 17.7 to 71.7 μmol.l cells^{-1}.h^{-1} and in the pre-dialysis patients range from 19 to 86.6 μmol.l cells^{-1}.h^{-1}.

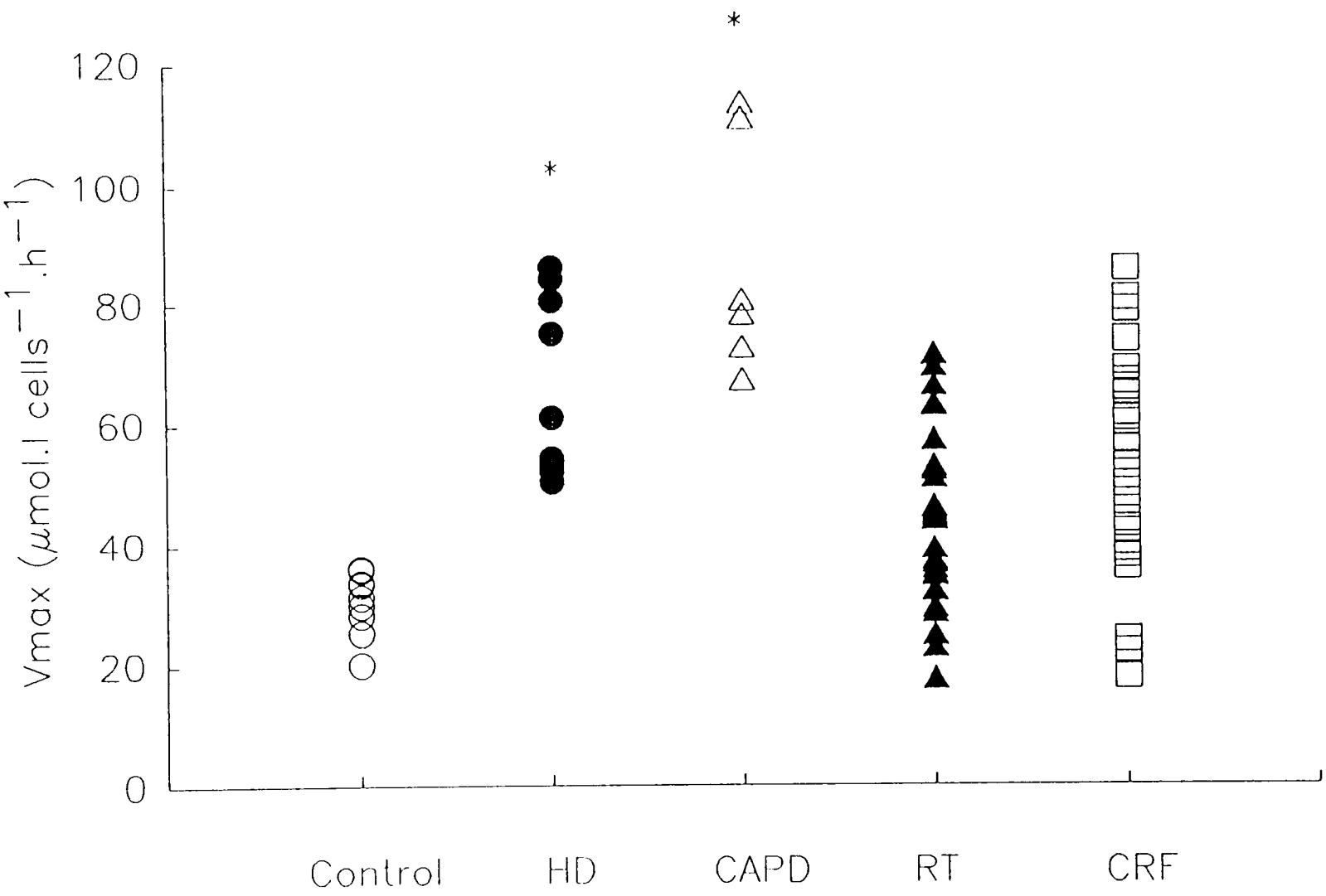

Figure 6.5. Values for the initial rate of erythrocyte choline uptake from standard saline containing 250 μM choline (V_{max}). The subject groups are controls, haemodialysis (HD), continuous ambulatory peritoneal dialysis patients (CAPD), renal transplant (RT) and pre-dialysis patients (CRF). Means ($\pm$ S.E.M) are presented in the text. * indicates a significant difference from the control group ($p< 0.001$).

Both groups also had great variability in their degree of renal function and this variability could account for the differences seen. Therefore a correlation between the V_{max} for choline influx and renal function (as represented by creatinine clearance, serum creatinine and urea) was sought.

Figure 6.6 represents a plot of V_{max} against creatinine clearance in the kidney transplant patients group. There was a significant negative correlation (Spearman, $p = 0.002$, $r = 0.55$) and the regression line is shown. There were also significant correlations between V_{max} and serum creatinine ($p = 0.012$, $r = 0.294$) and serum urea ($p = 0.015$, $r = 0.354$) (see figures 6.7 and 6.8). There was no significant correlation between V_{max} and any of the other parameters in Table 2.4. (Chapter 2) including patients' age (all $p > 0.05$).

The pre-dialysis patient values of V_{max} plotted against creatinine clearance values are shown in figure 6.9. There was a significant inverse correlation with the creatinine clearance ($p < 0.001$, $r = 0.693$) and also with serum creatinine ($p = 0.001$, $r = 0.483$)(fig. 6.10) and urea ($p < 0.001$, $r = 0.533$)(fig 6.11).

6.6. Discussion.

Red cell choline transport is via a well characterized, specific, high affinity system (Askari, 1966; Martin, 1968; Martin, 1972; Martin, 1977, Krupka and Deves, 1980) and has

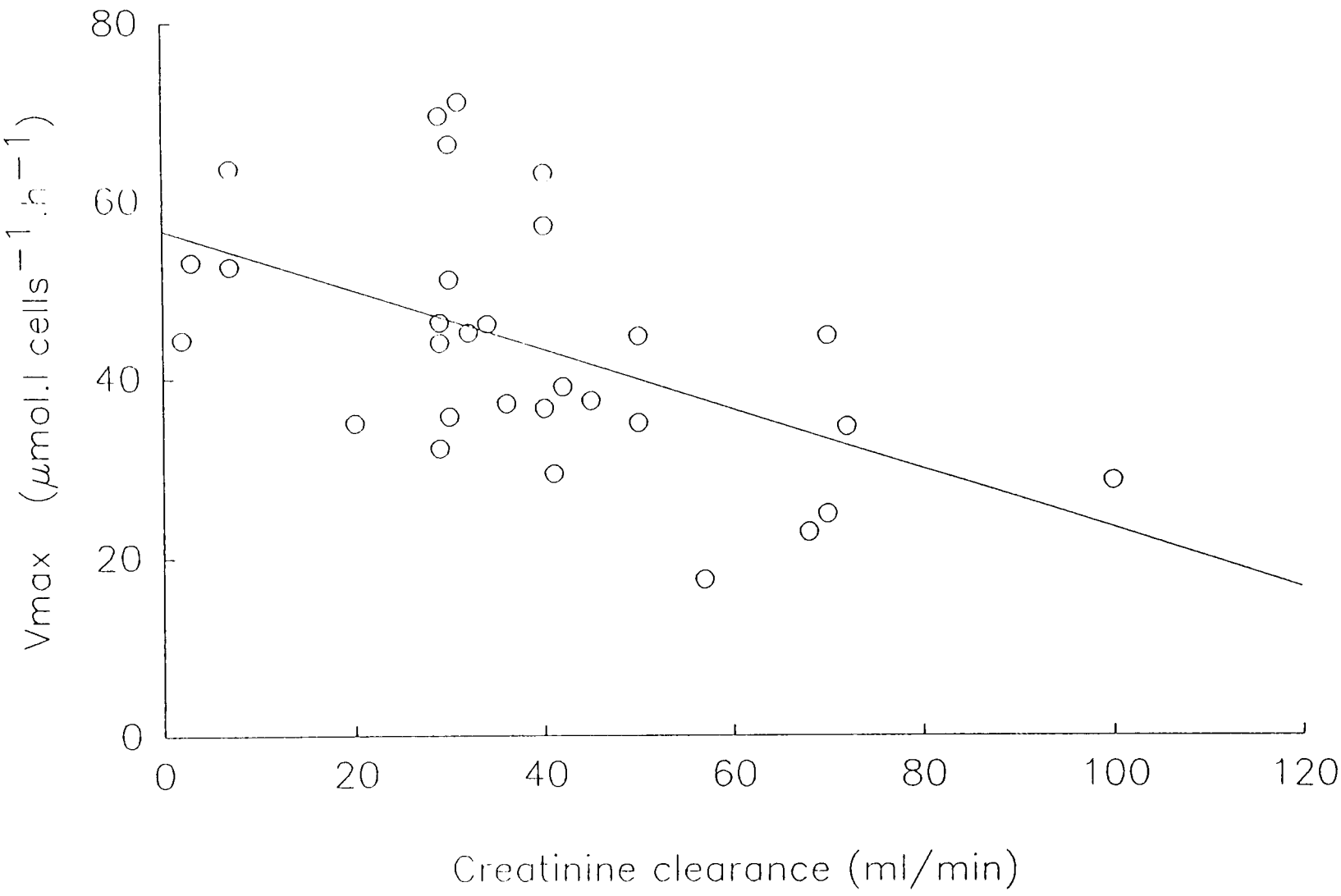

Figure 6.6. The correlation between choline transport (V_{max}) and creatinine clearance in erythrocytes from renal transplant patients. The linear regression line is V_{max} = 56.8 - 0.334 (clearance). At a clearance of 100 ml/min the line extrapolates to a V_{max} of 23.4 μmol.l cells^{-1}.h^{-1}. (r = 0.55, p = 0.002).

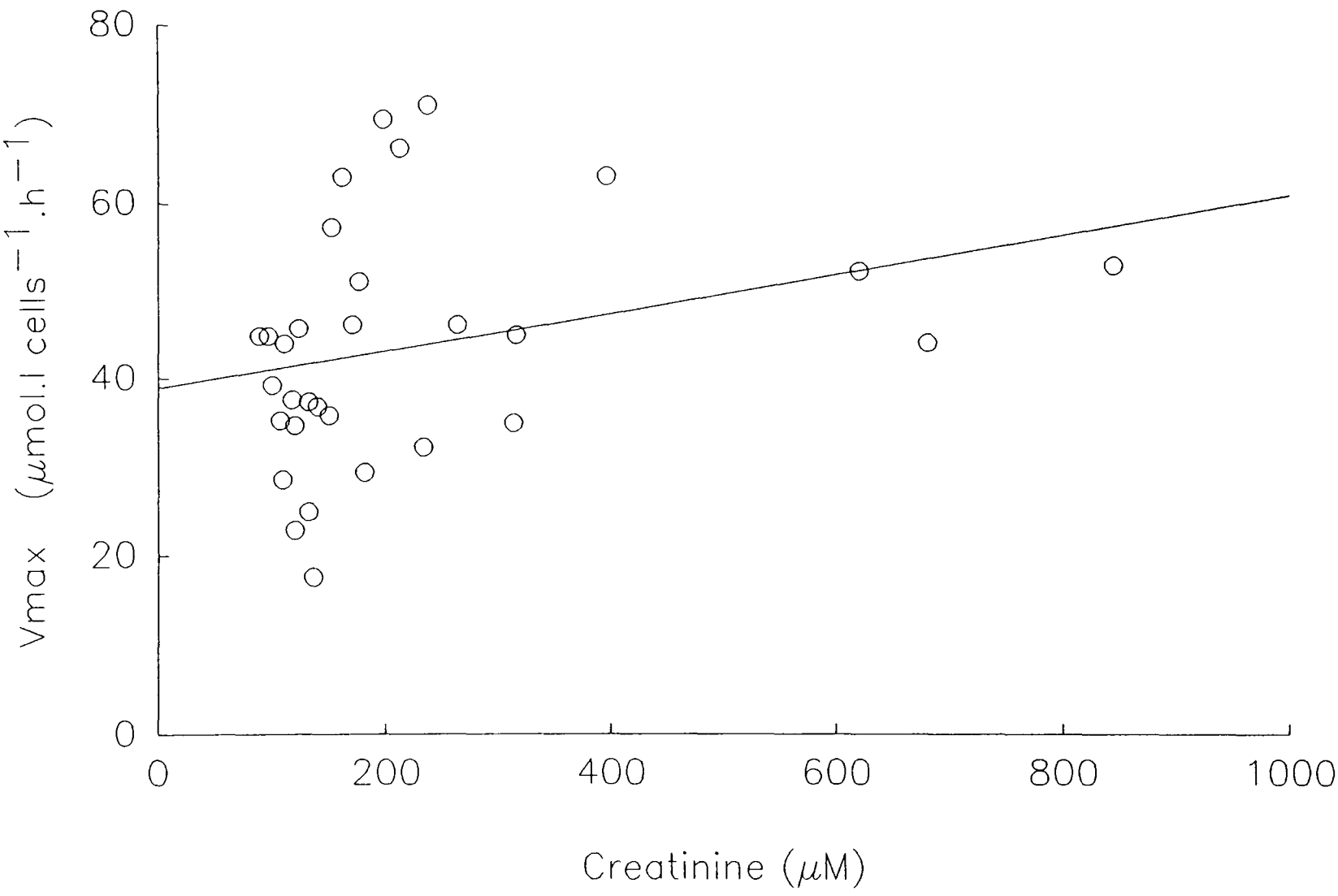

Figure 6.7. The correlation between choline transport (V_{max}) and serum creatinine in erythrocytes from renal transplant patients. The linear regression line is V_{max} = 38.9 + 0.022 (creatinine). At a creatinine of 1000 μM the line extrapolates to a V_{max} of 60.9 μmol.l cells^{-1}.h^{-1}. (r = 0.294, p = 0.012).

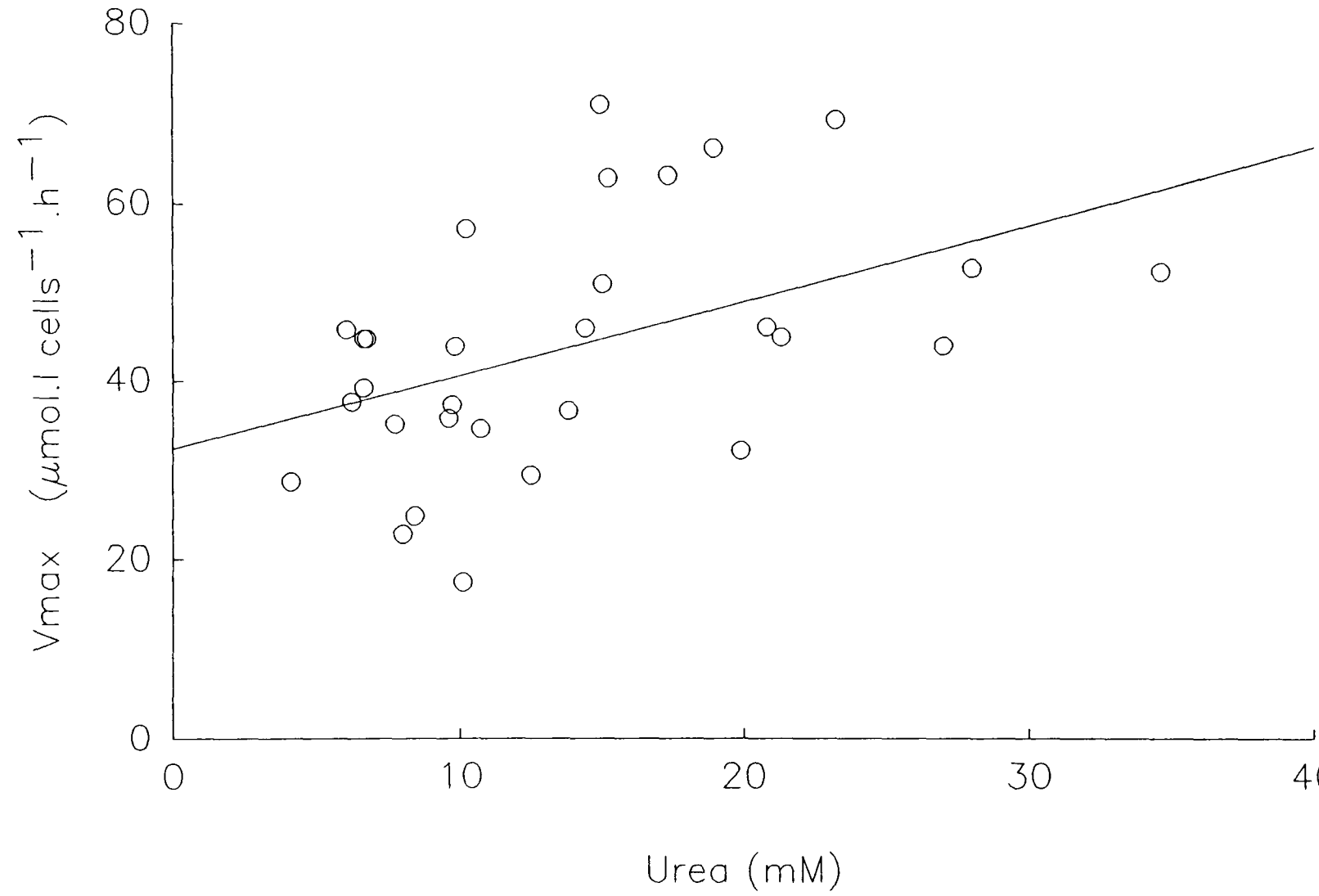

Figure 6.8. The correlation between choline transport (V_{max}) and serum urea in erythrocytes from renal transplant patients. The linear regression line is V_{max} = 32.2 + 0.855 (urea). At a urea of 40 mM the line extrapolates to a V_{max} of 66.4 μmol.l cells^{-1}.h^{-1}. (r = 0.464, p = 0.03).

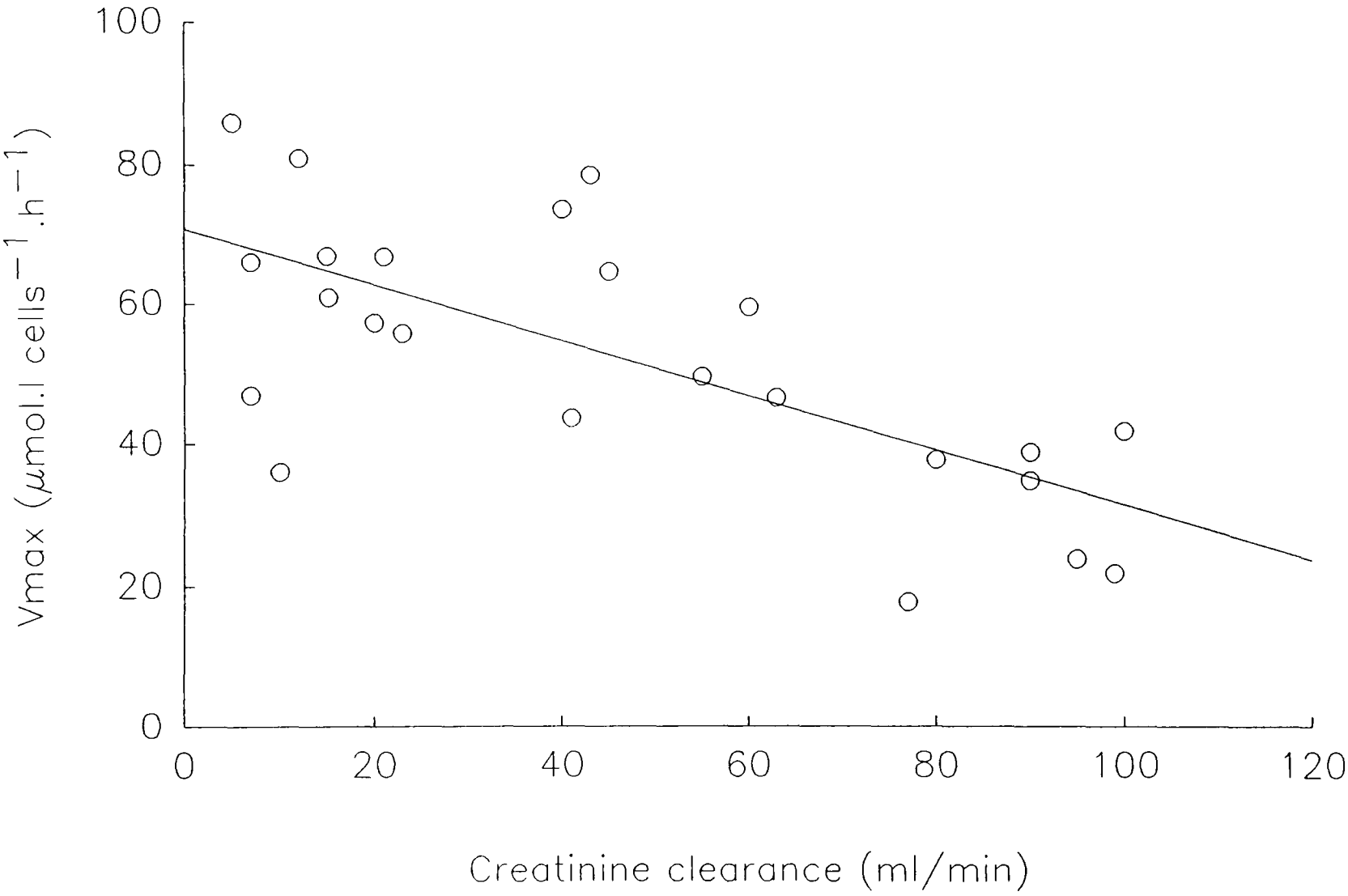

Figure 6.9. The correlation between choline transport (V_{max}) and creatinine clearance in erythrocytes from chronic renal failure patients not yet on dialysis. The linear regression line is V_{max} = 70.7 - 0.391 (clearance). At a creatinine clearance of 100 ml/min the line extrapolates to a V_{max} of 31.6 μmol.l cells^{-1}.h^{-1}. (r = 0.693, p < 0.001).

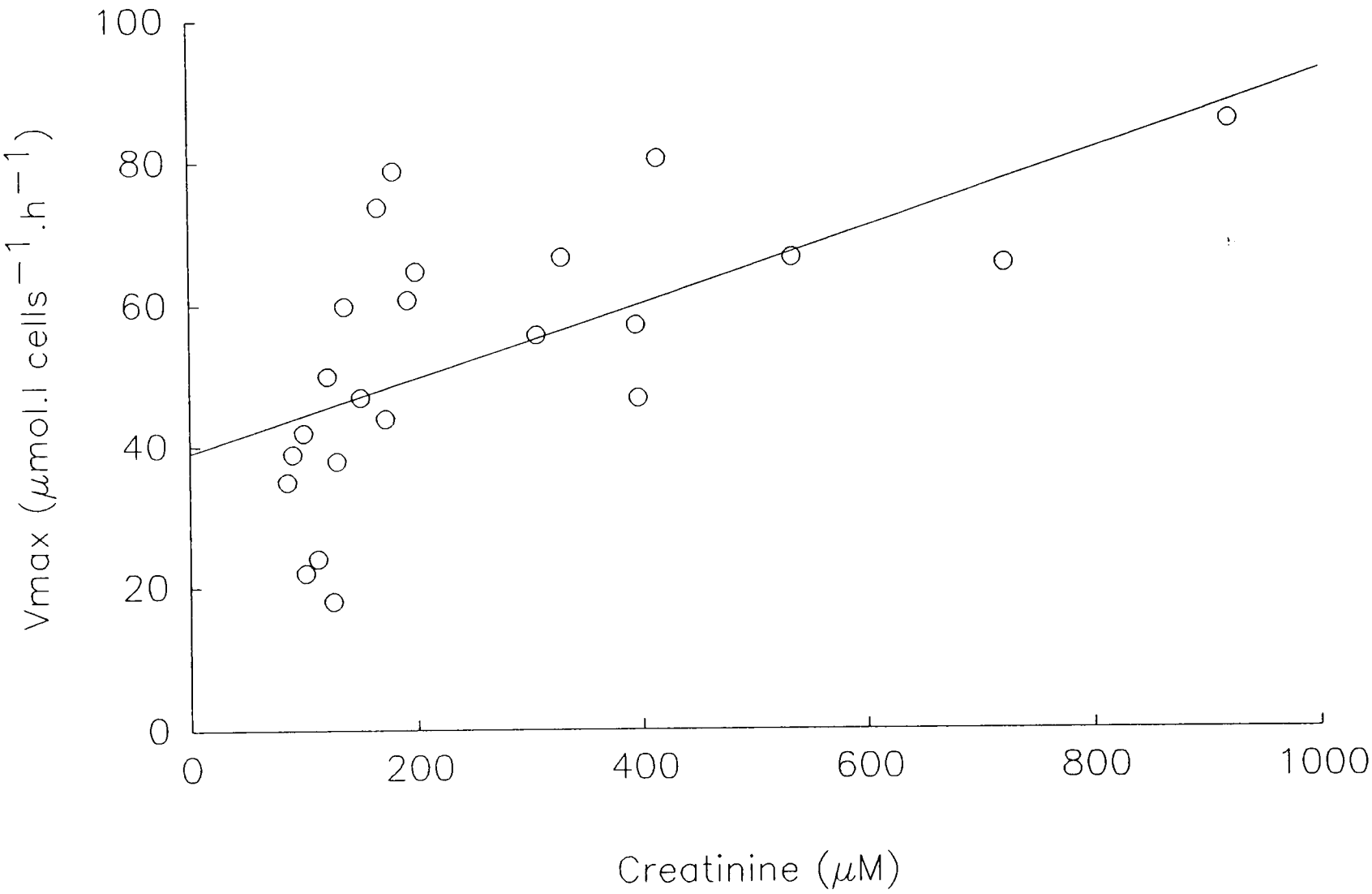

Figure 6.10. The correlation between choline transport (V_{max}) and creatinine in erythrocytes from chronic renal failure patients not yet on dialysis. The linear regression line is V_{max} = 41.3 + 0.040 (creatinine). At a creatinine of 1000 μM the line extrapolates to a V_{max} of 81.3 μmol.l cells^{-1}.h^{-1}. (r = 0.483, p = 0.001).

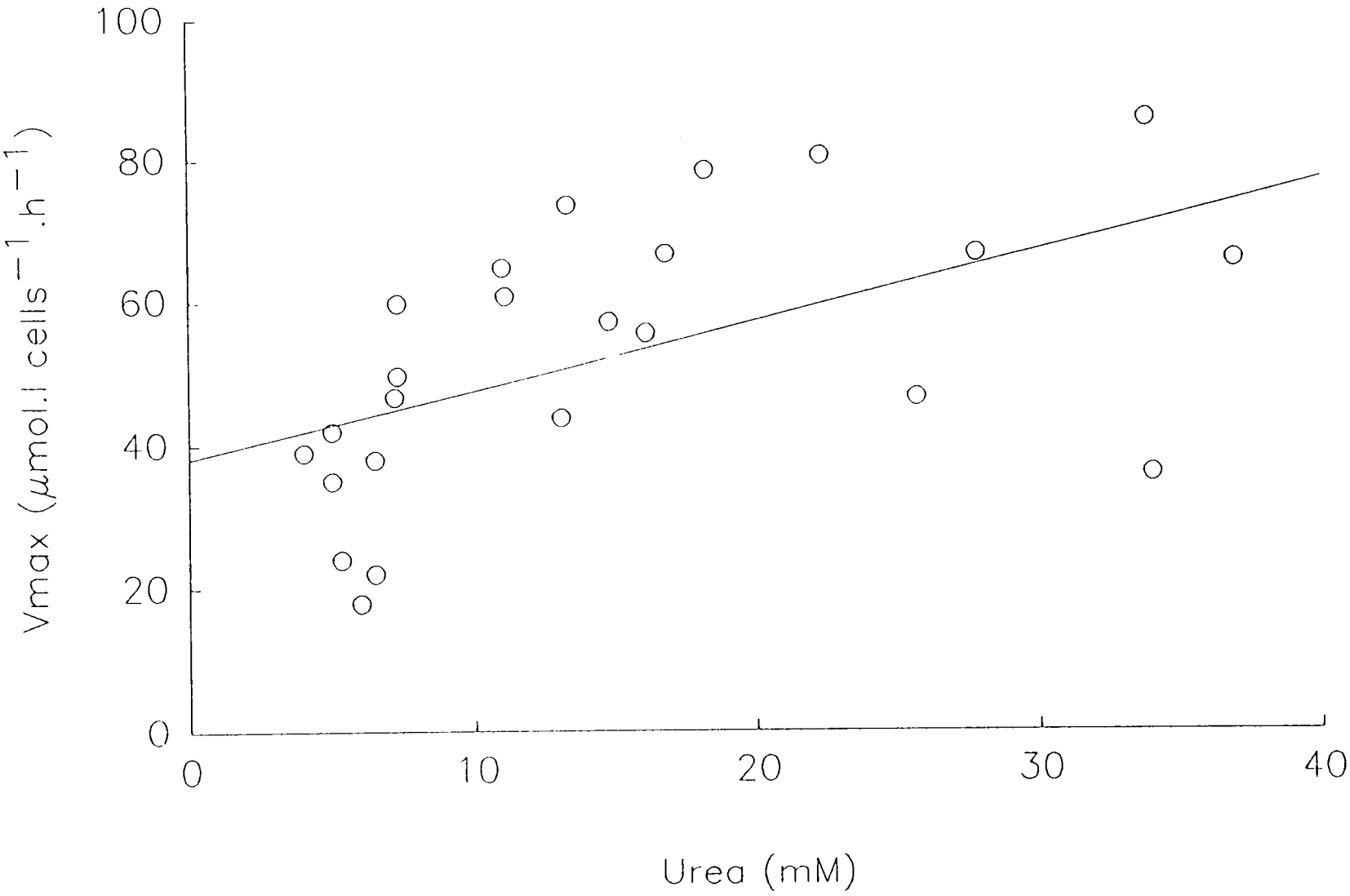

Figure 6.11. The correlation between choline transport (V_{max}) and serum urea in erythrocytes from chronic renal failure patients not yet on dialysis. The linear regression line is V_{max} = 37.9 + 0.984 (urea). At a urea of 40 mM the line extrapolates to a V_{max} of 77.2 μmol.l cells^{-1}.h^{-1}. ($r = 0.533$, $p < 0.001$).

proven to be an interesting system to study membrane transport in red cells of patients with renal failure. One advantage is that only 5 minutes incubation time is necessary to perform adequate influx measurements. A second advantage is that it has been shown to be a very reproducible measure since repeated sampling of the same donor gives very similar results, a fact also reported by Martin, (1972) and by independent research workers in the laboratory (data not shown). The zero-trans experiments V_{max} = 14.2 μmol.l cells^{-1}.h^{-1} and a K_m = 7.4 μM found in controls are in very good agreement with a V_{max} = 12.4 μmol.l cells^{-1}.h^{-1} and a K_m = 6.5 μM reported by Martin (1968). Also, in normal and uraemic red cells, choline transport obeys a simple Michaelis Menten equation as demonstrated by the fitting in figure 6.4 making the calculations and interpretation of the results easier since the problem of separate fitting of a linear component is not present.

Before the actual results are discussed, a brief mention will be made of exchange and zero-trans fluxes since these data are necessary for the correct understanding of the experimental findings.

In their normal environment, many carrier proteins perform exchange fluxes, i.e, they bind the substrate on one side of the membrane, cross the membrane, and release it. Then the same binding site binds again to the same substrate (or to another substrate for which the carrier has affinity) and

this carrier-substrate complex returns to the original side and releases it there (in the first case the process will be called homo-exchange and in the second case, hetero-exchange). This is the predominant mechanism in vivo for well characterized systems like many amino acid transporters (Harvey and Ellory, 1989) glucose (Lieb and Stein, 1972; Lowe and Walmsley, 1986) and also choline transport (Martin, 1968; Krupka and Deves, 1980). In fact, studies on choline transport have shown that reorientation of the carrier-substrate complex is 4-fold faster when the carrier is loaded than when it is empty (Martin, 1968; Krupka and Deves, 1980) a phenomenon known as trans-stimulation.

If the cell is completely depleted of its intracellular substrate (trans-side of the membrane) influx measurement will determine the behaviour of the carrier under zero-trans conditions, or in other words, without the stimulatory effect of its natural substrate(s). This analysis is particularly important in interpreting the above results, since the increased V_{max} exhibited by the uraemic cells used immediately after initial washing could have resulted from abnormal intracellular levels of choline in these cells, rather then a primary abnormality of the carrier. In certain experiments, therefore, cells were established to have reached zero-trans conditions on the basis that fluxes appeared to have reached a steady state after 4 hours incubation in saline and no apparent differences could be seen after this time.

The data presented above show that choline transport is abnormal in patients with renal impairment requiring dialysis. These patients exhibit an increased capacity for choline uptake. The increase appears greater in patients on CAPD than in those on haemodialysis. Kinetic analysis of the choline transporter in haemodialysis patients in zero-trans conditions shows an increased V_{max} and a reduced apparent affinity for choline binding compared to controls.

Furthermore, the results also demonstrate that erythrocyte choline uptake in pre-dialysis (CRF) and renal transplant patients correlates inversely with the degree of renal function (see figures 6.6 to 6.11). This correlation can, in the transplant patient data, be extrapolated to creatinine clearances of zero and 100 ml min^{-1} to produce V_{max} values of 56.8 and 23.4 μmol.l $cells^{-1}.h^{-1}$ respectively. In the pre-dialysis patient results, the same extrapolation would produce V_{max} values of 70.7 and 31.6 μmol.l $cells^{-1}.h^{-1}$ respectively. These numbers are in broad agreement with data obtained for dialysis patients and controls (figure 6.5) suggesting that a consistent relationship between renal function and choline transport exists in all of the groups studied.

Of the 31 transplant patients studied 19 had been transplanted for over 6 months. Separate analysis of this group and of the remaining 12 showed no differences from the overall picture of figure 6.6. There were too few results from

recently transplanted functioning kidney patients for conclusions to be drawn on the rate of recovery of choline transport abnormalities after successful transplantation.

The reasons for these abnormalities are not clear at the present moment but could include an increased number of choline transporters in the membrane, a true higher rate of activity by individual choline carriers in uraemic cells, that choline is transported by additional transport systems, or may represent simply an increased leakage to choline in the red cell membrane in uraemic cells.

It is unlikely that an increased number of transporters could account for the changes since this would be expressed by an increased V_{max} without any changes in K_m, while the experiments presented here showed that uraemic cells have higher values of V_{max} and K_m.

If changes in the choline transporter were due to a trans-stimulatory effect by increased intracellular choline levels in uraemic cells, experiments under zero-trans conditions would show no differences between uraemic patients' erythrocytes and controls. However, even when cells have been suspended in standard saline for 4 hours, the kinetic analysis of the choline transporter in zero-trans conditions confirmed the raised V_{max} for choline transport found in cells obtained from haemodialysis patients used immediately, and demonstrated abnormal choline binding affinity.

Whatever the reasons, choline transport seems to be

altered primarily due to changes in the characteristics of the individual transporters. It is possible that recruitment of a new low-affinity transport system in addition to the existing high-affinity system could explain the results, although this possibility cannot be confirmed with the present data.

A more general view about the abnormalities demonstrated in this system and their relevance in uraemia will be given in the general discussion in chapter 7.

CHAPTER 7

DISCUSSION

"I have been told that I tend to speak of the red cell as if it were a microcosm, and as if an understanding of its nature and properties would include an understanding of nearly everything else in the cellular world. To some extent this is true...". Eric Ponder, cited in Schatzmann, 1989.

7.1. Introduction.

This thesis has presented a study of selected red cell membrane transport systems, namely the Na/K pump, amino acid, nucleoside and choline transport systems in normal controls, patients with renal failure and patients with renal transplants. These results have been discussed individually in their respective chapters. The discussion that will follow

here will review these results in the context of uraemia, and will attempt to interpret the results in light of the possible effects that the uraemic environment may have in causing changes in the function of the transport systems studied.

7.2. The Na/K Pump.

Abnormal function of the Na/K pump will affect cell function seriously since high intracellular potassium and low intracellular sodium concentrations are needed for many enzymatic functions and cellular processes, including reabsorption of solutes by the kidney, uptake of nutrients by the intestine, nerve impulse propagation and other functions. Secondary active transport processes, like some amino acid transport systems and Na^+-dependent glucose transport depend on the Na^+ gradient generated by the pump. The re-uptake of certain neurotransmitters in the brain is also driven by secondary transport systems linked indirectly to the Na,K-ATPase activity (Sweadner and Goldin, 1980). Certain neurological manifestations present in uraemia could be due to an abnormal Na/K pump (Tyler, 1970; Minkoff, Gaertner, Darab et al, 1972; Cotton, Woodward, Carter and Knochel, 1979). It is therefore not surprising that many investigators have studied the Na,K-ATPase in relation to chronic renal failure.

The first reported abnormalities in erythrocyte cation

transport in uraemia were described by Welt, Sachs and McManus (1964) and linked with a decreased activity of the Na,K-ATPase (Welt, Smith, Dunn et al, 1967). Since then an increasing number of research groups have studied this problem and attempted to establish the nature of the defect (Villamil, Rettori and Kleeman, 1968; Cole, 1973; Kramer, Gospodinov and Kruck, 1976; Zannad, Royer, Kessler et al, 1982; Cheng, Kahn and Kaji, 1984; Kaji and Kahn, 1987). These abnormalities have been also reported in leucocytes (Edmonson, Hilton, Jones et al, 1975), brain (Minkoff, Gaertner, Darab et al, 1972) adipocytes and skeletal muscle (Druml, Kelly, May and Mitch, 1988). This last group found a diminished ^{86}Rb uptake and increased intracellular sodium in both these tissues from chronically uraemic rats. In adipocytes this was accompanied by a significant decrease in ^{3}H-ouabain binding; skeletal muscle showed normal binding and they concluded that the cellular response to uraemia is tissue specific. However, the literature is not unanimous and the activity of the Na/K pump has also been reported as normal (Corry, Tuck, Brickman et al, 1986; Corry, Lee and Tuck, 1987; Diez, Virto, Yep et al, 1986) or increased (Swaminathan, Clegg, Cumberbatch et al, 1982) in renal failure.

7.2.1. **Plasma Factors.**

The idea that mammals have an endogenous regulator of

the Na,K-ATPase came from the work of De Wardener and his group (1961) (for review, see Haupert, 1989) and has been implicated in the pathogenesis of primary hypertension.

The concept of a specific inhibitor of a major transport protein accumulating in uraemia, first proposed by Welt in 1964, is obviously attractive and has received increasing experimental support over the years. Welt, Smith, Dunn et al (1967) and Cole, Balfe and Welt (1968) were able to demonstrate that there was a factor in the uraemic plasma from patients with high intracellular sodium that would inhibit the enzyme activity of isolated Na,K-ATPase from normal cells. The defect was corrected when patients were haemodialysed for several weeks and their plasma would then lose the ability to induce the defect in ATPase activity.

The results of the studies in CRF and HD patients presented in this thesis favour the hypothesis that the abnormal membrane function seen was due to plasma factors, since plasma from non-dialysed chronic renal failure patients was able to induce the defect in normal erythrocytes, but the evidence is not compelling. In previous reports, control human erythrocytes incubated in uraemic plasma revealed a significant reduction in ouabain-sensitive efflux (Kramer, Gospodinov and Kruck, 1976). Zannad and collegues, (1982) were able to demonstrate a defect in the Na/K pump of erythrocytes of non-dialysed patients with acute renal failure, and the Na/K pump activity increased after a haemodialysis session

(Quarello, Boero, Guareno et al, 1985), supporting the idea of a plasma factor effect. The finding in this thesis that the subgroup of patients studied early after transplantation had normal erythrocyte Na/K pump function is consistent with the view that plasma factors, rather than intrinsic erythrocyte abnormalities are important in uraemia. These patients had not had a blood transfusion and their erythrocytes were essentially synthesized in the uraemic environment. Furthermore, the reversibility experiments presented in chapter 3, demonstrated that red cells from uraemic patients had the activity of the Na/K pump normalised when incubated in normal plasma, reinforcing the idea of plasma factors being involved in affecting the function of the Na/K pump in these patients.

Many research groups have reported the presence of digoxin-like substances in fresh plasma from normal volunteers (Hamlyn, Harris and Ludens, 1989), patients with liver diseases, acromegaly, preeclampsia (Graves and Williams, 1987), hypertension (Hamlyn, Ringel, Schaeffer et al, 1982; Gray, Hilton and Richardson, 1986) and in particular, in patients with renal failure (Graves, Brown and Valdes, 1983; Kelly, O'Hara, Mitch et al, 1986b; Graves and Williams, 1987; Walker, Bialy, Walker et al, 1987; Bisordi and Holt, 1989; Bosch, Hernando, Casado et al, 1989). In the data reported by Kelly, O'Hara, Mitch et al, (1986b) digoxin-like immunoreactivity (DLIS) and Na,K-ATPase inhibitory activity

were increased in hypertensive patients with moderate renal insufficiency and during dialysis but not in haemodialysis patients prior to a dialysis session, and this has similarities to an earlier report by Graves, Brown and Valdes (1983).

These substances have been identified as nonesterified fatty acids (linoleic and oleic acids) (Kelly, O'Hara, Canessa et al, 1985; Tamura, Kuwano, Kinoshita and Inagami; Kelly, O'Hara, Mitch and Smith, 1986a) and lysophospholipids (palmitoyl lysophosphatidylcholine) (Kelly, O'Hara, Mitch and Smith, 1986a) but these results have been challenged by Graves and Williams (1987) who pointed out that the dose-response curve of these compounds for Na,K-ATPase inhibition and antibody binding showed apparent affinities 4 to 5 orders of magnitude weaker than digitalis and related compounds. However, Bisordi and Holt (1989) do suggest that the circulating digitalis-like immunoreactive substances (DLIS) can be found in the lipid fraction of plasma, and "ouabain-like" factors isolated from the electric organ of Electrophorus electricus have been identified as non-esterified fatty acids (Bidart, Rossi, Renaud and Lazdunski, 1984). Other authors have suggested that dihydroepiandrosterone and other steroids might account for some of the digoxin-like activity present in the uraemic serum (Vasdev, Johnson, Longerich et al, 1987; Vasdev, Longerich, Prabhakaran et al, 1989). At the present time, the exact

nature of these DLIS is unknown. The *in vivo* biological activity and importance of substances detected by immunoreactivity as possible pump inhibitors is also open to doubt.

7.2.2. **Intrinsic Membrane Abnormalities.**

Lipid metabolism is abnormal in uraemia with patients characteristically presenting with elevated plasma triglyceride and VLDL and low HDL-cholesterol levels. These abnormalities show no correlation with the degree of renal impairment (Eknoyan, 1988). The precise mechanism(s) responsible for these abnormalities have not been completely defined (Chan, Varghese and Moorhead, 1981) but there are suggestions that the insulin resistance and hyperinsulinaemia present in renal failure may play an important part by increasing hepatic triglyceride synthesis and inhibiting lipoprotein lipase (Eknoyan, 1988).

Free fatty acids, unesterified cholesterol and plasma phospholipids are in a continuous exchange process with the lipids of the red cell membrane (Duhm, 1989). Renal failure patients have been found to have high plasma levels of saturated fatty acids together with reduced levels of linoleic and essential fatty acids (Norbeck and Walldius, 1982).

Recent reports indicate that the structural and compositional properties of the cell membrane can determine

the catalytic properties of membrane transport proteins (Deuticke and Haest, 1987; Carruthers and Melchior, 1988). Alterations in the lipid composition, fluidity or amount of saturated fatty acid in the red cell membrane have been demonstrated to affect the function of membrane transport systems like the Na/K pump (Claret, Garay and Giraud, 1978; Johannsson, Smith and Metcalfe, 1981; Yeagle, 1983; Yeagle, 1989; Dwight and Hendry, 1990), glucose transporter (Carruthers and Melchior, 1986) and other transport processes (Grunze, Forst and Deuticke, 1980; Cullis, DeKruijff, Hope et al, 1980; Kuypers, Roelofsen, Op Den Kamp and Van Deenen, 1984). For example, high membrane levels of cholesterol will inhibit the Na,K-ATPase while low levels of cholesterol will stimulate the enzyme (Yeagle, 1985; Yeagle, 1989).

It has also been suggested that the increased rate of red cell Na^+-Li^+ exchange in hypertensive patients might be related to plasma cholesterol and triglycerides with a negative correlation between plasma levels of HDL cholesterol and the exchange activity. In particular, the rate of activity of this system has been directly correlated with the mean saturation of membrane fatty acids (Duhm and Behr, 1986; Duhm, 1989). In uraemia, this system has been reported as showing no changes (Diez, Virto, Yep et al, 1986; Corry, Lee and Tuck, 1987) or increased activity (Trevisan, DeSanto, Laurenzi et al, 1986).

The idea that abnormal phospholipids and related

molecules are involved in changes in membrane transport systems has also been suggested for both volume-expanded states and uraemia (Tamura, Kuwano, Kinoshita and Inagami, 1985; Kelly, O'Hara, Mitch and Smith, 1986; Kelly, O'Hara, Mitch et al, 1986b). In fact, Kelly, O'Hara, Canessa et al (1989) have suggested that changes in non-esterified fatty acids (NEFA) in the membrane were responsible for increased ouabain-sensitive potassium flux after a haemodialysis session. Diez and collegues (1986) suggested that an intrinsic membrane abnormality must be considered in order to elucidate the exact mechanism involved in the alterations in the Na,K-pump in uraemia. Decreased levels of erythrocyte phosphatidylcholine and a decreased phosphatidylcholine : sphingomyelin ratio have been linked to a decreased membrane fluidity in haemodialysis patients (Komidori, Kamada, Yamashita et al, 1985) and membrane fluidity and function can be modified by changes in its lipid composition (Stubbs and Smith, 1984). Other groups have also found the erythrocyte membrane lipid composition in patients with renal failure to be abnormal, with an increased content of cholesterol and decreased content of phosphatidylcholine and sphingomyelin (Peuchant, Salles, Vallot et al, 1988).

The lack of progress over the last thirty years in identifying a single chemical species in the context of uraemia makes it likely that a complex change in plasma chemistry is involved. The idea that lipids are important in

this process fits well with this conclusion. Nevertheless, this possibility although attractive, should be taken in the context that numerous other possible molecular species might be involved and some will be mentioned briefly below.

7.2.3. **Hormones.**

Some hormones have been shown to affect the function of selective membrane transport proteins. The Na/K pump has been reported to have its activity decreased by thyroid hormones (Cole and Waddell, 1976) while others found it to be increased (Rosie, Standnert and Pollet, 1985). In animal models, thyroidectomy decreased the Na,K-ATPase activity by 25% in both the renal cortex and medulla (Katz and Lindheimer, 1973). The effect of thyroid hormones on the enzyme has also been reported to occur in other tissues, i.e. liver (Ismail-Beigi, Bissell and Edelman, 1979) and skeletal muscle (Ismail-Beigi and Edelman, 1970). Insulin appears to stimulate the Na/K pump (Czech, 1977; Rosie, Standnert and Pollet, 1985; Lytton, 1985) but insulin-mediated K^+ uptake is normal in uraemics (Alvestrand, Wahren, Smith and DeFronzo, 1984). Glucagon (Fehlman and Freychet, 1981) and vasopressin (Mendoza, Wigglesworth and Rozengurt, 1980) have been reported as able to stimulate the Na/K pump by increasing intracellular Na^+ concentration. Catecholamines also enhance Na,K-ATPase activity (Swann, Crawley, Grant and Maas, 1981; Bodemann,

Irmer, Schluter et al, 1985). Mineralocorticoids (Garg, Knepper and Burg, 1981) and glucocorticoids can increase the number of Na/K pumps in several tissues (Rodriguez and Klahr, 1980; Rodriguez, Sinha, Starling and Klahr, 1981; Kaji, Thakkar, Kahn and Torelli, 1981; Mujais, Sabatini and Kurtzman, 1984) an effect reported also for thyroid hormones (Lin and Akera, 1978). Parathyroid hormone (Bogin, Massry, Levi et al, 1982) and atrial natriuretic peptide appear to have no direct inhibitory effect on the Na,K-ATPase (Pollock, Mullins and Banks, 1983; Tripodo, Cole and MacPhee, 1984).

Abnormal levels of these hormones together with impaired target cell action have been reported in renal failure. It is possible that they could contribute and explain some of the membrane transport abnormalities present in renal failure as for example the role of insulin in modulating amino acid transport and this will be discussed below. However, at the present moment there is no evidence to link these abnormalities to the changes in the Na/K pump reported in this thesis.

7.2.4. **Other Substances.**

As already discussed in chapter 1, many other substances, including peptides, polyamines (Radtke, Rege, LaMarche et al, 1981), guanidines (Davidoff, 1974), phenols (Wardle and Wilkinson, 1976), myoinositol (Blumberg, Esslen and Burgi,

1978), hippuric acid (Boumendil-Podevin, Podevin and Richet, 1975), "middle-molecules" (Balestri, Biagini, Biagini and Giovannetti, 1972) and vanadate (Kaji and Kahn, 1987; Kaji, Goodman and Kahn, 1987; Hosokawa and Yoshida, 1989) are reported to be present in increased concentrations in uraemic plasma and may be able to affect membrane transport proteins. However, more research is needed to clarify the precise role these substances could have in the genesis of membrane transport abnormalities in uraemia.

7.2.5. **The Na/K Pump in Some Pathological States.**

Welt and co-workers (1967) were the first to report a decreased activity of the Na,K-ATPase in patients with severe burns, and terminal cancer. Since then changes in the Na,K-ATPase content or function from different tissues have been reported in other pathological states. Studies on erythrocytes from obese patients have produced contradictory reports, either exhibiting a higher number of Na/K pumps (DeLuise and Flier, 1982; Halperin, Brugnara, Kopin et al, 1987) or decreased Na,K-ATPase activity (Mott, Klimes and Clark, 1983) and decreased number of Na/K pumps (DeLuise, Usher and Flier, 1983). The reasons for these findings in obese patients are not clear but the changes are selective to the Na/K pump since other transport systems for potassium do not appear to be affected (Halperin, Brugnara, Kopin et al,

1987). Patients with chronic hypokalaemia, Bartter's syndrome and thalassaemia minor exhibited an increased number of Na/K pumps (Erdmann, Krawietz and Koch, 1979). The steeper potassium gradient across the red cell membrane is thought to be the stimulus in the chronic hypokalaemic state and also in Bartter's syndrome although these last patients have increased levels of circulating aldosterone. In these two conditions the defect can be slowly reversed (75-120 days) by correcting plasma potassium levels.

7.3. Amino Acid Transport.

Amino acid levels are abnormal in renal failure (Alvestrand, Furst and Bergstrom, 1983; Graziani, Cantaluppi, Casati et al, 1984). The aetiology of this is probably multifactorial and involves protein malnutrition, increased catabolism and, in patients on dialysis, loss of amino acids during the dialysis procedure. Nevertheless, the above suggestions do not entirely explain the abnormal amino acid profile of uraemia since changes are present in patients who are not malnourished (Bergstrom, Alvestrand and Furst, 1990) and in patients not yet on dialysis.

It is possible that the abnormal function of specific amino acid transport systems could contribute to these changes. In fact, this possibility had been suggested, in general terms, in 1967 by Welt and colleagues referring to

the Na/K pump: "it is conceivable that the membrane lesion described in erythrocytes is a more generalized phenomenon and involves many other cell types and conceivably other transport mechanisms" (Welt, Smith, Dunn et al, 1967).

The results presented in this thesis demonstrate part of this hypothesis to be correct. Why these amino acid transport systems behave abnormally in renal failure is unknown but an attempt to interpret these changes in the context of uraemia will be given now.

The y^+ system demonstrated an increased capacity for lysine transport in red cells from patients with chronic renal failure on haemodialysis. The increased lysine influx resulted directly from altered membrane properties.

One hypothesis to consider would be the effect of the hyperinsulinaemia characteristic of uraemia, and the results of insulin resistance with respect to glucose metabolism. This resistance does not extend to the stimulatory effect of insulin on cellular amino acid uptake (Alvestrand, DeFronzo, Smith and Wahren, 1988) and could explain increased insulin mediated amino acid transport. Although this may be of importance in vivo, it cannot account for the in vitro results.

An increased lysine uptake due to trans-stimulation seems unlikely as both plasma and intracellular lysine concentrations are reported to be normal or reduced in uraemia (Alvestrand, Furst and Bergstrom, 1983) and the relative increase in V_{max} in uraemic cells compared with

controls was seen even after intracellular amino acid depletion by preincubation. The conclusion from these experiments is that increased lysine influx results directly from altered membrane properties. A significant reduction in plasma levels of lysine associated with a significant increase in intracellular level of lysine in patients on CAPD has been recently reported (Lindholm, Alvestrand, Furst and Bergstrom, 1989). This observation of a high IC/EC gradient may not reflect amino acid depletion but rather a shift of free amino acids from the extracellular to the intracellular space. Such a shift could arise from altered membrane transport of lysine.

Erythrocyte lifetime in renal failure is reduced, and young erythrocytes are reported to exhibit increased amino acid transport capacities (Antonioli and Christensen, 1969; Benderoff, Blostein and Johnstone, 1978; Jones, Ellory, Young and Tucker, 1982). It is therefore possible to speculate that the increased capacity of the y^+ system seen in these experiments was related to the reduced mean age of the uraemic cells. However the results from the ASC and gly system studies indicated no increase in V_{max}. The increase in V_{max} for the gly system in young normal cells is reported to be marked (Antonioli and Christensen, 1969; Jones, Ellory, Young and Tucker, 1982). Furthermore, leucine transport is also reported to be higher in younger cells (Benderoff, Blostein and Johnstone, 1978), but transport through this system was normal in the uraemic cells studied. Therefore, it does not appear

possible to explain the overall behaviour of erythrocytes in renal failure as being simply due to their relative youth. The lack of an inverse correlation between L-lysine V_{max} and blood haemoglobin concentration is also indirect evidence against the role of cell age in causing the alterations in lysine transport. These data could also be compared with the results of the Na/K pump experiments since young red cells have been shown to have a higher number of Na/K pumps and higher K^+ transport capacity than old red cells (Joiner and Lauf, 1978). Since red cells from uraemic patients showed significant inhibition of K^+ influx and red cells from non-dialysed renal failure patients presented a reduced number of Na/K pumps, it seems difficult to attribute these changes to the presence of a younger red cell population in uraemia.

The increase in K_d observed in these L-lysine experiments in uraemia might indicate that erythrocyte amino acid transporters other than y^+ are also increased in number or turnover rate in renal failure. However, K_d showed considerable variability between subjects, and the difference between uraemic and normal groups in this study did not reach statistical significance. The biological importance of these changes in amino acid transport in uraemia is difficult to assess, but if they are present in other tissues they may contribute to the observed alterations in protein metabolism. The increased fluxes observed here could be due to an up-regulation of the lysine transporter system during

erythropoiesis due to the presence of a circulating uraemic inhibitor of amino acid transport. This possibility could be tested by measuring amino acid transport in uraemic plasma rather than in artificial media, but this is extremely difficult in practice due to the presence of many amino acids in plasma at physiological concentrations. Lysine is a precursor of L-carnitine (Siliprandi and Ciman, 1986) and plasma carnitine levels are reduced in renal failure (Bohner, Bergren and Eiklid, 1978). It has recently been suggested that L-carnitine deficiency could play a role in the pathogenesis of uraemia (Labonia, Morelli, Giminez et al, 1987). Carnitine is needed for the transport of fatty acids into the mitochondria and may therefore be also involved in the mechanism of the hypertriglyceridemia present in uraemia (Eknoyan, 1988). It is possible that an up-regulation of L-lysine transport occurs in uraemia as a response to a low concentration of L-carnitine, or that lysine transport is affected by alterations in membrane lipid which are secondary to carnitine deficiency.

The sodium-dependent influx of L-serine via the ASC system was significantly reduced in haemodialysis patients, through a decrease in V_{max}. There was also a decrease in K_m for L-serine in uraemic erythrocytes. The overall effect of the changes seen is that uraemic erythrocytes possess a reduced capacity for L-serine transport.

Plasma concentrations of L-serine are reported to be low

in untreated chronic renal failure and in patients on maintenance HD (Alvestrand, Furst and Bergstrom, 1983; Bergstrom, Alvestrand and Furst, 1990). Low concentrations of L-serine occur in skeletal muscle in CRF, and have been suggested to contribute to insulin resistance (Alvestrand, Furst and Bergstrom, 1983). The kidney is able to metabolise glycine to serine, but loses this ability in renal failure (Eknoyan, 1988). In the postabsorptive state the only organ which releases L-serine into the circulation is the kidney (Tizianello, Deferrari, Gariboto et al, 1987b). Accordingly it is possible that L-serine may become an essential amino acid in end stage renal failure.

The inter-organ transport of L-serine is clearly abnormal in renal failure. Renal release of L-serine is reduced to less than 40% of normal (Tizianello, Deferrari, Garibotto et al, 1987). The splanchnic uptake of L-serine in the postabsorptive state is reduced to about 25% of normal (Tizianello, Deferrari, Garibotto et al, 1987). In the postprandial state there is impaired hepatic uptake of L-serine resulting in excess spillage into the systemic circulation (Deferrari, Garibotto, Robaudo et al, 1987). Impaired membrane transport of L-serine could contribute to these changes, and the reduced erythrocyte transport capacity for L-serine found may be representative of changes in other tissues. However, hepatic uptake of L-serine is largely dependent on system A, which is not present in erythrocytes. System A transport has been

reported to be abnormal in hepatocytes (Maroni, Karapanos and Mitch, 1986a) and adipocytes (Druml, Kelly, May and Mitch, 1988) from acutely uraemic rats and could play a significant additional role in the altered inter-organ transport of L-serine. However, the first group failed to demonstrate any detectable abnormality in transport by the ASC system in muscle of acutely uraemic rats (Maroni, Karapanos and Mitch, 1986b). It is clear that any long term effect of the uraemic environment on cell membrane might not be detected when studying the acute situation.

System ASC appears to be relatively insensitive to adaptive regulation and hormonal control (Barker and Ellory, 1990). Therefore changes found in serine transport in haemodialysis patients are likely to represent an alteration in membrane properties due to other factors present in the uraemic environment.

Plasma glycine concentrations are normal in untreated CRF but appear to be increased in HD patients (Alvestrand, Furst and Bergstrom, 1983; Bergstrom, Alvestrand and Furst, 1990). There is impaired postprandial hepatic uptake of glycine, and it has been suggested that this represents a defect in system A (Deferrari, Garibotto, Robaudo et al, 1987). Cerebral glycine uptake is increased to twice normal in CRF patients (Tizianello, Defferrari, Garibotto et al, 1987a,b).

The results presented in this thesis demonstrate an

increased glycine affinity for the erythrocyte gly transporter in HD patients. It is not known whether the gly system is present in cerebral tissue, although an analogous high affinity, sodium and chloride-dependent glycine transport system has been described in rat brain synaptosomes and in C6 glioblastoma cells (Zafra and Gimenez, 1986). It has been suggested that the chloride-dependence of the gly system may parallel the role of glycine as a neurotransmitter in opening anion channels (Ellory, 1987). An alteration in the substrate affinity of glycine transporters in cerebral tissue could contribute to the increased cerebral glycine uptake in renal failure.

The L-leucine and L-tryptophan transport systems showed no changes in uraemia in relation to normal controls. The intracellular levels of leucine have been reported to be normal in uraemia (Furst, Alvestrand and Bergstrom, 1980).

There are therefore specific changes of selected membrane transport systems for amino acids, rather than general effects on membrane transport of amino acids in uraemia. Whether these changes reflect effects of circulating inhibitors (e.g. non esterified fatty acids) or represent alteration in transport system expression during erythropoiesis remains to be elucidated.

7.4. Uridine Transport.

The carrier-mediated transport of uridine in human red cells is a function of temperature and is altered by cell storage (Jarvis, Hammond, Paterson and Clanachan, 1983; Plagemann and Wohlhueter, 1984a,b; Cabantchik, 1989).

The carrier exhibits kinetic asymmetry after storage which is particularly marked at low temperatures. It has been suggested that this may be due to an altered lipid environment for the membrane transport protein (Plagemann and Wohlhueter, 1984a). Alterations of membrane lipid have been proposed to account for certain membrane transport changes in renal failure (Kelly, Canessa, Steinman and Mitch, 1989), and it was therefore of particular interest to know whether uridine transport was altered in renal failure patients. The results presented in this thesis showed clearly that uridine transport was normal in the renal failure subjects.

7.5. Choline Transport.

The data presented in this thesis showed that patients with renal impairment requiring dialysis exhibit an increased capacity for choline uptake. The increase appears greater in patients on CAPD than in those on haemodialysis. The increased V_{max} present in red cells from haemodialysis patients persisted

in cells under zero-trans conditions. They also demonstrate that erythrocyte choline uptake in non-dialysed renal failure patients and renal transplant patients correlates inversely with the degree of renal function.

It is not clear whether these alterations are a result of circulating factors acting directly on the transporter in mature red cells or factors affecting erythrocyte maturation in the bone marrow. As discussed previously, these alterations could have arisen through changes in the membrane lipid environment of the transporters, as suggested for the altered membrane Na/K pump function (Kelly, O'Hara, Mitch and Smith, 1986; Kelly, O'Hara, Canessa et al, 1989). Experiments on patients with acute renal failure and sequential studies on early successful transplantation could shed further light on this.

The clinical importance of altered membrane choline transport is unclear. The neuronal uptake of choline is Na^+-dependent, unlike the erythrocyte transporter (Haga and Noda, 1973). The erythrocyte defect in Na,K-ATPase activity in renal failure appears to be representative of other tissues (Edmondson, Hilton, Jones et al, 1975; Cotton, Woodward, Carter and Knochel, 1979; Frazer, Sarnacki and Arieff, 1985) but neuronal choline transport could be unaffected in renal failure. If neurones and other cells do exhibit abnormal uptake of choline then there is the possibility that this might alter cell function both through altered acetylcholine

levels and via altered phosphatidylcholine and sphingomyelin synthesis. There are reports of altered choline transport in erythrocytes and cultured neurones in lithium therapy and in Alzheimer's disease (Uney, Marchbanks and Marsh, 1985; Miller, Jenden, Cummings et al, 1986) but the pathophysiological significance of these changes is also unknown.

7.6. Conclusion.

This thesis presents a detailed study of selected cell membrane transport systems in relation to uraemia using the erythrocyte as a model. A variety of transport systems have been characterised in erythrocyte membranes and their kinetic properties have suggested that they are similar to the transport systems found in other tissues. They are the easiest and safest human cells to obtain, an important point when the study involves the cooperation of patients, and where other cell models, i.e. muscle or hepatocytes, would obviously present more difficulties because of an increased risk of damage to the patient. Lymphocytes have the problem that in order to isolate a reasonable number of cells a larger amount of blood would have to be taken from a group of patients who are already anaemic and also lymphocytes are unable to survive for more than a few hours out of the circulation. Red cells present the additional advantages of being easy to manipulate and since the whole cell is a single compartment, it does not

present the difficulties of multicompartment analysis of data obtained from studying more complex tissues. There are also advantages in studying transport in a model where the transported substance is not rapidly metabolised.

Eight systems were investigated, namely the Na/K pump, the y^+, ASC, gly, L and T amino acid transporters, and nucleoside and choline transport systems. The Na/K pump has in fact been the subject of intensive investigation and a large literature already exists regarding the effect of uraemia on its function. Nevertheless, a general approach that included patients in chronic renal failure before maintenance dialysis treatment was started, haemodialysis, CAPD and functioning kidney transplant patients has not been taken before. Furthermore, few studies have been performed in which the Na/K pump was investigated in conditions as near as possible to its normal environment. This is an important point since the presence of a putative inhibitor of the Na/K pump could have been lost, or its effect reversed, by incubating the cells in artificial media.

This thesis presents the first complete study on the five well characterised amino acid transport systems in human red cells, the first study on nucleoside transport and the first study of choline transport in patients with renal failure. This last system, in particular, was very rewarding, since the changes observed were very marked. It could be said that by looking at the rate of choline influx one could identify

patients who had severe renal impairment. It has also proven to be unique in that never before has been possible to correlate the activity of a membrane transport system with degrees of renal function.

The results presented in this thesis demonstrate that not all transport systems are affected in uraemia. Considering the unaltered function of nucleoside, leucine and tryptophan transport systems, the data eliminates the hypothesis of a generalised defect in the membrane and support the existence of a degree of selectivity in the range of transport abnormalities present in uraemia.

They also support the idea that plasma factors may be involved in causing a derangement of membrane transport protein function since the studies on the Na/K pump demonstrated that the defect could be induced in normal cells by incubating them in uraemic plasma, and that the inhibition of the pump was reversible by incubating uraemic red cells in normal plasma.

To test this idea as far as amino acid transport is concerned would be difficult. Apart from the fact the plasma contains several amino acids, their concentrations differ among individual controls, and also between controls and renal failure patients. Therefore, in order to calculate the rate of influx of a specific amino acid it would be necessary to determine this particular amino acid concentration in each individual plasma sample. Even then, the presence of other

amino acids in the plasma will affect the transport rate of the studied amino acid, making it difficult, if not impossible to interpret a given result.

In the case of choline, very recent experiments performed in red cells obtained from transplant patients with good renal function soon after transplantation also suggest that a plasma factor is involved since some of these patients presented choline influx rates similar to normal controls (data not shown), but these are preliminary findings and further studies on choline transport rates as a function of time after transplantation should shed more light on this problem.

Nevertheless, it is also possible that the uraemic environment could have a direct effect on the membrane at the early stages of erythrocyte maturation. This is supported by the fact that non-dialysed patients have a reduced number of Na/K pumps and that in these patients the K^+ uptake defect can not be corrected, at least over two hours of incubation in normal plasma. The fact that some amino-acid transporters behave abnormally, despite the fact that the experiments were performed in cells suspended in standard saline, and therefore without the presence of a possible uraemic factor, also support the idea of an intrinsic membrane abnormality in these patients.

It has been argued that membrane changes in uraemia (and certain other disease states) can be related to the presence of a circulating inhibitor of the sodium pump or "endogenous

ouabain". The data presented in this thesis demonstrate that the changes of membrane transport in renal failure are too widespread to be explained in this manner. The membrane transport of a variety of solutes is clearly abnormal and a range of transport systems are involved. The identification of the plasma factors concerned has certainly not proved easy. Nevertheless the markedly abnormal data for choline transport are of particular interest as they should facilitate the investigation of putative plasma substances.

In conclusion, some transport systems in the membranes of erythrocytes from patients with renal failure present interesting changes. Many of these changes have not been previously reported. These studies represent a small step in better understanding the pathophysiology of the uraemic syndrome.

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