LEUCOCYTE SODIUM TRANSPORT: PROBLEMS OF MEASUREMENT AND STUDIES IN URAEMIA

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ABSTRACT

Leucocytes were used to study cellular sodium (Na_{WBC}) and potassium (K_{WBC}) content and sodium transport (sodium flux rate, FR and sodium pump rate constant, RC).

In the first part of this work, a method was developed for reliable counting of the cells, thus avoiding weighing and permitting more estimations of cation content on a given volume of blood. This allowed identification of methodological factors which altered Na_{WBC}. After isolation, an incubation of at least 30 minutes was required before Na_{WBC} became stable. Intermittent gentle resuspension of the cells during incubation gave the lowest values for Na_{WBC}. Mixing by inversion, or centrifugation at 200g for 6 minutes significantly increased Na_{WBC}.

Sodium transport was assessed by measuring the increase in Na_{WBC} during a 20 minute incubation with ouabain. The rise was linear over this time and equal to FR. FR divided by baseline Na equalled RC. Ethanol used as a solvent for ouabain was found to affect sodium influx at concentrations > 17mmol/l.

Using these methods, no effects of age or sex were found on Na_{WBC}, FR or RC, but K_{WBC} was higher in females ($p=0.05$). These methods were then used to investigate suggestions that chronic renal failure (CRF) in man is associated with release of a circulating sodium pump inhibitor which raises intracellular sodium.

In 14 CRF patients who had never been dialysed, Na_{WBC} was raised compared with controls ($p<0.001$) but this could not be attributed to reduced RC. In 15 continuous ambulatory peritoneal dialysis (CAPD) patients, Na_{WBC} was less than in the undialysed group ($p=0.05$), due

to a better matching of FR and RC. In the CAPD group only, Na_{WBC} correlated with plasma urea ($p=0.005$) and creatinine ($p=0.031$) concentrations.

Eighteen regular haemodialysis patients were studied immediately before and after a standard haemodialysis (HD). Na_{WBC} ($p=0.078$), FR ($p=0.006$) and RC ($p=0.071$) were lower at the end of dialysis. No correlations were found between these changes and measures of the biochemical or volume changes produced by HD.

Simultaneous measurements in erythrocytes (RBC) showed opposing abnormalities in sodium content. In undialysed patients, sodium was low compared with controls and CAPD patients. HD was associated with a rise in Na_{RBC}.

These studies provided evidence in opposition to the theory that intracellular sodium is raised in patients with CRF due to the presence of a volume-related circulating sodium pump inhibitor. The presence of very different abnormalities in RBC and WBC questions the assumption that changes in sodium transport in either of these cell types can be extrapolated to more relevant but less accessible cells.

THEORETICAL BACKGROUND

PREFACE

Welt et al (1964) first suggested that uraemia might be associated with disturbed intracellular cation homeostasis when they reported raised sodium concentrations and diminished sodium pump activity in erythrocytes from some uraemic patients. This report characterised two major and still unresolved problems associated with these disturbances - why were they present in only a proportion of patients, and did they have pathogenetic significance?

A further stimulus to the investigation of intracellular cation content was given by Baron (1969) who was the first to measure the cation content of leucocytes and who pointed out the potential interest in measuring electrolyte content in cells rather than plasma.

Bricker (1972) proposed an attractive theory to explain some of the widespread abnormalities of cell function seen in uraemia. His basic principle was of a "trade-off" in which various responses to diminishing renal function had both beneficial and harmful effects. He pointed out that some major adaptation must be required to maintain urinary sodium excretion as glomerular filtration rate and therefore the quantity of filtered sodium falls progressively, and he proposed that the mechanism for this was increasing production of a sodium pump inhibitor. Released in response to mild sodium retention, this inhibitor would act on renal tubular cells, reducing sodium reabsorption and therefore maintaining natriuresis in the face of a decreased filtered sodium load. The trade-off was due to sodium pump inhibition in other tissues. In nerve and muscle cells decreased trans-membrane potential would lead to a decreased threshold for activation and therefore neuromuscular irritability. In arteriolar

smooth muscle cells increased sodium content would inhibit sodium-calcium exchange and in turn increase intracellular calcium, cell contractility, peripheral resistance and therefore blood pressure (a more detailed hypothesis linking sodium, calcium and hypertension was later proposed by Blaustein (1977)). As the rise in sodium was expected to exceed the fall in internal potassium, water would be drawn into cells producing swelling and generalised cellular dysfunction.

This theory stimulated much research into cellular cation transport but probably also had a deleterious trade-off in that results which at best were compatible with the presence of a circulating sodium pump inhibitor were taken as proof of its existence. A wealth of contradictory data has been published since then and the abnormalities of intracellular cation content and transport in uraemia remain scarcely better defined than at the time of Welt's paper in 1964. Most of the work performed has used erythrocytes, but a limited amount of widely quoted work in leucocytes has never been repeated.

In my work I was particularly interested in refining existing methods of measuring cation content and transport in leucocytes, using these methods to identify abnormalities of intracellular cations in uraemic patients, and correlating any abnormalities with standard clinical measurements of disease. As the work progressed it became clear that the development of a refined method for measuring leucocyte cations was going to become a significant part of the total undertaking. In the introduction that follows there are reviews of previous work done in developing leucocyte cation assays, the findings in uraemia and other diseases, and the use of cells other

than leucocytes to investigate cation transport in uraemia. A large part of the latter concerns red cells, both because they have been most widely used and because part of the work included in this thesis involved the use of red cell cation measurements.

CONCEPTS IN THE REGULATION OF INTRACELLULAR CATION CONTENT

Interpretation of abnormalities in intracellular cation concentrations or fluxes is aided by the formation of a background theory of how these are regulated and how abnormalities in some components will affect others. As biological systems tend to respond to changes by minimising their effect as far as possible, it can be difficult to deduce the original abnormality which produced observed changes, and to differentiate between that original abnormality and its consequences.

Before considering cation transport several important assumptions must be made when the concept of intracellular cation content is used. The many reports on leucocyte cations (and the far greater number on erythrocytes) assume, or hope, that the findings in these cell types apply to cells generally or at least to other specific but inaccessible cells e.g. arteriolar smooth muscle cells in essential hypertension, and those cells plus nerve, skeletal muscle and renal proximal tubular cells in uraemia. When it comes to expressing the quantity of an intracellular substance, several different denominators can be used - cell water, cell protein or nitrogen, dry or wet cell weight, or number of cells. Concentrations (per unit volume or weight) or contents (per number of cells) can be used interchangeably, as explained in the introduction to the "Development of Improved Methods" section. In giving a value for the intracellular concentration of some substance, the implicit assumption is that all of this amount is able to participate in metabolism and, with regard to transport, is freely exchangeable.

The possibilities that different compartments of a cell might have different cation contents, or that the total amount of cation might not be equally available to participate in metabolism, have been generally ignored.

These problems were all known when leucocytes were first mooted as typical cells with which to study the generalised intracellular state (Baron and Roberts, 1963). The existence of such a thing as a typical or model cell is, of course, another important assumption.

In discussions of leucocyte cation transport, the following overall model seems to have been generally accepted. The leucocyte is regarded as a small homogeneous container of sodium, potassium and water, the relative concentrations of sodium and potassium being inverted to those in plasma. This container is surrounded by a membrane which is freely permeable to water and has an inherent passive influx of sodium down its concentration gradient. This influx is matched by a membrane bound pump (Na-K-ATPase or the "sodium pump") which sends three sodium ions out of the cell and two potassium ions into the cell for every ATP molecule consumed. Abnormal sodium content can therefore be due to abnormal pump activity or abnormal passive flux. Potassium content is closely related to trans-membrane potential and is not predominantly set by sodium pump activity. Perhaps because the aim of many of the early experiments was to look for evidence of sodium pump inhibition, abnormal passive influx seems to have been relatively ignored as a producer of disturbed cation content.

The sodium pump has been clearly shown to be a membrane bound enzyme which is specifically inhibited by ouabain and other cardiac glycosides (Sweadner and Goldin, 1980). Active sodium efflux and

potassium influx can be measured using radioactive methods and these reveal that there are components of sodium efflux and potassium influx which are independent of the sodium pump. The exact nature of these remains unclear and they are accommodated in the general theory as being of little significance and not producing net movements of either cation i.e. the radiolabelling is revealing movement in one direction only of a sodium-sodium or potassium-potassium exchange.

Active sodium efflux rate in the physiological range is considered to equal the product of the intracellular sodium concentration and the rate constant for the sodium pump. Invariably in leucocytes, the latter two have been measured and the former calculated from their product. For such a calculation to be valid sodium content and rate constant should be measured under similar conditions. The value of the rate constant represents a combination of the affinity of the sodium pump for its substrate (sodium ions) and the number of available pumps.

Using a model similar to this, Patrick and Hilton (1979) theorised on how changes in cell sodium pumping or leak would be manifest in measurements of cation content and transport. Impaired sodium pump availability (i.e. a lower rate constant) without change in influx would lead to an elevation in intracellular sodium, until a new steady state was reached when the product of the raised sodium concentration and the lower rate constant equalled the unchanged influx rate. Potassium content would fall initially due to decreased pumped influx in the face of unchanged efflux, until the new steady state was reached. They stated that the decreased potassium content at this point would gradually return towards normal.

The state of partial sodium pump inhibition, therefore, would

result in decreased rate constant, raised intracellular sodium, normal sodium efflux and influx rates, and near normal potassium content. On the other hand, a rise in passive leak of sodium into the cell would raise internal sodium until the sodium influx rate was equalled by the product of the unchanged rate constant and the elevated sodium content. As this represents an overall increase in the total sodium pump activity, potassium influx would be higher and intracellular potassium would accumulate. However, intracellular potassium concentrations are closely linked to trans-membrane potential and cannot be accurately predicted from consideration of sodium pump activity alone.

A very clear exposition of the correlations between measured values for sodium content and transport and their likely implications in terms of membrane leak and sodium pumping was given by Cumberbatch and Morgan (1981). They emphasised that cells should be considered to be in steady state and therefore sodium efflux rate must equal influx rate. A finding of low efflux rate must also indicate low passive influx and either value is a measure of membrane leak. The rate constant, being the flux rate divided by the internal sodium concentration, is therefore an indication of sodium pump activity at a given sodium concentration, and the sodium concentration is an indication of the matching of pump efflux to passive influx. It is clear that an isolated measurement of flux rate or rate constant cannot be adequately interpreted unless sodium concentration is also known. Not all workers have agreed with this model and abnormal flux rates are sometimes taken as indicative of a primary abnormality of sodium pump activity. Cumberbatch and Morgan's model is used to interpret measurements of flux rate and rate constant in this thesis.

Although this theory is helpful in trying to assess how observed changes might have come about, there are some flaws. Perhaps the major one is the apparent assumption that the cell has a fixed rate constant which reflects the number of available sodium pumps, and that although sodium flux rate is altered by altered internal sodium, the rate constant is not. If this is true at all, it is so only over a very brief period of time. It is now clear that membrane sodium pumps can be readily increased or decreased and this regulation is probably mediated through alterations in internal sodium concentration (Fambrough et al, 1987). Therefore any alteration bringing about a change in internal sodium will cause an alteration in the number of sodium pumps in such a way as to counteract the change in sodium. A low rate constant measured in isolation does not therefore necessarily imply inhibition of the sodium pump, but could equally reflect down-regulation of sodium pumps secondary to decreased passive influx, and an increase in this influx would produce an elevation in sodium content and rate constant. When sodium pumps are inhibited, the normal cellular response is to make more pumps - it is not at all clear that in a state of chronic exposure to a sodium pump inhibitor a marked decrease in sodium pump rate constant should be observable.

Another problem is the creation of a clear division between active sodium efflux and passive sodium influx. Both of these depend on the integrity of the cell membrane and both require energy (the influx indirectly to maintain membrane structure). It is quite likely that factors affecting one will affect the other. The description of passive influx as a leak is perhaps unfortunate in suggesting that this influx is undesirable. Cells can use the sodium gradient to

transport substances into the cell by linking them with sodium ions and it is possible that the cells therefore manipulate this influx depending on their metabolic requirements. Some of the sodium movements are due to sodium-hydrogen and sodium-calcium exchangers and the degree of net sodium movement they produce is closely linked to extra and intracellular hydrogen and calcium ion content. If these alter sodium content they may also ultimately alter sodium pump numbers.

Although a simple model of cell sodium pumping is helpful in trying to make sense of different patterns of disordered function, it is clear that cellular transport processes are complex, numerous and inextricably interlinked. Great care must be taken in attributing abnormalities to any single source.

Summary and definitions.

The amount of cations in cells has conventionally been described as a concentration of moles of cation per unit wet or dry weight. In this work, cations have been measured as moles per number of cells, i.e. as a content. In effect, these give similar answers as is explained in the introduction to "Development of Improved Methods".

The sodium flux rate, often referred to simply as flux rate, is taken to be a measure of passive sodium influx or leak. It is assumed that the cells are in steady state and this rate therefore equals the amount of sodium being pumped out through the sodium pump. This value divided by the intracellular sodium content/concentration equals the sodium efflux rate constant, which will usually be referred to simply as rate constant. This is taken as a measure of the number of functional sodium pumps in the cell membrane or "sodium pump availability". An increase in rate constant is therefore assumed to

reflect an increased availablity of sodium pumps, and an increase in flux rate to reflect an increase in passive sodium influx.

THE DEVELOPMENT OF METHODS FOR MEASURING LEUCOCYTE CATION CONTENT AND TRANSPORT

The first report of the use of human leucocytes as typical cells whose internal cation content could be taken as indicative of the overall intracellular environment was published by Baron and Roberts (1963). They used leucocytes because, being nucleated, they were more likely to be typical cells than erythrocytes and, for routine analyses, were easier to obtain than muscle biopsies. They added a 6% dextran (mol. wt. 200,000 - 275,000) solution to defibrinated blood in a ratio of 1:4 to sediment the red cells. The leucocyte rich supernatant was removed after 30 minutes and spun at 300 g for 5 minutes, the authors noting that longer or more vigorous centrifugation resulted in cell death, and residual erythrocytes in the resulting cell pellet were lysed by the addition of 1.8 ml of distilled water for 10 seconds. Isotonicity was restored by the addition of hypertonic modified Hartmann's solution and then centrifugation for 3 minutes at 300g left a cell pellet which was dried at 110°C to constant weight. The dried cells were lysed with 0.1N HNO_3 and sodium and potassium estimated by flame photometry. No attempt was made to estimate or correct for trapped extracellular fluid, or for the red cell ghosts which were seen to be present. The ratio of leucocyte sodium to potassium allows comparisons of results using different units of measurement, and in this study averaged 0.61. Although the method used can, with the benefit of hindsight, be seen to be crude and to grossly overestimate sodium content, it provided the outlines on which current methods are based. It is also worth noting the reproducibility, with the coefficients of variation

from 28 subjects being 7.8% for sodium and 6.2% for potassium. This compares favourably with all reports since, reflecting both the constancy of extracellular fluid trapping in this method and the difficulties involved in estimating or avoiding such trapping in later methods.

In 1964, Block and Bonting isolated leucocytes from normal controls and patients with leukaemia and made the important observation that the sodium content fell and the potassium content rose during a one hour incubation in Tyrode's buffered salt solution at 37°C, presumably reflecting recovery from the isolation procedure (Block and Bonting, 1964). They also showed that incubation with ouabain at 2×10^{-4}M caused sodium to rise and potassium to fall, and demonstrated the presence of a ouabain sensitive Na-K-ATPase system whose activity was related to active cation transport rates.

In 1969 a modified version of Baron and Roberts' method was described (Baron and Ahmed). Heparinised rather than defibrinated blood was used, red cell ghosts were removed as much as possible, but most importantly, trapped extracellular fluid volume in the final leucocyte pellet was estimated by the addition of radio-iodinated serum albumin. Because of the high sodium content of ECF and the low intracellular sodium, the calculation to allow for ECF introduced considerable potential for error and this was reflected in the large range for 30 measurements from 20 controls - mean sodium concentration (Na_{WBC}) was 22.2 $\pm$ 6.5(SD) mEq/kg wet weight. The mean sodium potassium ratio was 0.25 and no mention was made of how long the cells were incubated after the isolation procedure. Calculating trapped ECF therefore gave an answer considerably closer to the truth but also considerably less precise. Potassium content (K_{WBC}) was

lower in men than in women, but there was no significant difference in Na_{WBC}.

Further evidence of the effect of the isolation procedure on intracellular cation content was provided in the same year (Lichtman and Weed, 1969). They used a different procedure to separate red from white cells and destroyed the red cells by the addition of 150mM NH_4Cl. This did not entirely eliminate red cells but reduced them to less than 5% of the total cell number. The white cells were incubated for varying lengths of time in Hanks buffered salt solution, and at the end of this they were centrifuged and then washed in choline chloride to avoid the problem of ECF contamination. Rather than weighing the dried cell pellet prior to cation measurement, they counted the concentration of cells in their suspensions and expressed sodium and potassium as moles per cell. The sodium and potassium concentrations changed markedly during the incubation - sodium fell to 50% of the value at the start of the incubation when it was measured at 60 minutes and remained constant at 90 and 120 minutes, whilst potassium rose to 130% of its original value at 60, and 160% at 90 and 120 minutes. The authors recommended leaving cells for 90 to 100 minutes to recover from the isolation procedure, and in their stable cells the sodium potassium ratio was 0.24. Despite avoiding having to estimate and allow for trapped sodium, there was considerable variation in their measurement of sodium concentration - a mean of 12 $\pm$ 2.7(SD) x 10^{-15} mol/cell from 9 controls.

Methods for measuring the movement of potassium and sodium in and out of leucocytes were developed by Hilton and Patrick (Hilton and Patrick, 1973). The cells were isolated according to the method of Baron and Ahmed and were finally suspended in TC199 with an

osmolality of 280 - 290 mosm/kg and a pH of 7.35 to 7.45. Once again the method did not mention if the cells were given time to recover from the isolation procedure, and although the authors pointed out that internal and external cation concentrations must be stable to allow meaningful estimates of flux rates, they provided no data regarding the stability of intracellular sodium and potassium content under their experimental conditions.

Sodium efflux rate constant was measured by adding $^{22}NaCl$ to the cell suspension for 20 minutes. The cells were then centrifuged at 160g for 3 minutes and washed to remove extracellular ^{22}Na before being resuspended in TC199 with or without ouabain at a concentration of 10ug/ml. Over the next 20 minutes aliquots were taken, centrifuged, and the supernatant removed as completely as possible before measuring radioactivity per unit dry cell weight. The natural logarithms of these values, plotted against time, appeared to be linear and the slope of this line was taken to equal the rate constant for sodium efflux (NERC). NERC was considerably decreased in the presence of ouabain, and the difference between total NERC (tNERC) and the ouabain resistant NERC (orNERC) was the ouabain sensitive NERC (osNERC). Mean osNERC in normal controls was 3.2 $hour^{-1}$. This value can be considered to be representative of the sodium pump availability, and the ouabain sensitive sodium efflux rate (osNER) equalled osNERC multiplied by Na_{WBC}. This rate of active efflux equals the passive influx if the cells are in equilibrium, a measure of sodium leak down its concentration gradient. However, Na_{WBC} was not measured in these experiments, probably because of the large quantities of blood required to obtain enough leucocytes to weigh with reasonable accuracy. The calculated value for osNERC was

multiplied by their mean laboratory value for Na_{WBC} but this had little meaning because the former had been measured at 37°C and the latter at room temperature. This method, with minor variations, has been widely used since to measure sodium pump rate constant in leucocytes, often without concomitant measurement of Na_{WBC}.

Sodium influx rate was measured by adding ^{22}Na to the incubation medium for 7.5 minutes and then centrifuging and washing the cells before measuring radioactivity. The calculation of influx required a knowledge of the NERC (to allow in the calculation for the amount of labelled sodium which entered the cell but was then pumped out), and the authors found a mean value of 359 (SD128) mmol/kg dry weight/hr. This was considerably lower than the calculated mean value for NER of 487 mmol/kg dry weight/hr, and although this may have meant that the cells were not in equilibrium, the value for NER may have been inaccurate for the reasons described above.

Potassium influx and efflux were measured in similar fashion using ^{42}K as the radio-isotope. Influx was measured over a 28 minute incubation period with or without ouabain and efflux was measured over 40 minutes after a 40 minute incubation in the presence of the isotope to load the cells. Potassium influx and efflux agreed better than sodium fluxes but only a small proportion of influx appeared to take place through the sodium-potassium pump - 99 out of a total of 346 mmol/kg dry weight/hr.

The same authors investigated the effect of differing extracellular osmolality on leucocyte cation concentration (Patrick and Hilton, 1973). The osmolality of the incubating solution was altered between 249 and 345 mosmol/kg by varying the sodium chloride concentration, and the cells were incubated for an average of 30 minutes. ECF

osmolality had clear effects on intracellular sodium and potassium, with sodium content going up and potassium content down as osmolality fell. These changes were not balanced and the total cation content and intracellular water increased as external osmolality decreased, reflecting cell swelling. Similar experiments in erythrocytes showed that the ECF osmolality, at least in the range 249 to 345 mosmol/kg, had no effect on internal sodium or potassium content per unit dry cell weight. This study could not differentiate between the effects of hyponatraemia and low osmolality, nor could it explain why sodium and potassium content altered, and these questions were the subject of another report (Hilton and Patrick, 1974) which showed that the abnormalities were due to the low osmolality and that a solution with sodium concentration of 103 mmol/l but osmolality restored to 285 mosmol/kg by the use of choline chloride gave similar results to the standard incubation medium. They found that in the hypo-osmolar solution, passive sodium influx was increased and that this rather than a diminution in sodium pump activity was responsible for elevating intracellular sodium.

The same methods were used to study leucocytes in various disease states and the results of these are discussed later.

Negendank and Collier (1976) demonstrated a dose reponse curve for the effect of ouabain on lymphocyte sodium and potassium concentration, finding maximal activity at concentrations of 10^{-6} M and higher.

Patrick et al (1978) measured the effect of differing zinc concentrations in the incubation medium on leucocyte sodium concentration and transport. They found that both tNERC and osNERC increased as external zinc concentration increased. However this was

matched by a rise in sodium influx and there was no detectable effect of external zinc on Na_{WBC}. The authors could not explain this abnormality, and it can now be viewed as one of the first indications that internal sodium regulation cannot be simply described by entirely independent passive influx and active efflux, the latter of which is the product of internal sodium concentration and a static rate constant. Perhaps of equal interest in this paper were the values for Na_{WBC} - approximately 80 mmol/kg dry weight - and sodium influx - approximately 220 mmol/kg dry weight/hr - compared with values from the same laboratory using apparently the same method in earlier papers of 116 mmol/kg dry weight and 359 mmol/kg dry weight/hr. It is possible, although difficult to be sure, that the changes in the later paper were due to leaving a 30 minute incubation period after isolation before Na_{WBC} and fluxes were measured. Indeed, in the paper on zinc, the authors stated that after 30 minutes incubation at 37°C "the sodium, potassium and water content of the cells have reached equilibrium" but this was not referenced and no data were provided in support. It may be that the authors meant that rates of sodium influx and efflux, and potassium influx and efflux were similar rather than that serial measurements of Na_{WBC} and K_{WBC} showed stability. Indirect evidence that the shift in results was a consequence of allowing the cells to recover from the effects of separation is provided in a later paper (Hilton et al, 1981). Using similar methods to their previous work, the authors incubated cells for 60 minutes in incubation media containing variable amounts of sodium, and on this occasion the mean control value for Na_{WBC} was 56.5 mmol/kg dry weight. They found that very low external sodium concentrations lowered Na_{WBC} and K_{WBC}, passive

sodium influx and active sodium efflux. Interestingly the fall in the latter was due to decreased Na_{WBC} and a fall in osNERC - evidence that this "constant" can in fact change over periods of one hour or less.

Further information regarding the effects of the methods used to isolate the cells on the values obtained for intracellular cation content and transport has appeared in the last few years, often as adjuncts to the methods section of papers reporting abnormalities of leucocyte sodium transport in various disease states. Poston et al found tNERC to be unchanged by a three hour pre-incubation (although of course the twenty minute incubation in ^{22}Na was always necessary) (Poston et al, 1982a) and also reported that osNERC was lower if Ficoll/Triosil was used to sediment red cells rather than dextran (Poston et al, 1982b). Ng et al (1988) found that the sodium and potassium content of leucocytes isolated using Ficoll/diatrizoate was only about 75% of that of cells isolated with dextran.

Because of the need to measure Na_{WBC} and osNERC under the same conditions, Heagerty isolated leucocytes at 37°C, using Plasmagel rather than dextran which he found ineffective at this temperature. He expressed the radioactivity of the cells per unit protein to reduce the error inherent in weighing very small samples when measuring NERC. The cells were allowed to incubate in TC199 for 30 minutes prior to being washed in ice cold isotonic magnesium chloride to remove ECF contamination when measuring cation content (Heagerty et al, 1982). This method gave quite different results for normal controls compared with those mentioned above - Na_{WBC} 38.5 mmol/kg dry wt, K_{WBC} 213 mmol/kg, sodium potassium ratio 0.18, and osNERC 1.47 hr^{-1}. These values changed a little in later reports,

e.g. Na_{WBC} 44, K_{WBC} 366, ratio 0.12 and osNERC 1.37 (Heagerty et al, 1986a), but were no closer to those from other centres. Whether or not these results more or less accurately reflected the true state of leucocyte cation concentration and transport, Heagerty's group showed that their values for osNERC in leucocytes correlated with osNERC in arteriolar smooth muscle cells ($r = 0.64$, $p < 0.01$) (Aalkjaer et al, 1986). No measurements of Na_{WBC} were reported in that study.

The picture has become no clearer recently despite the attempts of Ng and co-workers to identify factors which could explain disparate results. They suggested that Heagerty's low value for osNERC may have been due to the use of lithium rather than sodium heparin but provided no data to support this (Ng and Hockaday, 1986a), and found that both os- and or-NERC were quite different if the cells were incubated in their own plasma rather than tissue culture fluid (Ng et al, 1985a). OsNERC was lower in plasma which led them to suggest that there were inhibitors of the sodium pump present in normal plasma. They could not explain the difference in orNERC. This group used dextran to sediment red cells, allowed a 30 minute recovery period in TC199 after isolation and washed the cells in ice cold $MgCl_2$ prior to cation estimation - a method very similar to those previously used - and they stated that placing cells in ice cold $MgCl_2$ for up to 30 minutes did not lead to a "substantial" loss of sodium (Ng et al 1987a). Their normal results have again been different from those from other centres - Na_{WBC} 27.8 mmol/kg dry wt., K_{WBC} 332.7 mmol/kg (ratio 0.084) and osNERC 2.35 $hour^{-1}$ (Ng et al, 1988). They explained their low Na_{WBC} as being due to allowing the cells to recover from the isolation procedure, stating that, because isolation was carried out at room temperature, sodium efflux decreased but influx remained

constant, thus raising internal sodium. These statements were not supported by data or references and did not explain why their sodium values were lower than those of Heagerty who isolated the leucocytes at 37°C and allowed a 30 minute recovery period.

Ng and Hockaday (1986b) also showed that their measurement for osNERC correlated with leucocyte Na-K-ATPase activity (r_s = 0.75, $p < 0.001$). Alam et al (1978) did not find such a correlation but used a different technique for Na-K-ATPase estimation.

None of these groups appear to have been able to measure Na_{WBC} as accurately and reproducibly as NERC and this may partly have been due to problems in accurately weighing the very small final leucocyte pellet. One group bypassed this by counting the cells rather than weighing them but gave no details of how they did this or any problems encountered. Their values for controls were 3.9 (SD1.0) for Na_{WBC} and about 24 for K_{WBC} in mmol/10^{12} cells, i.e a sodium potassium ratio of 0.16 (Boon et al, 1985).

With current methods, therefore, the accuracy with which Na_{WBC} can be measured seems considerably less than that for NERC, as judged by coefficients of variation for the measurements in normal controls. Although it is possible that this reflects a greater in vivo range of internal sodium concentration compared to sodium pump activity, on theoretical grounds one might have expected the opposite to be true. Because sodium efflux rates have been calculated from the product of NERC and Na_{WBC}, this inaccuracy has also applied to flux rate measurements. The second obvious problem has been the large discrepancy in results from different centres, even in normal controls. The way in which the cells are isolated, the time for which they are incubated and the constituents of the incubation fluid have

all been shown to alter cation concentration and transport and it seems likely that inevitable artefacts are introduced in the process of rendering the cells suitable for analysis. It is not known if these artefacts have equal effects on normal and abnormal cells.

LEUCOCYTE CATION CONTENT AND TRANSPORT IN URAEMIA.

Leucocytes were first used to investigate intracellular cation content in chronic renal failure in 1963 (Roberts and Baron, 1963). No effort was made to remove or allow for trapped extracellular fluid which may be the explanation for the finding of lowered leucocyte sodium concentration (Na_{WBC}) in uraemia, a finding not supported by later studies.

Patrick et al (1972) used a variation of the method of Baron and Ahmed (1969), estimating and allowing for trapped ECF by the use of radio-iodinated albumin, to measure leucocyte potassium concentration (K_{WBC}) in 16 undialysed patients with chronic renal failure and 18 regular haemodialysis patients. The uraemic group had plasma creatinine levels ranging from 11 to 30 mg/100ml (i.e. 1000 to 2800 umol/l approx.) and were deliberately heterogeneous with regard to drug treatment and general health, and the haemodialysis patients were receiving 30 hours dialysis weekly on Kiil dialysers, blood samples for analysis being taken immediately before dialysis. The authors found dextran did not separate the leucocytes from the erythrocytes in uraemic blood and therefore used a methylcellulose/diatrizoate density gradient. In addition to K_{WBC}, erythrocyte sodium and potassium concentration (Na_{RBC} and K_{RBC}) and total body exchangeable potassium (Ke) were measured.

Ke fell below the lower 95% confidence limit of predicted normal values in 6 of 9 undialysed and 6 out of 17 haemodialysis patients. K_{WBC} was significantly lower than normal in the undialysed group but the same as normal in the haemodialysis group, whereas K_{RBC} was significantly higher than normal in both the uraemic and

haemodialysis groups, a finding attributed to the inverse relationship between haemoglobin concentration and K_{RBC} (Maizels 1936). The authors concluded that the low K_{WBC} in undialysed patients probably reflected generalised potassium depletion and might become a better method for assessing potassium balance than measurements of plasma potassium or Ke.

The same group went on to publish the two major and much cited papers regarding leucocyte cations and uraemia. In the first (Patrick and Jones, 1974), cells were prepared in the fashion described above, and Na_{WBC} and K_{WBC} were measured. Thirty measurements were made on 19 undialysed patients (plasma creatinine concentrations ranged from 460 to 2570 umol/l,mean = 1330), and 15 pre-dialysis measurements in regular haemodialysis patients dialysing for 30 hours weekly. Control values were derived from 59 normal staff and students.

Mean Na_{WBC} (mmol/kg dry wt.) was 104 (SD 30.7) in controls, 123 (43.8) in the undialysed group and 85(42.6) in haemodialysis patients. There was a tendency therefore for Na_{WBC} to be elevated in the uraemic group which only just reached statistical significance ($p = 0.05$), and the apparent lowering of Na_{WBC} in haemodialysis patients was not significant compared with controls. The K_{WBC} results were different from their previous findings. Although K_{WBC} was again significantly low in the undialysed group (332 (49) mmol/kg v. 377 (31) in controls, $p<0.001$), in this study it remained equally low in the haemodialysis group (324 (46)). Sodium/potassium ratios were 0.27 in controls, 0.37 in undialysed and 0.26 in regular dialysis patients. In four patients studied before and after the onset of regular haemodialysis there were quite marked falls in Na_{WBC}. No correlation was found in the undialysed group between Na_{WBC} and

plasma creatinine, urea, sodium, or bicarbonate, but the authors commented that the highest Na_{WBC} concentrations were found in the patients who were "most ill". They also made the interesting observation that most of the undialysed patients with high Na_{WBC} were hypertensive whereas most of the dialysis patients with low Na_{WBC} were normotensive, an observation that led to the use of leucocytes to measure intracellular sodium in essential hypertension.

The second of these influential papers (Edmondson et al, 1975a) measured not only Na_{WBC} but also total and ouabain sensitive sodium efflux rate constants (t and osNERC) by the method of Hilton and Patrick (1973). Measurements were made on 16 undialysed uraemic patients with a mean plasma creatinine of 1470 umol/l and 16 regular haemodialysis patients. Eight patients were common to both groups, i.e. were studied before and after the start of regular dialysis.

Control values for Na_{WBC} were the same as in the preceding paper. 14 undialysed patients had Na_{WBC} measured and the mean value was identical to that in the 30 patients reported in the previous paper. In only 3 of the 14 was Na_{WBC} higher than the mean plus two standard deviations of the control group. The mean control value for osNERC was 3.03 (SD 0.49) derived from 30 volunteers as opposed to 1.82 (0.48) in the 11 undialysed patients in whom it was measured. This difference was highly statistically significant and indeed osNERC in the undialysed patients was below the control mean in every case. In the 16 haemodialysis patients mean osNERC was 2.54 (0.52) - a figure significantly higher than in the undialysed group. In the eight patients measured before and after starting dialysis, osNERC rose in seven and did not change in one. As Na_{WBC} was measured at room temperature, calculation of sodium efflux rates was not possible.

There was no correlation between osNERC and plasma creatinine, creatinine clearance or blood urea in the undialysed group. The authors concluded that a dialysable chemical which inhibited the sodium pump was circulating in the blood of uraemic patients, that this was supportive evidence for the theory that intracellular sodium was elevated because of a circulating sodium pump inhibitor in the uraemic state and that this elevated sodium was responsible for some of the disordered cell functions of uraemia (Bricker 1972).

There has been no further evidence that Na_{WBC} in uraemia is elevated because of inhibition of the sodium pump, and the studies of Patrick and Jones, and Edmondson et al cannot be regarded as conclusive proof. Although the demonstration of lowered osNERC appeared convincing, Na_{WBC} lay within the normal range in many of the patients, and there was no correlation between Na_{WBC} and osNERC in the 11 patients in whom both were measured. Furthermore mean osNERC was only partially corrected in the dialysis group, but Na_{WBC} was reduced to below normal levels. These findings suggested that passive sodium flux as well as active sodium pumping was altered in the uraemic and dialysis patients, but flux rates could not be calculated. There must also be some doubt about the validity of the Na_{WBC} measurements which were made at room temperature and apparently without allowing the cells any time to recover from the isolation procedure. This is a likely explanation for the high control value for Na_{WBC} compared with later work from the same laboratory.

Another methodological problem may have been the use of different media to isolate leucocytes in the controls compared with the uraemic groups. It is clear that the uraemic leucocytes were isolated using a diatrizoate and methylcellulose mixture, but it is not clear if

this mixture or dextran was used for the controls. The control groups for leucocyte cation measurements in three papers seem to have been identical (Edmondson et al, 1974; Patrick and Jones, 1974; and Edmondson et al, 1975a). In the first of these papers dextran was used to isolate leucocytes from the patients under investigation whereas diatrizoate/methyl cellulose was used in the latter two. No specific mention is made of the methods used in the controls but the standard method in that laboratory seems to have been to use dextran. This problem is an important one in view of later reports regarding the effect of different isolation media (Poston et al, 1982b; Ng et al, 1988). It is possible that the low osNERC in the uraemic group was partly due to this, although the higher values in the dialysis group and the upward change in the patients studied before and after commencing dialysis would not be affected by this problem. In a short paper in 1978 from the same laboratory (Michael et al), osNERC in controls and uraemic patients was measured using different concentrations of zinc in the incubation medium. It seems clear in this report that diatrizoate/methyl cellulose was used to isolate cells in both the control and the uraemic group and although osNERC was lower in the uraemic group, the magnitude of the difference was about half of that reported in 1975. Na_{WBC} was not measured in this study and the significance of the abnormal rate constants cannot therefore be interpreted.

Jones et al (1980) compared Na_{WBC} in eight diabetic and eight non-diabetic patients all of whom were on regular haemo- or peritoneal dialysis, using similar methods to those described above. Na_{WBC} (mmol/kg dry wt.) was 143.4 (SD 68.6) in the diabetics and 76.2 (30.3) in the non-diabetics, and no other data were provided. As they

had also found inter-dialytic weight gain to be greater in diabetics than non-diabetics, they suggested that the raised intracellular sodium might in some way be responsible for excessive thirst in that group of patients. Due to the paucity of data it is difficult to draw any conclusions from this paper.

Although no further work appears to have been done on leucocyte cations in uraemia, Mansell et al (1981) measured leucocyte ATP concentrations in several different uraemic groups. They found significant reductions in ATP content in undialysed uraemic and regular haemodialysis patients, but ATP levels were closer to normal in the dialysis group. ATP levels fell progressively as serum creatinine rose above 700umol/l in the undialysed group and correlated inversely with serum phosphate concentrations. In acute renal failure ATP levels were again diminished and were returned towards normal by dialysis or resalination. The authors speculated that the low ATP levels might either provide an inadequate energy supply and therefore limit sodium pumping capacity, or reflect inhibited sodium pumping reducing the demand for ATP production. It is of interest to note that, on finding raised erythrocyte ATP levels in uraemia, Kramer et al (1976) proposed that this also reflected decreased sodium pump activity, leading on this occasion to an accumulation of ATP due to decreased utilisation!

There would appear to be sufficient doubts for further investigation to be required before accepting that studies in leucocytes support the hypothesis that uraemia is associated with a state of raised intracellular sodium concentration secondary to sodium pump inhibition. In the reports to date, Na_{WBC} was measured at room temperature and was not elevated in the majority of patients,

there were problems concerning the way in which the cells were isolated prior to measurement of rate constant and no measurements of passive sodium influx were made.

FACTORS WHICH MAY INFLUENCE LEUCOCYTE CATION CONTENT AND TRANSPORT IN VIVO.

Biological variation.

Very little work has been published about natural biological variation in leucocyte cation content and transport. In more recent reports authors have tended to match groups for age, sex and sometimes weight, but this was not the case for the work in uraemic patients (Edmondson et al,1975a; Patrick and Jones, 1974). Although there appear to have been no papers published specifically addressing the question of the effects of age and sex on leucocyte cation transport and content, some authors have mentioned differences or lack of them in papers chiefly concerned with other problems.

Baron and Ahmed (1969) in their much cited description of the basic technique for measuring Na_{WBC} and K_{WBC} reported that K_{WBC} was significantly lower in men but found no significant difference in Na_{WBC}. Their subjects were all aged between 20 and 40. Araoye et al (1978) confined their observations to men but in their results presented Na_{WBC} by age groups, split into decades from less than 30 to greater than 60. There was no hint in the controls of any effect of age on Na_{WBC} but the numbers in each group were small, ranging from 3 to 9.

Heagerty in his reports always took great care to age and sex match different groups but did not specifically mention evidence that this was necessary. In his thesis (Heagerty, 1986) however, he reported a slight increase in Na_{WBC} and total sodium efflux rate (tNER) in women but no difference in total (t), ouabain sensitive (os) or ouabain resistant (or) sodium efflux rate constant (NERC). When he compared controls of different sex in age matched groups this difference

disappeared and he found slight negative correlations between age and Na_{WBC} and K_{WBC}. The former depended for significance on one value in a 70 year old. In young controls (n = 25, mean age 25 years) he found a negative correlation between age and tNERC and orNERC but not osNERC.

More recently Ng et al (1987a) have also found different values in male and female controls. Like Heagerty they found higher Na_{WBC} and NER in women but this time the difference was highly significant. They did not say whether or not their male and female controls were age matched, but they did state that all the premenopausal female controls were in the follicular phase of the menstrual cycle and none were taking the contraceptive pill. They were unaware of previous reports of age or sex affecting leucocyte sodium values. In another paper chiefly concerning the effect of obesity on NERC, no correlation was found between age and osNERC in subjects whose age ranged from 18 to 65 (Ng and Hockaday 1986c). In their large series of controls, Poston's group have found no effect of age or sex on leucocyte cation content or transport (L.Poston, personal communication).

Although unlikely to have a major effect, it remains unclear whether or not age and sex have to be taken into account when studying white cell cation content and transport, despite the fact that these cells have been studied in various disease states for more than ten years. This apparently remarkable omission is perhaps explained by the continuing difficulties in analysing these cells - even in the recent paper of Ng et al quoted above, the coefficient of variation in normal controls for Na_{WBC} was 34%, no better than in the original reports.

Ng's group have also identified diet and obesity as further factors which may alter Na_{WBC} content and transport (Ng and Hockaday, 1986b). They measured t, os and orNERC in 11 controls after a 12 hour fast and then again 2 hours after an oral glucose load, and found a significant rise in osNERC from 1.97 (SD 0.25) to 2.44 (0.19). The rise in individual controls correlated with a concurrent fall in non-esterified fatty acid (NEFA) levels. At the same time there was a significant fall in orNERC, but unfortunately measurements of Na_{WBC} were not made and the significance of the observed alterations remains unclear. In 23 fasting controls in whom Na_{WBC} and NEFA were measured there was no correlation between the two, whereas in 24 fasting controls there was a highly significant correlation between osNERC and NEFA. The authors concluded that changes in osNERC due to altered NEFA levels were unlikely to be mediated through changes in Na_{WBC}. This statement is not necessarily true - if NEFA levels had altered Na_{WBC} (e.g. by altering passive influx), within two to three hours the cell might have altered the number of functioning membrane sodium pumps and therefore osNERC, returning Na_{WBC} to normal or nearly so (Fambrough et al, 1987; Doucet, 1988). Furthermore, the lack of a relationship between NEFA and Na_{WBC} implied that changes in osNERC did not alter Na_{WBC}. This would again lead to the conclusion that NEFA must have affected sodium influx. Evidence that the alterations in osNERC produced alterations in Na_{WBC} would have supported the conclusion that NEFA are potent inhibitors of the sodium pump.

These findings were challenged by Poston et al (1987) who repeated the experimental method of Ng in 11 normal subjects but found no evidence that an oral glucose load altered osNERC.

The effect of dietary supplementation with linoleic acid on leucocyte sodium transport was investigated by Heagerty et al (1986b) in 22 healthy controls. They found rises in Na_{WBC}, osNERC and osNER, only the last of which reached statistical significance. A plausible explanation for this is that all these changes were the result of a rise in passive sodium influx due to alterations in the membrane caused by the dietary linoleic acid.

An association between obesity and NERC has also been reported (Ng and Hockaday 1986c, Ng et al 1987a). In the first of these studies mean osNERC in 12 obese but otherwise normal controls was 2.72 (SD 0.45) compared with 2.31 (0.47) in 35 non-obese controls. This finding was confirmed in the second study and shown to be independent of sex differences. The higher osNERC in the obese was not accompanied by significantly lower Na_{WBC} or higher flux rates, one of which might have been expected. It is impossible to say, therefore, if the observed change in osNERC was a primary phenomenon leading to lowered Na_{WBC} which the assay was not sensitive enough to detect, or a response to raised sodium influx which again remained undetected, or indeed a combination of the two.

Drug treatment.

Several drugs have been shown to alter leucocyte cation content and transport. The first paper to address the question of the effect of drugs on intracellular cation content compared Na_{WBC} and K_{WBC} in two groups of patients receiving diuretics - for heart failure in one group and for non-cardiac reasons in the other group (Edmondson et al, 1974). Although Na_{WBC} was high and K_{WBC} low in the cardiac group and normal in the non-cardiac group no attempt was made to match the type or dose of diuretic in the two groups. Na_{WBC} and K_{WBC}

were quoted for each patient and in some cases the former exceeded the latter. It now seems likely that such a state could not exist in viable leucocytes in vivo and that these abnormalities were induced by the experimental methods used at that time. It is important therefore to note that this paper used the same methods and same control group as the influential report on leucocyte cation content in uraemia and dialysis (Patrick and Jones, 1974). A further difficulty in interpreting any possible effect of diuretics was that all the cardiac group were taking digoxin, and it seems that no conclusions can now be drawn from this paper regarding the effect of drugs on intracellular cation content.

A more useful study (Araoye et al, 1978) followed Na_{WBC} and K_{WBC} in patients with essential hypertension before and after the introduction of four drugs. Na_{WBC} was high in the hypertensives and fell significantly after the introduction of hydrochlorothiazide or reserpine but not after methyldopa or hydralazine, although all four drugs lowered blood pressure. None of the drugs had a significant effect on K_{WBC} although hydrochlorothiazide did produce the expected fall in serum K. This study therefore dissociated the presence of elevated Na_{WBC} from elevated blood pressure: hydrochlorothiazide or reserpine lowered elevated Na_{WBC}, and this effect was unlikely to be mediated through lowered blood pressure, as Na_{WBC} remained high after methyldopa or hydralazine.

Poston et al (1981a) found a rise in osNERC in seven essential hypertensives treated for at least four weeks with a combination of a thiazide diuretic, amiloride and a beta adrenergic blocker, although it was unclear whether this was a return to normality or a non-specific stimulation of osNERC as the magnitude of the change did

not appear to correlate with the degree of abnormality before treatment. In 34 hypertensives on a variety of treatments, mean osNERC was similar to normal in those receiving diuretics and similar to values for untreated hypertensives in the group whose treatment did not include diuretics. The scattergrams revealed large overlaps between the groups. Nevertheless these results suggested that thiazide diuretics elevate osNERC in essential hypertension. Milner et al (1984) gave 5mg of bendrofluazide daily for one week to 14 controls and 14 normotensive relatives of patients with essential hypertension (NTR), and found a significant elevation in osNERC in the NTR but not controls. As NTR had originally had low osNERC this had the effect of returning them to the normal range. Despite the differences in osNERC, there were no differences in Na_{WBC} in the two groups, and no change after diuretic. This paper is confirmation of a restorative effect of thiazide diuretics on low osNERC.

Gray et al (1984) studied the effect of verapamil treatment in essential hypertension and found a significant elevation in previously low osNERC and a significant fall in previously elevated Na_{WBC}. The latter was not due solely to the former as they also found a significant fall in sodium influx after treatment. They followed up this work with studies of the in vitro effect of verapamil on leucocytes from normal volunteers, using incubation fluid with and without calcium (Gray et al 1985). In the presence of calcium, verapamil significantly increased t- and os-NERC without altering Na_{WBC} or sodium influx. This reflected the insensitivity of the measurements as an increase in osNERC must reduce Na_{WBC} unless influx increases. In the absence of calcium verapamil had no effects but in the absence of verapamil, the presence of calcium was

associated with significantly higher osNERC, and significantly lower Na_{WBC} and sodium influx.

There do not appear to have been any other studies of the effect of in vivo drug administration on leucocyte sodium transport, the most surprising omission perhaps being that of digoxin in view of the debate concerning circulating sodium pump inhibitors or digoxin-like substances in essential hypertension and uraemia.

Steroids might also be expected to alter leucocyte sodium pump availability as there is evidence suggesting they are general stimulators of sodium pumping (Charney et al, 1974, Kaji et al, 1981). Ng et al (1985b) reported stimulation of leucocyte sodium pumping (measured as Na-K-ATPase activity) by the addition of dexamethasone to the fluid in which the cells were incubating. Elevated endogenous cortisol levels were associated with increased osNERC and decreased Na_{WBC} (Ng et al, 1988).

In conclusion, there have been no studies which define normal biological variation of leucocyte cation content and transport. Although there is evidence suggesting that sex and age may have some effect this comes from studies not designed to look specifically at these questions. There is conflicting evidence regarding the very important question of whether diet and time in relation to meals have any effects and no evidence regarding other possible sources of variation such as the time of day. (The recent findings of effects of endogenous glucocorticoids make a diurnal variation in sodium transport possible).

There is reasonable evidence that some drugs, particularly thiazide diuretics and verapamil, alter sodium transport but it is not clear if this is a non-specific effect or one confined to restoring

abnormal values in specific disease states.

LEUCOCYTE CATION CONTENT AND TRANSPORT IN DISEASE STATES OTHER THAN URAEMIA.

Leucocytes have been used to investigate possible abnormalities of intracellular cation content and transport in several disease states other than uraemia. Without doubt the most extensive work has been in the field of essential hypertension.

Essential hypertension.

Abnormal intracellular sodium handling has long been considered potentially important in the pathogenesis of hypertension and after finding that Na_{WBC} was particularly high in hypertensive uraemic patients, Edmondson et al (1975b) measured Na_{WBC} and osNERC in 17 patients with essential hypertension, comparing the results with 17 age and sex matched controls. Na_{WBC} was increased by an average of 29% and osNERC decreased by an average of 30% in the hypertensive group. Although these findings suggested that essential hypertension was related to sodium pump inhibition and raised intracellular sodium content,no correlation existed between Na_{WBC} and osNERC, or between either of these and blood pressure. Poston et al (1981b) used similar methods in 9 essential hypertensive patients and found a remarkable average increase in Na_{WBC} of nearly 100%, osNERC being about 70% of normal. In this study, cells from normal controls were also incubated in serum from other controls or hypertensives and the hypertensive serum reduced osNERC to similar values to those in cells from hypertensive patients. No results were presented concerning the effect of such incubations on Na_{WBC}. The same types of incubation produced no reduction in red cell sodium pump activity. No correlation between blood pressure and Na_{WBC} or osNERC was reported.

Evidence that these abnormalities were more directly linked to blood

pressure was presented by Gray et al (1986) who incubated healthy leucocytes in serum from hypertensive patients. They found that the inhibitory effect of serum on control cells' osNERC correlated directly with osNERC in the same patient's own leucocytes and that the magnitude of this correlated with diastolic blood pressure. They also demonstrated a correlation between the ability of serum to raise Na_{WBC} in normal cells and the diastolic blood pressure of the patient from whom the serum was derived.

Taken together these three reports would seem to provide convincing evidence that a serum factor is present in patients with essential hypertension, in proportion to the magnitude of the blood pressure, which decreases sodium pump activity and raises intracellular sodium. These results were considered indirect evidence for the theories of de Wardener and MacGregor (1980) and Blaustein (1977) who proposed that essential hypertension was caused by a circulating sodium pump inhibitor. This inhibitor, by raising intracellular sodium in vascular smooth muscle cells would in turn raise intracellular calcium, contractility, peripheral resistance and therefore blood pressure. Leucocyte studies from other workers have not substantiated these findings however. Heagerty et al (1982) found the lowering of osNERC to be present in normotensive relatives of hypertensive patients, and therefore divorced the sodium pump abnormality from the presence of hypertension, suggesting that inhibition of sodium pumping was not mechanistically important in raising blood pressure. The magnitude of abnormalities found by these workers was considerably less than those described above and indeed when Heagerty finally collated all his results (Heagerty, 1986) the differences in sodium content and transport between hypertensives and controls did

not reach statistical significance.

These extremely discrepant findings are not easily resolved and the problems are described in review articles by Swales (1983), Weissberg (1983), Hilton (1986) and Bing et al (1986). Detailed discussion of this subject is not relevant to this thesis but an interesting view of the question of intracellular sodium in hypertension was given by Friedman (1983), who pointed out that in animal models of hypertension, sodium transport was almost invariably increased and sodium content sometimes lowered, because of the sodium pump stimulatory activity of aldosterone and glucocorticoids. He suggested that the presence of a sodium pump inhibitor was not causal in essential hypertension but rather released as a homeostatic response to the elevated blood pressure. A recent review (Simon, 1989) has suggested that intracellular sodium is not raised in essential hypertension. The raised sodium found by some workers is attributed to abnormal membrane permeability which causes sodium to rise more than in normal cells during experimental preparation.

Liver disease.

Severe liver disease has been reported to alter leucocyte cation transport. Alam et al (1977) reported up to threefold increases in Na_{WBC} in patients with fulminant hepatic failure and confirmed these findings in a later paper (Alam et al 1978) and also found greatly reduced values for osNERC. The authors themselves in the latter paper pointed out that calculated sodium efflux was nearly twice sodium influx. This imbalance effectively invalidates the measurement of either rate constant or sodium content (or both) - the net rate of sodium efflux, if continued for 45 minutes, would have removed all sodium from the leucocytes, and the calculation of rate constant

assumes the cells to be in steady state.

In a later study, Sewell et al (1981) investigated patients with cirrhosis and hepatic encephalopathy. This time calculated values of sodium influx and efflux rates were more closely matched. Although Na_{WBC} was nearly identical in the patients and controls, there was a significant reduction in osNERC in the patients. It must be assumed that there was therefore a matched decrease in sodium influx, and although it was suggested that the decreased osNERC reflected the accumulation of some toxic substance in these patients, it could equally well be viewed as a compensatory downregulation of sodium pump activity to compensate for decreased influx.

Thyroid and adrenal disease.

Khan and Baron (1987) studied the effect of hyper- and hypothyroidism on intracellular cation content and transport, using both erythrocytes and leucocytes. In leucocytes from hyperthyroid patients, osNERC was significantly increased without altering Na_{WBC}. In erythrocytes from the same patients osNERC was significantly reduced and Na_{RBC} raised. The erythrocytes therefore showed a typical picture of the effects of reduced sodium pump activity whereas the leucocytes showed an elevation in active sodium pumping matched by an increase in passive influx. In cases where measurements were available before and after treatment, abnormalities in both cell types returned to normal, and the opposite patterns were seen in hypothyroidism. The leucocyte abnormalities were compatible with those reported in skeletal muscle (Kjeldsen et al, 1984) and renal cortical cells (Lo and Edelman, 1976) and this suggests that the erythrocyte abnormalities reflected an atypical response to circulating factors which affected sodium transport. These results

therefore have very serious implications for the widespread use of erythrocytes as model cells with which to investigate sodium transport in other diseases.

Ng and co-workers investigated the effects of states of cortisol deficiency and excess on leucocyte sodium transport and found that in adreno-cortical insufficiency, osNERC was decreased and Na_{WBC} increased (Ng et al, 1987b), whereas in Cushing's syndrome osNERC was increased and Na_{WBC} decreased (Ng et al, 1988). In both cases, the abnormalities of leucocyte sodium transport disappeared after correction of the underlying endocrine disturbance. In the latter paper, osNERC was directly correlated with 24 hour urinary free cortisol excretion, but did not correlate with fasting plasma insulin levels. This was indirect evidence that cortisol itself may have been responsible for altering sodium pump activity. The presence of hypertension in some of the patients with Cushing's disease and increased sodium pump activity was an interesting contrast to the reports of low sodium pump activity and raised intracellular sodium in essential hypertension.

These last studies were unusual in that they appeared to show a straightforward alteration in active sodium pumping, associated with the expected changes in intracellular sodium concentration. In the other disease states studied, the abnormalities found are usually more complex and can only be explained on the basis of abnormalities in membrane influx and active sodium pumping. Possible explanations are that in these other diseases the primary abnormality is in membrane permeability and the pump changes are compensatory, that the disease causes inextricably linked alterations in both membrane structure and pumping activity (limitations of energy supply and

abnormalities in lipid metabolism are theoretical producers of such changes), or that the disease is associated with multiple metabolic disturbances which separately affect membrane structure and pump activity. The clear cut effects seen in adreno-cortical excess and insufficiency may be because these states are associated with isolated abnormalities of substances which directly regulate the size of the pool of active membrane Na-K-ATPase.

CATION CONTENT AND TRANSPORT IN URAEMIA IN CELLS OTHER THAN LEUCOCYTES.

Erythrocyte cation content and transport in uraemia.

The great majority of studies of intracellular cations in uraemia have been performed with erythrocytes, the relative ease with which they can be studied apparently outweighing the potential disadvantages. The two obvious problems are that erythrocytes, being non-nucleated, cannot be considered typical cells, and that because of the anaemia associated with renal failure, the average age of circulating red cells is different from normal, making it difficult to attribute observed differences solely to the uraemic environment.

Undialysed uraemic patients

The first report came from Welt et al (1964). They measured erythrocyte sodium (Na_{RBC}), the influx of radiolabelled potassium (^{42}K) in the presence and absence of ouabain (as indicative of sodium pump activity), membrane ATPase activity, and erythrocyte ATP content. No details about the patients were given other than that they were uraemic. 25% of them had Na_{RBC} higher than the highest control. Sodium pump activity was not measured in all patients, but in some with a high Na_{RBC} there was evidence of impaired pump function. Membrane alkali-metal sensitive ATPase activity was greatly diminished in uraemic red cell ghosts but ATP content was increased. The major problem in interpreting this study is the complete lack of patient information. Only 1 in 4 patients had raised Na_{RBC} but it was not stated if they had any distinguishing clinical features.

Villamil et al (1968) studied 37 controls, 37 uraemics and 37 patients with chronic non-uraemic debilitating disease (mostly

metastatic cancer). They measured Na_{RBC}, K_{RBC} and radioactive sodium efflux rate constant (NERC) in freshly prepared cells and also in cells kept at 4°C and cells stored cold and then rewarmed. In fresh cells there were no differences in red cell cation content between groups, but NERC was decreased in the uraemic group. In the cold or rewarmed cells Na_{RBC} was higher in the uraemic group. Cross incubations of uraemic or normal cells in normal or uraemic plasma provided no evidence for a plasma factor which altered Na_{RBC}. Although it is impossible to know the clinical significance of changes which only became apparent under such artificial conditions, the authors pointed out that the raised Na_{RBC} in the refrigerated cells was indicative of an abnormality in passive membrane leak of sodium, as active sodium efflux would not occur at 4°C. The reduction in NERC cannot be taken as a measure of sodium pump availability as the total rather than ouabain sensitive portion was measured.

Further evidence of raised Na_{RBC} in a minority of uraemic patients was provided by Smith and Welt (1970). Na_{RBC} was normally distributed in 96 controls but positively skewed in 113 uraemic patients. Again there was no suggestion of any distinguishing features in the patients with the high Na_{RBC}. In some of them, osNER and ouabain sensitive membrane ATPase were reduced. These abnormalities were reversed slowly once haemodialysis was commenced, the authors commenting that they could not discern between recovery of defective red cells or their replacement by new, healthy cells from the bone marrow. In an effort to identify a uraemic toxin responsible for these abnormalities the cells were incubated in solutions containing urea, creatine, creatinine, guanidine or guanidino-acetic acid but none had any effect on active sodium transport.

Francavilla et al (1972) measured the specific activity of Na-K-ATPase in red cells from 16 controls, 16 patients with chronic renal failure and 8 patients with acute renal failure. In each case they made a "membrane" preparation using red cell ghosts and a more highly purified "enzyme" preparation. There were no abnormalities of specific activity in any of the enzyme preparations but in 4 of the 16 "membrane" preparations in the chronic renal failure group, specific ATPase activity was markedly reduced. There were no abnormalities in the acute renal failure group. The authors postulated two explanations for their findings - that the low values in the membrane preparation were due to an inhibitor which was removed from the enzyme in the more purified preparation, or that in uraemia, some patients had a decreased amount of normally functioning membrane.

Cole (1973) studied 20 patients with chronic renal disease of varying severity (serum creatinine concentration ranged from 3.0 to 14.2 mg/100ml, i.e. 280 to 1300 umol/l approx.) and compared them with 20 controls. As in most of these studies no attempt was made to match the age of the patients and controls. The mean Na_{RBC} was significantly higher in the uraemic group but this difference was entirely dependent on three patients with extremely high Na_{RBC}. Membrane ouabain sensitive ATPase was measured in all patients, in each case being expressed as a fraction of the value in control cells in the same experiment. The mean activity in the uraemic cells was 0.62 of that in control cells, and in 19 of the 20 the uraemic value was less than the mean control value. The three patients with very high Na_{RBC} had marked reductions in ouabain sensitive ATPase activity, but there was no comment on any possible correlation in the

whole group between Na_{RBC} and ATPase activity. No correlation was found between ATPase activity and various biochemical indices of the severity of the uraemia or drug treatment but low Na_{RBC} was associated with low CO_2 combining power in serum which the author suggested may have reduced passive sodium influx.

A similar spectrum of patients was studied by Kramer et al (1976). Na_{RBC} was elevated in 7 of 13 uraemic patients with serum creatinine concentrations ranging from 1.6 to 14 mg/100ml(i.e 150 to 1300 umol/l approx.), with no correlation between the former and the latter. ^{22}Na efflux rate and rate constant were reduced in the uraemic group and these abnormalities could be reversed by incubation in normal plasma, or induced in normal cells by incubation in uraemic plasma. Assuming the cells to have been in a steady state, these abnormalities were indicative not only of decreased active sodium pumping but also of decreased passive sodium influx. Specific Na-K-ATPase activity was slightly decreased in 6 of 16 uraemics but when enzyme kinetic studies were performed the affinity constant of the reaction was increased in 11 of the 16 which the authors interpreted as being compatible with the presence of a competitive inhibitor. The values for this constant correlated positively with serum creatinine concentrations. The authors found elevated ATP levels in the uraemic cells, perhaps the most consistent abnormality in the many studies of uraemic erythrocytes, and suggested that they may have reflected decreased utilisation.

A review of studies of cation transport in uraemia (Mansell and Grimes, 1979) stressed the superficially similar findings from various centres without trying to resolve any of the differences. They drew useful attention to the gradual decrease in most enzyme

activities as red cells age and the consequent difficulties in interpreting abnormalities due to the haemolytic component of uraemic anaemia which results in decreased average cell age. They commented on the specialised and simplified metabolism of red cells and suggested that white cells might be more predictable in their responses.

Sigstrom (1981) studied uraemic children and although they were divided into small subgroups he drew some interesting conclusions. Na-K-ATPase activity did not correlate with serum creatinine concentration but, for a given creatinine level, tended to be lower in children with slowly rather than rapidly progressive disease. In patients followed longitudinally, enzyme activity fell as creatinine rose. Significant falls in activity were only seen in the late stages of uraemia, and in some patients they were not mirrored by appropriate changes in Na_{RBC}, suggesting that passive sodium influx was also altered. Haemodialysis acutely increased Na_{RBC} without altering Na-K-ATPase activity, again suggesting altered passive flux, while repeated dialysis resulted in elevation of previously low Na-K-ATPase activity. Successful renal transplantation was followed by a drop in RBCNa and rise in Na-K-ATPase activity. Steroids are known to enhance sodium pump activity (Charney et al, 1974; Kaji et al, 1981; Ng et al, 1985b and 1988) but the author suggested that as the patients were taking only small doses of steroid the changes were due to the removal of uraemic toxins. The major flaw in this report was the small number of patients- 15 in all, with only three or four in the various treatment subgroups rendering the information largely anecdotal.

Cumberbatch and Morgan (1981) gave a lucid account of the

implications of abnormalities of the various measurements of cation transport. In addition to using radioactive tracer methods, they measured the change in Na_{RBC} after the addition of ouabain and by timing this could calculate flux rate and therefore rate constant. The two methods gave similar results. In 16 "healthy" uraemic out-patients, all of whom were taking diuretics and whose serum creatinine averaged 578umol/l, they found a diminution in mean Na_{RBC} associated with decreased flux rate and a normal rate constant. It is unlikely that this finding, unusual at that time, was due to their different methods, for in patients with hypokalaemia or on digoxin they found the theoretically expected elevation of Na_{RBC} and fall of rate constant with unchanged flux rate. They also considered the inter-relations between the various measures of cation transport and in controls found the Na_{RBC} to correlate inversely with the rate constant, which in turn correlated closely with flux rate. This second correlation explained the weak negative inverse correlation between flux rate and Na_{RBC}. The tight link between flux rate and rate constant was also found in the different patient groups studied and the authors suggested that although this could have been artefactual, it possibly hinted at the presence of a membrane structure which somehow linked passive influx and active efflux. It had previously been suggested that in healthy controls active pumping was the determinant of Na_{RBC} (Smith, 1972) but this study found a more complex relationship in that rate constant and flux rate moved up and down together over a fivefold range for rate constant, and correlated inversely to Na_{RBC}.

The same laboratory looked more closely at uraemic erythrocytes by measuring ouabain sensitive Na-K-ATPase activity and tritiated

ouabain binding capacity (OBC) as well as cation content and transport (Swaminathan et al, 1982). In 15 out-patients with chronic renal failure of similar severity to that of Cole (1973), they found no difference in mean ouabain sensitive sodium flux rate constant or Na-K-ATPase activity compared with controls. Mean Na_{RBC} was reduced in association with decreased sodium flux rate and a decreased OBC. They then split the uraemic group into those with low Na_{RBC} and those with very low Na_{RBC}. The low group had low flux rates and were more uraemic than the very low group who had normal flux rates but an elevated rate constant. It appeared therefore that as uraemia progressed a state of low Na_{RBC} secondary to increased pump activity gave way to a state of less low Na_{RBC} secondary to decreased passive sodium influx but nearly normal pump activity. These results were clearly at odds with most previous published work. The authors pointed out that the differences could not be attributed to different degrees of uraemia in other studies and suggested that the explanation might lie in differences in methodology, in particular that other studies had incubated cells in artificial media whereas they studied sodium transport in whole blood and the cells were therefore more likely to be influenced by whatever factors in uraemia altered membrane permeability and transport.

Brod et al (1984) measured Na_{RBC} and ouabain sensitive potassium influx rate in 66 patients with renal disease only 11 of whom were labelled uraemic (glomerular filtration rate <30 ml/min.). Mean active potassium influx rate (a measure of sodium pump activity) was considerably lower than in normal controls but Na_{RBC} was not different from normal. These patients therefore had low sodium pump activity and presumably also low passive sodium leak. These findings

contrasted with the presence of elevated Na_{RBC}, slightly low leak and markedly low pump activity in the non- uraemic renal disease patients. This latter combination was much more in keeping with some external inhibition of sodium pumping, whilst the results in the uraemic group suggested a compensatory reduction in pumping to prevent Na_{RBC} falling in the presence of decreased passive influx. The authors, however, felt that the changes in the uraemic group were suggestive of the presence of a circulating sodium pump inhibitor.

Studies to this point had concentrated on undialysed uraemic patients with no consensus emerging on a consistent pattern of change, and no link between those with particularly abnormal sodium content or transport and the severity of their uraemia. Because of differing patient selection and varying methodology, it is impossible to accurately summarise the findings in the literature, but Table 1 indicates the variety of findings with regard to erythrocyte sodium content.

TABLE 1. Reported abnormalities of erythrocyte sodium content in undialysed uraemic patients.

Authors	Change in Na_{RBC}
Welt et al '64	Increase
Villamil et al '68	No change
Smith & Welt '70	Increase
Cole '73	Increase
Kramer et al '76	Increase
Cumberbatch & Morgan '81	Decrease
Swaminathan et al '82	Decrease
Brod et al '84	No change
Kariya et al '86	Decrease

Patients receiving dialysis

Zannad et al (1982) assessed sodium pump activity by measuring ouabain sensitive rubidium influx in 11 established haemodialysis patients, comparing the results with age matched controls. Rubidium influx was significantly reduced in the dialysis group and this was taken as evidence for a toxic effect of uraemia on the sodium pump. As Na_{RBC} was not measured the findings could equally well have represented down-regulation of sodium pumping in response to decreased passive influx similar to the findings of Brod et al (1984). Walter et al (1983) measured both osNERC and Na_{RBC} in 21 haemodialysis patients and a group of controls but provided no details regarding the age or sex characteristics of the groups. The blood samples were taken three days post dialysis and significant elevations of Na_{RBC} and lowering of K_{RBC} and osNERC were found in

the dialysis group. OsNER was also low in this group suggesting that although pump availability was significantly reduced, there was also a less marked drop in sodium influx. In dialysis patients and controls, Na_{RBC} was inversely related to osNERC, one of the few findings common to most studies. The abnormalities in intracellular cation content and transport are some of the most marked in any studies of dialysed or undialysed uraemic patients and although the authors mentioned the presence of conflicting data from other studies they could not identify explanatory differences in methods or patient selection.

Izumo et al (1984) performed detailed studies on 13 haemodialysis patients and 24 younger controls. They measured Na_{RBC}, ouabain sensitive ^{86}Rb uptake, and ^{3}H-ouabain binding sites (OBS) before and after a four hour dialysis and also in normal cells incubated in pre- and post-dialysis serum. The number of OBS, which they took as a measure of the number of membrane sodium pumps, was not different from controls and did not change over dialysis. Rb uptake, a measure of pump activity, was decreased before dialysis and although it increased over dialysis it remained below normal. For a given number of OBS, Rb uptake was lowest before dialysis, suggesting diminished efficiency of the sodium pumps at this time. The change in Rb uptake over dialysis did not correlate with any alterations in serum biochemistry but was related to the amount of fluid removed at dialysis, and all these abnormalities were also produced in control cells incubated in uraemic plasma. These findings seemed to support the existence of a circulating inhibitor of sodium pumps, the concentration of which was related to alterations in ECF volume. However, no elevation of Na_{RBC} was found before or after dialysis and

no link could be found between Na_{RBC} and pump activity as measured by Rb uptake. The only possible explanations were that either the techniques used were insufficiently precise to record alterations in Na_{RBC} or that the alterations in pump activity were matched by alterations in passive sodium influx. If the latter were true it is possible that the primary change was in fact decreased sodium entry and the pump numbers had been down-regulated to maintain a near normal intracellular sodium. As the original theories which linked sodium pump inhibition to the pathogenesis of the uraemic state required an elevation of intracellular sodium, the failure to demonstrate this cannot be easily overlooked. A final point from this work is that if the changes in Rb uptake were due to a circulating inhibitor, it must have bound loosely to the sodium pump otherwise it would also have reduced the measured number of OBS.

A study of apparently similar patients producing quite different results was reported by Cheng et al (1984). Na_{RBC} and K_{RBC}, OBS and ^{42}K influx were measured in 45 haemodialysis patients and 32 younger controls. All samples were taken immediately before a dialysis session. Na_{RBC} was raised in 16 of the 45 dialysis patients but no differences in K_{RBC} were found. OBS were decreased in the dialysis group, and correlated inversely with Na_{RBC} in both patients and controls. Normal cells were not affected by incubation in plasma from patients with high Na_{RBC} and low OBS. Ouabain sensitive K influx rate was the same in all groups, but for a given Na_{RBC} was lower in the dialysis group. In cells loaded with sodium, maximal K influx was decreased in dialysis patients, in proportion to the decrease in OBS, i.e. K turnover per pump site was the same in controls and dialysis patients. These results suggested that pump activity was diminished

secondary to a decreased number of pump sites. Sodium influx rate was unchanged as measured by the increase in Na_{RBC} during an incubation in the presence of ouabain, the results being similar in controls and patients. When red cell age was determined by glucose-6-phosphate dehydrogenase activity, OBS fell with increasing age in controls and patients. The differences in OBS between the two groups were most marked in the youngest cells.

Serial studies were performed in some patients and produced conflicting results. In 13 dialysis patients with high Na_{RBC}, previous measurements "before the onset of end-stage renal failure" had revealed normal Na_{RBC}. In 2 dialysis patients with high Na_{RBC}, repeat estimations at a later date revealed normal Na_{RBC} and increased OBS. In 3 patients with high Na_{RBC} before the onset of dialysis, this became normal after 6 but not 3 weeks of regular dialysis. No changes in OBS or Na_{RBC} were found over a single dialysis of four hours. The authors concluded that they had found no evidence for a circulating sodium pump inhibitor, but that Na_{RBC} was raised in uraemia because of a decrease in the number of sodium pumps and this decrease was probably due to reduced synthesis of pumps in the early part of the red cell lifespan. This decreased synthesis was due to some aspect of the uraemic state and was sometimes but not always corrected by dialysis. There is no obvious explanation for the marked differences in the findings of this study and that of Izumo et al mentioned above.

Quarello et al (1985) looked at the effect of 4 hours of acetate or bicarbonate dialysis on red cell sodium content and transport in 15 patients. Na_{RBC} and K_{RBC} did not change over dialysis but osNER and osNERC both rose and the magnitude of the change correlated with the

amount of fluid removed at dialysis, and not with changes in serum creatinine, urea, sodium or potassium. They concluded that the results were compatible with but not proof of the presence of a sodium pump inhibitor released in response to alterations in volume load. In response to this paper Diez et al (1986) published their findings of reduced Na_{RBC} in 14 haemodialysis patients, secondary to decreased leak with no change in rate constant, when compared with 27 normal controls. These results are not necessarily incompatible with those of Quarello et al as the latter did not quote values for normal controls and Diez et al did not measure pump activity before and after dialysis. No data were provided to allow comparison of the patient groups and adequacy of dialysis in the two studies.

Kariya et al (1986) studied 11 undialysed patients with varying degrees of renal failure (serum creatinine ranged from 230 to 929 umol/l), 16 regular haemodialysis patients immediately before a dialysis and 16 age matched controls. All drug treatment was stopped for one week prior to the study and 6 of the undialysed and 10 of the dialysed patients were labelled as hypertensive. Na-K-ATPase inhibitory activity in plasma as well as Na_{RBC} and osNERC were measured. Plasma from both patient groups possessed significant Na-K-ATPase inhibitory activity but osNERC was normal in both groups. Na_{RBC} was decreased in the undialysed group and even more so in the dialysed group. The degree of Na-K-ATPase inhibition was related to mean blood pressure, Na_{RBC} and (inversely) osNERC in both patient groups. Arterial blood pH, which was not significantly different in the two patient groups, correlated with Na_{RBC} and this effect was not apparently mediated through altered sodium pump activity. When all the patients were split according to the presence or absence of

hypertension, the hypertensive group had greater levels of Na-K-ATPase inhibition, lower osNERC and higher Na_{RBC}. The latter was still lower than Na_{RBC} in controls, however, and the "normotensive" group had higher blood pressure than controls. These results superficially lend support to the theory that hypertension in uraemia is related to a circulating sodium pump inhibitor but the finding of subnormal Na_{RBC} implied the presence of a large alteration in passive influx and is incompatible with the theories of Blaustein and Bricker which require intracellular sodium to rise following sodium pump inhibition. The authors suggested no alternative mechanism to link decreased sodium pump activity to hypertension.

Further studies of Na_{RBC} in dialysis patients were reported by Corry et al (1986). In a mixed group of 36 CAPD and haemodialysis patients mean Na_{RBC}, K_{RBC} and osNER from sodium loaded cells (a measure of pump availability) were no different than in 29 controls. There were no differences between CAPD and haemodialysis patients, hypertensive and normotensive patients, or between pre and post dialysis samples with regard to sodium content or transport. They concluded that their results reflected adequate dialysis, and less well dialysed or undialysed uraemic patients would have raised Na_{RBC} due to decreased sodium pump activity. In an effort to integrate the findings of Izumo et al (1984) and Cheng et al (1984) they suggested that adequately dialysed patients would have normal Na efflux but diminished K influx. The implications of this are not entirely clear - if they are referring to the respective rate constants, this would require the sodium pump to be fully active in pumping sodium out but simultaneously less active at pumping potassium in to the cell. If, on the other hand, they are referring to flux rates, then, on the

basis that the cells are in steady state, the implied abnormality is actually in potassium leak out of the cell. It is also worth noting that only one of the 36 patients but 18 of 29 controls were female. In a later study from the same laboratory (Corry et al, 1987), Na_{RBC}, K_{RBC}, and ouabain sensitive sodium efflux from cells loaded to several different sodium concentrations were the same in controls and 14 haemodialysis patients. It was again suggested that this reflected optimal dialysis of these patients.

Another study of similar patients was that of Engelhardt et al (1987). They measured Na_{RBC}, K_{RBC}, and the rise in Na_{RBC} during incubation with ouabain which they called the ouabain resistant sodium uptake and can be taken as an indication of passive flux. The ratio of this to Na_{RBC} was calculated and is equivalent to osNERC or pump availability. 27 haemodialysis patients were studied before and after a dialysis session and they were split into hypertensive and normotensive groups, the former being defined as those taking antihypertensive drugs. 5 of the latter group were taking frusemide. In both groups Na_{RBC} rose significantly over dialysis, due to an increase in passive sodium influx. Na_{RBC} was lower in the hypertensives and this was due to increased active sodium efflux. The change in Na_{RBC} over dialysis was related to changes in pH, bicarbonate and base excess in presumably arterial blood in both groups and the change in sodium flux rate related to changes in pH and bicarbonate in the hypertensive group only. The change in flux rate also correlated with change in rate constant. Taking frusemide had no effect on RBC values. The finding of altered Na_{RBC} over dialysis is at odds with most of the studies mentioned above but is perhaps not surprising as previous workers had shown Na_{RBC} to

correlate with pH and bicarbonate concentrations (Cole, 1973; Wieth, 1969) and these inevitably alter during dialysis. This paper quoted mean pre and post dialysis pH values and the changes did not seem excessive. Previous reports did not give this information so it is not possible to know if the changes were of a comparable magnitude.

In a study principally designed to measure the effects of L-carnitine supplementation on erythrocyte sodium transport (Labonia et al, 1987), Na_{RBC} was raised in 8 normotensive haemodialysis patients compared with the same number of age and sex matched controls. Sodium efflux rate in the presence and absence of ouabain from cells loaded with sodium was measured as an indicator of sodium pump availability, and was decreased in the dialysis patients. The administration of carnitine increased this value without altering Na_{RBC}. The authors concluded that as improvement in sodium pump activity did not alter Na_{RBC}, sodium pump inhibition was not responsible for the original elevation in Na_{RBC}.

In a rare study of children on haemodialysis, Desanto et al (1987) found no significant abnormality of mean Na_{RBC}. In 6 patients studied before and after a dialysis session, Na_{RBC} fell in 5.

Cheng et al (1987) studied 19 patients who underwent 48 hours of peritoneal dialysis once weekly and compared them with 13 undialysed uraemic patients with similar levels of urea but lower plasma creatinine concentrations. In 38 studies Na_{RBC} rose over dialysis on 35 occasions, but K_{RBC} was unchanged. No such changes were seen in the undialysed patients measured on two occasions 48 hours apart. The only significant correlation with the change in Na_{RBC} was change in plasma potassium, and in particular fluid removal over dialysis was not related. The authors wondered if the observed changes reflected

the effect of external potassium concentration on sodium pump activity. In vitro studies suggest that at the levels of potassium present in these patients (around 4 mmol/l) large alterations in external potassium concentration would be required to alter pump activity - the sodium pump is half-maximally stimulated at external potassium concentrations of 1-2 mmol/l and is 80 to 90% stimulated at 4mmol/l. Whatever the explanation for the relationship, the results were against the presence of a sodium pump inhibitor which was removed by dialysis or the production of which was related to volume changes.

Like the results in undialysed patients, those in dialysis patients need careful interpretation and close consideration of patient selection and methodology. A simplified summary of the reported alterations in Na_{RBC} in dialysis patients is given in Table 2.

TABLE 2. Reported abnormalities of erythrocyte sodium content in dialysis patients.

Authors	Change in Na_{RBC}
Walter et al '83	Increase
Izumo et al '84	No change
Cheng et al '84	Increase
Diez et al '86	Decrease
Kariya et al '86	Decrease
Corry et al '86	No change
Corry et al '87	No change
Labonia et al '87	Increase

Other methods of assessing erythrocyte sodium content and transport

The inhibitory effect of uraemic plasma on oubain binding in normal red cells was studied by Deray et al (1986). Significant inhibition was found in the pre-dialysis plasma of patients undergoing regular haemodialysis but not in patients with renal insufficiency not on dialysis or in the post-dialysis plasma of the dialysis patients. The authors suggested that this "digitalis like" activity was present in response to hypervolaemia but gave no evidence to confirm that the pre-dialysis patients were in such a state or that the non-dialysed group were normovolaemic. It is also possible that the substance was removed by dialysis. There were no apparent differences in digitalis-like activity in anephric patients, suggesting that the digitalis-like factor was not of renal origin but as only two dialysis patients had marked digitalis-like activity, one being anephric, the data cannot be regarded as conclusive.

A recent development which may improve the reliability of intracellular ion measurements is the use of nuclear magnetic resonance (NMR) techniques. Monti et al (1986) measured Na_{RBC} in 41 haemodialysis patients and 16 controls. The values in controls were normally distributed and agreed well with those achieved by conventional methods. In the uraemic group the mean Na_{RBC} was increased but probit analysis suggested two distinct groups of roughly equal size, one with normal Na and one with raised Na. There may also have been a small third group with low Na. This was a preliminary paper which did not try to find clinical differences according to Na_{RBC}.

Discussion

More than 20 years after Welt's first description, the

abnormalities of red cell cation transport induced by uraemia are still in dispute. Virtually all possible variations of sodium content, active efflux and passive influx have been found in apparently similar groups of patients. Is it possible to integrate these disparate findings? Differences may have arisen either in patient selection or in methodology and these will be discussed in turn.

Uraemia is the end stage of a number of different diseases and it is highly likely that the type of patients studied varied significantly even though their level of renal function was roughly similar. In most of the above studies no mention is made of underlying renal disease, drug treatment (digoxin, diuretics and steroids all being potentially capable of altering sodium transport), blood pressure, state of hydration or degree of acidosis. In the case of dialysis patients the list of variables can be extended to include the duration and nature of maintenance dialysis treatment and the adequacy of dialysis with regard not only to biochemistry but also fluid balance.

In a recent review, Kaji and Kahn (1987) sought to explain some of the observed anomalies and concluded that there were a group of uraemic patients in whom cell sodium was raised as a consequence of decreased numbers of active sodium pumps with normal passive influx, and that this abnormality was corrected by maintenance dialysis. They suggested that different results from other studies were due to differing degrees of uraemia or less effective dialysis. There may well be such a group but they form a minority of patients even in most of the studies able to find such abnormalities. Although it is true that this pattern has been found in some studies where the

average degree of uraemia was greater than in other studies where no such pattern was found, it is remarkable that a correlation between the degree of biochemical uraemia and observed sodium transport abnormalities is virtually never found within individual studies. Perhaps the likeliest explanation is that red cell sodium transport, both active and passive, is affected by a wide range of factors which are likely to vary in patients with renal failure. As it is virtually impossible to control for all these factors, correlations are obscured. Generally the numbers of patients studied have been insufficient to allow multivariate analysis of the results. If it is accepted that pH (Engelhardt et al, 1987; Kariya et al, 1986; Cole, 1973), blood pressure (Engelhardt et al, 1987), degree of uraemia (Sigstrom, 1981), fluid balance (Quarello et al, 1985) and plasma potassium (Cheng et al, 1987) are all in some way associated with alterations in sodium transport then the problem of meaningfully comparing different groups of patients reported in the literature, even without considering drug treatment or disease type, becomes virtually impossible.

Consideration of methods gives further possible explanations for differing results. Age and sex matching of patients and controls has been rare, and of course such matching between patients in different studies would be purely fortuitous. Perhaps the major methodological problem has been the lack of definition of the age of the erythrocytes studied. At the end of the cell isolation procedure, centrifugation sends the oldest cells to the bottom of the tube leaving the youngest at the top. The different ages may give quite different results (Cheng et al, 1984) and no comparison of results between studies is possible without such information. In many studies

it is likely that the red cells were sampled from a mixed population rather than one stratified according to age.

Although a variety of different methods have been used to measure sodium pump activity and passive leak, it is quite possible that these have little to do with the differing results. The interpretation of these has been variable, however. Many studies have hoped to find evidence for sodium pump inhibition and the results are presented with this in mind. Abnormalities of sodium influx seem at least as common but are less often discussed.

No definite conclusions can be drawn from these studies. It seems likely that Na_{RBC} in uraemia can be altered by changes in sodium influx and efflux and that many factors alter these, the final picture in individual patients reflecting the exact state of that patient at the time of the study. The results generally have not supported the presence of a circulating sodium pump inhibitor as a standard feature of uraemia, nor have any specific uraemic toxins been implicated in altering sodium transport.

Cation content and transport in other tissues in uraemia.

Intracellular cations and the sodium pump have been studied most often in red or white blood cells in uraemia but there is some evidence for abnormalities in other tissues.

Minkoff et al (1972) measured ouabain-inhibitable Na-K-ATPase activity in brain homogenates from rats rendered symptomatically uraemic by bilateral nephrectomy and found it to be reduced when compared with sham operated animals. Such findings were absent from the study of van den Noort et al (1968), but they had left their animals for a shorter period after nephrectomy. Minkoff et al also found the Na-K-ATPase activity of normal brain homogenates to be

reduced by incubation in serum from uraemic rats.

Kramer et al (1974) rendered rats uraemic by bilateral ureteral ligation and measured Na-K-ATPase activity in jejunum, midgut, duodenum and ileum, finding it reduced in only the first two. The authors claimed that this was evidence for a state of generalised sodium pump inhibition in uraemia but could offer no explanation for the normal pump activity in duodenum and ileum.

In an extensive study, Cotton et al (1979) measured skeletal muscle cation content and transmembrane potential (Em) in dialysed and undialysed uraemic patients. In the undialysed patients, Em was low in those with a creatinine clearance of less than 6.3ml/min, and below this level Em and creatinine clearance were linearly related. In a group of patients studied before commencing and then after 7 weeks of haemodialysis initially low Em, high sodium, and low potassium content were all corrected. When dialysis hours were then reduced Em fell again. The authors pointed out that these abnormalities were present in almost all of the patients studied unlike red or white blood cell abnormalities which were frequently absent in significant numbers of patients. As no direct measurements of sodium transport were made, the findings were compatible with depressed sodium pump activity or increased passive permeability.

Bergstrom et al (1983) reported values for muscle intracellular electrolytes in large numbers of uraemic patients- 102 undialysed uraemics, 27 haemodialysis patients and 27 peritoneal dialysis patients. Intracellular sodium was markedly increased in all groups compared with controls and intracellular potassium was elevated when expressed per unit cell solids but reduced per unit intracellular water in the two dialysis groups. In all groups intracellular

potassium content correlated with plasma potassium. No measurements of cation transport were made in these studies and so no conclusions can be drawn about the cause of the observed abnormalities.

These studies serve to confirm that intracellular cation abnormalities are present in uraemia in cells other than erythrocytes and leucocytes but do little to define the reasons for these abnormalities, nor do they implicate them in producing the symptomatic disturbances of the uraemic state. It is not clear to what extent abnormalities in rats rendered acutely anephric can be extrapolated to patients with chronic renal failure. The studies did not confirm that blood cells can be taken as typical of other cells as no comparative studies were performed.

EXPERIMENTAL WORK 1

DEVELOPMENT OF IMPROVED METHODS FOR MEASURING LEUCOCYTE CATION CONTENT AND TRANSPORT

INTRODUCTION

On reviewing established methods used to measure leucocyte cation content and transport, it became apparent that the major shortcomings were the large volumes of blood required, the difficulty in measuring sodium content accurately, and the lack of evidence that internal cation concentrations were stable under experimental conditions. The latter problem probably reflected the first two, and the first two seemed likely to be related to the need to weigh the cells. These problems are discussed in detail in the review of previous work.

The initial aim was to use established methods to isolate the leucocytes but to count rather than weigh them, on the assumption that a small number of leucocytes could be counted more accurately than they could be weighed. The second aim was to develop a method for measuring net sodium movement due to the sodium pump by directly measuring changes in cell sodium content.

Counting cells would not allow estimation of intracellular water, and cation concentration per unit cell water, or per unit dry cell weight, would not be calculable. In practice, however, the measurement of cation content per cell is likely to give a similar answer (Simon, 1989). If, for example, intracellular sodium content doubled and there was no change in the concentration of other osmotically active species, this doubling would be apparent if sodium was measured per cell, or per unit cell weight. The increase in sodium concentration per unit cell water would be very slightly less because the resulting small force for an increase in intracellular osmolality would have caused water to enter the cell to keep intra- and extracellular osmolality equal. The major theoretical drawback to

the use of cell numbers is that a matched change in sodium and potassium content may represent a change per unit cell weight or a change in cell size. The change in cell size would have to be a genuine gain or loss of normal cell contents, and there is no theoretical reason to believe that such changes are likely to occur in these type of experiments. Most short term changes in cell size are changes in cell water secondary to changes in extracellular osmolality, and as all the intended work was to take place using an artificial extracellular fluid of fixed osmolality, such changes would not arise. Any concern over the confounding effect of cell size would of course only be relevant when changes in sodium and potassium content were of similar magnitude.

The other potential disadvantage of the measurement of cell content is that it limits comparison with previous reports. However, there is no consensus in previous work as to true leucocyte sodium and potassium concentration or sodium transport rates. Sodium potassium ratios and sodium efflux rate constants can be directly compared with previously reported values.

By measuring changes in cell sodium when sodium pump activity was blocked, it was hoped that the previous practice of measuring sodium efflux rate constant by a radioactive method under different experimental conditions from the measurement of intracellular sodium concentration could be avoided.

This section describes the problems that occurred in attempting to develop such methods, the ways in which they were resolved and the methods finally used. For the sake of clarity, the different parts of the method are described in the order in which they are performed rather than the order in which problems were discovered and solved.

MATERIALS AND EQUIPMENT

10 ml. plastic tubes containing lithium heparin (Laboratory Sales (UK) Ltd.)

15 ml. conical plastic centrifuge tubes (Boehringer Corporation Ltd.)

4ml. plastic tubes (Sarstedt)

Unsheathed caps for above (Teklab)

Sheathed caps for above (Sarstedt)

Plastic 1ml. Pasteur pipettes (Alpha Laboratories)

Coulter counter S plus IV

IL 943 flame photometer (Instrumentation Laboratory)

Osmometer (Roebling Automatik, Camlab)

pH meter (Radiometer Ltd.)

Coolspin centrifuge (MSE)

Minifuge T centrifuge (Heraeus)

TC199 tissue culture fluid (Northumbria Biologicals Ltd.)

Hepes buffer (Northumbria Biologicals Ltd.)

Sodium bicarbonate (BDH Chemicals)

Dextran (mol. wt. 500,000 approx.)(Sigma Chemicals)

Ethanol (BDH Chemicals)

Ouabain (Sigma Chemicals)

Concentrated TC199 solution was diluted with distilled water, with 2 grams of sodium bicarbonate and 20 millimoles of Hepes buffer added per litre of final solution. The final osmolality of this solution was 285 to 295 mosmol/kg, final sodium concentration was 135 to 145 mmol/l and final potassium concentration was 4 to 5 mmol/l. Immediately prior to use this solution was bubbled with 5% CO_2 / 95%

O_2 mixture until the pH was in the range 7.40 to 7.50. This solution is referred to as "TC 199".

"Double strength TC 199" solution with an osmolality of approx. 580 mosm./kg. was prepared by using half the volume of distilled water for dilution. This solution was always gassed before use as described for the standard TC 199 solution.

"Dextran solution" was prepared by dissolving dextran powder in TC199 to give a 6% w/v solution.

i) Isolation of leucocytes.

Venous blood was taken into lithium heparin tubes and within 15 minutes dextran solution was added to give a blood to dextran solution ratio of 6:1. Mixing was performed by several gentle inversions and the tubes were left standing at a 60° slope for 30 minutes at room temperature to allow red cells to sediment. The leucocyte-rich supernatant was then removed and spun at 250g for 3 minutes. This produced a bilayered pellet with roughly equal volumes of red cells at the bottom and white cells on top.

ii) Lysis of residual erythrocytes.

a) Initially, the red cells in the bilayered pellet were lysed by the addition of 1ml of distilled water for 10 to 12 seconds, following which isotonicity was restored by the addition of the appropriate volume of M NaCl. In the resulting suspension, white cells were counted by Coulter counter and manual counts of red and white cells were performed by staff of the haematology laboratory at Freeman Hospital on fresh wet preparations of the cell suspensions. In each case 100 consecutive cells were counted. This was done on 16 consecutive cell suspensions, and red cell numbers (as a percentage of total cells) were compared with the sodium content of the final pellet expressed as a fraction of the potassium content. (Red cell counts could not be accurately measured below levels of 20 x 10^9 cells/l by the Coulter counter, a figure close to the amount of red cells in these preparations).

Red cell count as a percentage of total cell count correlated closely with sodium potassium ratio, $r_s = 0.81$, $p < 0.001$ (Fig M1).

b) In a second experiment the supernatant obtained after dextran

sedimentation was deliberately contaminated with three different amounts of red cells, the rest of the experiment then being performed as above. Sodium potassium ratios and the degree of red cell contamination were compared.

The sodium potassium ratios (mean of four values for each level of contamination) were 2.7, 0.84 and 0.25 with red cell contamination in the final cell pellet of 95%, 50% and 30% respectively.

c) In an effort to minimise red cell contamination the method of performing the hypotonic shock was changed. The bilayered pellet was first resuspended in a small amount of residual supernatant and then 3 ml. of distilled water were added and the contents agitated for 10 to 12 seconds, before isotonicity was restored by the addition of double strength TC199. The osmolality of the TC199 was measured before this stage to allow calculation of the amount required to restore osmolality to 290 mosm/kg. Thirty consecutive samples were prepared in this fashion and the degree of red cell contamination determined by manual counting as described above.

27 samples were free of red cells, one had 2% and two 4% contamination.

iii) Preparation for incubation.

The leucocyte suspension obtained by the above method was centrifuged at 250g for 3 minutes and the supernatant discarded. Any slight rim of pink discolouration around the top of the cell pellet was removed by gentle suction and the final white cell pellet resuspended in TC199, to a cell concentration of 3 to 10 x 10^6 cells/ml. This suspension was then divided into separate tubes for incubation, with 1.3 ml. in each tube.

iv) Cell counting.

a) The initial method used for counting was to add exactly 1.3 ml. of $MgCl_2$ to each cell pellet after the first $MgCl_2$ wash and spin at the end of the incubation period (see vi, Measurement of sodium and potassium content), and then take a 200ul. aliquot for counting. When more than one incubation time was used in a single experiment, the early counting samples were kept refrigerated until the end of the experiment and then all counts were performed by Coulter counter. Cell counts were measured in this fashion after varying incubation times in seven experiments and are shown plotted against incubation time in Fig. M2. Although leucocyte suspensions from individual subjects had been divided into equal aliquots, the measured cell count varied between samples depending on the incubation time (and therefore also the time between taking a counting sample and putting it through the Coulter counter).

b) In an effort to find a stable method for counting the cells, the initial pure leucocyte suspension was split into three tubes, with 2ml in each tube. Human serum albumin solution, 0.2ml, was added to one tube at the outset. This tube, plus one of the other two, was incubated at 37°C for up to 220 minutes with occasional samples being taken directly from the tube into the Coulter counter. The third tube was kept in ice and again, occasional samples were taken and counted. This procedure was carried out on two separate cell preparations.

The cell counts in TC 199 under these conditions are shown in Fig.M3. The highest counts were from suspensions to which albumin had been added. The steps in the lines at 90 minutes for cells kept at 37°C without albumin were due to a three minute period when three consecutive counts were made without returning the cells to the

incubation block, thus allowing them to cool towards room temperature.

v) Effect of handling during incubation.

Initially all incubating tubes in an experiment were handled in the same way at a given time, but this was not standardised between experiments. Handling was intermittent and varied from a gentle resuspension of the cells to several inversions of the tubes.

a) Intracellular potassium contents (K_{WBC}) were compared after varying incubation times in three experiments where samples from two subjects, and one where samples from three subjects were being incubated simultaneously. In each experiment, all incubating tubes were handled in the same way, but no effort was made to standardise handling between experiments.

K_{WBC} during incubation in these experiments is shown in Fig.M4. Variations in K_{WBC} matched within but not between experiments.

b) To further investigate the effect of handling during incubation, leucocyte suspensions were prepared in the usual fashion using 30ml. of whole blood from each of 14 healthy volunteers. Each suspension was divided between 6 separate tubes and all were incubated at 37°C for one hour. During this hour, one pair of tubes was left untouched, one pair was gently agitated at 15 minute intervals to keep the cells in suspension, and one pair was mixed by 3 inversions of the tubes at the same time intervals. At the end of the hour the cells were washed and sodium content (Na_{WBC}) and K_{WBC} measured (see below). The statistical significance of effects of handling on cation content was determined by non-parametric analysis of variance for related samples (Friedman test). Where handling was found to have an effect, groups were directly compared by Wilcoxon matched-pairs test.

The effect of the three different types of handling on cation content are shown in Fig.M5. Na_{WBC} in all 14 subjects was lower after gentle resuspension (R) than if the cells were left undisturbed (U)($p < 0.001$). These two ways of handling had no significant effect on K_{WBC} although there was a tendency for extreme values in the undisturbed group to be closer to the mean in the gently resuspended group. Sodium potassium ratio fell in all 14 subjects after gentle resuspension, the average fall being 0.010 ($p < 0.001$).

Repeated inversion (I) also produced a significant fall in Na_{WBC} but this was associated with a similar size of fall in K_{WBC} content such that there was no change in sodium potassium ratio.

Mean values (and standard deviation) in each group are shown in Table M1.

Table M1. The effect of handling on leucocyte cation content.

	Sodium	Potassium	Ratio
Undisturbed	3.49 (0.44)	31.1 (4.3)	0.113 (0.014)
Resuspended	3.16 (0.40)	30.9 (3.2)	0.103 (0.012)
Inverted	2.83 (0.45)	26.6 (3.1)	0.108 (0.016)

All values are mean (standard deviation) in nmol/10^6 cells.

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vi) Measurement of sodium and potassium content.

At the end of the incubation period, the cell suspensions were immediately centrifuged (at 4°C) for 2 minutes at 700g. The supernatant was carefully removed by suction and the cell pellet was rapidly washed twice with 2ml of ice cold isotonic $MgCl_2$. After the final centrifugation and removal of supernatant, 1 ml of 1.5mM caesium chloride was added to the final cell pellet and this served both to lyse the cells and to act as an internal standard for the

flame photometer. After 60 minutes the cell lysate was centrifuged at 700g for 5 minutes to compact the cell debris in the bottom of the tube and leave a clear supernatant for aspiration into the flame photometer, which estimated sodium and potassium concentration in mmol/dl. From this value and the known concentration of cells in the suspension, intracellular sodium and potassium content were calculated in nmol/10^6 cells.

vii) Sodium contamination.

In initial experiments, values for sodium content in duplicate tubes were often widely disparate, although potassium contents were well matched.

a) To investigate the possibility that significant sodium contamination of the tubes was occurring during the $MgCl_2$ washes, standard caesium chloride solution was added to ten empty tubes which were stoppered, great care being taken not to touch the rim of the tube or the stopper. All stoppered tubes were shaken and the stoppers then removed from two, touched by a finger, replaced, and the tubes shaken again. The stoppers were removed from two other tubes and the contents inverted once using a finger as stopper. Sodium content of all tubes was then measured.

In the six untouched tubes, sodium concentration in every case was 0.0 mmol/dl. In the two which had been touched on the stopper, sodium concentration was 0.7 and 1.2 mmol/dl, and in the two inverted over a finger sodium concentration was 2.3 and 2.8 mmol/dl.

b) During the repeated removal and replacement of stoppers in the final washing procedures it proved impossible not to inadvertently touch the inside of the unsheathed stoppers initially used. These were replaced by sheathed stoppers which protected the inside of the

stopper from accidental handling. The insides of these stoppers were rinsed with distilled water at the first and last stages of the cell washing procedure. This method was used in 112 consecutive paired leucocyte samples and sodium contents were measured in the usual fashion.

Sodium content in 45 pairs was identical, the difference was 5% or less of the lower value in 79 pairs and greater than 10% in 8 pairs. If 10% is taken as the level representing significant sodium contamination, this occurred in 8 of 224 or 3.6% of estimations.

viii) Changes in cation content during incubation.

a) Leucocyte suspensions were made in the usual fashion from 9 volunteers, and 2 to 5 serial measurements of sodium and potassium content were made over 0 to 230 minutes after the start of incubation. All cells were gently resuspended every 20 minutes during incubation.

There was a marked fall in Na_{WBC} during the first part of the incubation (Fig.M6). Apparent constancy of Na_{WBC} was reached after 30 to 60 minutes. Changes in K_{WBC} were less marked (Fig.M7).

b) In the second part of this investigation, sodium and potassium contents were measured in cells obtained from healthy volunteers. In 16 cases there were sufficient cells to allow measurement of cation content after incubation for 65 $\pm$ 15 minutes and then 165 $\pm$ 15 minutes. In 12 cases values were obtained at only the initial time point, and in two cases at only the later time point.

Between one and three hours, mean Na_{WBC} and K_{WBC} did not alter significantly (Na_{WBC} 3.28 (SD 0.49) and 3.23 (0.45) and K_{WBC} 31.4 (2.3) and 32.4 (2.1) nmol/10^6 cells)(t-test). However, in the 16 paired samples there was a mean rise in potassium of 1.1 nmol/10^6

cells between one and three hours and this was significant (paired t-test, $p = 0.007$).

ix) Effect of centrifugation.

Leucocyte suspensions were prepared from 10 healthy volunteers in the usual fashion and split equally between four tubes prior to a one hour incubation. The cells were kept in suspension by occasional gentle agitation. At the end of the incubation two of each set of tubes were centrifuged at 200g for 3 minutes, resuspended in the supernatant and then spun again at 200g for 3 minutes. Cell washing and estimation of sodium and potassium content were then performed in the usual fashion. The washing was begun immediately after centrifugation in 7 and after a 5 minute recovery period in 3 of the pairs of tubes.

In all 10 cases, Na_{WBC} was higher in cells which had been centrifuged compared with their controls ($p < 0.001$, Fig.M8). The difference was less marked when a 5 minute recovery period had been allowed after the centrifugation. K_{WBC} was not significantly affected by centrifugation.

x) Changes in sodium content during incubation with ouabain.

For measurements of sodium transport by the ouabain-sensitive sodium pump, the rise in Na_{WBC} following addition of ouabain was measured. In order to establish a time period over which this rise was linear, leucocyte suspensions from seven subjects were prepared in the usual fashion and divided into 1.3ml aliquots. After a one hour incubation during which the cells were gently resuspended at intervals, 0.4 ml of ouabain in TC199 solution, pre-warmed to 37°C, was added to all but two of the aliquots and gently mixed, such that the final ouabain concentration was 10^{-4}M. The ouabain had been

previously dissolved in 50% ethanol. Na_{WBC} in the remaining two aliquots was determined as a baseline. At varying time intervals Na_{WBC} in the remaining aliquots was measured. The time of exposure to ouabain was considered to be from the moment of addition to the time the first centrifugation at the start of the washing procedure reached full speed.

The increase in Na_{WBC} with time was linear over the time intervals used (Fig.M9). As would be expected from blocking active sodium-potassium exchange, K_{WBC} fell during incubation with ouabain (Fig.M10).

b) Measurement of ouabain dose-response.

Leucocyte suspensions in TC 199 were prepared from 11 healthy volunteers in the normal fashion and divided into three to five pairs of tubes depending on the cell yield. (Cell counts in the TC 199 had to exceed 3 x 10^9/l to form a pellet following centrifugation which was of sufficient size to permit washing, and to release enough sodium to be measurable by the flame photometer). All tubes were incubated for one hour, and the cation content in one pair was then measured as a baseline. A ouabain/ethanol solution was prepared as described in x a) above. Successive tenfold dilutions in 0.4ml of TC199 were prepared from this. In all 11 cases, the ouabain solution was added to a pair of tubes to give the same final concentrations as in x a). The tenfold dilution was added to a further pair of tubes in seven cases, the hundredfold dilution in nine cases and the thousandfold dilution in five cases. The tubes were incubated for exactly twenty minutes and then cation content was determined. Fractional increases in Na_{WBC} from baseline were calculated.

These increases at differing concentrations of ouabain/ethanol are

shown in Fig.M11. The increases in Na_{WBC} were less with 10^{-4} M ouabain/ 170 mM ethanol than with 10^{-5} M ouabain/ 17 mM ethanol, $p < 0.01$ by Wilcoxon matched-pairs test.

c) To separate the effects of differing concentrations of ouabain and ethanol on Na_{WBC}, a similar experiment to x b) was performed. Three pairs of leucocyte suspensions were prepared from each of seven healthy volunteers. Ouabain was added to two pairs to give final concentrations of 10^{-4} and 10^{-5} M with a final ethanol concentration of 17 mM. A further three pairs of suspensions were prepared from each of four volunteers and ouabain was added to two of those pairs to give a final concentration of 10^{-4} M. The final ethanol concentration was 170 mM in one pair and 17 mM in the other. Again, the fractional increase in Na_{WBC} compared with baseline over a twenty minute incubation was calculated.

The fractional increases in Na_{WBC} with constant ethanol concentration and a tenfold difference in ouabain concentration and with constant ouabain concentration and a tenfold difference in ethanol concentration are shown in Fig.M12. The differences in increase in Na_{WBC} with constant ethanol concentration were not significant by Wilcoxon matched-pairs test. No statistical tests were performed on the small number of observations in the constant ouabain concentration group, in which Na_{WBC} increased more in each case when 17 mM was the final ethanol concentration.

d) The effect of ethanol itself on Na_{WBC} was investigated in cell preparations from 11 healthy volunteers. The experiments were similar to those described above, with ethanol added to give final concentrations of 17 mM($n = 8$), 170 mM($n = 11$) and 850 mM($n = 3$). In each case the ethanol was added in 0.4 ml of TC 199, and the

incubation continued for twenty minutes.

The measured values for Na_{WBC} are shown (Fig.M13) for control cells, and those with ethanol added to a concentration of 17 or 170 mM. Ethanol concentration had a significant effect on Na_{WBC} ($p < 0.01$, Kruskaal-Wallis non-parametric analysis of variation) and direct comparison of the effect of 170 mM ethanol with controls ($p = 0.003$) and 17 mM ethanol ($p < 0.01$) showed that the drop in Na_{WBC} in the cells exposed to the higher ethanol concentration was highly significant (Wilcoxon matched-pairs test). The addition of ethanol at a concentration of 850 mM resulted in very large and variable increases in Na_{WBC} - 3.50 to 8.80, 2.25 to 2.90 and 2.44 to 10 nmol/10^6 cells.

xi) Adequacy of cell disruption in caesium chloride.

In order to determine whether the time allowed was sufficient for all intracellular sodium and potassium to be released into the hypotonic caesium chloride, seven cell suspensions were made and washed in the usual fashion and then disrupted in caesium chloride. After 30 minutes they were centrifuged at 700g for 5 minutes and the sodium and potassium concentrations of the supernatant were determined by flame photometry. The tubes were then stoppered and left overnight. Next day they were again spun at 700g for 5 minutes and the cation concentrations of the remaining supernatant measured by flame photometry. The results are shown in Table M2.

Table M2. Changes in measured sodium and potassium concentration over 24 hours from leucocytes disrupted in hypotonic caesium chloride.

Preparation	After 30 minutes	After 24 hours	% change
	Sodium concentration (mmol/dl)		
1	2.0	2.0	0
2	1.9	1.9	0
3	2.1	2.2	+4.8
4	1.6	1.7	+6.3
5	2.9	3.0	+3.4
6	3.3	3.4	+3.0
7	4.3	4.5	+4.7
		Mean change (%)	+3.2
	Potassium concentration (mmol/dl)		
1	21.0	21.6	+2.9
2	21.3	22.0	+3.3
3	20.9	21.5	+2.9
4	20.3	21.0	+3.4
5	19.2	20.0	+4.2
6	17.7	18.3	+3.4
7	17.3	17.9	+3.5
		Mean change (%)	+3.4

VALIDATION AND REPRODUCIBILITY STUDIES.

i) Accuracy of division of cell suspension.

As all analyses were performed in duplicate in the final accepted method, an assessment of the accuracy of the division of the cell suspension was obtained by comparing the potassium content of duplicate tubes. This also included any variations in loss of cells during the washing procedure. In 112 consecutive pairs of samples, the difference in potassium content as a percentage of the lower value was 2.5% or less in 85 pairs and greater than 10% in only 2 pairs.

ii) Reproducibility of the measurement of sodium and potassium content and sodium transport.

a) Intra-assay variation.

For sodium and potassium content, this was calculated from 19 consecutive duplicate experiments, and the results are shown in Table M3.

Intra-assay coefficients of variation for the measurements of sodium flux rate and rate constant were calculated from 15 consecutive duplicate experiments (the baseline value for sodium was not duplicated, therefore when flux rates were identical, rate constants were also identical). The values are shown in Table M4.

b) Inter-assay variation.

Day-to-day variation was calculated from repeat experiments on six volunteers, the gap between experiments always being at least one week. Sodium potassium ratios are also shown because day-to-day variations in cell counting would alter sodium and potassium equally, leaving the ratio unchanged. The values and calculated inter-assay coefficients of variation are shown in Table M5.

Table M3. Duplicate measurements of Na_{WBC} and K_{WBC} from 19 consecutive experiments.

Subject	Na_{WBC}	K_{WBC}
1.	3.42, 3.08	28.82, 29.36
2.	3.45, 3.11	27.57, 27.92
3.	3.25, 3.08	32.18, 33.53
4.	3.34, 3.34	32.63, 32.78
5.	4.12, 4.22	38.11, 38.52
6.	4.12, 4.12	41.61, 40.17
7.	4.25, 4.13	30.17, 31.75
8.	3.38, 3.64	32.18, 32.75
9.	3.38, 3.38	31.46, 33.02
10.	3.26, 3.42	32.63, 33.41
11.	3.91, 4.08	33.25, 35.05
12.	4.74, 4.74	34.88, 33.58
13.	4.53, 4.33	28.94, 29.46
14.	3.00, 3.15	29.75, 29.63
15.	3.29, 3.15	33.12, 33.35
16.	3.04, 3.04	33.93, 33.93
17.	2.69, 2.69	29.75, 29.58
18.	3.43, 3.73	29.75, 29.93
19.	3.28, 3.28	26.39, 27.17
CV%	3.46	2.02

All values in nmol/10^6 cells.

Table M4. Duplicate measurements of sodium flux rate and rate constant from 15 consecutive experiments.

Subject	Flux rate	Rate constant
1.	4.01, 3.56	1.12, 1.00
2.	1.71, 1.02	0.51, 0.31
3.	2.04, 2.04	0.63, 0.63
4.	4.31, 4.68	1.03, 1.12
5.	3.56, 3.87	0.85, 0.93
6.	4.29, 3.90	1.22, 1.11
7.	2.73, 3.12	0.81, 0.92
8.	4.16, 4.16	1.25, 1.25
9.	3.68, 3.68	0.92, 0.92
10.	3.70, 4.32	0.78, 0.91
11.	3.24, 3.24	1.06, 1.06
12.	2.78, 1.92	0.86, 0.60
13.	2.45, 2.45	0.81, 0.81
14.	2.67, 3.74	0.99, 1.40
15.	4.02, 4.02	1.23, 1.23
CV%	10.4	10.7

Flux rates in nmol/10^6 cells/hour, rate constants in hour^{-1}.

Table M5. Inter-assay coefficients of variation in 6 subjects for leucocyte cation content, sodium flux rate and sodium flux rate constant.

Subject	Na_{WBC}	K_{WBC}	Ratio	Flux rate	Rate constant
1. a.	2.33	31.5	0.074	2.45	1.05
b.	2.46	34.9	0.070	2.88	1.17
2. a.	2.66	28.4	0.094	2.75	1.03
b.	2.81	30.0	0.094	3.51	1.25
3. a.	2.42	27.0	0.090	4.11	1.70
b.	2.38	31.1	0.077	4.11	1.73
4. a.	2.44	29.4	0.083	2.82	1.16
b.	2.74	31.6	0.087	2.73	1.00
5. a.	2.63	34.5	0.076	2.85	1.08
b.	2.28	31.2	0.073	2.54	1.11
6. a.	2.37	33.1	0.072	3.09	1.30
b.	2.69	32.6	0.083	3.20	1.19
CV %	6.9	6.3	7.4	8.7	7.9

Cation contents in nmol/10^6 cells, flux rates in nmol/10^6 cells/hour and rate constants in $hour^{-1}$.

c) Variation in measurement of sodium and potassium content over a prolonged time interval.

Two healthy volunteers had repeated estimations of leucocyte cation content throughout the time the work desribed in this thesis was performed. The values are shown plotted against the date of the test in Fig.M14. The vertical line represents the date at which a new jar of dextran powder was used to make up dextran solution. All

experiments before that date were performed using a single previous jar of dextran, and all experiments after used the same new jar of dextran. The changes in the two individuals were also seen in the mean (SD) values in healthy volunteers for Na_{WBC}, K_{WBC}, flux rate and rate constant which with the first and second batches of dextran were respectively, 3.28 (0.45) and 2.58 (0.31); 31.1 (2.7) and 31.5 (1.9) (all nmol/10^6 cells); 3.32 (0.94) and 3.25 (0.72) nmol/10^6 cells/hour and 1.02 (0.25) and 1.26 (0.25) $hour^{-1}$. There was no dextran left in the old jar to allow a direct comparison of cation contents using the two different batches of dextran.

CONCLUSIONS AND DISCUSSION

Despite intending to make relatively small alterations to a previously established method for measuring leucocyte cation content, many difficulties were encountered. In view of the variability in previously published values for leucocyte cation content, both between and within centres, it is perhaps not surprising that it was difficult to establish a reliable method. The inability to measure sodium content with reasonable accuracy may be one reason why all previous measurements of sodium flux rate constant were derived from a radioactive method which did not require measurement of internal sodium content, and there is no previous work in leucocytes with which to compare our method for calculating flux rate and rate constant. The relevance of the methodological problems discovered in this work are discussed below, with regard both to previously published work and to the development of a method for use in later work.

Lysis of residual erythrocytes.

The results clearly showed that not only was great care required to remove all erythrocytes, but that failure to do so resulted in a significant increase in the sodium potassium ratio of the final cell pellet. This was unexpected as the sodium potassium ratio of healthy red cells is less than that of leucocytes. However, the "erythrocytes" left after the hypotonic shock must have been partially disrupted and then resealed with a highly abnormal internal sodium potassium ratio of approximately 3:1. By resuspending the mixed red and white cell pellet prior to addition of distilled water, by using an adequate volume of water, and by removing all

visible traces of a red rim at the top of the white cell pellet after the hypotonic shock, it proved possible to minimise the effects of this problem. Although very occasionally up to 4% red cell contamination was present, it can be seen from Fig.M1 that contamination at this level would be unlikely to affect the measurement of sodium content.

Cell counts.

When cells were kept in $MgCl_2$ prior to counting, cell counts were not constant, but followed a pattern of an early fall followed by a gradual rise (Fig.M2). As the potassium content in each tube from an individual was constant over these time intervals, it seemed clear that this was an error in counting rather than a true change in cell numbers.

When a single counting sample was taken at the time of division of the cell suspension into individual tubes for incubation it was not technically possible to always count the samples immediately. It became apparent that the cell counts in a single tube fell very quickly at room temperature. This fall in cell count was probably due to cells starting to stick to the sides of the tube. When counts were made in TC199 with and without a small amount of albumin, the results showed that, compared with the addition of albumin, simple incubation or chilling underestimated the cell count and this gradually worsened with time (Fig.M3). The graphs also showed how quickly the counts fell as the tubes started to cool if albumin was not present, suggesting that once cells stuck to the side of the tube because of this, they remained stuck despite rewarming.

The standard method of counting used thereafter was to take a 0.5ml aliquot at the time of division of the final cell suspension

into a tube containing 0.05ml human serum albumin. This was kept at 37°C until it could be counted, which was usually within ten minutes, although as shown in Fig.M3 the counts remained constant for at least three hours. This count, corrected for the dilutional effect of the added albumin, was taken as being equal to the cell concentration in the other tubes derived from the same suspension. As the sodium potassium ratio is independent of the cell count, the observation that these ratios were no less variable than the calculated values for sodium and potassium content suggests that this was a reproducible method for counting the cells..

Effect of handling during incubation.

The matched alterations in potassium content in cell preparations incubated and handled together (Fig.M4) suggested that handling in some way altered cation content. The comparison of different types of handling (Fig.M5) showed the importance of standardising cell handling during the incubation. It is worth stressing that the inversions were not done vigorously. It seems reasonable to assume that the values in the gently resuspended cells were least prone to artefactual error. The higher sodium in the undisturbed group may have been due to the accumulation of products of metabolism or exhaustion of nutrients in the fluid surrounding the cells, both of which were remedied in the gently resuspended group. The matched drop in sodium and potassium in the inversion group possibly reflects severe membrane damage to a proportion of the cells resulting in free exchange of the contents with the magnesium chloride washing solution. Loss of cells is a less likely explanation as cell counts in suspensions handled in a similar fashion remained stable over this time period.

Sodium contamination.

At an early stage of developing the method there were occasional inexplicably high values for Na_{WBC} and therefore duplicate tubes were always used for any measurement. This confirmed that although K_{WBC} usually duplicated very well (less than 5% difference) this was not the case for Na_{WBC}. As the average sodium concentration of the 1ml samples prepared for flame photometry was 1.5 to 2.5 mmol/dl it seemed possible that the errors could have been due to sodium contamination. This could have been due to inadequate washing, contamination during the washing procedure or contamination during the addition of caesium chloride. Having shown that significant sodium contamination could be produced by touching the inside of the cap, sheathed caps which could be easily removed and replaced without risk of touching any part that could come into contact with the contents were used. Duplicate tubes were always used and the mean of the two sodium concentrations taken as the correct value unless the difference between the duplicates was greater than 10% of the lower value, in which case the lower value was taken. This method would not detect significant but roughly equal contamination in both tubes of a pair. However, using the figures obtained, the probability that both tubes of a pair would be contaminated by greater than 10% was 0.00126, or 1 in 784 pairs.

Changes in cation content during incubation.

The preparation of the leucocyte suspension for incubation was carried out at room temperature and involved considerable handling and centrifugation of the cells. It seemed likely therefore that time would be required for the cells to recover from these insults. This proved to be the case with large falls in Na_{WBC} in the first 30

minutes of incubation (Fig.M6). The Na_{WBC} in samples from healthy volunteers became stable within 30 to 60 minutes and remained so for at least three hours. The statistically significant rise in K_{WBC} in paired cell samples from the same subject at one and three hours was small, about 3% of the one hour value. It is not clear if this change represented further recovery of the cells or a gradual change induced by the incubation medium.

In view of these results, incubation times were always carefully measured and cells from normal controls left for at least 45 minutes to recover from the isolation procedure. The finding of relative stability of cation content in cells from healthy volunteers after about 45 minutes incubation did not of course necessarily imply that cells from ill patients would behave in the same fashion.

Effect of centrifugation.

The demonstrable effect of different types of cell handling on cation content raised the possibility that centrifugation of the cells would, at least temporarily, upset sodium and potassium content. As established methods for calculating sodium efflux rate constant involve measuring radiolabelled sodium content sequentially over 15 to 20 minutes immediately following centrifugation of the cells, this was an important consideration. The centrifugation rate and duration was chosen to be similar to that used in determining rate constant by the radio-labelled sodium method (Hilton and Patrick,1973). These experiments did not reveal if the effect of centrifugation on internal sodium content (Fig.M8) was due to an increase in passive sodium leak or decreased sodium pump activity. Whichever it was, during centrifugation sodium influx must have exceeded efflux, and as the cells recovered efflux must have exceeded

influx. It is not possible to say from these results whether or not the radioactive method for calculating rate constant would definitely be affected by centrifugation. If the observed change in sodium content was due to altered passive sodium influx, the measurement of rate constant would be unaffected. If, however, the centrifugation had some transient effect on the sodium pump itself, then the rate constant would be altered. As the method used in this study to determine sodium flux rate and rate constant was not complicated by problems with centrifugation, this topic was not pursued.

Changes in sodium content during incubation with ouabain.

It was decided to try to develop a non-radioactive method for the measurement of sodium pump activity both for simplicity and to avoid the potential problems associated with measuring cell transport immediately after centrifugation, an unavoidable component of the radioactive method. The method was based on that sometimes used in red cells but not previously in leucocytes. Ouabain in a quantity sufficient to block all sodium pump activity was added to a cell suspension and the consequent rise in sodium concentration measured. The rate of this rise would be due to passive and unopposed sodium influx. As previous experiments had confirmed the cells to be in steady state, this influx must have been exactly equal to sodium efflux via the sodium pump until the ouabain was added. This flux rate, divided by the baseline sodium value, would equal the sodium pump rate constant. This, of course, is the same equation which is used to calculate the flux rate from the product of the sodium concentration and the rate constant when the latter is measured by the radioactive tracer method. The method assumes that ouabain does not alter sodium influx.

To justify the use of this method, it was necessary to show that the rise in sodium content with ouabain was linear over the time period used to measure the change, and that the concentration of ouabain used had a maximal effect on the sodium pump. In previous leucocyte sodium transport experiments, the cells have been exposed to ouabain at concentrations between 2×10^{-4} and 10^{-6} M. Ouabain is not soluble in water at the higher concentrations needed to prepare solutions which, when added to cell suspensions, will give the final concentrations mentioned above, and an ethanol water mixture is needed. None of the previous reports of the use of ouabain in leucocytes have mentioned the quantity of ethanol used to dissolve the ouabain and therefore added to the cells.

Based on the results in Fig.M9. a standard 20 minute incubation in ouabain was adopted to assess sodium transport through the sodium pump. This was long enough to allow accurate measurement of both the time interval and the increase in sodium content, and short enough to lie clearly within the time of linear rise in sodium content after the addition of ouabain.

The initial attempts to construct a dose response curve for the effect of ouabain suggested that 10^{-4} M ouabain had less effect on the sodium pump than 10^{-5} or 10^{-6} M ouabain (Fig.M11). There was an obvious loss of effect at 10^{-7} M. Further experiments showed that the apparently reduced effect of the highest ouabain concentration was related not to the concentration of ouabain but to the concentration of ethanol in the cell suspensions (Fig.M12). Experiments with the addition of ethanol alone showed that at certain concentrations it resulted in a lowering of intracellular sodium (Fig.M13). In larger concentrations it had a drastic effect and caused very large rises in

intracellular sodium. As the lowering effect was of similar magnitude both in the presence and absence of ouabain, it is likely that most of the effect was due to changes in sodium influx rather than in sodium pump activity.

Adequacy of cell lysis with caesium chloride.

The consistent slight increase in measured cation content when the cells were left in caesium chloride for 24 hours may have reflected a further release of cations from the cells over this period, or some evaporation of water despite the tubes being stoppered. As the changes were slight, and as they affected sodium and potassium content equally, it was decided to measure sodium and potassium concentrations one hour after addition of caesium chloride.

Validation and reproducibility.

Most of the early reports of leucocyte sodium content and transport, including those on uraemic patients, gave no data concerning the reproducibility of their results, but the normal ranges for these measurements were large, the standard deviation frequently being 25% or more of the mean. In this work, the finding of slightly lower inter-assay than intra-assay coefficients for flux rate and rate constant probably reflected the larger numbers in the intra-assay group.

Heagerty in his thesis (1986) found intra-assay coefficients of variation of 11% for ouabain sensitive flux rate and 13.3% for ouabain sensitive flux rate constant. The values for sodium and potassium were 5% and 3.5% respectively but this only involved duplication of the handling of the final dried cell pellet. These coefficients are of a similar order to those in the work reported here.

His values for day-to-day variation in 12 subjects were 13% for sodium, 7% for potassium, 14% for ouabain-sensitive rate constant and 24 % for ouabain sensitive sodium efflux rate. These are again of a similar order to those found in this study with the exception of the flux rate, which has a larger variability due in part to having to be calculated from the sodium concentration and the rate constant. Ng et al (1988) reported, in an unspecified number of subjects, inter-assay coefficients of variation of 10% for sodium, 5% for potassium and 6% for rate constant, with no value quoted for flux rate.

The methods used in this thesis therefore gave comparable reproducibility to that of previous methods.

Comparison with previous methods and results.

The sodium potassium ratios obtained with this method are lower than all others in the literature with the exception of Ng et al (1988) who found a mean ratio of 0.084 in controls. Other reported mean ratios from controls include those of Heagerty et al (1986a) 0.12, Hilton et al (1981) 0.145 and Khan and Baron (1987) 0.21.

The results of the current studies have suggested several possible explanations for the differences between more recent results. Firstly, higher sodium potassium ratios might reflect incomplete removal of red cell ghosts. None of the papers give much detail of how the hypotonic shock was carried out. As the finding of such high sodium content in erythrocytes might not be expected, there may have been insufficiently close attention to their removal of erythrocytes. The use of smaller volumes of leucocytes in the current method may however have aggravated the problem of removing the superficial rim of "erythrocytes" left after the hypotonic shock.

The changes in Na_{WBC} induced by handling did not seem sufficiently

large to explain the wide differences in sodium potassium ratio between centres. The effect of, for instance, a shaking waterbath was not measured in this study but it is quite possible that the constant agitation produced by these could have some effect on cation content. Failure to scrupulously standardise handling within a given centre may have contributed to the very large range of values for Na_{WBC} in normal controls in some reports.

In many of the early reports of Na_{WBC}, the cells were not reported to have been given any time to recover from the isolation procedure, which was often carried out at room temperature. Too rapid analysis of the cells after isolation could well explain the very high sodium potassium ratios reported in some papers, e.g. 0.27 (Patrick and Jones, 1974), 0.22 (Forrester and Alleyne, 1981) and 0.21 (Khan and Baron, 1987). It is of particular relevance to this thesis that the papers on Na_{WBC} in uraemic patients mention no allowance for recovery after isolation at room temperature.

The likeliest explanation for the sudden shift in values for Na_{WBC} and rate constant in this work (Fig. M14) seems to have been the use of a new batch of dextran powder. Unfortunately, as the previous batch was completely used, this could not be proven beyond doubt. The two batches of dextran came from the same source (Sigma Chemicals) at a different time and had different batch numbers and slightly different average molecular weights (490,000 and 500,000). This could theoretically have had differing effects on leucocyte membrane transport, either by the slight differences in the nature of the dextran or by the presence of different membrane affecting impurities. Interestingly, a similar batch effect of dextran on leucocyte sodium content and transport has also been found by

Poston's group (L.Poston, personal communication).

There are reports in the literature that the method used to separate the leucocytes can alter the cation content and transport (Ng et al 1988, Poston et al 1982b). If this were the cause, the magnitude of the effect would be sufficient to explain differences in Na_{WBC} between the first half of this work and that of Ng, whose control sodium potassium ratios have been very similar to those found with the second batch of dextran. The use of Plasmagel rather than dextran would be a likely explanation for the slightly higher sodium potassium ratios found in Heagerty's work. All these results were expressed per unit weight of cells, but direct comparison with cation content is possible with a small number of papers where results were expressed per number of cells. Lijnen et al (1988) found K_{WBC} of a similar magnitude to values in this work (about 25 nmol/10^6 cells) but Na_{WBC} was about twice that of the current control values. Very little detail of methodology was provided but Plasmagel was probably used to separate the leucocytes. Boon et al (1985) separated lymphocytes with Ficoll/sodium diatrizoate and although sodium potassium ratio was about 0.16 the actual cation contents were tiny compared with those in this work and that of Lijnen - Na_{WBC} was 3.9 mmol/10^{12} cells - a thousandfold difference. This was perhaps more likely to have been due to a simple error in calculation rather than a methodological difference or a difference between lymphocytes and mixed leucocytes.

Comparisons with rate constants from other studies are difficult because no other workers have directly measured net sodium influx in the presence of ouabain. As some sodium pump activity results in sodium-sodium exchange which would be detected by the radio-isotope

method only, the current method might be expected to give a rather lower value for rate constant. The values obtained in this study have indeed been lower than others. The effect of not measuring sodium sodium exchange could explain the difference from the results of Heagerty's group (osNERC about 1.4 $hour^{-1}$). Other workers have reported osNERC of greater than 2 and there are no obvious explanations for this. Although centrifugation and ethanol both affected Na_{WBC} it is not clear to what extent these effects might alter rate constant as measured by the radioactive method. Because of uncertainty as to the mode of the ethanol effect and the complete lack of data in the literature concerning the concentrations of ethanol used, it is impossible to know if this could be responsible for some of the variations in rate constant between centres. It is interesting that over a relatively small range of concentration the sodium lowering effect turns into a large sodium raising one. Even if all the activity of ethanol was independent of the sodium pump, large changes in sodium influx could alter the rate constant as calculated by the radioactive method.

The effect of ethanol is also of interest in view of the association between chronic heavy alcohol consumption and hypertension (Lian, 1915; Matthews, 1976). The mechanism of action of ethanol on blood pressure has not been defined, but ethanol has been reported to alter sodium transport. Dog skeletal muscle sodium content and Na-K-ATPase activity were increased by the addition of ethanol to their diet, a change attributed to increased membrane permeability to sodium (Ferguson et al, 1981) and ethanol treated rats showed increased brain Na-K-ATPase activity (Israel et al,1970). In man, alcoholism has been associated with increased erythrocyte Na-K-ATPase activity

(Israel et al, 1970) and active sodium efflux (Gobel et al,1987) consistent with the findings in the animal studies. Green and Baron (1986) found reduced ^{22}Na efflux rate constant in leucocytes in the presence of ethanol at 80 and 120 mM but did not appear to measure sodium content when ethanol was present. Clearly the effects of a 20 minute incubation in ethanol cannot easily be extrapolated to the effects of chronic alcohol consumption, but the results in the current work suggest that studies of the effects of alcohol consumption on leucocyte sodium transport may be of interest.

Changes in leucocyte cation content and transport in individuals during prolonged follow-up.

It is clear from Fig.M14 that Na_{WBC} and sodium potassium ratio in one of the subjects were much more constant than in the other. Na_{WBC} of the less constant subject was not simply more variable but appeared to change gradually with time. Both subjects were apparently healthy throughout the period and took no medicines. Both were male. The only obvious differences were that the subject with more constant Na_{WBC} had a more constant diet (rarely, if ever, eating out, and never drinking alcohol), and took regular exercise throughout the year (cycling 8 miles to work), whereas the other subject took more exercise in the winter (football and squash) than the summer (golf), ate out once or twice a week and drank variable amounts of alcohol. Physical training has been reported to reduce Na_{RBC}, possibly by altering passive sodium influx, and also to alter plasma lipid concentrations (Hespel et al, 1988). It is impossible to identify the factors responsible for the leucocyte alterations and time did not permit further investigation of this interesting finding.

NOTE.

Some of the work described in this section has been published (Main et al, 1989) and a copy of the paper is appended at the end of the thesis.

FINAL METHOD.

The method used for investigations of leucocyte cation content and transport in uraemia was as follows.

Whole blood was taken into lithium heparin tubes and gently mixed by inversion. Within 15 minutes, 6% dextran in TC199 solution was added to give a blood to dextran solution ratio of 6 to 1, and the tubes were left for 30 minutes at room temperature to allow the erythrocytes to sediment. The leucocyte rich supernatant was then removed and spun at 250g for 3 minutes. The supernatant was discarded except for about 0.5 ml., sufficient to allow resuspension of the mixed red and white cell pellet in the bottom of the tube. Distilled water (3ml. per tube containing leucocytes from up to 12 ml. of the original leucocyte rich supernatant) was added and mixed thoroughly with the cells for 10 to 12 seconds, at which point a precise volume of approximately double strength TC 199 was added to restore osmolality to 290 mosm/kg. The cell suspension was then spun at 250g for 3 minutes, the supernatant discarded, and any visible red rim at the top of the white cell pellet removed by suction. The cell pellet was then resuspended in TC 199 and divided into separate tubes in 1.3 ml. aliquots. A 0.5 ml. aliquot of the original suspension was placed in a separate tube with 0.05 ml. of human serum albumin solution and kept at 37°C until being counted twice in a Coulter counter model S plus IV.

The tubes containing the cell suspensions were incubated at 37°C for at least 60 minutes, being gently agitated every 15 minutes. Duplicate tubes were used for any single measurement of cation content. To measure sodium transport, 0.4 ml. of ouabain dissolved in ethanol and TC 199 was added to each 1.3 ml. tube to give final

ouabain and ethanol concentrations of 10^{-4} M and 17 mM respectively. Mixing was ensured by gentle agitation at the time of addition and again after 10 minutes.

At the end of the desired incubation period, the cell suspensions were immediately centrifuged at 750g for 2 minutes at 4°C., the supernatant removed by suction, and the cells resuspended in ice cold $MgCl_2$. This washing procedure was repeated before a final centrifugation under the same conditions and removal of all supernatant. The caps of the tubes were rinsed in distilled water at the beginning and end of the washing procedure.

The cells were disrupted by the addition of 1 ml. of 1mM caesium chloride to each pellet. After one hour, the suspension was centrifuged to sediment the lysed cells and the sodium and potassium concentration of the supernatant measured by IL 943 flame photometer.

From the results, intracellular cation concentrations were calculated in nmol/10^6 cells, sodium influx rate in nmol/10^6 cells/hour and ouabain sensitive sodium flux rate constant in $hour^{-1}$.

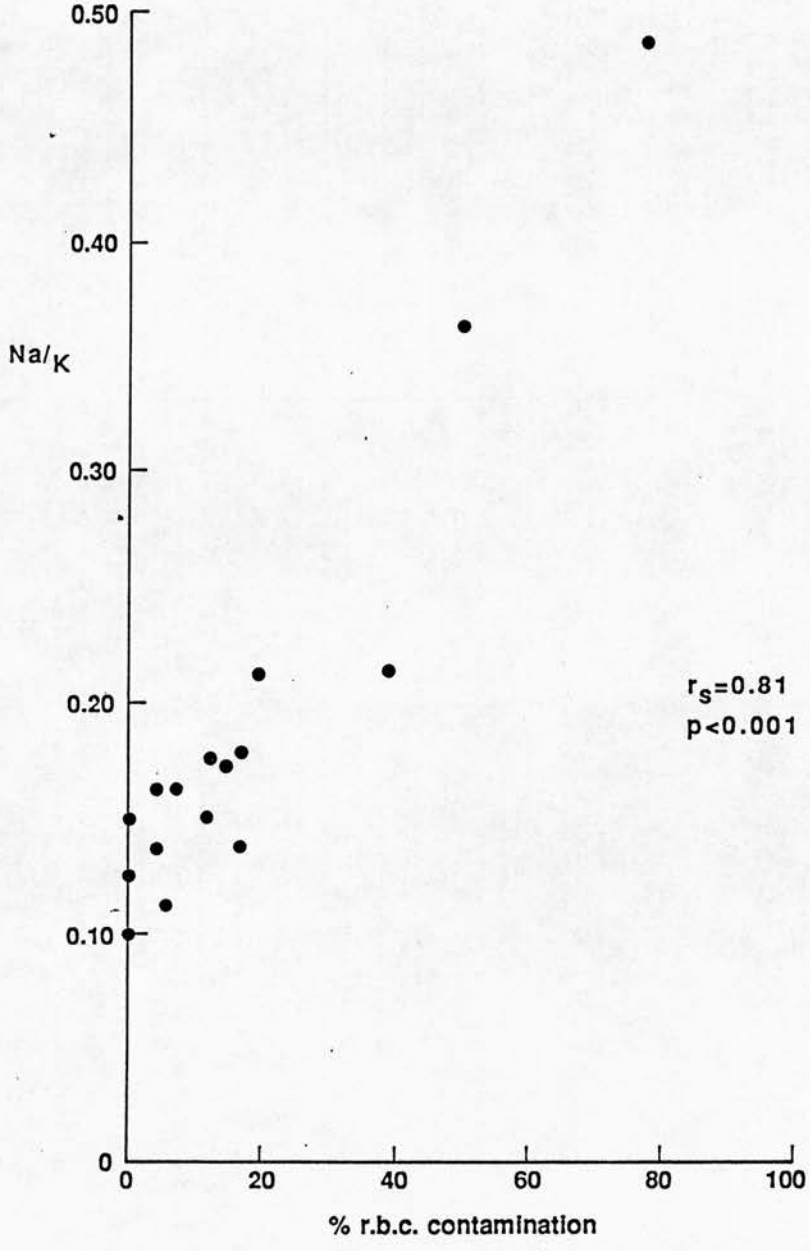

Figure M1. The effect of red cell contamination on Na/K ratio in the final cell pellet.

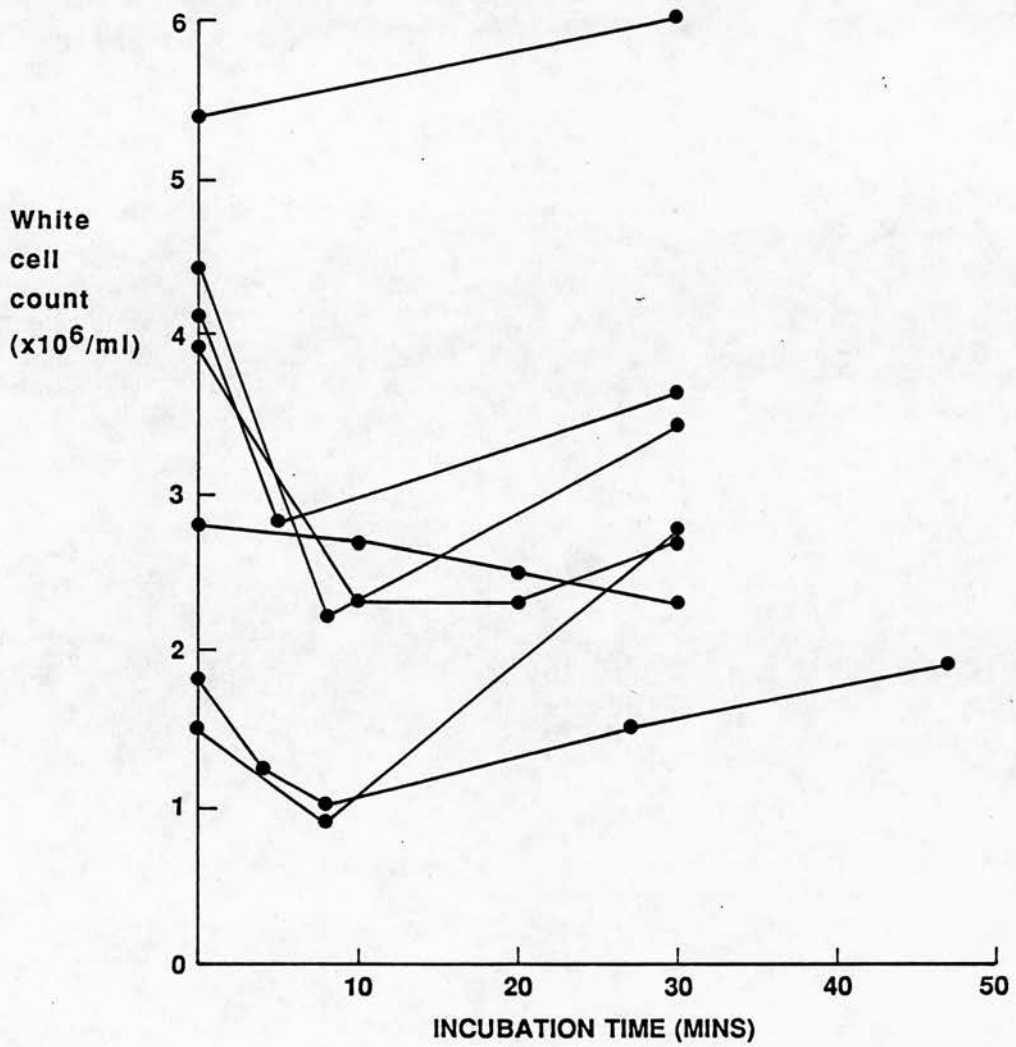

Figure M2. Effect of time of sampling on measured white cell counts. Lines join counts obtained at different times from a single cell suspension.

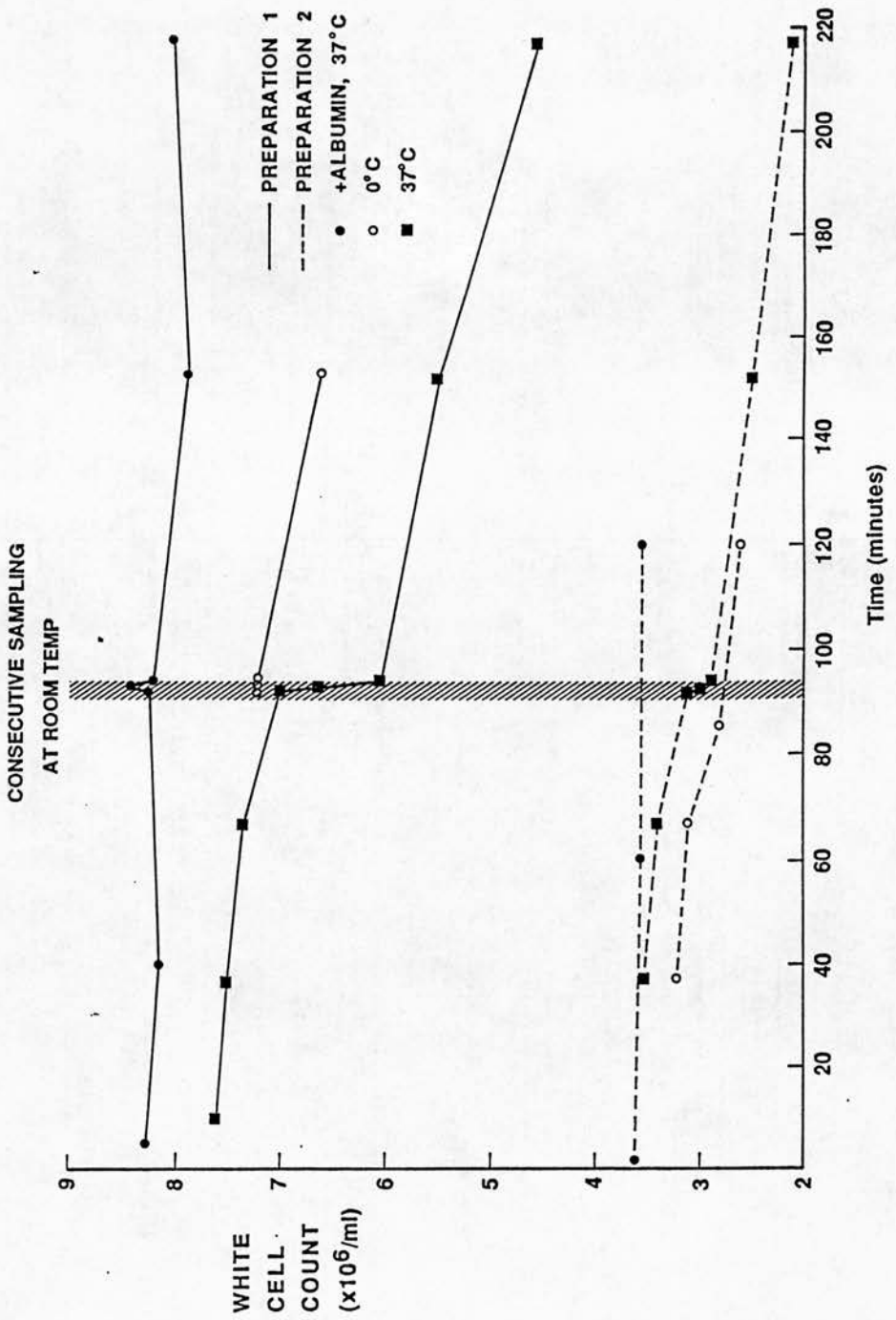

Figure M3. Effects of albumin and temperature on measured white cell count.

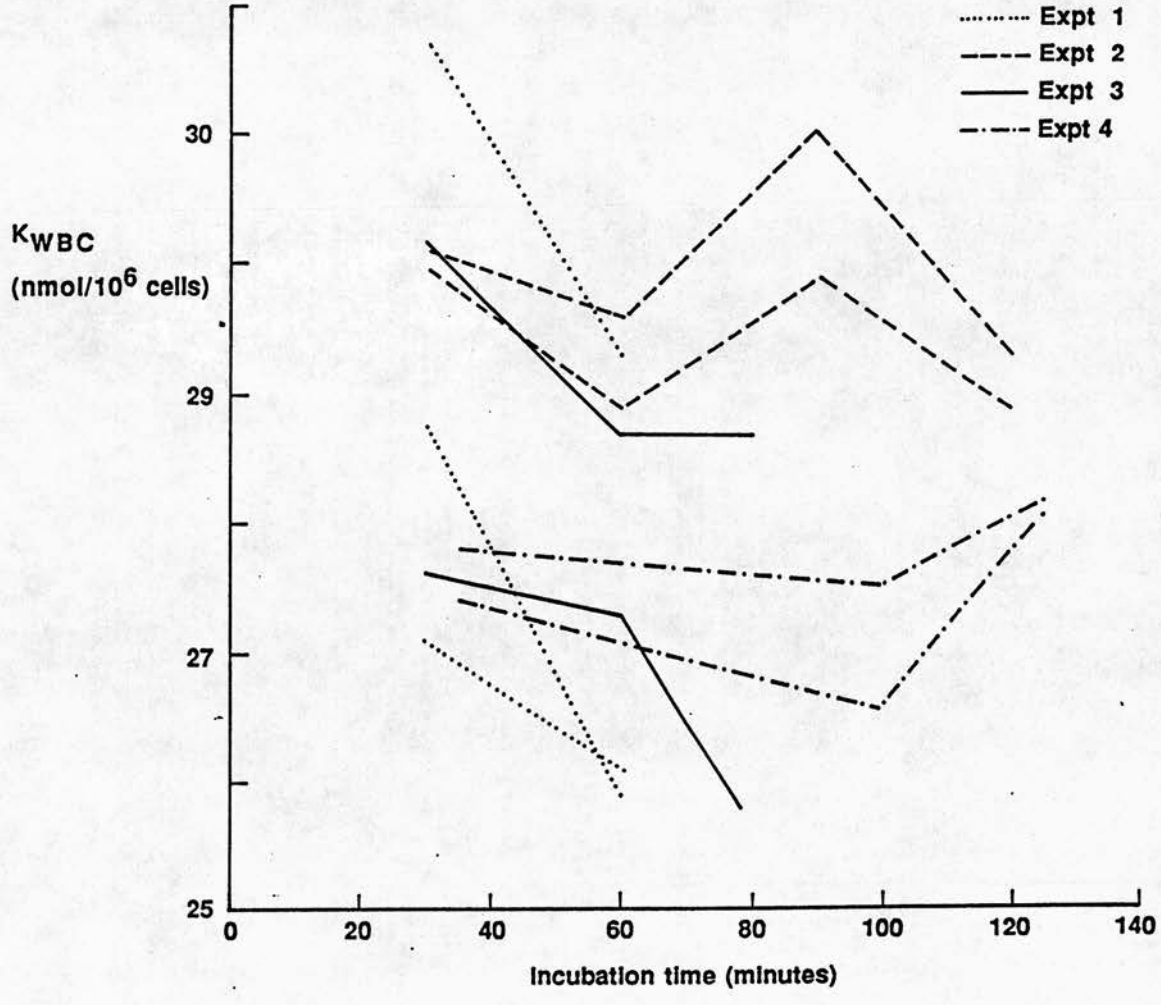

Figure M4. Variations in K_{WBC} within and between experiments.

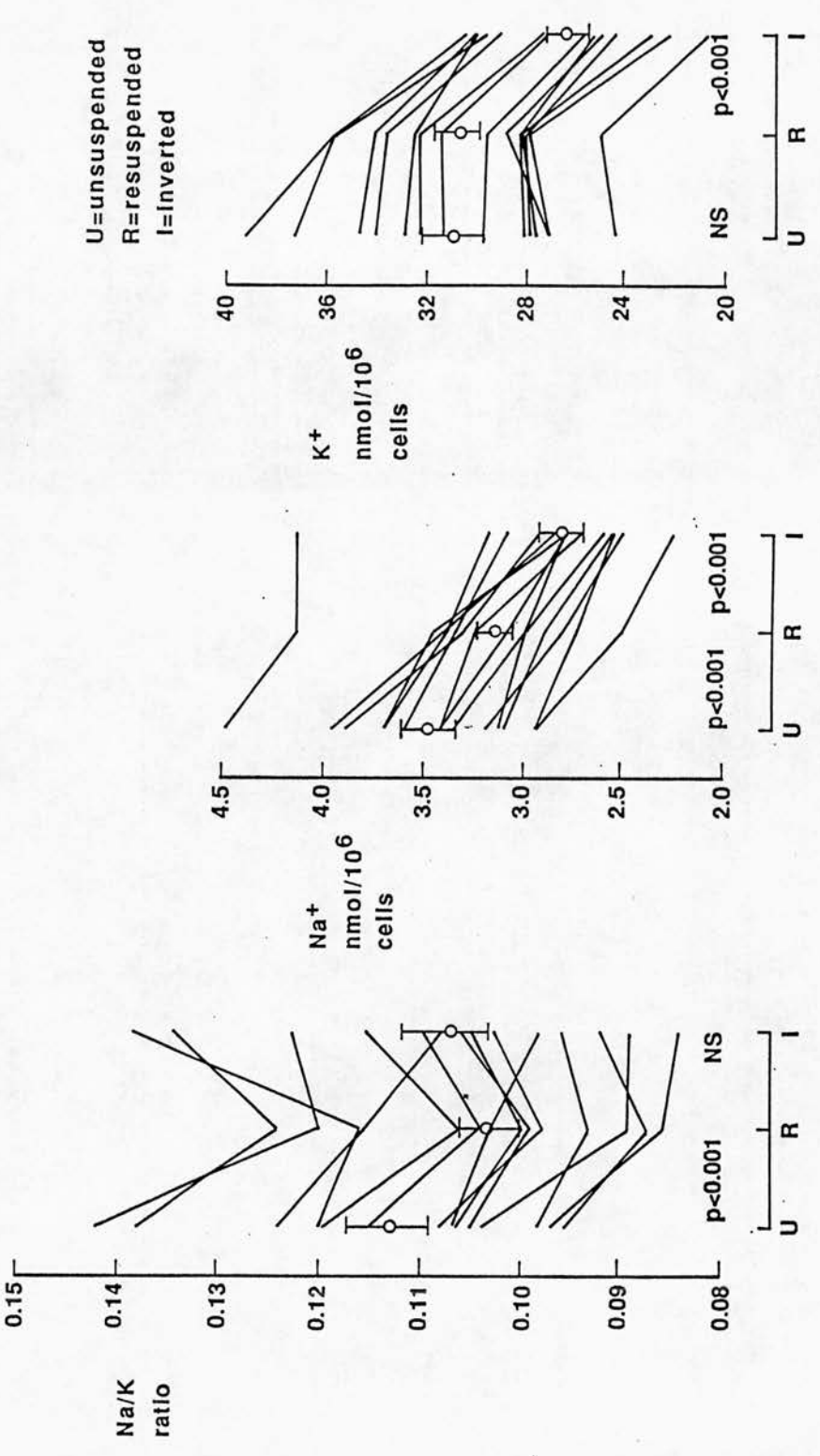

Figure M5. Effect of different handling on leucocyte cation content.

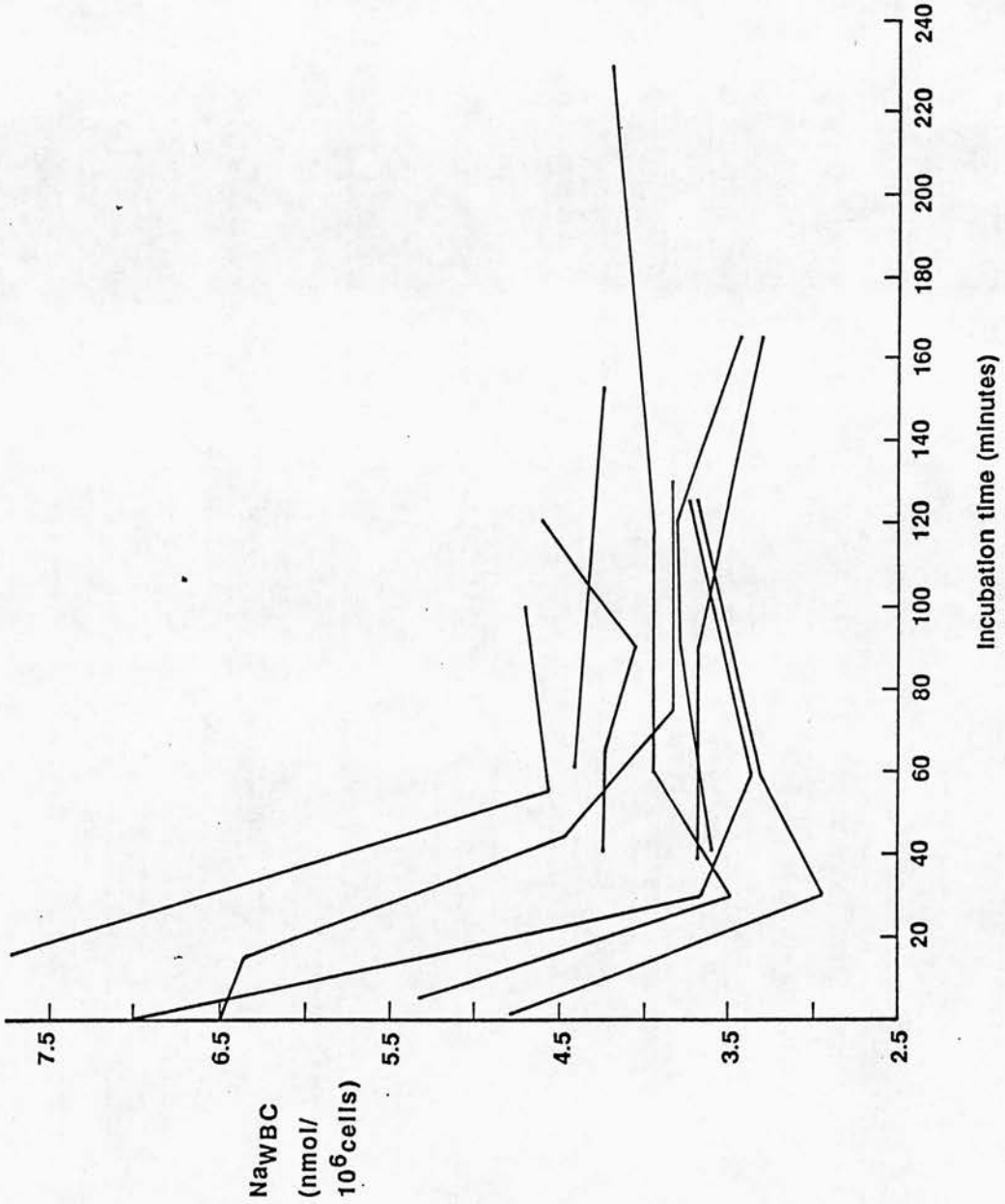

Figure M6. Effect of incubation time on Na_{WBC} (healthy volunteers).

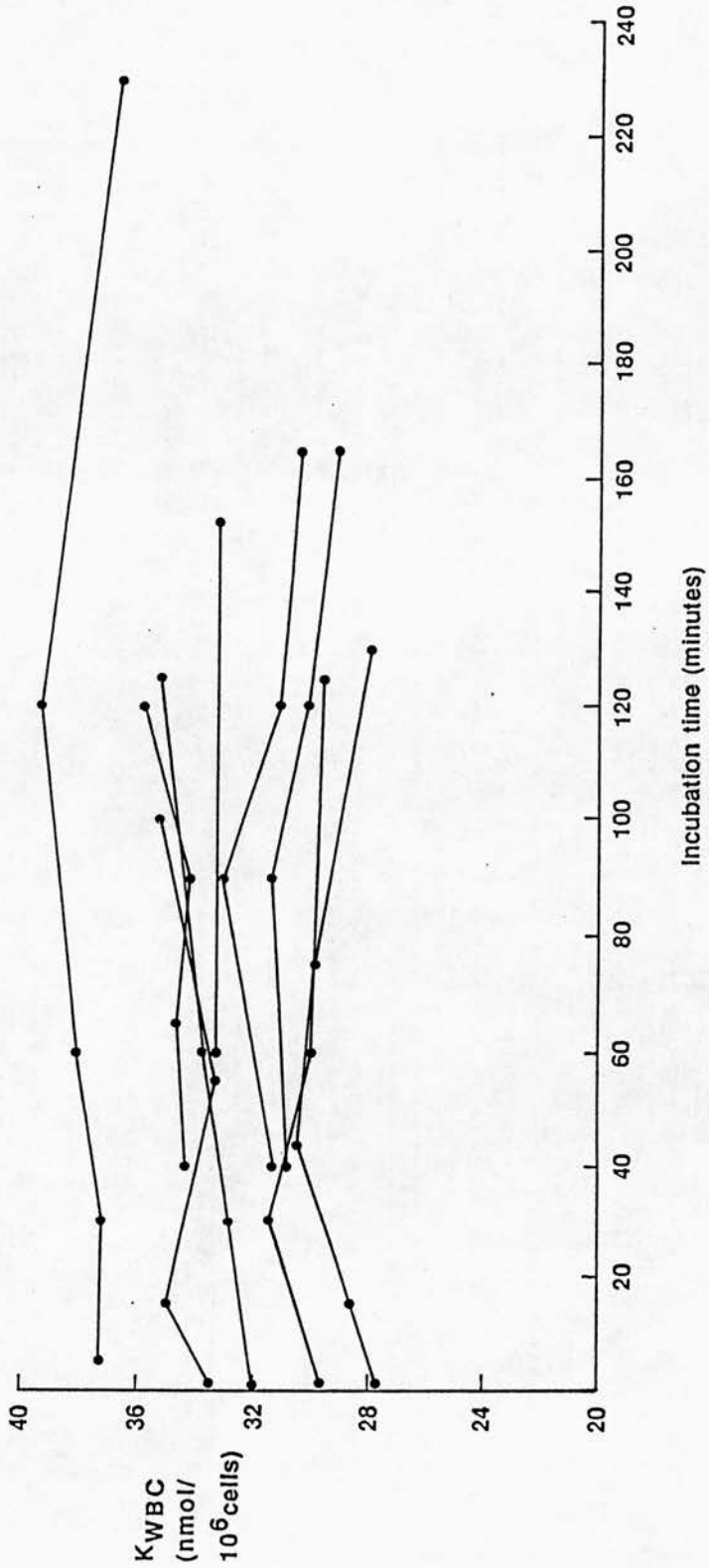

Figure M7. Effect of incubation time on K_{WBC} (healthy volunteers).

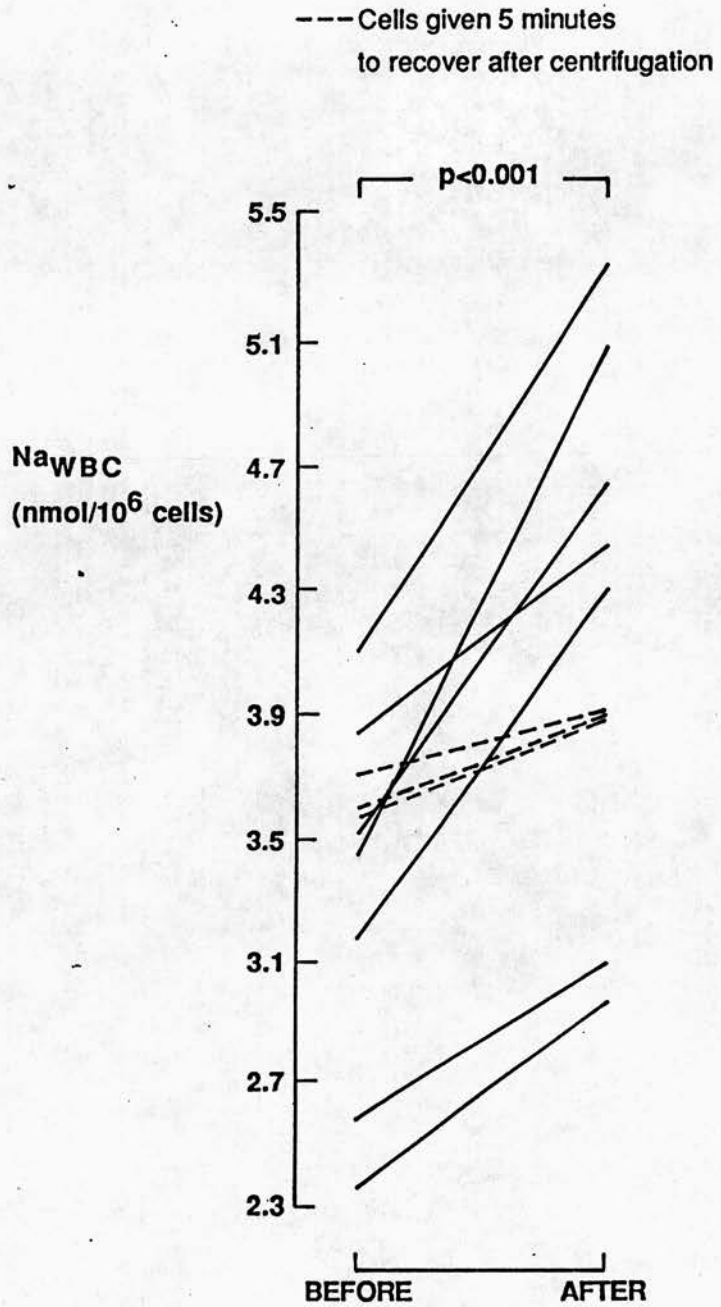

Figure M8. Effect of centrifugation on Na_{WBC}.

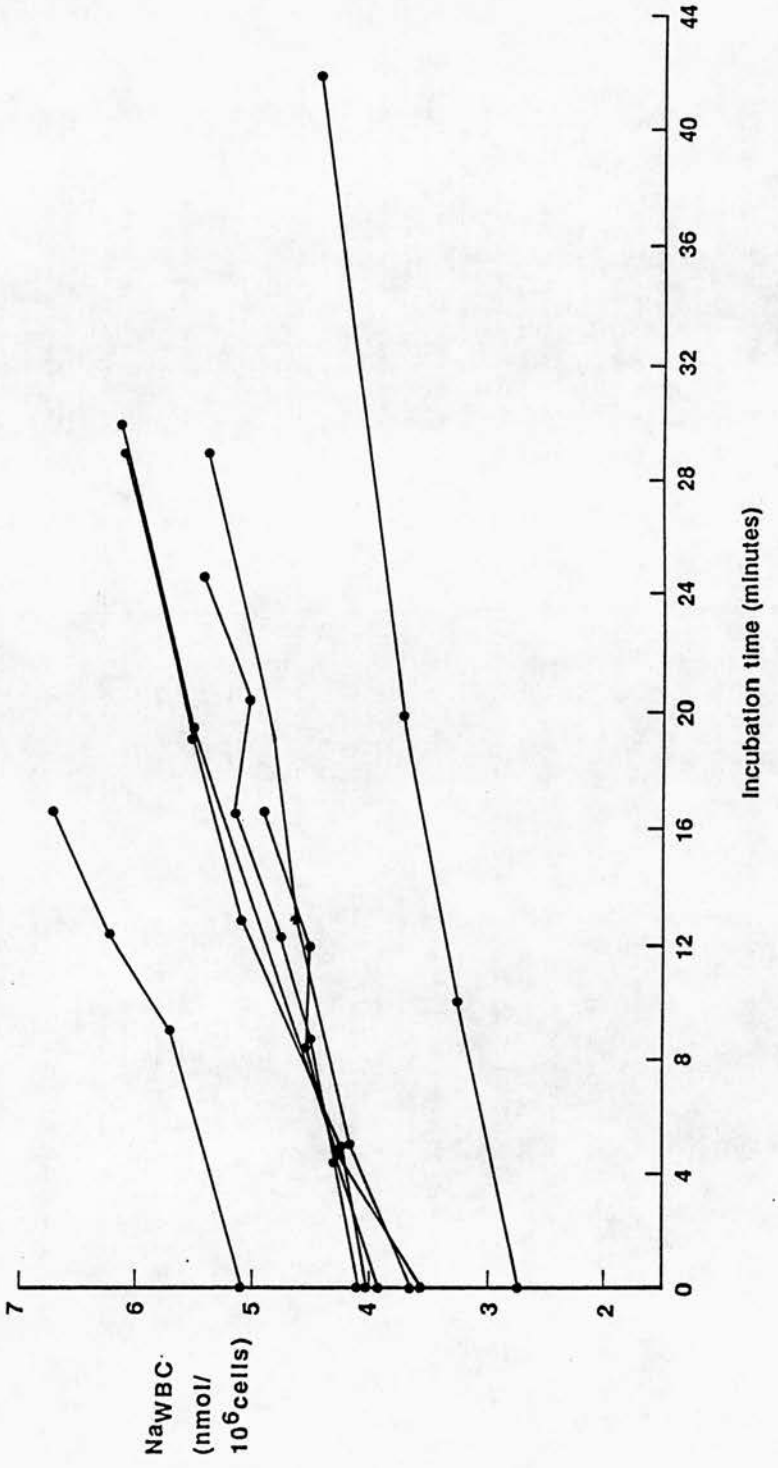

Figure M9. Rise in Na_{WBC} during incubation with ouabain (10^{-4}M).

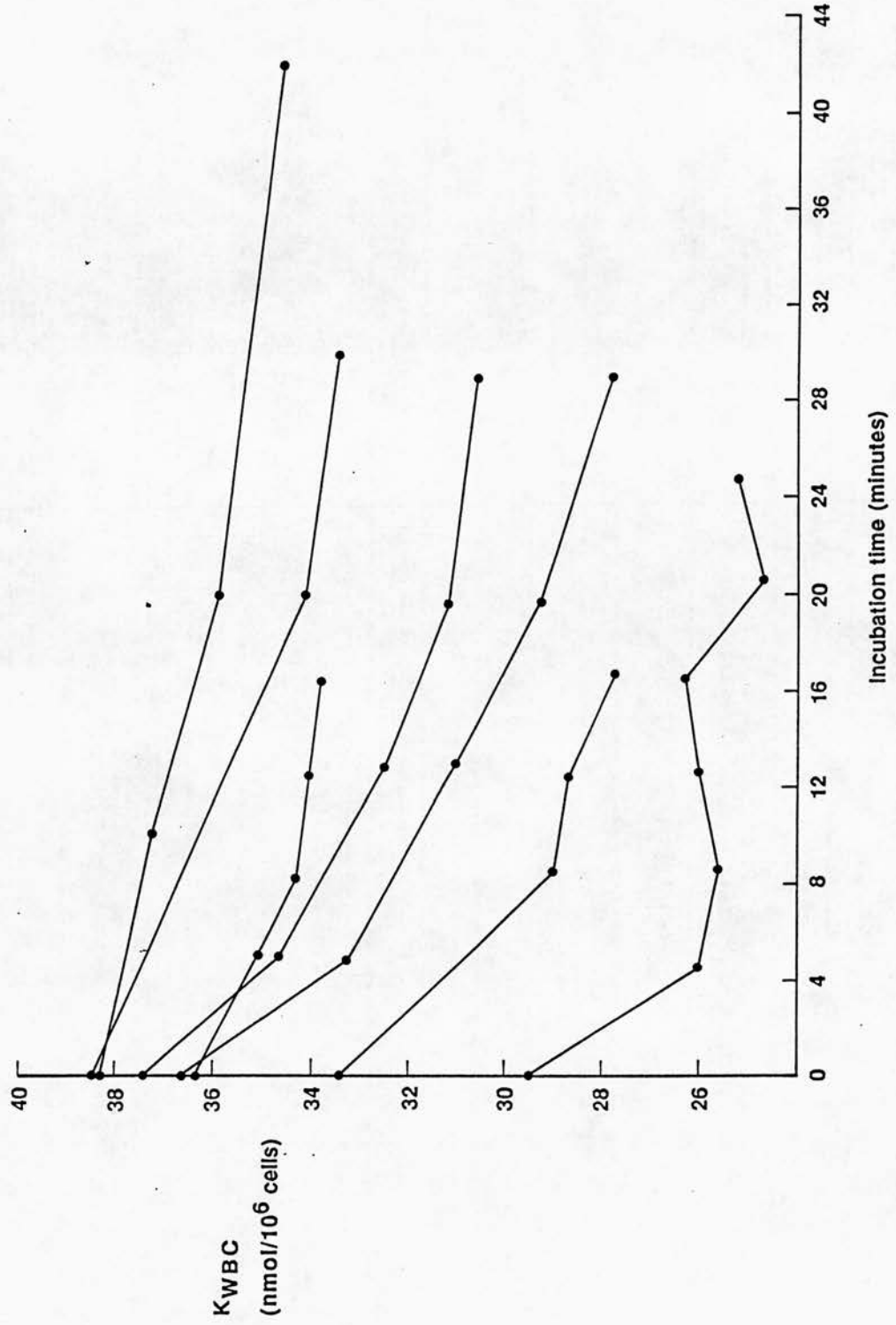

Figure M10. Fall in K_{WBC} during incubation with ouabain (10^{-4}M).

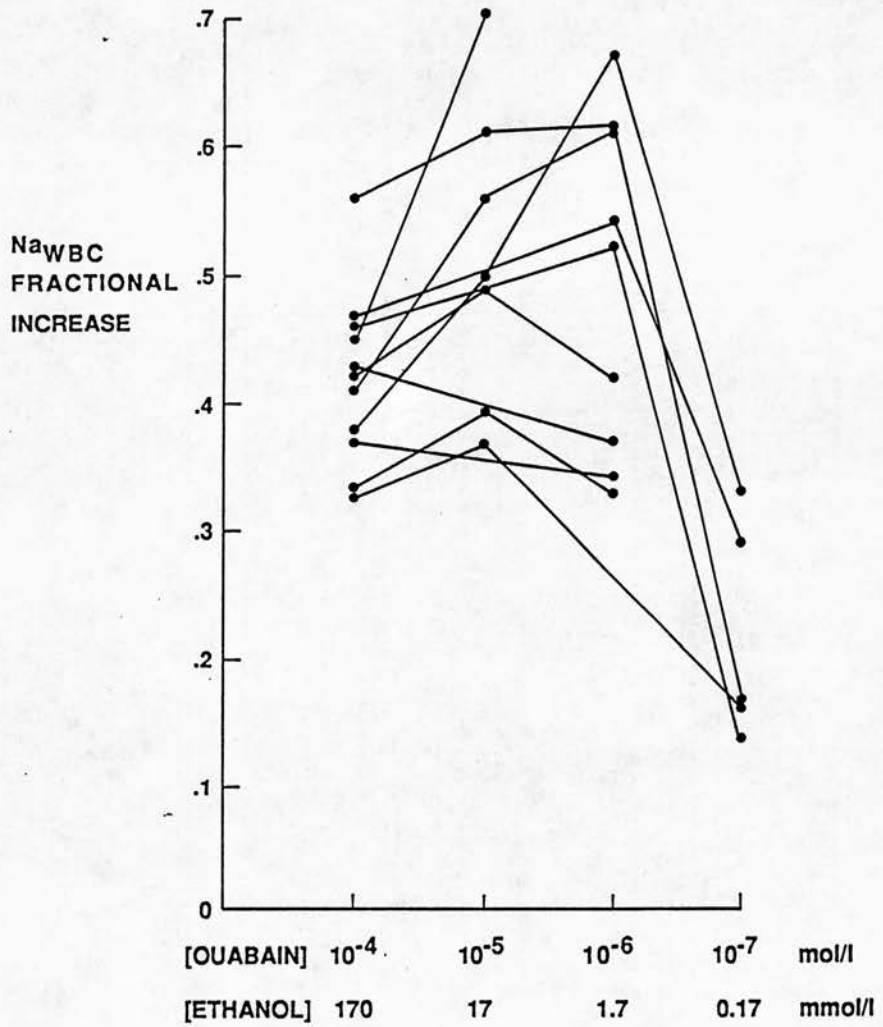

Figure M11. Increase in Na_{WBC} produced by 20 minute incubations with serial dilutions of ouabain and ethanol.

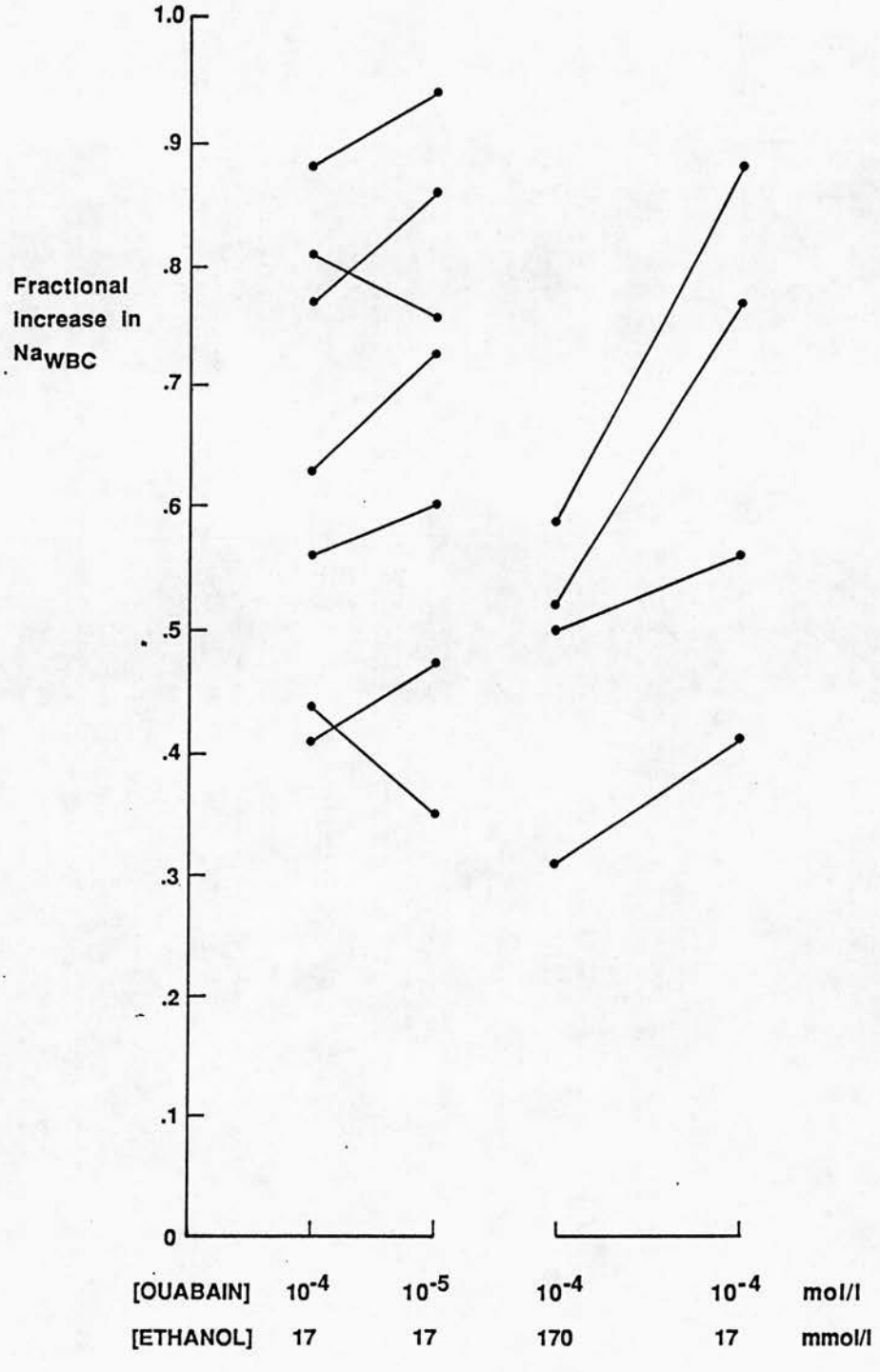

Figure M12. Increase in Na_{WBC} produced by 20 minute incubations with differing concentrations of ouabain and ethanol.

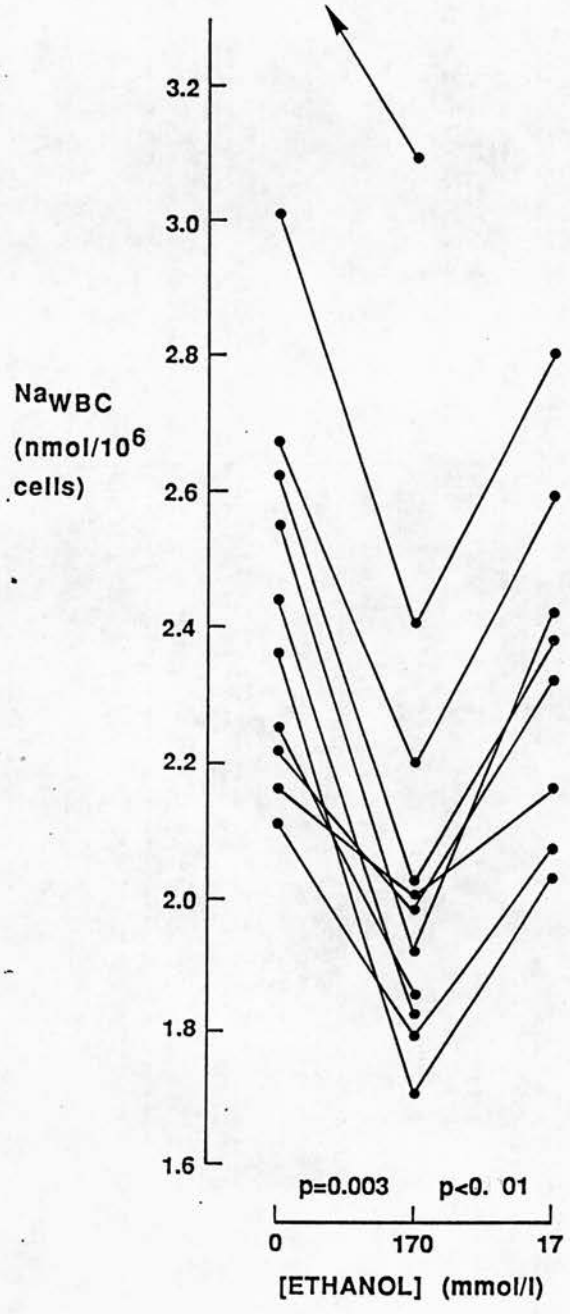

Figure M13. Effect of different concentrations of ethanol on Na_{WBC}.

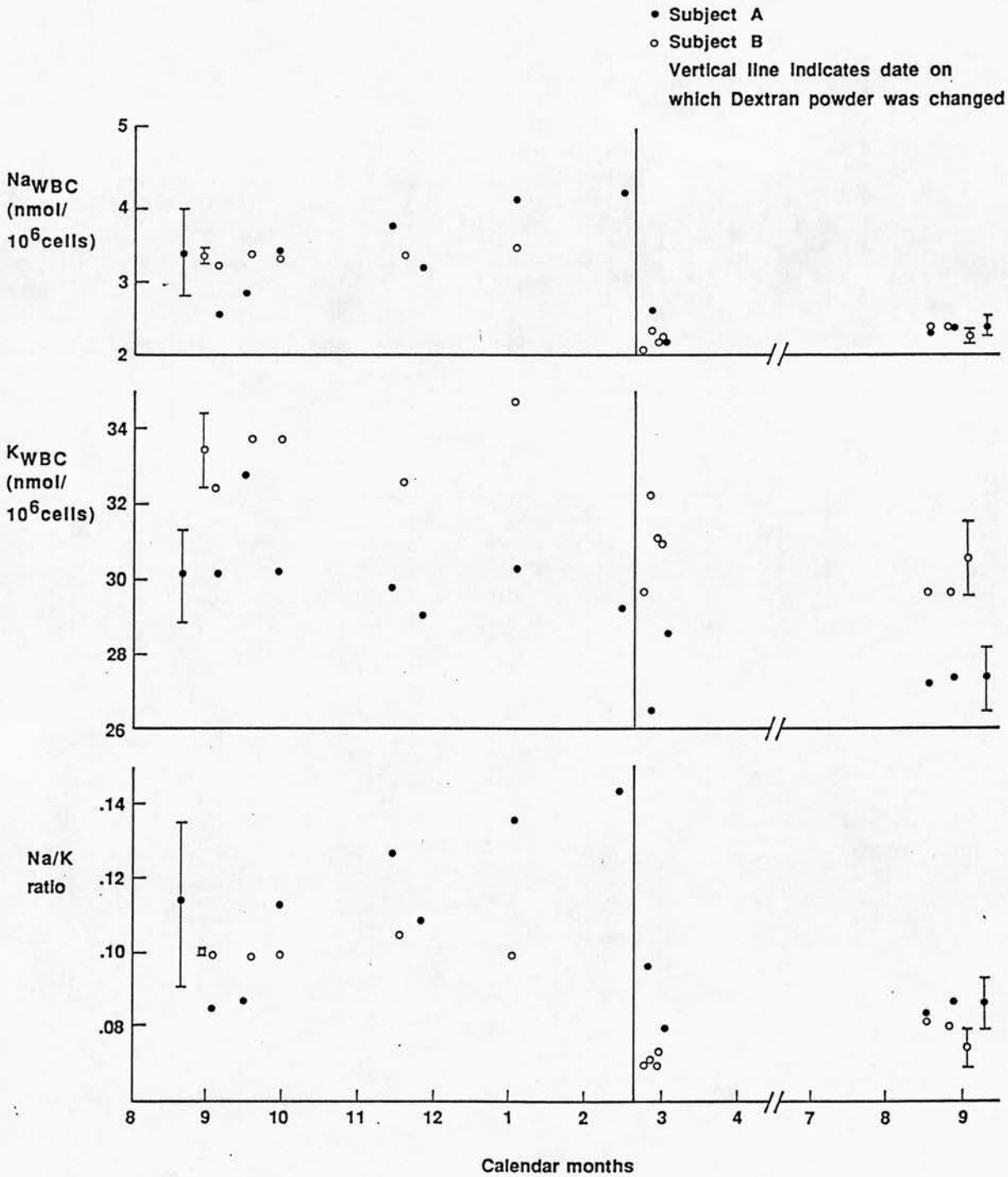

Figure M14. Repeated measurement of leucocyte cation content in 2 healthy subjects. Bars indicate mean ± 1 SD with two different batches of dextran.

EXPERIMENTAL WORK 2

STUDIES IN CHRONIC RENAL FAILURE IN MAN

PRELIMINARY STUDY: THE EFFECT OF INCUBATION ON THE CATION CONTENT OF LEUCOCYTES FROM SUBJECTS WITH CHRONIC RENAL FAILURE

Aim

As the studies done during the development of the method showed that Na_{WBC} in cells from control subjects took time to reach stability after the commencement of incubation, a similar study was required to discover how long Na_{WBC} in cells from uraemic patients took to reach stability.

Method

From 2 to 8 measurements of Na and K were made during incubations of up to 180 minutes in leucocytes obtained from 10 patients (3 female) whose ages ranged from 40 to 72. All patients had chronic renal failure with serum creatinine concentrations ranging from 400 to 1000 umol/l, but none had been dialysed. No patients were suffering from systemic infection, diabetes mellitus, uncontrolled hypertension or clinically significant fluid depletion or overload. No patients were taking steroids or digoxin but all continued on their normal medications.

Results

Na_{WBC} tended to fall steeply during the first hour of the incubation, but changes after this time were much less marked (Fig S1). Trends in K_{WBC} were less distinct, although in cases where measurements were made in the early part of the incubation, K_{WBC} invariably rose during the first hour (Fig. S2).

For statistical analysis, the values were divided into three groups:- those measured between 0 and 59 minutes (n=14), 60 and 119 minutes (n=13), and 120 to 180 minutes (n=12). By Kruskall-Wallis non-parametric analysis of variance, no difference in K_{WBC} was

evident. There was a highly significant difference in Na_{WBC} ($p<0.005$) and when groups were compared directly by Mann-Whitney U-test, the values at the earliest times were significantly higher than at the middle ($0.01<p<0.02$) or later ($p<0.01$) times. There was no significant difference in Na_{WBC} between the middle and later times.

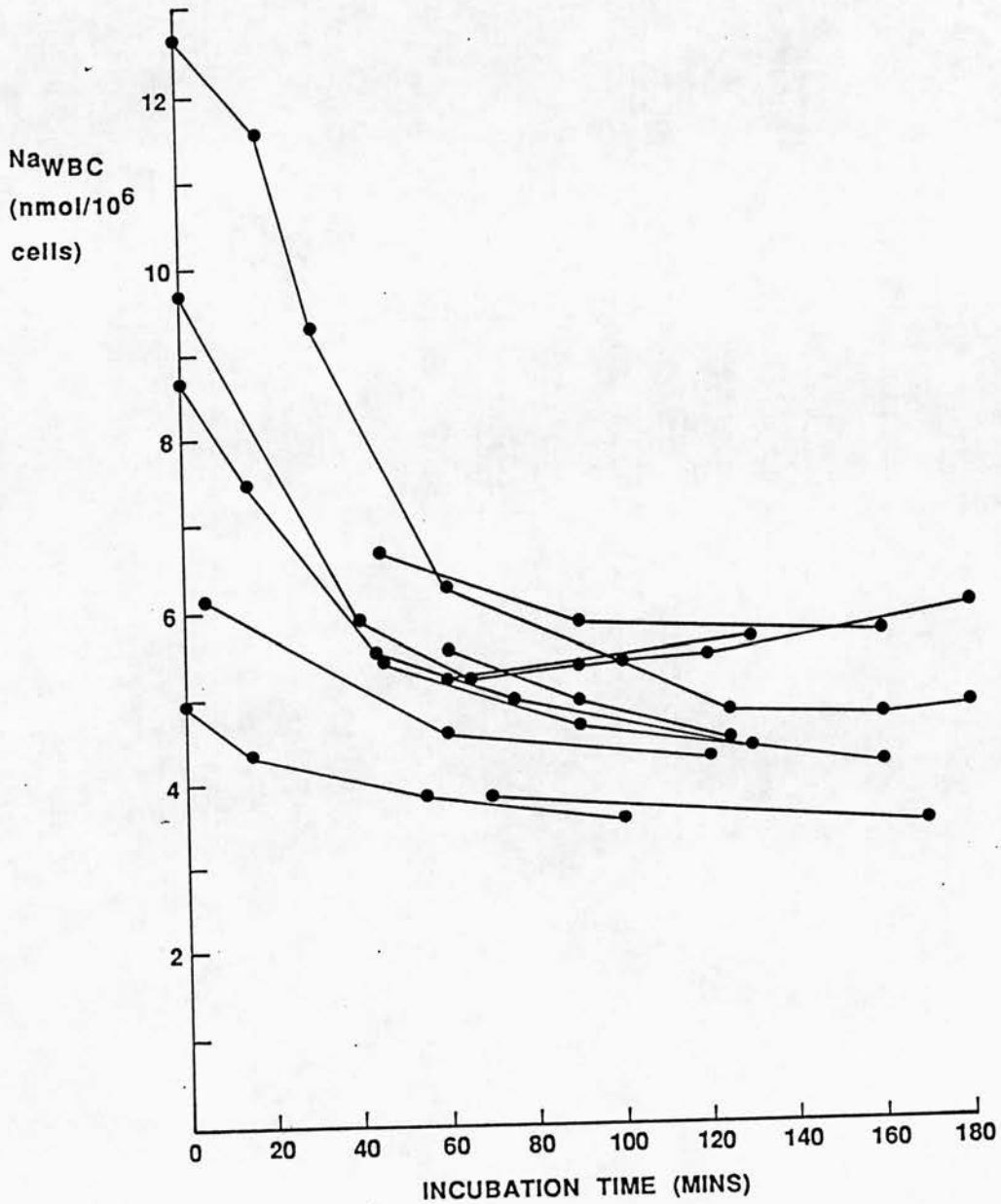

Figure S1. Effect of incubation time on Na_{WBC} in uraemic subjects.

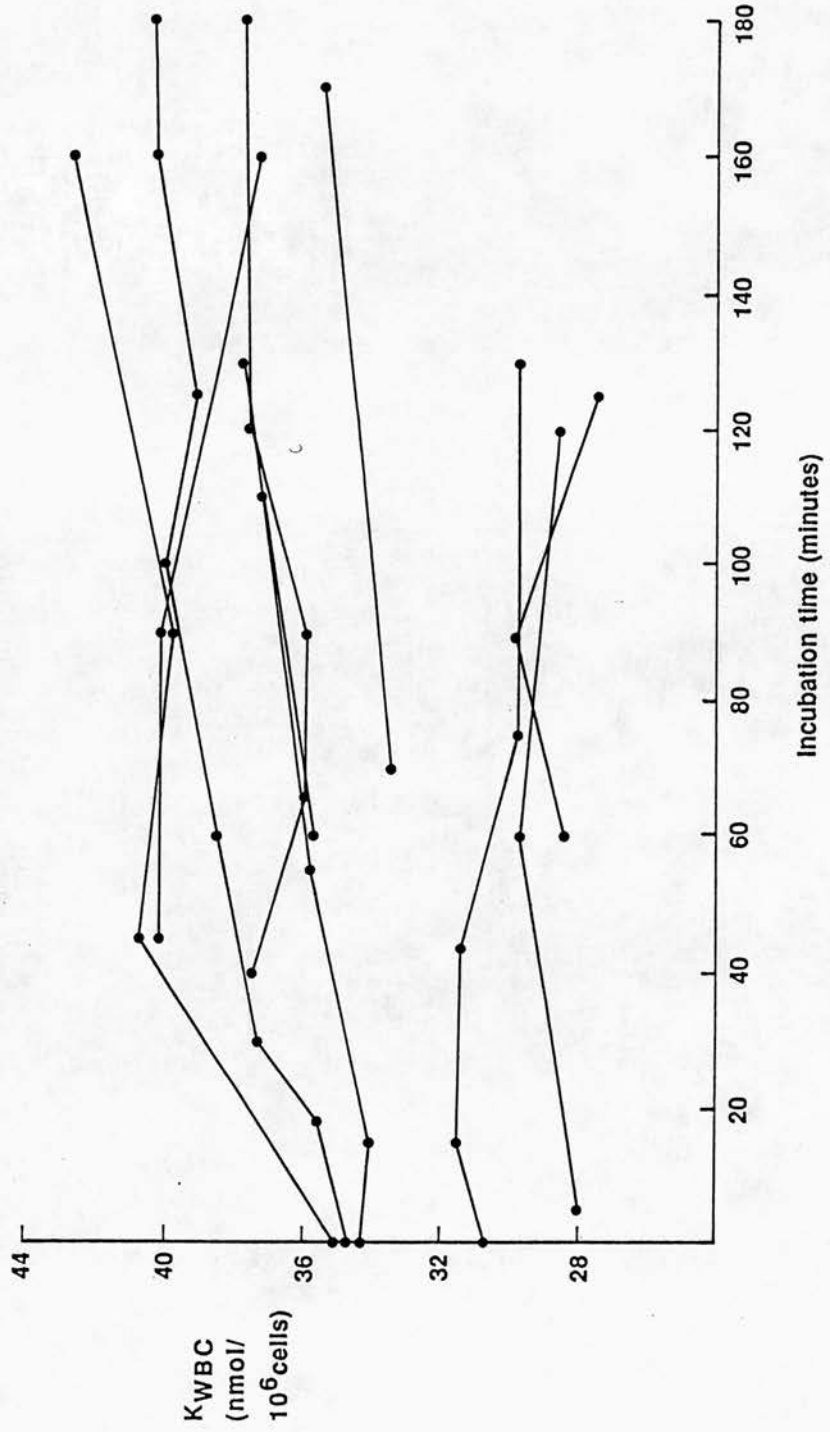

Figure S2. Effect of incubation time on K_{WBC} in uraemic subjects.

LEUCOCYTE AND ERYTHROCYTE CATION CONTENT AND TRANSPORT IN HEALTHY VOLUNTEERS, UNDIALYSED URAEMIC, AND CONTINUOUS AMBULATORY PERITONEAL DIALYSIS PATIENTS

INTRODUCTION

As the findings of Patrick and Jones (1974) and Edmondson et al (1975a) had never been confirmed in further studies, and because of the potential problems related to those studies, it was decided to measure leucocyte cation content and transport in a group of patients with chronic renal failure who had never been dialysed (CRF group). As well as a comparison group of healthy volunteers, a number of continuous ambulatory peritoneal dialysis patients (CAPD group) were also studied - there do not appear to have been any reports about leucocyte cations in CAPD patients. Unlike haemodialysis patients, these patients dialyse continuously and therefore tend to have constant plasma biochemistry and little fluctuation in weight, ECF volume or blood pressure. It was intended to study about 20 of each type of patient but due to the sudden shift in measurements of cation content and transport which occurred following the use of a new batch of dextran, it was not possible to achieve these numbers. (The problem with dextran is described in more detail in the "Development of Improved Methods" section).

As the preliminary, uncontrolled study described above had suggested that Na_{WBC} might be elevated in some undialysed uraemic patients, and as previous work by Thomas et al (1989) from this laboratory had shown Na_{RBC} to be reduced in such patients, simultaneous studies of erythrocytes and leucocytes were performed to allow a direct comparison of alterations in the two cell types.

The aims of this study therefore were; to compare leucocyte cation content and transport in undialysed patients with chronic renal failure, CAPD patients and healthy volunteers; to seek associations between any abnormalities of cation content and measurable clinical indices of renal failure; to observe changes in cation content and transport during the progression of uraemia and commencement of dialysis in individual patients; to further examine the stability of leucocyte cation content and transport during incubation in tissue culture fluid; and to simultaneously compare alterations in leucocyte and erythrocyte cation content and transport.

METHODS

a) Leucocyte cation content and transport

Leucocytes were isolated from 30 ml. of whole blood and their cation content and transport were measured using the final method as described at the end of the "Development of Improved Methods" section. Analyses were routinely performed after a one hour incubation in TC 199, but in some cases an additional aliquot of cells was incubated for a further two hours. In a few cases the initial cell yields allowed only sufficient cells for cation content but not transport measurements. All leucocyte separations in this study were done with dextran from the same batch.

b) Erythrocyte cation content and transport

The method used was that of Thomas et al (1989). In each case, the erythrocytes came from the same blood sample, in lithium heparin tubes, from which leucocytes were isolated. Two 3ml. aliquots of whole blood were incubated at 37°C for two hours, ouabain having been added to one aliquot to a final concentration of 10^{-4}M. At the end of this time the cells were centrifuged at 2,500g and washed in ice cold

isotonic magnesium chloride 3 times to remove plasma contamination. The erythrocytes were then centrifuged at 15°C at 10,000g for 20 minutes then 15,000g for 15 minutes. Cells were taken from the top tenth of the erythrocyte column, weighed, and disrupted in 5ml of 1.5mM CsCl. The sodium and potassium concentrations were then determined by flame photometry as for the leucocytes. Final cation concentrations were calculated as mmol/kg wet weight.

c) Patients and controls

Control values for leucocyte cation content were derived from 35 healthy volunteers (19 male), with a median age of 30, range 21 to 50, and for sodium transport from 26 of this group. All believed themselves to be suffering from no illness. Six women were taking the oral contraceptive pill, but no others were known to be taking drugs. The quoted normal values for erythrocyte cation content and transport were derived from 30 healthy volunteers (15 male), median age 61, range 42 to 79. None were taking any drugs at the time they were studied.

Fourteen patients with chronic renal failure (CRF) (8 male) were studied. Median age was 53 (range 18 to 72). All had had chronic renal failure for at least 6 months and none had ever been dialysed. Five were being treated for hypertension at the time they were studied. Leucocyte cation content was measured in all and sodium transport in 11. Simultaneous measurements of erythrocyte cation content and transport were carried out in 12 of these patients (6 male).

Fifteen continuous ambulatory peritoneal dialysis (CAPD) patients were studied (12 male). Median age was 58 (range 29 to 72). They had been on CAPD for 2 to 67 months (mean = 21 months). Five were

receiving treatment for hypertension. None had had an episode of peritonitis within one month prior to being studied. Leucocyte cation content was measured in all and sodium transport in 11. Simultaneous measurements of erythrocyte cation transport were carried out in 10 of these patients (8 male).

No patients studied had clinical evidence of fluid overload and all had diastolic blood pressures of 100mm. Hg or less at the time of the study. No patients had had significant peripheral or pulmonary oedema, a systemic infection, or a blood transfusion in the month prior to study. In both groups, drug treatment was noted but not discontinued at the time of study, and no patients were taking steroids, digoxin or immunosuppressive drugs.

The causes of renal failure in the two groups are shown in Table S1.

d) Longitudinal studies

Leucocyte cation content was measured shortly before and then two or three times after the commencement of dialysis in four patients. Leucocyte sodium transport and erythrocyte sodium content and transport measurements were made after commencing dialysis in all four patients, and shortly before dialysis in three. No patient had had a blood transfusion during the study period or in the month beforehand.

e) Plasma biochemistry

Standard plasma biochemical indices of the severity of uraemia were measured by AU5000 multi-channel analyser from a venous blood sample taken at the time of study.

f) Statistics

Comparisons within the control group were by t-test and Pearson's linear correlation. Comparisons of the two patient groups and the control group were by non-parametric analysis of variance (Kruskaal-Wallis) and then Mann-Whitney U-tests where appropriate. The effect of incubation time in the two patient groups was assessed by Wilcoxon matched-pairs test. Spearman's rank correlations were used to assess possible relationships between leucocyte cation content and plasma biochemical indices of the severity of uraemia. When simultaneous erythrocyte and leucocyte measurements were made, direct comparisons between the groups were by Mann-Whitney U-test.

Table S1. Causes of renal failure in undialysed uraemic (CRF) and CAPD patients.

DIAGNOSIS	CRF group	CAPD group
Chronic glomerulonephritis	4	5
Small kidneys, cause unknown	4	3
Hypertensive nephrosclerosis	3	1
Chronic pyelonephritis	1	2
Polycystic kidney disease	1	1
Renal vascular disease	0	2
Bilateral hydronephrosis	1	0
Diabetic nephropathy	0	1

RESULTS

a) Leucocyte cation content and transport

The results from the healthy volunteers are shown in Table S2.

Table S2. WBC cation content and transport in healthy volunteers.

	All	n	Males	n	Females	n
Na_{WBC}	3.28(0.45)	35	3.26(0.40)	16	3.31(0.49)	19
K_{WBC}	31.1(2.7)	35	30.1(2.74)	16	31.9(2.5)	19
Na/K ratio	0.106(0.014)	35	0.108(0.014)	16	0.104(0.014)	19
Flux rate	3.32(0.94)	26	3.15(0.57)	11	3.45(1.14)	15
Rate constant	1.02(0.25)	26	0.98(0.20)	11	1.05(0.28)	15

Results are expressed as mean (standard deviation). Na and K are in nmol/10^6 cells, flux rate in nmol/10^6 cells/hour and rate constant in $hour^{-1}$.

No correlations between age and the various measures of cation content and transport were found.

When the results were analysed by sex, the only difference which reached statistical significance was in K_{WBC}, which was higher in the female group ($p=0.05$). The mean results for the women taking the oral contraceptive pill were similar to the results in the rest of the group (Na_{WBC} = 3.24 (0.65), K_{WBC} = 32.6 (2.1) (n = 6 for both), flux rate = 3.39 (0.98) and rate constant = 1.04 (0.25) (n = 4).

Results for leucocyte cation content and transport in both patient groups and healthy volunteers are shown in Table S3. When Na_{WBC} in the three groups was compared by non-parametric analysis of variance, a significant difference was found ($p<0.005$). Direct comparison between groups showed significant differences between the

undialysed group and both the control group ($p < 0.001$) and the CAPD group ($p = 0.05$).

Table S3. Leucocyte cation content and transport in controls (NC), undialysed uraemic (CRF) and CAPD patients.

	NC	CRF	CAPD
Na_{WBC}	3.31(2.36-4.20)	4.02(3.07-6.05)	3.26(2.63-5.05)
K_{WBC}	30.7(25.1-38.1)	30.8(26.7-35.8)	31.7(25.6-36.3)
Flux rate	3.27(1.35-5.07)	3.37(2.37-6.39)	3.84(2.34-4.83)
Rate constant	1.01(0.36-1.42)	1.02(0.44-1.37)	1.12(0.83-1.73)

Values expressed as median (range). Na and K are in nmol/10^6 cells, flux rate is in nmol/10^6 cells/hour and rate constant in hour^{-1}.

Actual values for Na_{WBC} from all three groups are plotted in Fig. S3. Na_{WBC} was above the "normal" mean in all but two of the undialysed patients, and greater than the highest control value in 6 of the 14. Of the three patients with hypertensive nephrosclerosis (i.e. probable essential hypertension), two had Na_{WBC} above the normal range. (Actual values 4.27, 6.05, and 3.22). Patients being treated for hypertension at the time of the study are indicated separately and had a wide range of Na_{WBC}.

The distribution of Na_{WBC} in the CAPD group was closer to that in the healthy volunteers, half the patients having values below the normal mean and only 3 of the 15 had Na_{WBC} greater than the highest control value. Again, the patients receiving treatment for hypertension had a wide range of values for Na_{WBC}.

Where patients were taking frusemide or B-blockers, these are indicated in Fig. S3. The spread of values in patients taking either

type of drug was similar to the spread in those not taking those drugs.

There was no significant difference in K_{WBC} in the three groups by non-parametric analysis of variance. Actual values for K_{WBC} are shown in Fig. S4. The patients taking B-blockers or frusemide are spread throughout the range.

Actual values for sodium flux rate are shown in Fig S5. Although there was a tendency for flux rates in both patient groups to be in the upper half of the range for healthy volunteers, no significant difference was present by non-parametric analysis of variance. In the CRF group there was a tendency for those with the lowest flux rates to be on B-blockers or frusemide.

Actual values for sodium flux rate constant are shown in Fig S6. No significant difference was found by non-parametric analysis of variance. One of the healthy volunteer and two of the CRF group had markedly low values. There was no obvious effect of frusemide or B-blockers.

Repeat measurements available after a three hour incubation were compared with those after the one hour incubation by Wilcoxon Matched-Pairs test. In the CRF group, paired data for Na, K and Na/K ratio were available from 11 patients, and for flux rate and rate constant from 9 patients. In the CAPD group there were 15 pairs of cation content results and 9 pairs of cation transport results. No statistically significant alterations between one and three hours of incubation were found for any measurement in either group.

Plasma biochemistry in the two groups is shown in Table S4.

Table S4. Plasma biochemistry in CRF and CAPD patients.

	CRF	CAPD	
Sodium (mmol/l)	139 (3)	137 (2)	
Potassium (mmol/l)	4.38 (0.60)	4.23 (0.93)	
Bicarbonate (mmol/l)	16.1 (3.4)	26.4 (3.2)	***
Urea (mmol/l)	30.0 (14.1)	17.7 (5.0)	**
Creatinine (μmol/l)	702 (221)	917 (280)	*
Corrected calcium (mmol/l)	2.18 (0.21)	2.59 (0.20)	***
Phosphate (mmol/l)	2.16 (0.62)	1.70 (0.53)	*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

All results expressed as mean (standard deviation).

Correlation coefficients were calculated between the various leucocyte cation measurements and plasma urea and creatinine concentrations. No significant correlations were found in the CRF group, or with sodium flux rate or rate constant in the CAPD group. In that group however, significant correlations between plasma biochemistry and leucocyte cation content were found, as shown in Table S5.

Na_{WBC} is shown plotted against plasma urea in Fig. S7.

b) Simultaneous measurements in erythrocytes and leucocytes

The two patient groups were subsets of those described above, and parameters of plasma biochemistry and differences between the groups were similar to those of the complete groups. Results in the two patient groups are shown in Table S6.

Table S5. Correlations between plasma biochemistry and WBC cation content in 15 CAPD patients.

	Na_{WBC}		Na/K ratio	
	r_s	p	r_s	p
Urea	0.69	0.005	0.72	0.002
Creatinine	0.56	0.031	0.67	0.007

Table S6. Leucocyte and erythrocyte cation content and transport in CRF and CAPD patients.

	CRF	p	CAPD	Controls
Leucocytes				
Na	3.93 (3.07-6.05)	*	3.14 (2.63-5.05)	3.28(0.45)
K	31.5 (26.7-34.1)		31.5 (26.7-36.3)	31.1(2.7)
Flux rate	3.32 (2.37-6.39)		3.80 (2.34-4.74)	3.32(0.94)
Rate constant	1.00 (0.44-1.37)		1.07 (0.83-1.73)	1.02(0.25)
Erythrocytes				
Na	3.38 (2.60-4.28)	**	4.41 (3.09-5.51)	5.31(0.95)
K	95.5 (88.0-101.7)		95.4 (87.6-98.7)	98.7(3.5)
Flux rate	1.45 (1.07-2.53)		1.52 (1.39-1.69)	1.64(0.23)
Rate constant	0.41 (0.31-0.59)	*	0.35 (0.28-0.55)	0.32(0.09)

Values in the two patient groups are expressed as median (range). Mean (standard deviation) values from controls are shown as comparison. * indicate significance level when the two patient groups are compared directly: * $0.02 < p < 0.05$, ** $p < 0.01$. Na_{WBC} and K_{WBC} are in nmol/10^6 cells, WBC flux rate is in nmol/10^6 cells/hour and rate constant in $hour^{-1}$. Na_{RBC} and K_{RBC} are in mmol/kg wet weight, RBC flux rate is in mmol/kg/hour and rate constant in $hour^{-1}$.

As in the complete groups, Na_{WBC} was significantly lower (and therefore closer to values in controls) in the CAPD than the undialysed group, but the differences in flux rate and rate constant were not statistically significant. Na_{RBC} was significantly higher in the CAPD than the undialysed group, and again this was closer to the control mean. In erythrocytes, the differences in flux rate were not significant but the rate constant was significantly lower in the CAPD group, and again closer to the normal mean. K_{RBC} was similar in both groups.

c) Longitudinal studies

Leucocyte and erythrocyte sodium, flux rate and rate constant in 4 individual patients are shown in Fig. S8. Leucocyte values are those obtained after a one hour incubation.

Patient WG was a 63 year old male with polycystic kidney disease. Before dialysis his plasma urea and creatinine concentrations were 63 mmol/l and 1190 umol/l respectively, and he felt nauseated and unwell. There was no clinical evidence of fluid overload, and the first post dialysis measurements were made after a total of seven hours of haemodialysis administered in two sessions on consecutive days. At this time his urea and creatinine were 35.7 mmol/l and 736 umol/l, and he felt better. Maintenance haemodialysis was continued and the third and fourth measurements were made 12 and 14 days after starting dialysis. The third measurement was immediately after a dialysis session (urea and creatinine 22.0 mmol/l and 529 umol/l) and the fourth measurement just before the next dialysis session (urea and creatinine 36.7 mmol/l and 763 umol/l). Na_{WBC} fell immediately after dialysis and then further at 12 days, but rose between dialysis sessions. No sodium transport or erythrocyte

measurements were made before starting dialysis. The rise in Na_{WBC} between dialysis sessions was associated with a rise in WBC flux rate and a fall in WBC rate constant. Na_{RBC} fell slightly in the same interval, in association with rises in RBC flux rate and rate constant. This patient was taking no medicines during the study period.

Patient MS was a 66 year old lady with longstanding rheumatoid arthritis who presented with end-stage renal disease and was found by ultrasound scan to have two small kidneys. She was tired but not ill or overloaded with fluid. Plasma urea was 52.8 mmol/l and creatinine 938 umol/l. Intracellular cation measurements were made immediately prior to insertion of an abdominal Tenckhoff catheter, ten days later whilst she was an in-patient on continuous cycling peritoneal dialysis (two litre exchanges hourly) with urea and creatinine of 25.7 mmol/l and 862 umol/l, and 18 days after that when she was established on CAPD, four two-litre exchanges every 24 hours, with a urea of 35.8 mmol/l and creatinine of 1159 umol/l. She was on no regular medication throughout this time (her rheumatoid disease at this stage required only occasional simple analgesia and she took no non-steroidal anti-inflammatory drugs).

Her Na_{WBC} was not raised before dialysis and changed little. Na_{RBC} was relatively low and remained so after commencing dialysis. RBC flux rate and rate constant increased over the study period by similar amounts.

Patient HC was a 64 year old male with end-stage renal failure of unknown cause, but a past history of hypertension and a cerebro-vascular accident. Throughout the study period his blood pressure was controlled by metoprolol 50 mg. daily. He was studied immediately

prior to Tenckhoff catheter insertion (urea 50.2 mmol/l, creatinine 799 umol/l), and 4, 6 and 17 days after insertion, during which time he became established on CAPD. His urea and creatinine during this time were stable at around 22 mmol/l and 650 umol/l respectively. His Na_{WBC} fell throughout the study, most steeply initially, and this was associated with an initial rise in WBC rate constant and a later fall in WBC flux rate. Na_{RBC} rose immediately after dialysis and remained stable. This was associated with an initial rise in RBC flux rate and fall in RBC rate constant, then later matched fluctuations in both measurements.

Patient BC was a 34 year old male who had both hypertensive nephrosclerosis and tubulo-interstitial nephritis on renal biopsy. Throughout the study he took atenolol 100 mg daily and allopurinol 300 mg daily. Until commencing dialysis he took sodium bicarbonate 6 gms daily. He felt nauseated and tired but otherwise well and his plasma urea and creatinine were 36.5 mmol/l and 986 umol/l. He was studied again after a three hour haemodialysis session (urea now 25.1 mmol/l, creatinine 760 umol/l) and then 14 days after that whilst on CAPD (urea 20.9 mmol/l, creatinine 925 umol/l). Na_{WBC} was normal throughout the study period, and Na_{RBC}, which was closest to normal of the three patients in which it was measured before starting dialysis, rose only slightly.

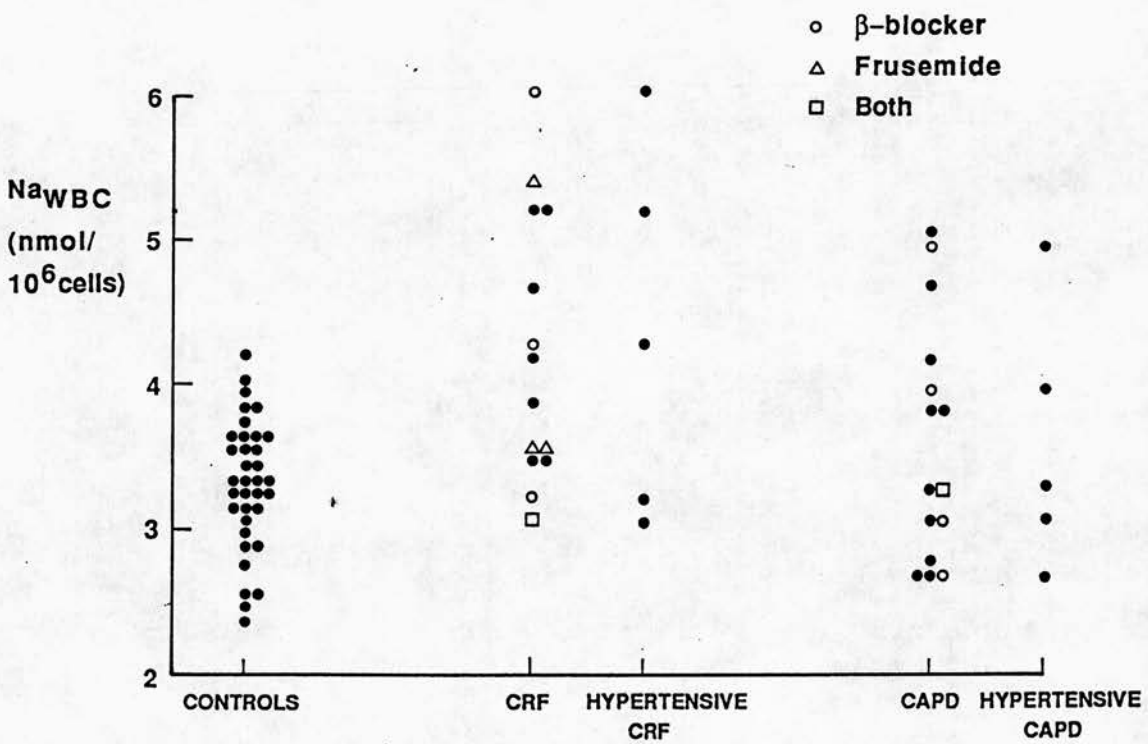

Figure S3. Na_{WBC} in controls, undialysed uraemic (CRF) and CAPD patients. (Patients in the hypertensive groups also appear in the parent groups).

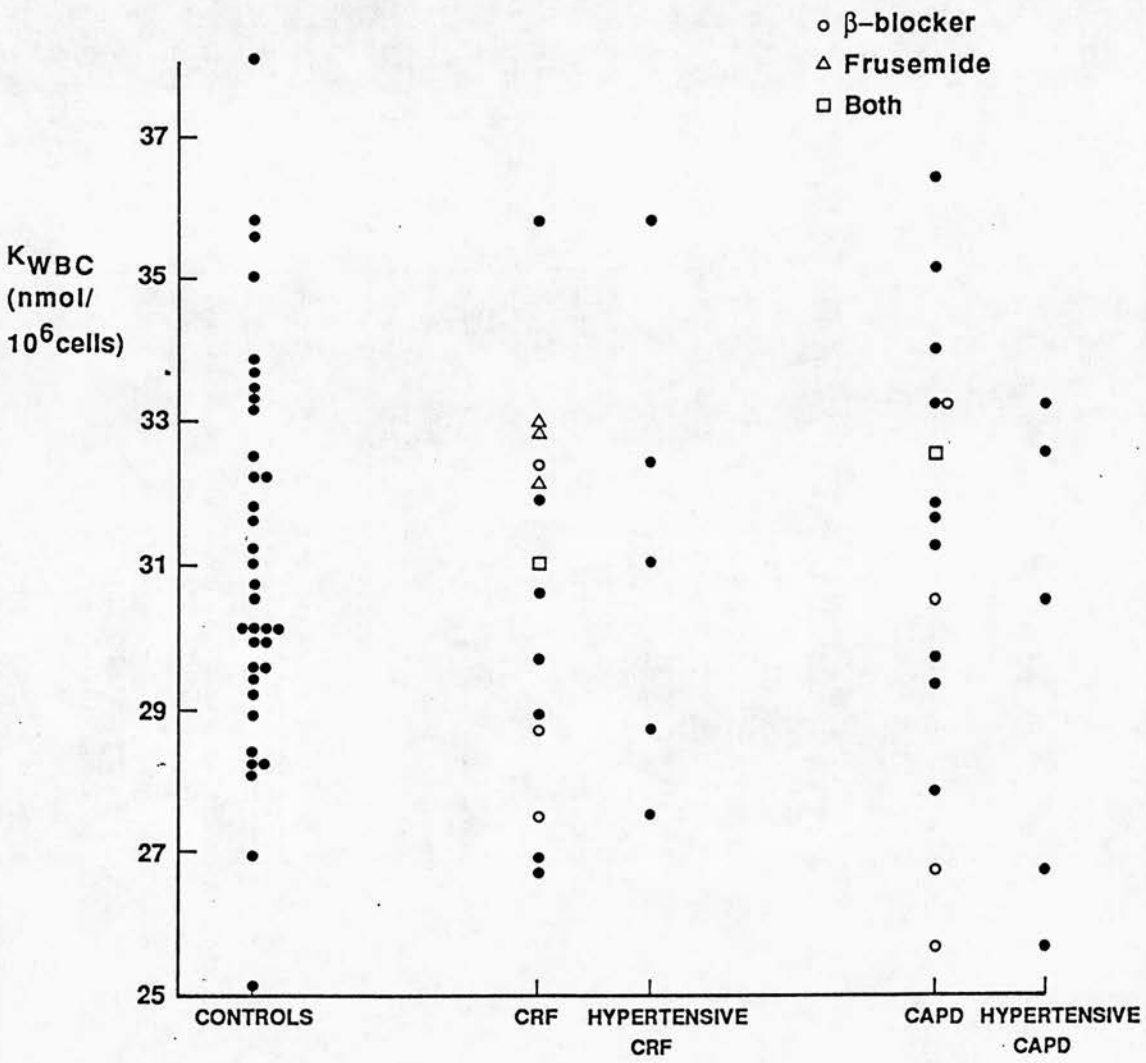

Figure S4. K_{WBC} in controls, undialysed uraemic (CRF) and CAPD patients. (Patients in the hypertensive groups also appear in the parent groups).

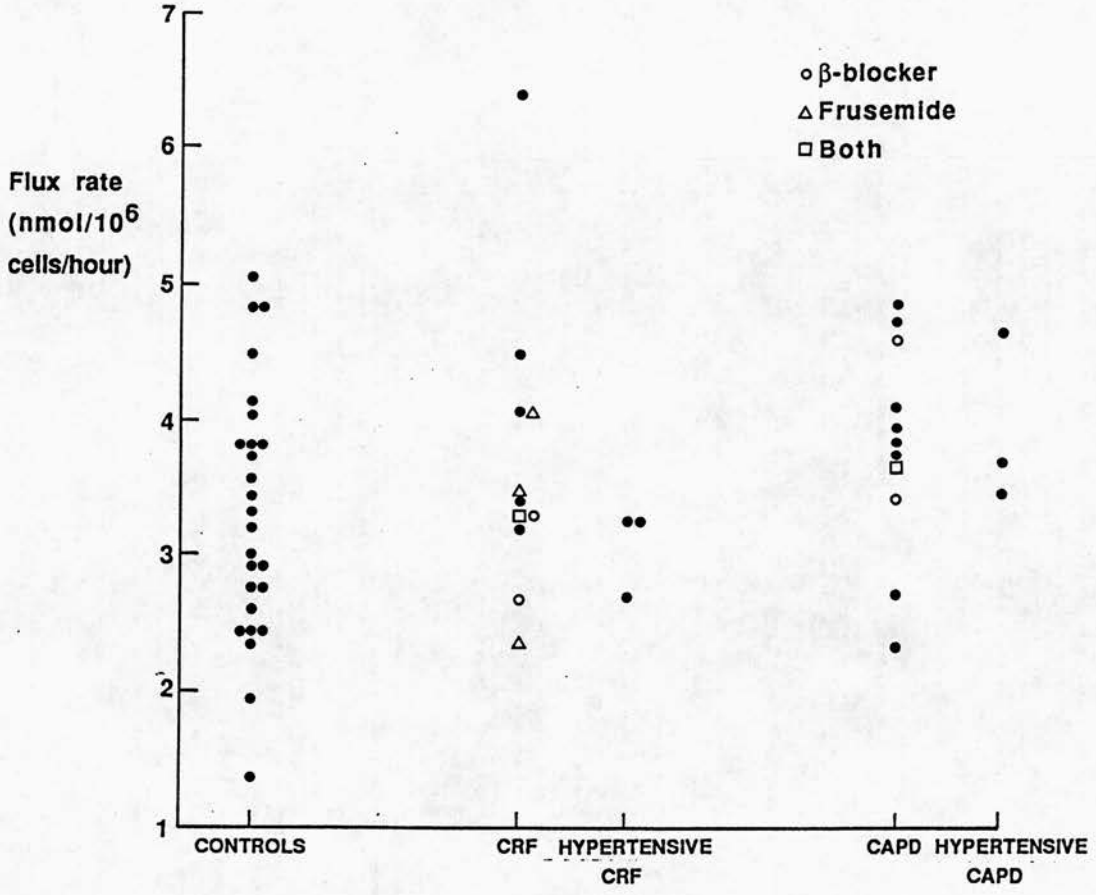

Figure S5. Leucocyte sodium flux rate in controls, undialysed uraemic (CRF)and CAPD patients. (Patients in the hypertensive groups also appear in the parent groups).

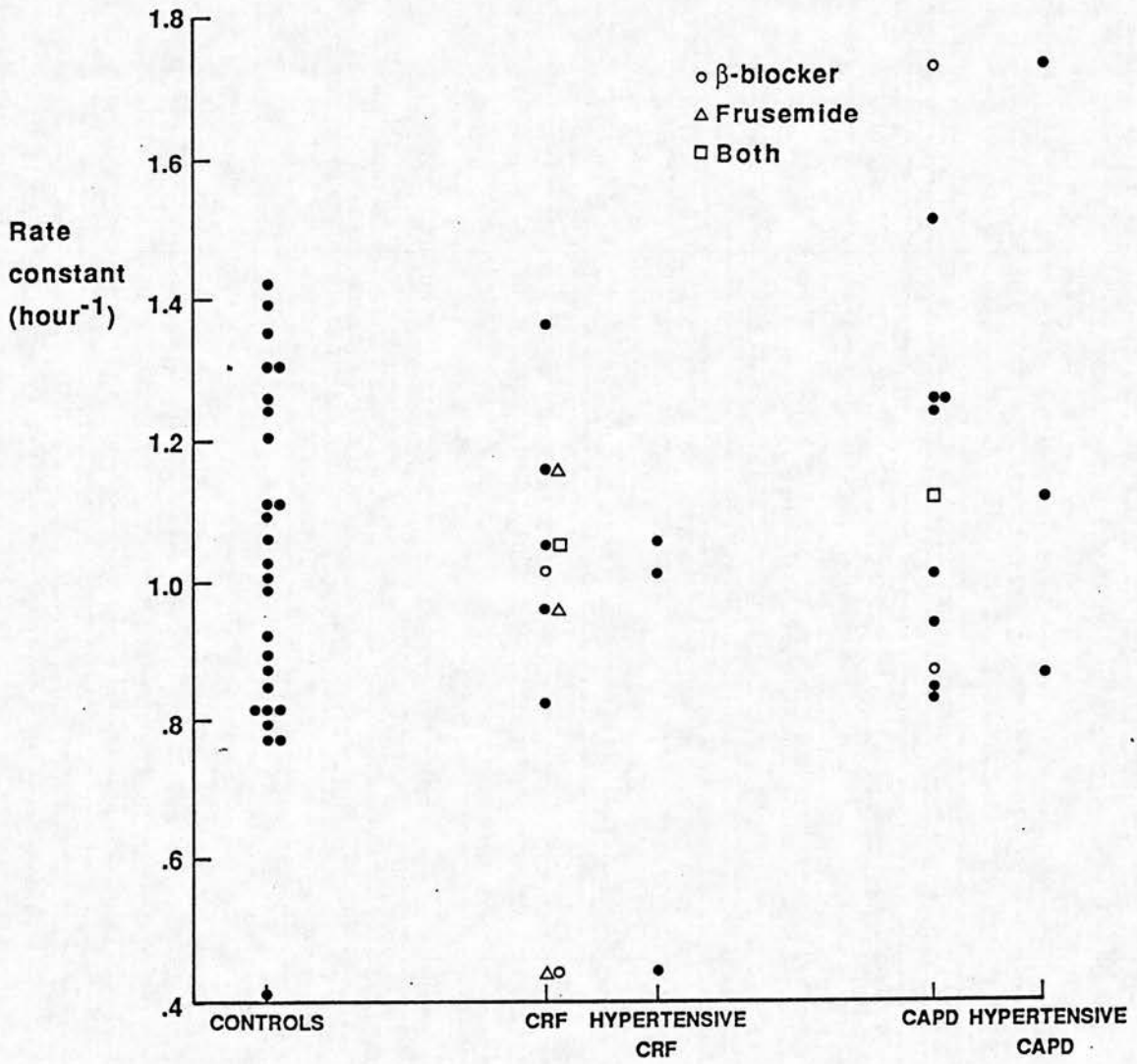

Figure S6. Leucocyte sodium flux rate constant in controls, undialysed uraemic (CRF) and CAPD patients. (Patients in the hypertensive groups also appear in the parent groups).

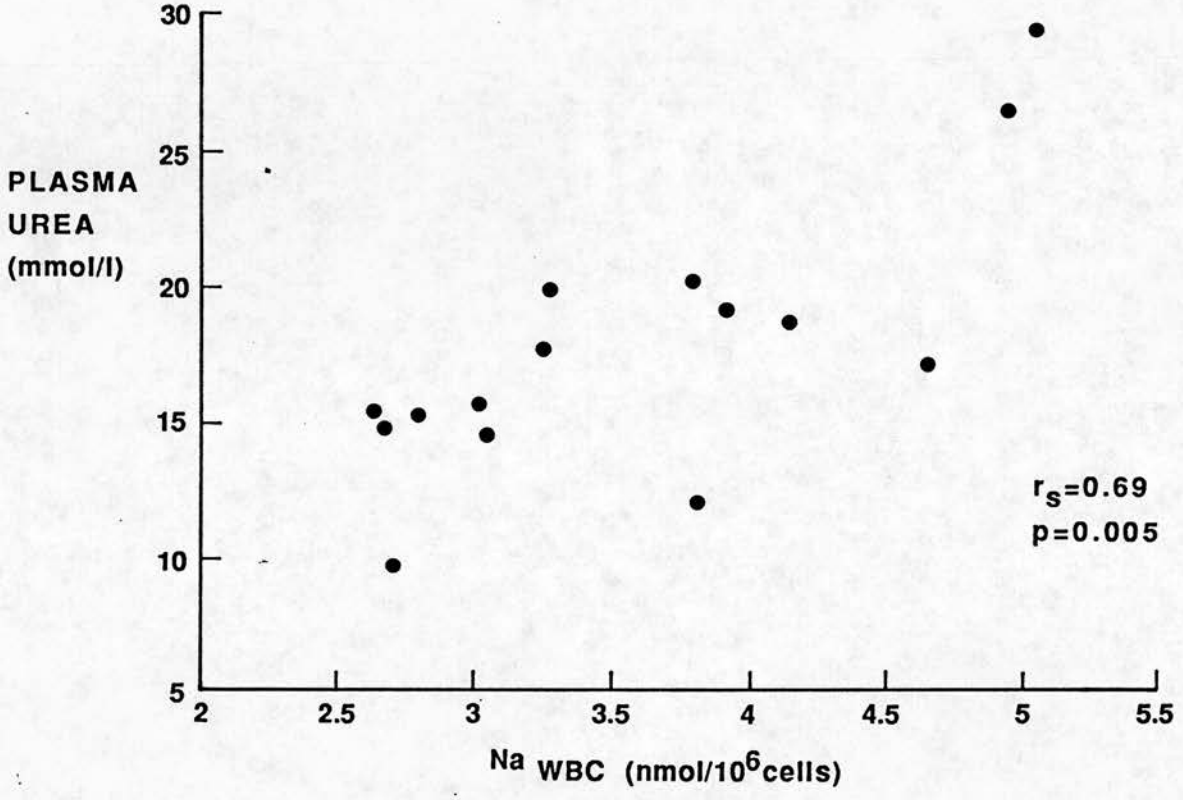

Figure S7. Na_{WBC} and plasma urea concentration in CAPD patients.

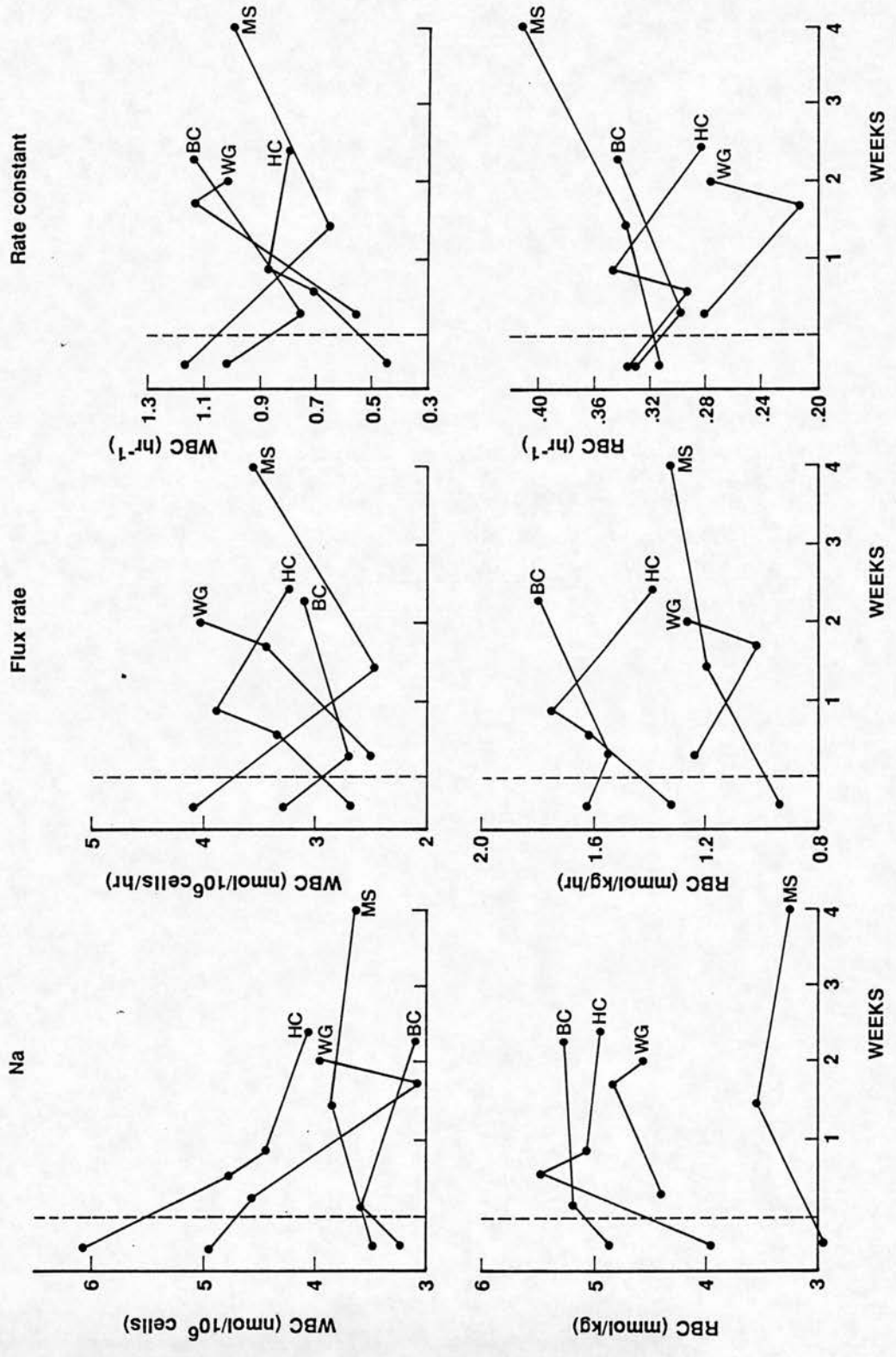

Figure S8. Longitudinal studies of leucocyte and erythrocyte sodium content and transport. Time is from commencement of dialysis, indicated by vertical dotted lines.

ERYTHROCYTE AND LEUCOCYTE CATION CONTENT AND TRANSPORT IN HAEMODIALYSIS PATIENTS

INTRODUCTION

There have been no reports of the effect of a haemodialysis session on leucocyte cation content and transport, and conflicting reports of the effect of haemodialysis on erythrocyte cations. The principal aim of this study was to measure cations in both cell types before and after a standard haemodialysis session, and to compare the changes, if any, in the different cell types. The effect of incubating red cells in either whole blood or tissue culture fluid was also studied.

The changes induced by haemodialysis are widespread and interlinked but can be split into two groups - ECF volume changes, and changes in the biochemical degree of uraemia. The alterations in these two groups produced by a dialysis session are largely independent of each other, and a secondary aim was to try to relate any changes in intracellular cations to either the ECF volume changes or the biochemical changes occurring over the dialysis session. As indicators related to ECF volume, patient weight as a percentage of his or her "dry" weight was calculated, and blood pressure and atrial natriuretic peptide (ANP) concentrations were measured. Plasma creatinine concentrations and dialysis index were used as measures of the biochemical degree of uraemia and adequacy of dialysis, the dialysis index being calculated according to the formula, index = change in plasma urea concentration during dialysis/0.5 x sum of pre- and post-dialysis plasma urea concentrations (Probst and Binswanger, 1988).

METHODS

Leucocyte cation content and transport

Leucocyte sodium and potassium content, and ouabain sensitive sodium transport were measured using the method described in the "Development of Improved Methods" section. All experiments on patients and controls were performed using the same batch of dextran.

Erythrocyte cation content and transport

Erythrocyte cation content and transport were measured in two ways. One method was identical to that used in the studies on undialysed and CAPD patients, the cells being incubated as whole blood. The other method involved an initial separation of the erythrocytes from whole blood by centrifugation at 700g, washing once with isotonic NaCl solution, and then incubation in TC199 identical to that used for the leucocyte incubations, with and without the addition of ouabain. Incubation times and washing procedures were the same as in the standard method.

Biochemistry

Urea and creatinine concentrations in plasma were measured by AU5000 autoanalyser.

Atrial natriuretic peptide concentrations in plasma were measured by taking whole blood into pre-cooled lithium heparin tubes containing sufficient trasylol to give a final concentration of 50 kIU/ml. After centrifugation at 4°C, plasma was removed and stored at -70°C. Samples were analysed within one month using a radioimmunoassay kit for alpha-hANP (Peninsula Laboratories).

Patients

Twenty hospital haemodialysis patients (10 male) were studied. All had had end-stage renal failure for at least 12 months and had been

on haemodialysis for at least 4 months (see Table S7). None had had a blood transfusion in the three months prior to study or a clinically significant infection in the one month prior to study. Patients continued on their normal drugs but none were receiving steroids or diuretics. Three patients were receiving digoxin at the time of study. All had been receiving this drug for at least three months and had digoxin levels within the therapeutic range. One patient was receiving a beta-blocker (atenolol) during the study.

Table S7. Patient details.

Age (years)	Median 62, range 24 to 73
Duration of ESRF (months)	Mean 56.1, SD 39.5
Duration of haemodialysis (months)	Mean 38.7, SD 32.2

Diagnosis	n
Chronic pyelonephritis	6
Small kidneys, cause unknown	6
Chronic glomerulonephritis	3
Polycystic kidney disease	2
Renal vascular disease	2
Scleroderma	1

All patients were studied immediately before and after a regular haemodialysis session. Common to all dialyses was the use of Hemoflow E2 cuprophan hollow fibre dialysers (Fresenius). Dialysate was made up to give final concentrations as follows:- sodium 136 mmol/l, potassium 2.0 mmol/l, bicarbonate 30 mmol/l, acetate 2.0 mmol/l, calcium 1.5 mol/l, magnesium 0.375 mmol/l and chloride 106 mmol/l. At each occasion, 45 ml. of blood was taken, usually from an

arterio-venous fistula, for the investigations listed above. Patients were weighed and had their blood pressure taken before and after the dialysis session, and their weight gain since the previous dialysis and clinically assessed "dry weight" were also noted.

All patients consented to having extra blood taken for these studies.

Controls

Comparative values for leucocyte cations were measured in 18 healthy volunteers (9 male), with a median age of 47 years (range 31 to 59). No subjects were taking medicines at the time of the study.

Comparative values for erythrocyte cations were measured in 30 healthy volunteers (15 female) aged 42 to 79 (median 61).

Statistics.

Intracellular cation measurements in the haemodialysis patients were not normally distributed and therefore comparisons before and after dialysis were by Wilcoxon Matched-Pairs test and Mann-Whitney U-test. Spearman Rank correlation coefficients were calculated between potentially related variables.

RESULTS

Leucocytes

Leucocyte cation contents were measured in 18 patients and the results before and after dialysis are shown in Table S8. Only the change in flux rate over dialysis reached statistical significance, p = 0.006 (Wilcoxon Matched-Pairs test). The changes in Na_{WBC} and rate constant did not quite reach statistical significance (p = 0.078 and 0.071 respectively), although the failure of the latter to reach significance was due to one very high post dialysis value. For Na_{WBC}, K_{WBC}, flux rate and rate constant, pre-dialysis values are plotted

against post-dialysis values in Figs S9-12. These show that the distribution of Na_{WBC} after dialysis had become closer to that in the controls and that there was very little change in K_{WBC}. The change in flux rate was such that before dialysis the majority of values lay above the mean of the controls and after dialysis most values lay below this mean. At both times, however, the majority of values were well within the mean ± 2 standard deviations of the controls. The three patients who were taking digoxin and the one taking atenolol are indicated on these plots, and were not obviously different from the others. The change in flux rate correlated with the change in rate constant, $r_s = 0.55$, $p = 0.019$ (Fig S13).

Table S8. Leucocyte cation content and sodium transport in 18 haemodialysis patients.

	Haemodialysis		Controls
	Before	After	
Na	2.84(2.33-4.57)	2.64(2.13-3.17)	2.58(0.31)
K	32.3(25.5-37.1)	30.9(25.9-36.2)	31.5(1.9)
Na/K	0.091(0.069-0.132)	0.085(0.068-0.108)	0.082(0.010)
Flux rate	3.85(2.33-6.70)	2.94(2.19-5.72)	3.25(0.72)
R.Constant	1.31(0.85-2.10)	1.19(0.81-2.40)	1.26(0.25)

Cation contents are in nmol/10^6 cells, flux rates in nmol/10^6 cells/hour and rate constants in $hour^{-1}$. Patient values are shown as median (range), control values as mean(standard deviation).

Erythrocytes.

Erythrocyte cation content in cells incubated in whole blood was measured before and after dialysis in 19 patients, and sodium

transport in all but one of these. In erythrocytes incubated in TC199, cation content was measured in 18 patients and sodium transport in 13 of that group. Results are shown in Table S9.

Table S9. Erythrocyte cation content and sodium transport before and after haemodialysis.

Whole blood incubation

	Haemodialysis		Controls
	Before	After	
Na	4.41 (3.27-7.69)	5.48 (4.18-7.33)	5.31(0.95)
K	97.3(91.3-107.7)	96.7(92.3-112.5)	98.7(3.54)
Flux rate	1.76 (1.51-2.45)	1.71 (1.34-2.53)	1.64(0.23)
R.constant	0.384(0.229-0.595)	0.331(0.240-0.445)	0.321(0.016)

TC199 incubation

	Haemodialysis	
	Before	After
Na	4.50 (3.33-7.14)	5.09 (3.90-7.53)
K	96.3 (90.5-109.3)	95.9 (90.9-111.2)
Flux rate	1.63 (1.33-1.96)	1.39 (0.99-1.78)
R.constant	0.359 (0.245-0.589)	0.269 (0.157-0.370)

Cation concentrations are in mmol/kg wet weight, flux rates in mmol/kg/hour and rate constants in $hour^{-1}$. Patient values are expressed as median (range). Control values are expressed as mean (standard deviation).

In cells incubated in whole blood, changes in Na_{RBC} ($p<0.001$) and rate constant ($p=0.002$) were significant. These also changed significantly in cells incubated in TC199 ($p = 0.007$ and $p = 0.003$

respectively), and in this medium, there was also a significant change in flux rate ($p = 0.003$) (Wilcoxon Matched-Pairs test). Pre- against post-dialysis plots of Na_{RBC}, flux rate and rate constant for both incubation methods are shown in Figs S14 to S19. These show that Na_{RBC} and rate constant in cells incubated in whole blood were closer to control values after dialysis. As in the leucocytes, there was no obvious effect of digoxin or atenolol. Over dialysis, the change in flux rate correlated with the change in rate constant (for cells incubated in blood, $r_s = 0.74$, $p < 0.001$ (Fig S20) and for cells incubated in TC199, $r_s = 0.83$, $p < 0.001$).

Although different methods of incubation produced significant differences in some of the measurements, values obtained from one type of incubation correlated well with those from the other type (Table S10), and pre-dialysis values from the two incubation methods are plotted against each other in Figs S21 to 23.

Only for rate constant did the changes in pre-dialysis cells resulting from incubation in TC199 appear similar to those that occurred across dialysis in cells incubated in blood. However, when the changes produced in these two ways were compared statistically, the only significant correlation was for the changes in potassium concentration ($r_s = 0.52$, $p = 0.029$).

Comparison of leucocytes and erythrocytes.

Only flux rates changed significantly in both cell types over dialysis, being lower after dialysis in erythrocytes and leucocytes. Rate constants also fell in both cell types, but the change was only significant in erythrocytes. Sodium increased in erythrocytes and fell in leucocytes. Despite being in opposite directions, changes in sodium content in both cell types were towards normal. In both cell

types, change in flux rate over dialysis correlated with change in rate constant (Figs S13 and S20).

Table S10. Comparison of erythrocyte indices obtained from two different incubation media.

	Significance of difference	Rank correlation	
Before dialysis	p	r_s	p
Na	0.877	0.80	<0.001
K	0.029	0.93	<0.001
Flux	0.003	0.83	<0.001
Rate constant	0.055	0.71	0.006
After dialysis			
Na	0.163	0.67	0.002
K	0.615	0.69	0.002
Flux rate	0.001	0.73	0.005
Rate constant	0.003	0.63	0.021

Significance of differences assessed by Wilcoxon Matched-Pairs test.

Spearman correlation coefficients were calculated between cation content and transport measurements in leucocytes and erythrocytes. Only measurements of rate constant before dialysis showed any evidence of being related in the different cell types. For leucocytes with erythrocytes incubated in TC199, r_s = 0.66, p = 0.028, and for leucocytes with erythrocytes incubated in whole blood, r_s = 0.46, p = 0.071.

Intracellular electrolytes, plasma biochemistry and fluid changes.

Pre- and post-dialysis values for plasma concentrations of creatinine and the chosen indicators of volume status are shown in

Table S11. Median dialysis index was 0.77 (range 0.39 to 1.09).

Table S11. The effect of dialysis on plasma creatinine, ANP and concentrations, weight and blood pressure.

	Pre-dialysis	Post-dialysis	p
Creatinine (umol/l)	871(729-1160)	430(290-685)	<0.001
ANP (pg/ml)	204(56-335)	163(36-200)	0.001
Weight %	103(100-107)	100(99-102)	0.002
Mean BP (mm Hg)	98(57-165)	83(45-117)	0.006

All values are shown as median (range). Weight is expressed as a percentage of estimated "dry weight" for each patient. Statistical comparisons by Wilcoxon matched-pairs test.

Weight, mean blood pressure, ANP and creatinine concentrations all fell significantly during dialysis. Spearman correlation coefficients were calculated between these variables and dialysis index. (Table S12). Before and after dialysis, indices related to ECF volume did not correlate with dialysis index or plasma creatinine concentrations. When correlation coefficients were calculated between changes over dialysis, measures of volume change correlated significantly with dialysis index and change in plasma creatinine. Changes in ANP concentration correlated with changes in weight and blood pressure but not creatinine or dialysis index.

For measurements before and after dialysis and the change over dialysis, Spearman correlation coefficients were calculated between the indices of dialysis listed in Table S12 and intracellular sodium and potassium content, flux rate and rate constant in leuc ocytes and erythrocytes incubated in blood. The only correlation found with

$p<0.01$ was between erythrocyte rate constant and weight% after dialysis ($r_s=0.60$, $p=0.009$)(Fig S24).

Table S12. Spearman correlation coefficients between blood pressure, weight, dialysis index (DI), plasma ANP and plasma creatinine concentrations.

Pre-dialysis.	DI	Creatinine	ANP	Weight %
Mean BP	-0.22	- 0.17	0.48*	0.01
Weight %	0.36	0.04	0.48*	
ANP	0.17	- 0.39		
Creatinine	-0.03			
Post-dialysis.	**DI**	**Creatinine**	**ANP**	**Weight %**
Mean BP	0.06	0.06	0.36	- 0.52*
Weight %	-0.05	0.22	- 0.31	
ANP	0.02	- 0.36		
Creatinine	-0.85***			
Change.	**DI**	**Creatinine**	**ANP**	**Weight %**
Mean BP	-0.52*	0.49*	0.51*	0.48*
Weight %	-0.55*	0.47*	0.59**	
ANP	-0.27	0.20		
Creatinine	-0.98***			

* $p<0.05$, ** $p<0.01$ *** $p<0.001$.

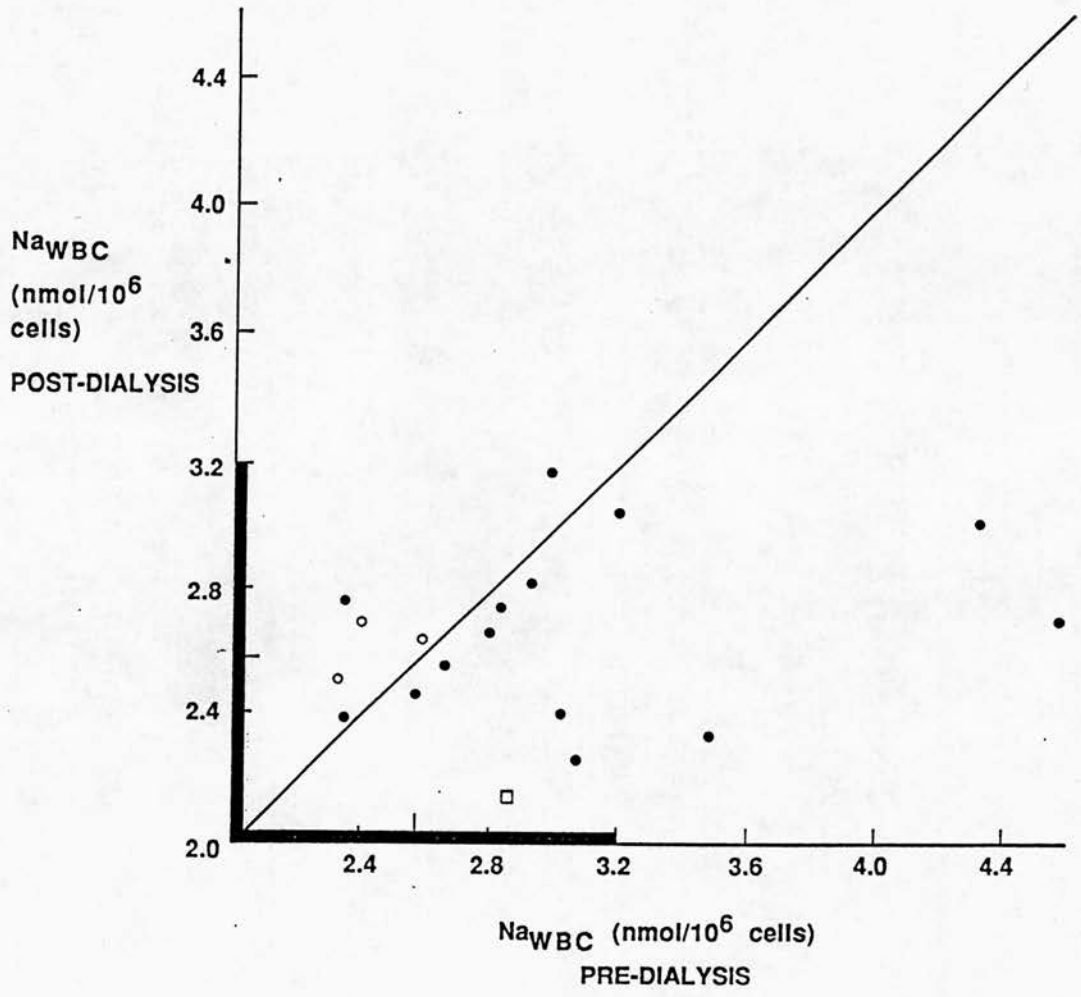

Figure S9. Na_{WBC} before and after haemodialysis. Open circles = on digoxin, open square = on atenolol, thick bars = mean $\pm$ 2SD from controls.

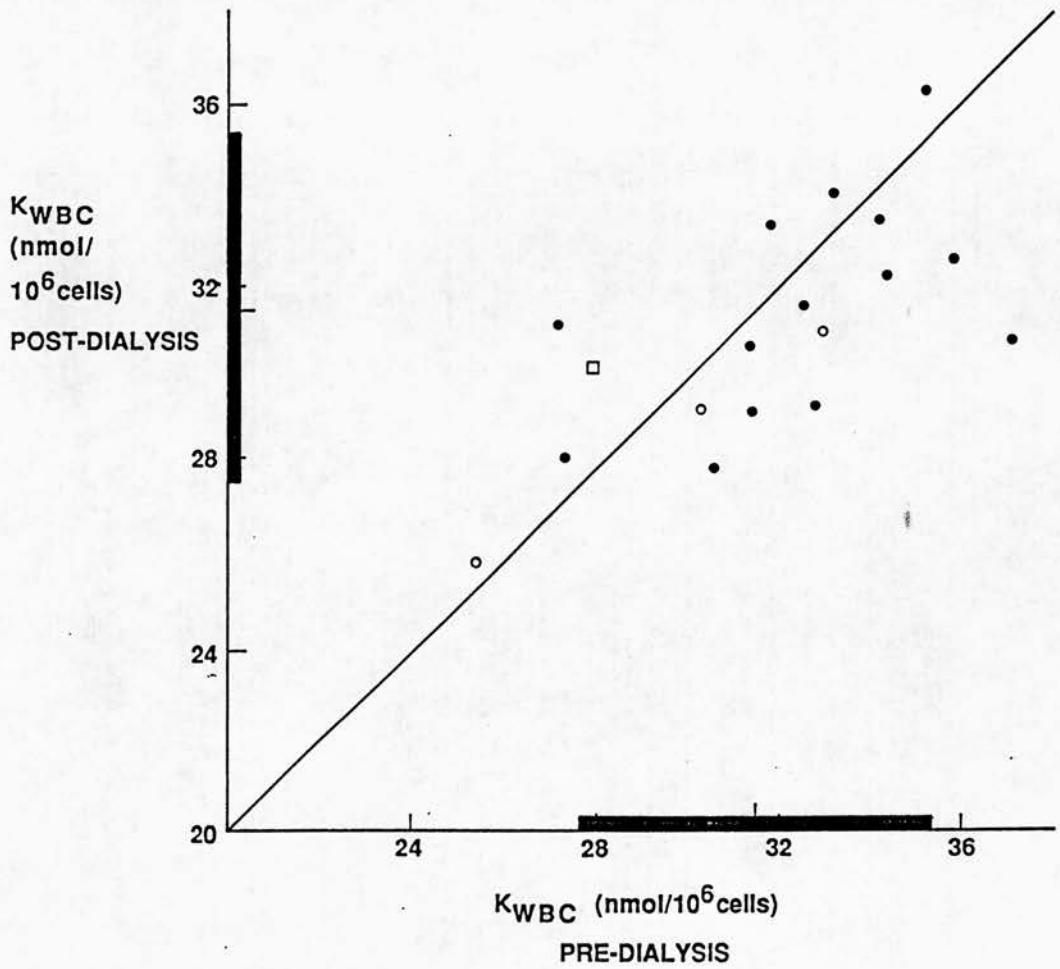

Figure S10. K_{WBC} before and after haemodialysis. Open circles = on digoxin, open square = on atenolol, thick bars = mean $\pm$ 2SD from controls.

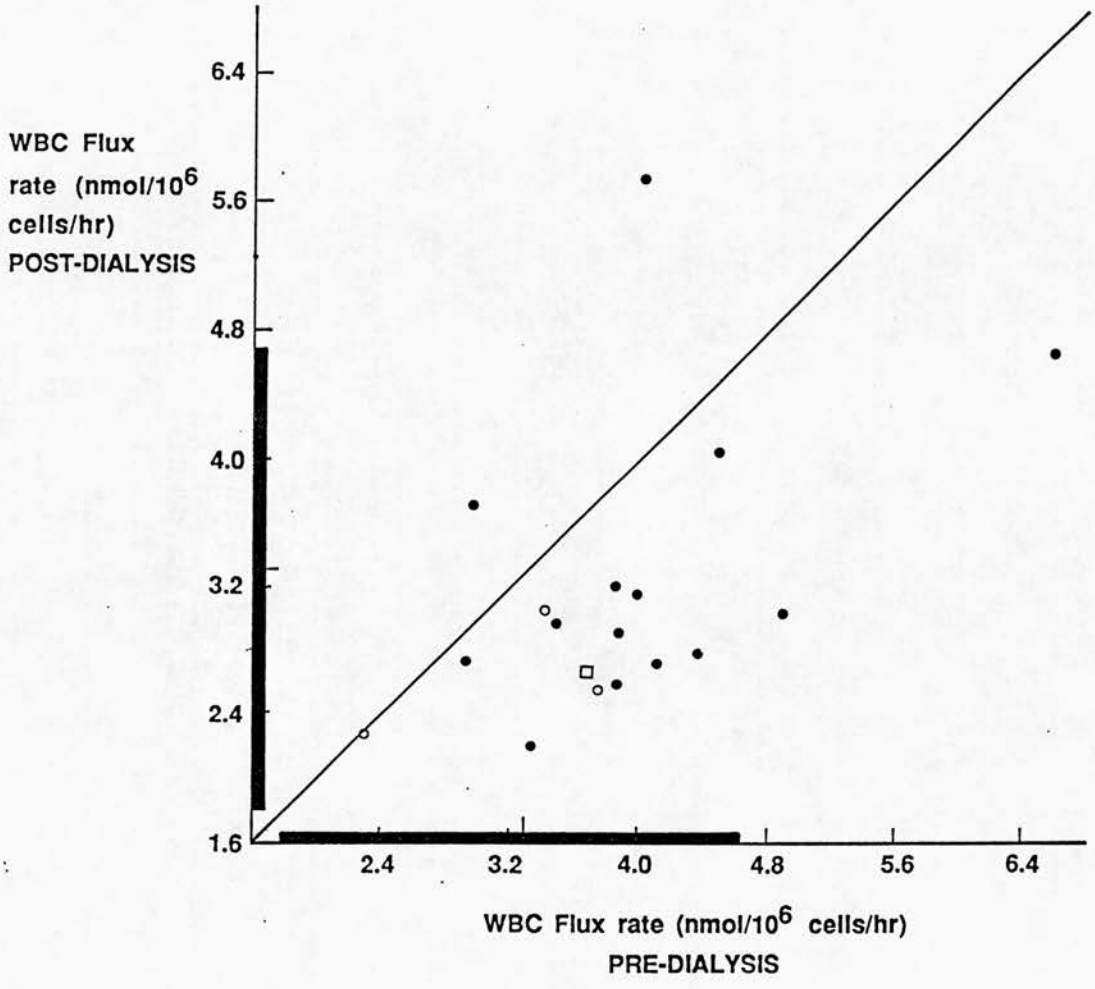

Figure S11. Leucocyte sodium flux rate before and after haemodialysis. Open circles = on digoxin, open square = on atenolol, thick bars = mean ± 2SD from controls.

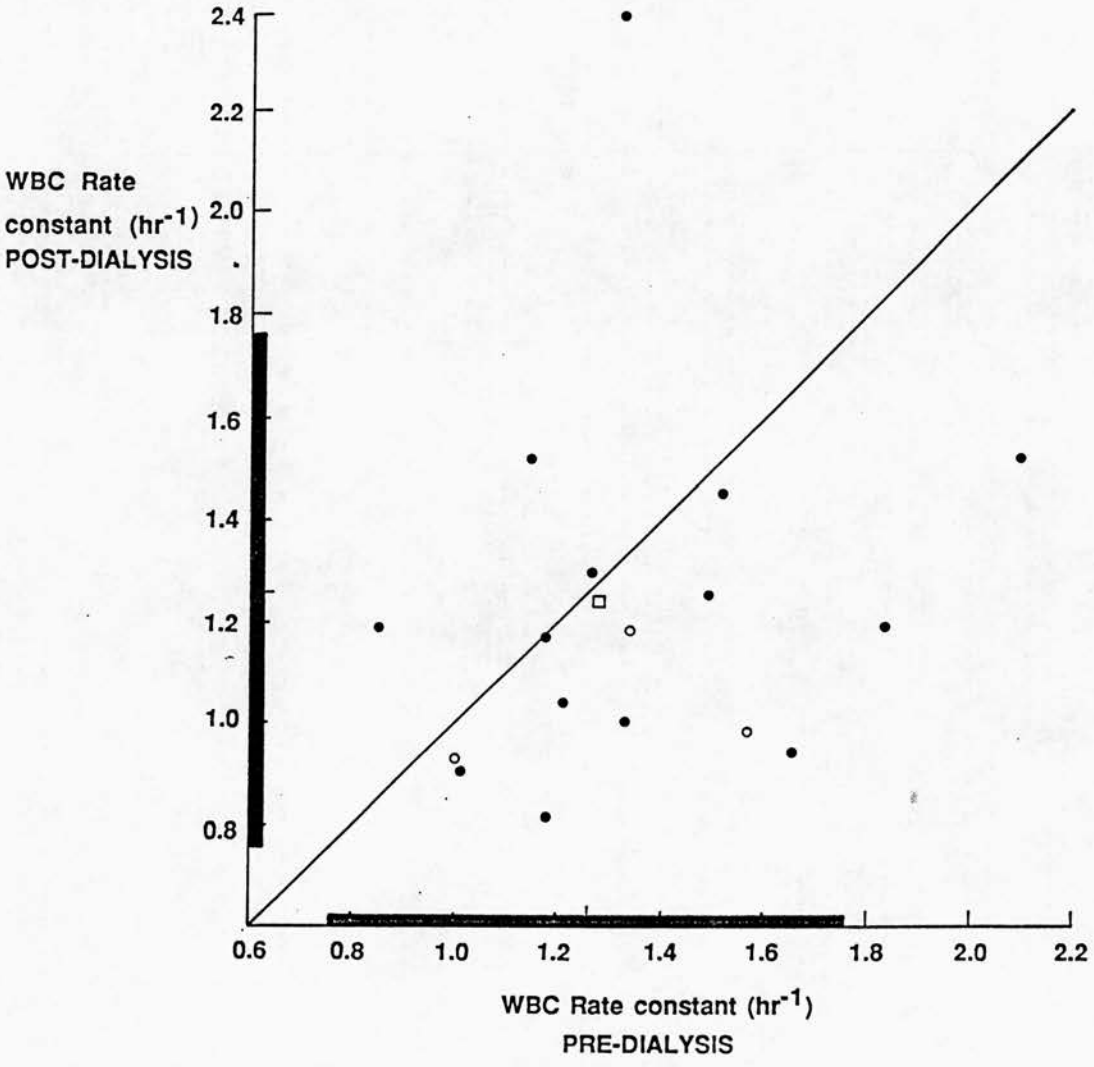

Figure S12. Leucocyte sodium flux rate constant before and after haemodialysis. Open circles = on digoxin, open square = on atenolol, thick bars = mean ± 2SD from controls.

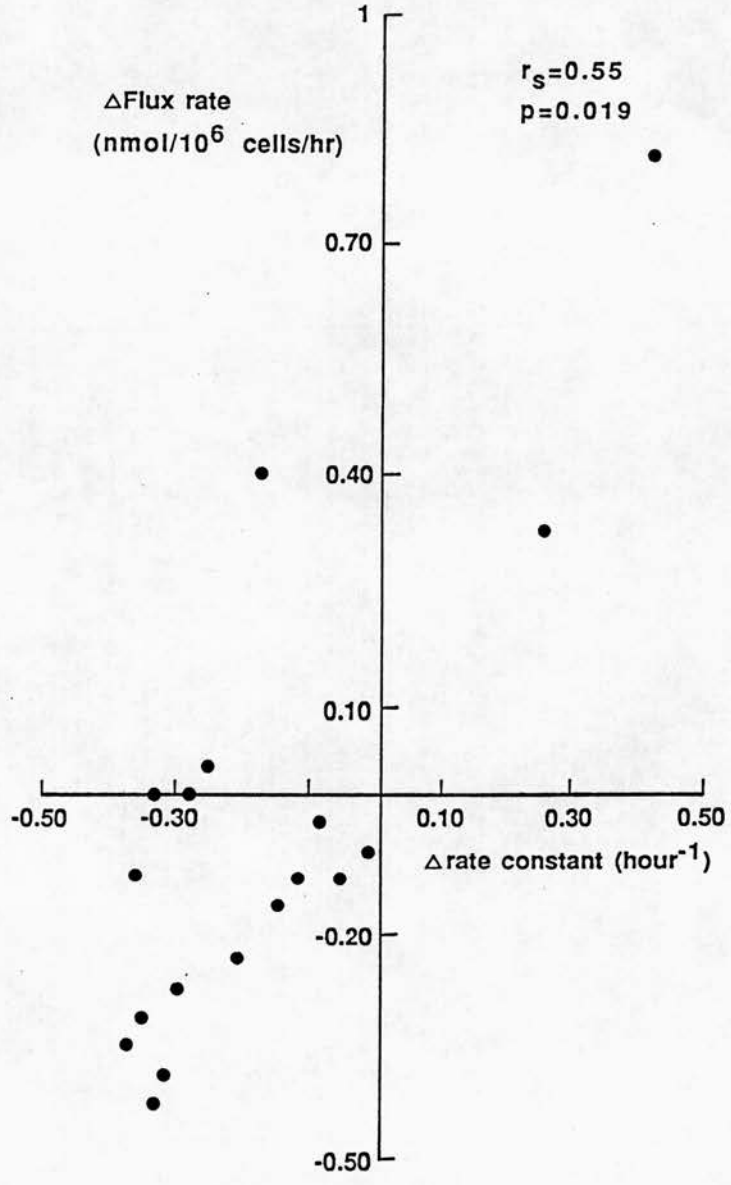

Figure S13. Changes in leucocyte sodium flux rate and rate constant over haemodialysis.

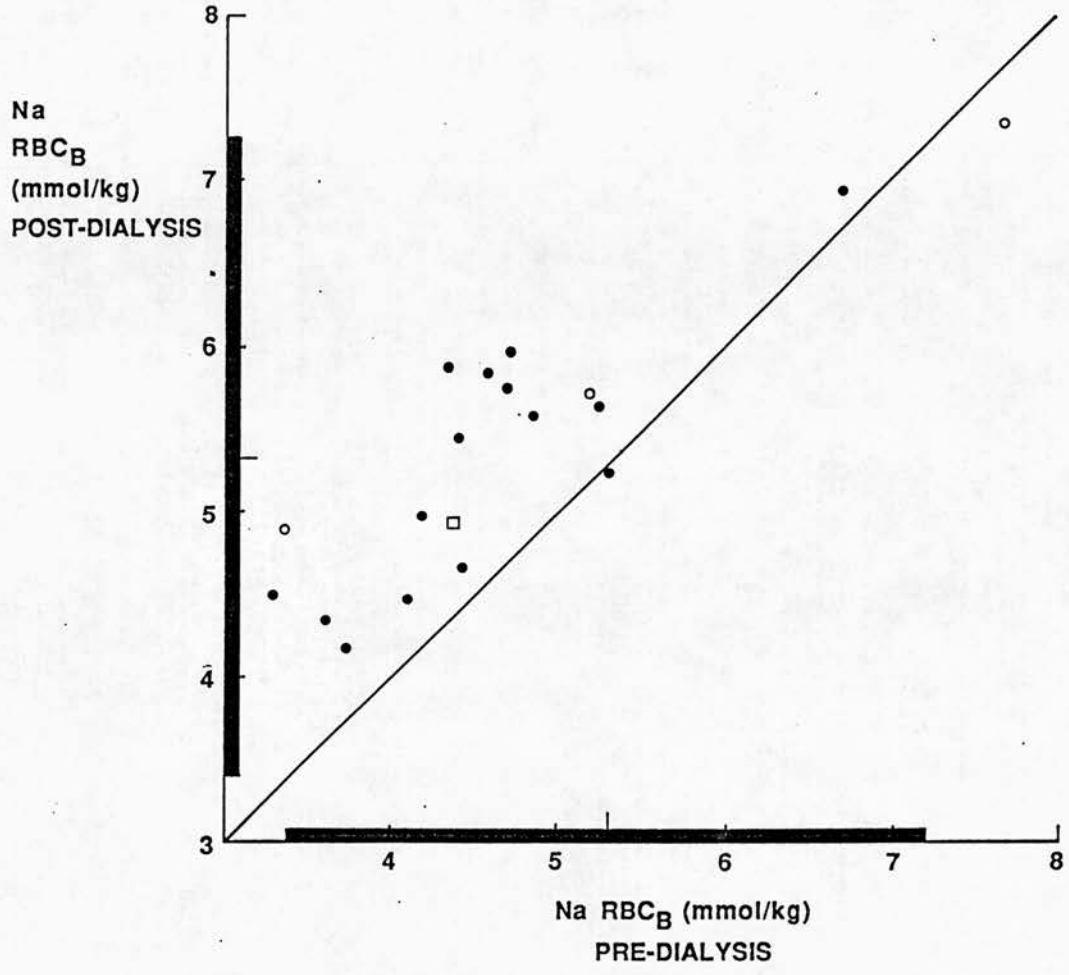

Figure S14. Na_{RBC} (whole blood incubation) before and after haemodialysis.Open circles = on digoxin, open square = on atenolol, thick bar = mean $\pm$ 2SD from controls.

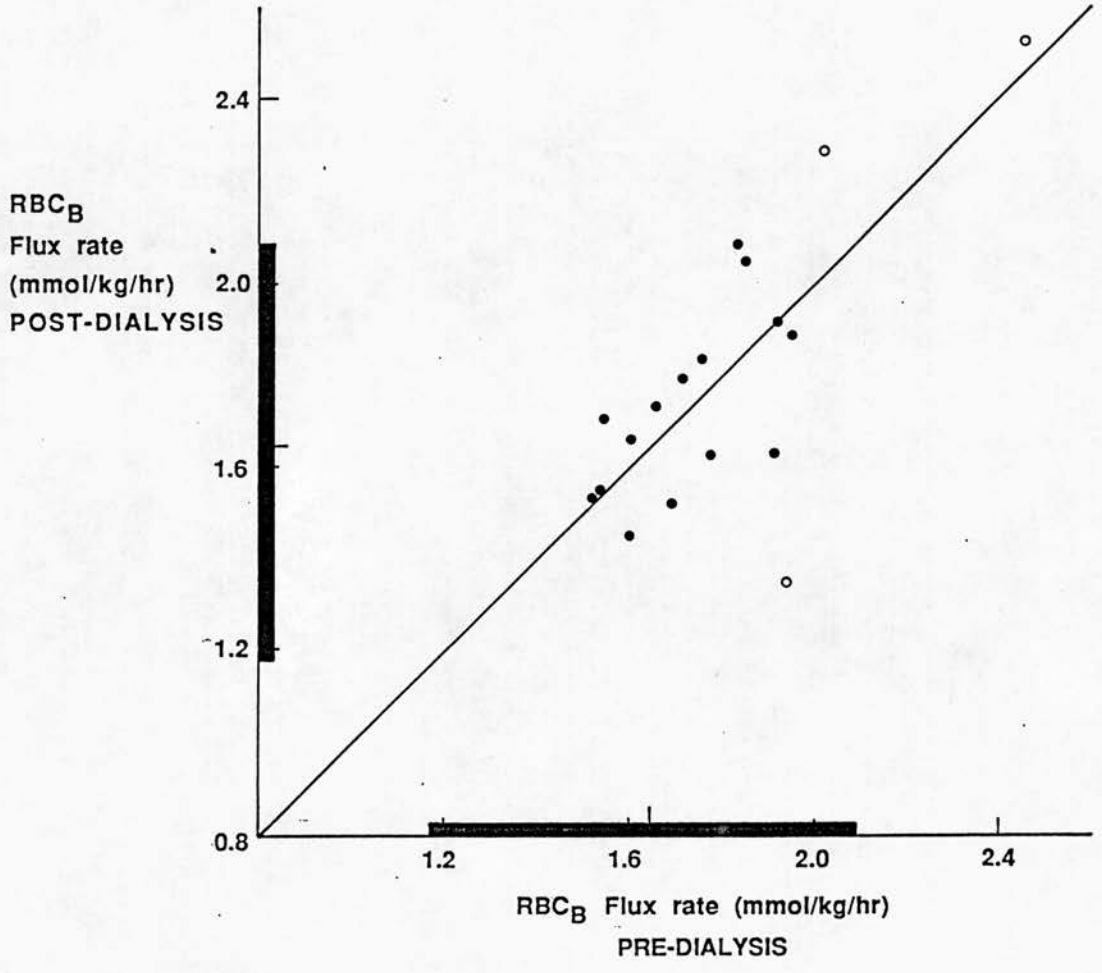

Figure S15. Erythrocyte sodium flux rate(whole blood incubation) before and after haemodialysis.Open circles = on digoxin,thick bar = mean ± 2SD from controls.

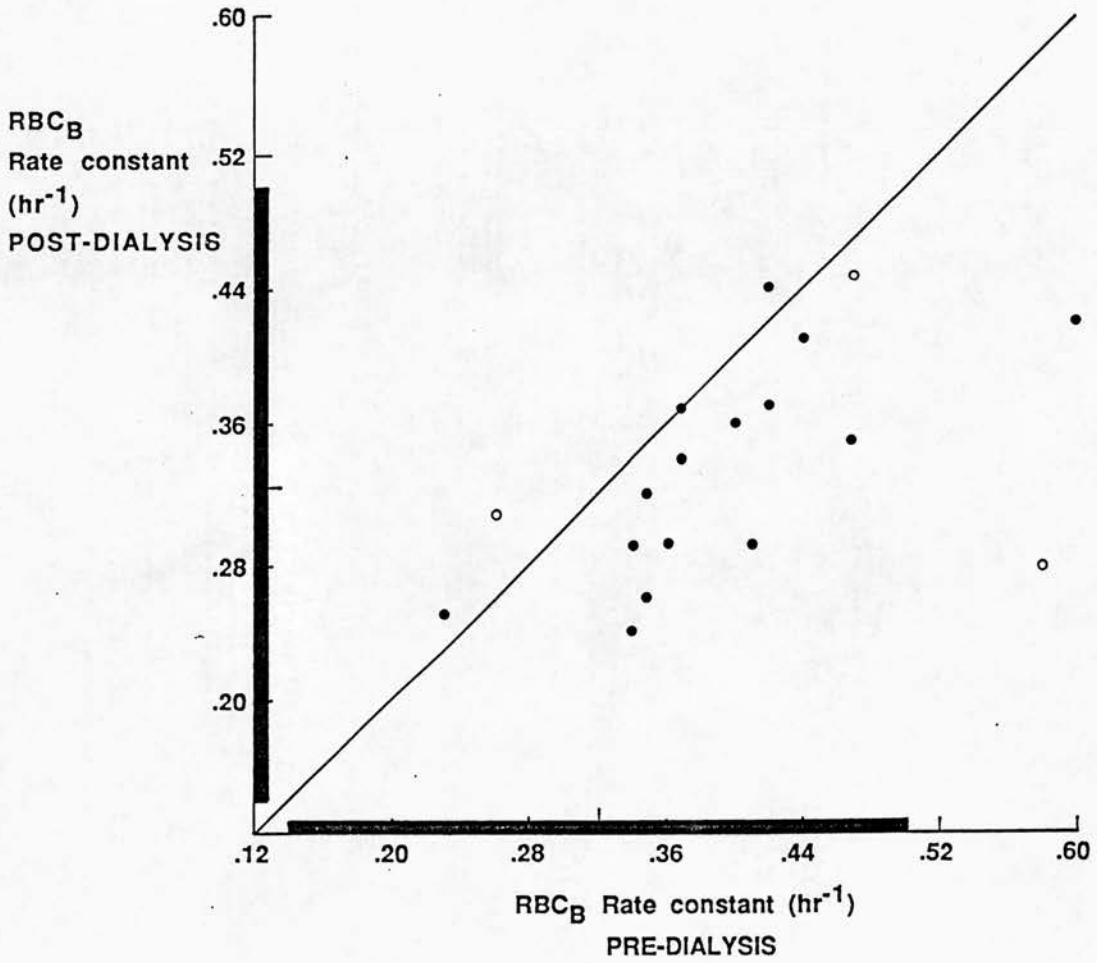

Figure S16. Erythrocyte sodium flux rate constant(whole blood incubation) before and after haemodialysis. Open circles = on digoxin,thick bar = mean $\pm$ 2SD from controls.

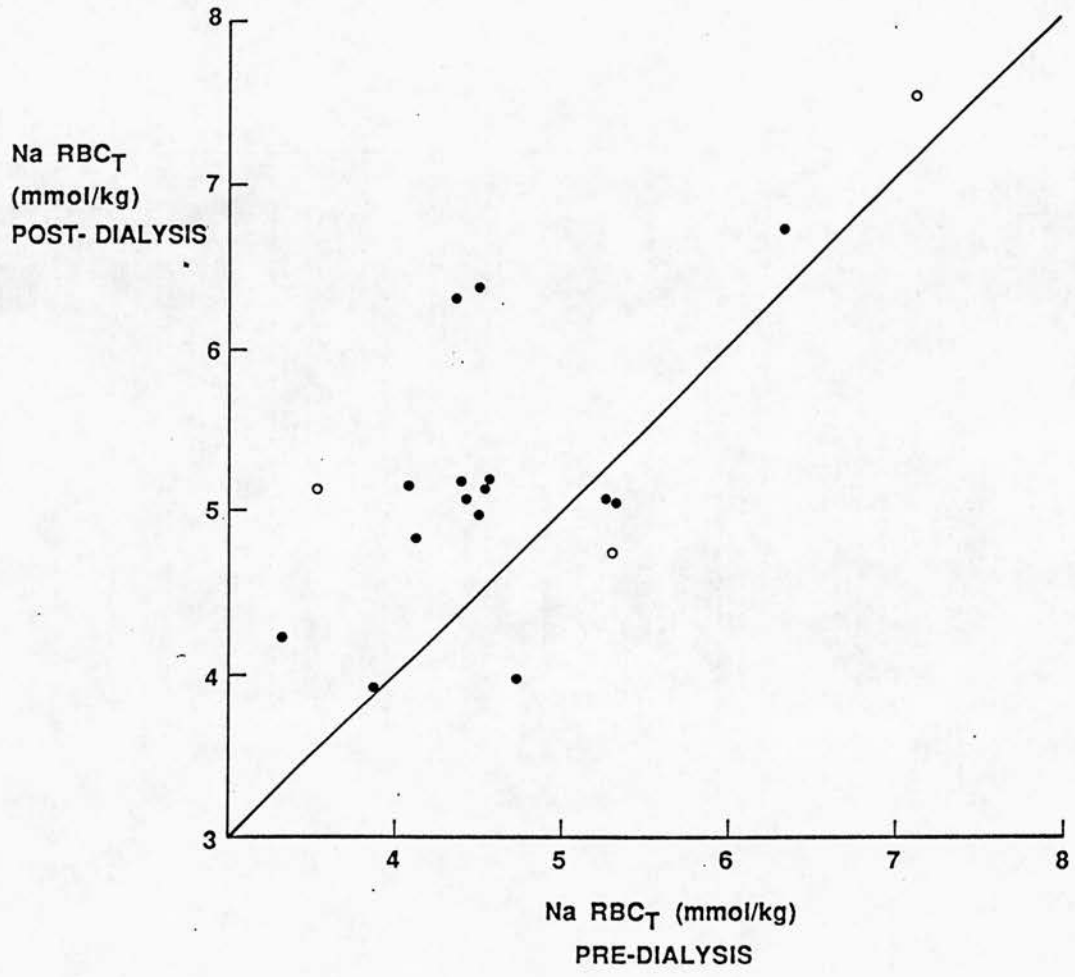

Figure S17. Na_{RBC} (TC199 incubation) before and after haemodialysis. Open circles = on digoxin.

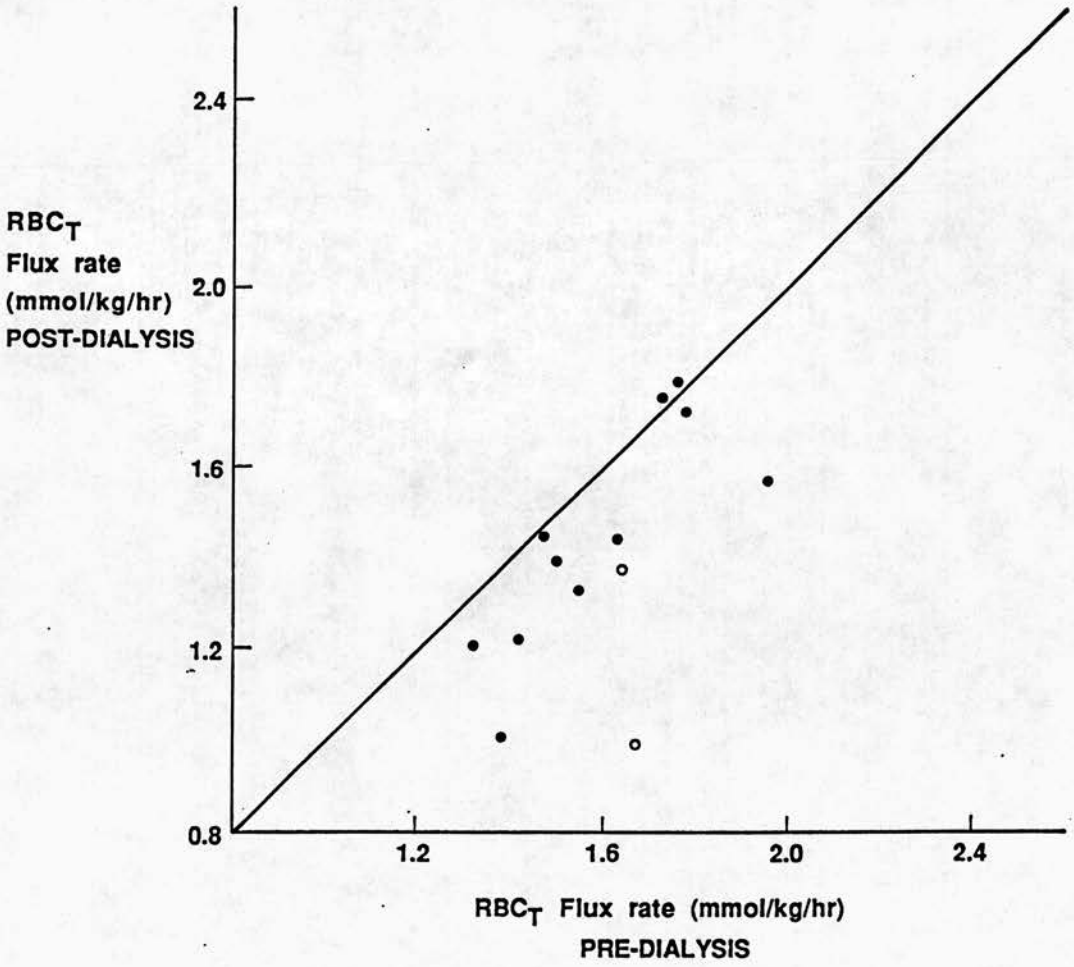

Figure S18. Erythrocyte sodium flux rate (TC199 incubation) before and after haemodialysis.Open circles = on digoxin.

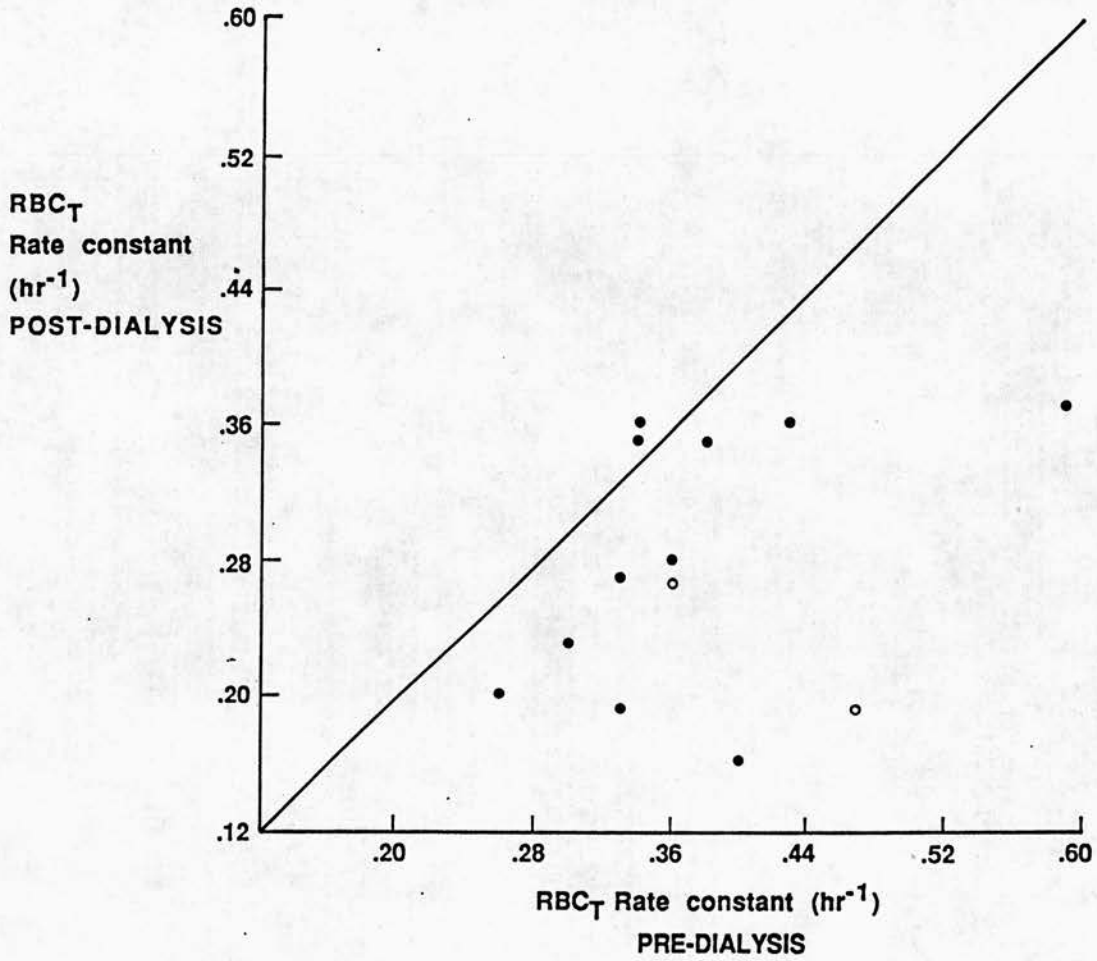

Figure S19. Erythrocyte sodium flux rate constant(TC199 incubation) before and after haemodialysis.Open circles = on digoxin.

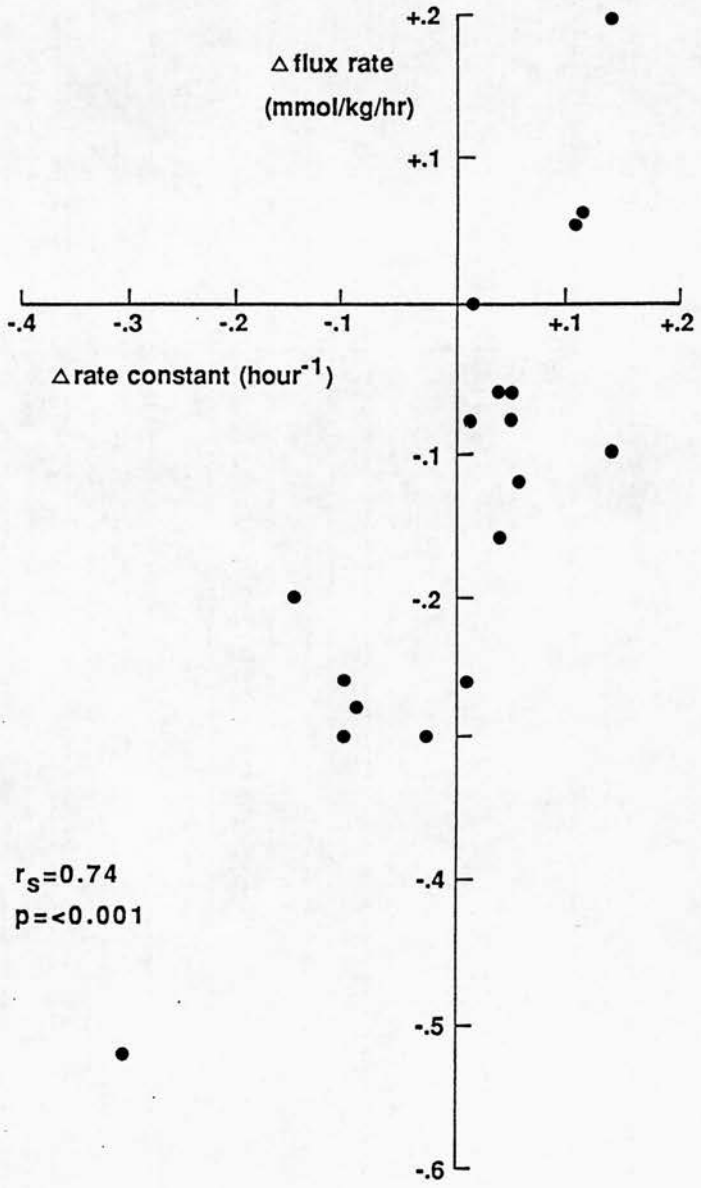

Figure S20. Changes in erythrocyte flux rate and rate constant over haemodialysis.

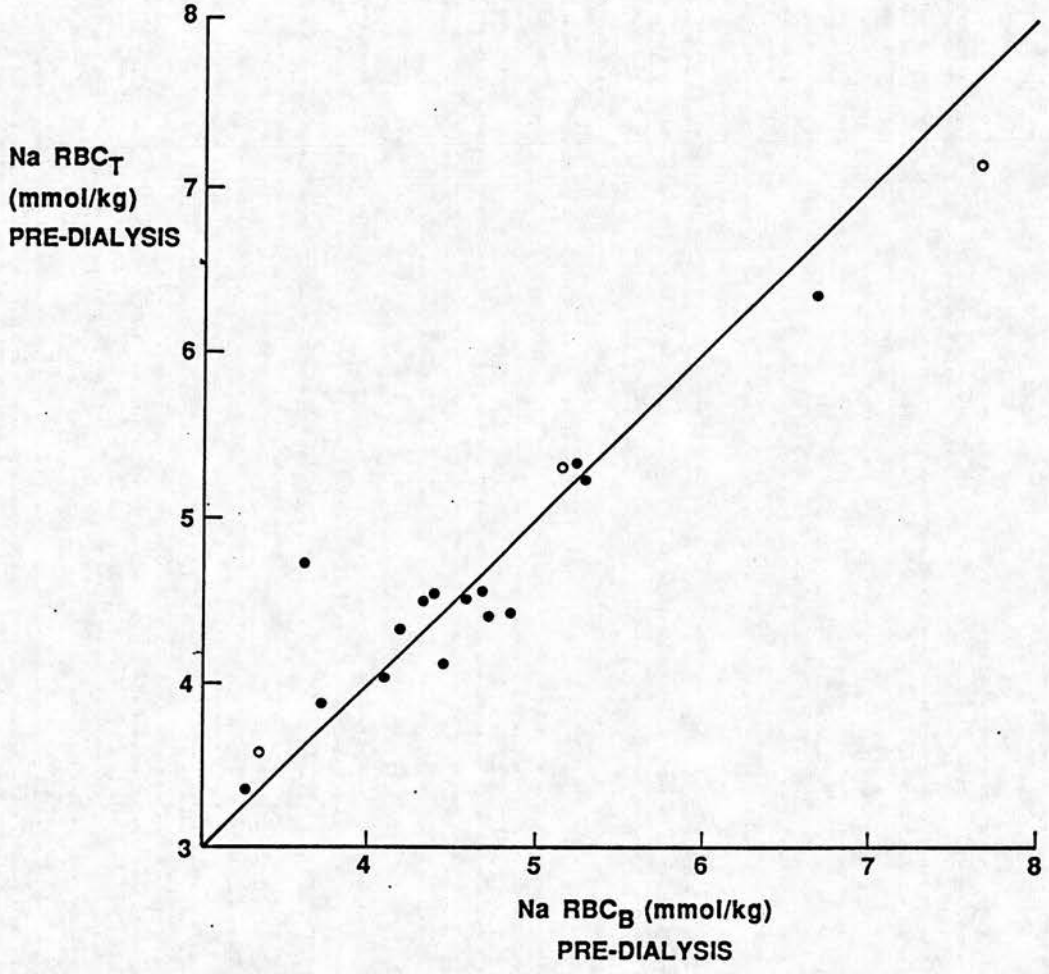

Figure S21. Pre-dialysis Na_{RBC} from cells incubated in whole blood (RBC_B) and TC199 (RBC_T). Open circles = on digoxin.

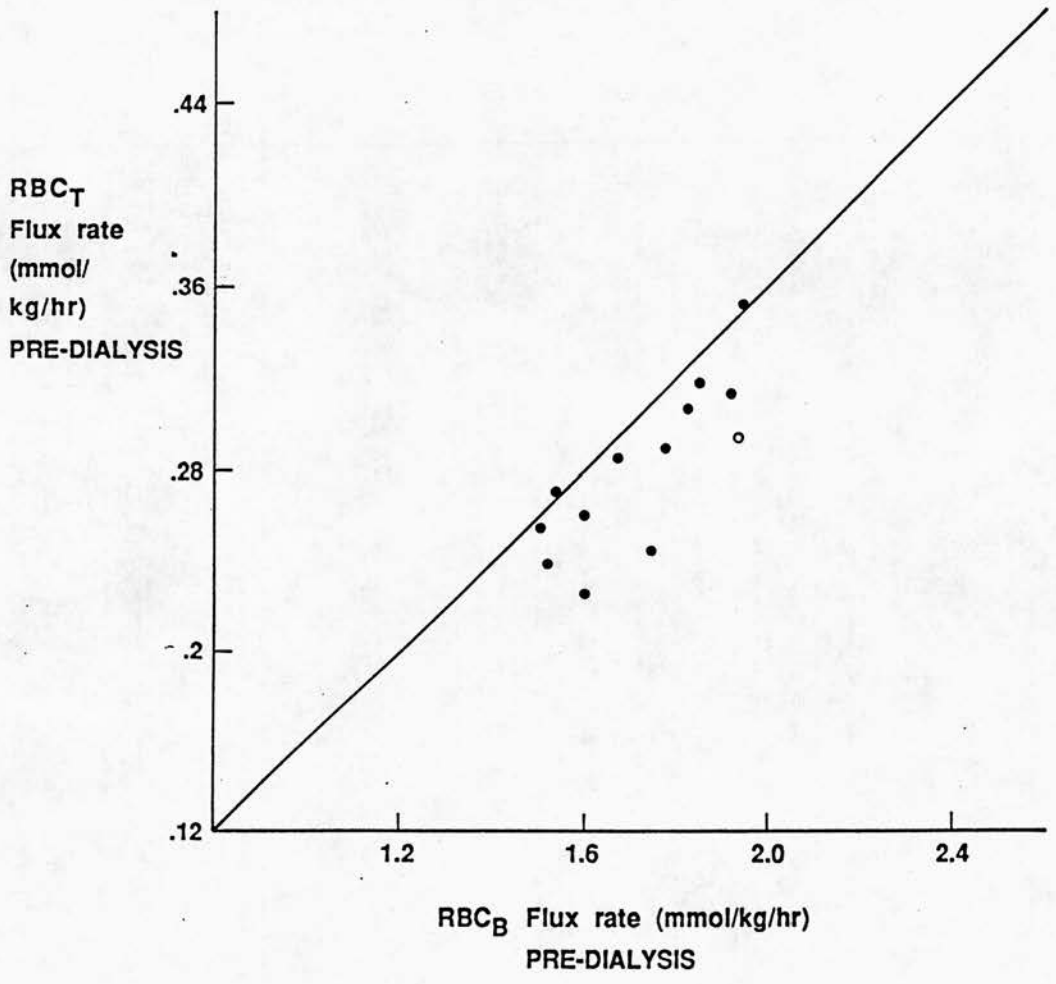

Figure S22. Pre-dialysis erythrocyte sodium flux rate from cells incubated in whole blood (RBC_B) and TC199 (RBC_T). Open circle = on digoxin.

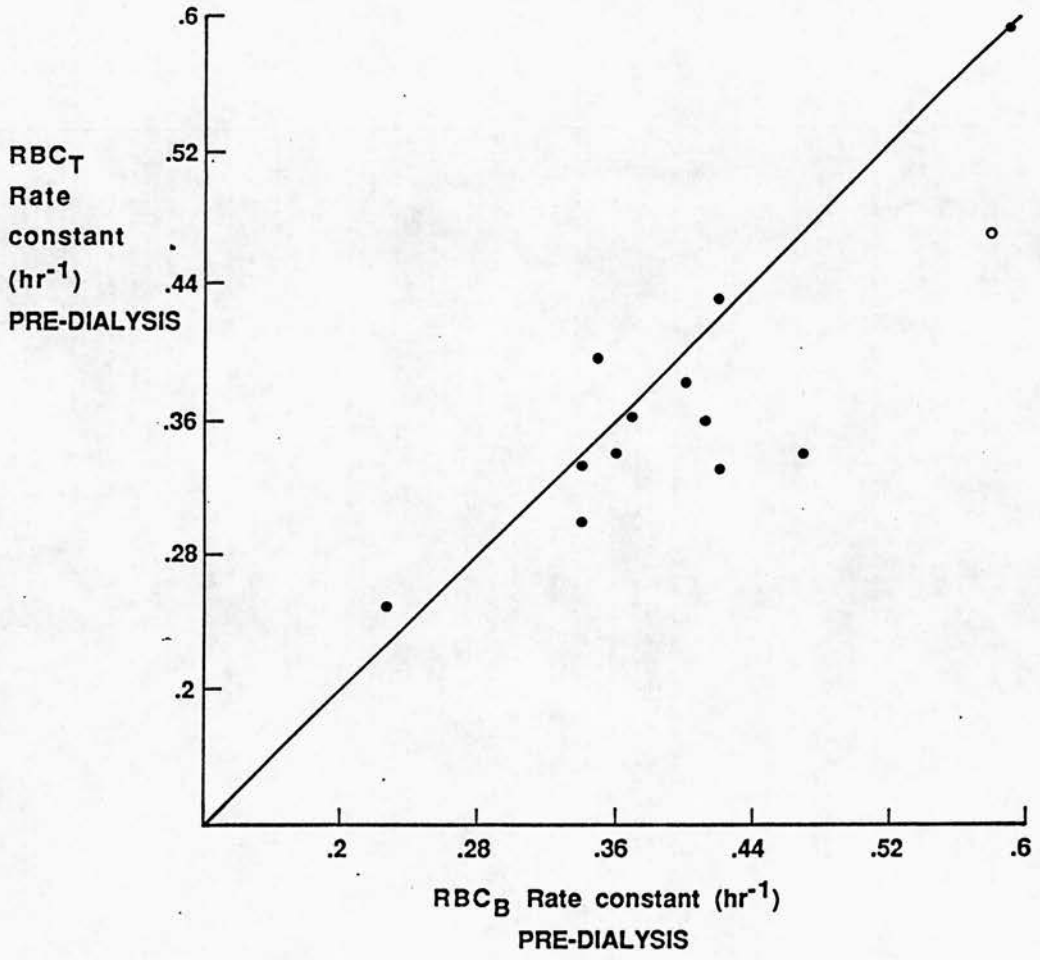

Figure S23. Pre-dialysis erythrocyte sodium flux rate constant from cells incubated in whole blood (RBC_B) and TC199 (RBC_T). Open circle = on digoxin.

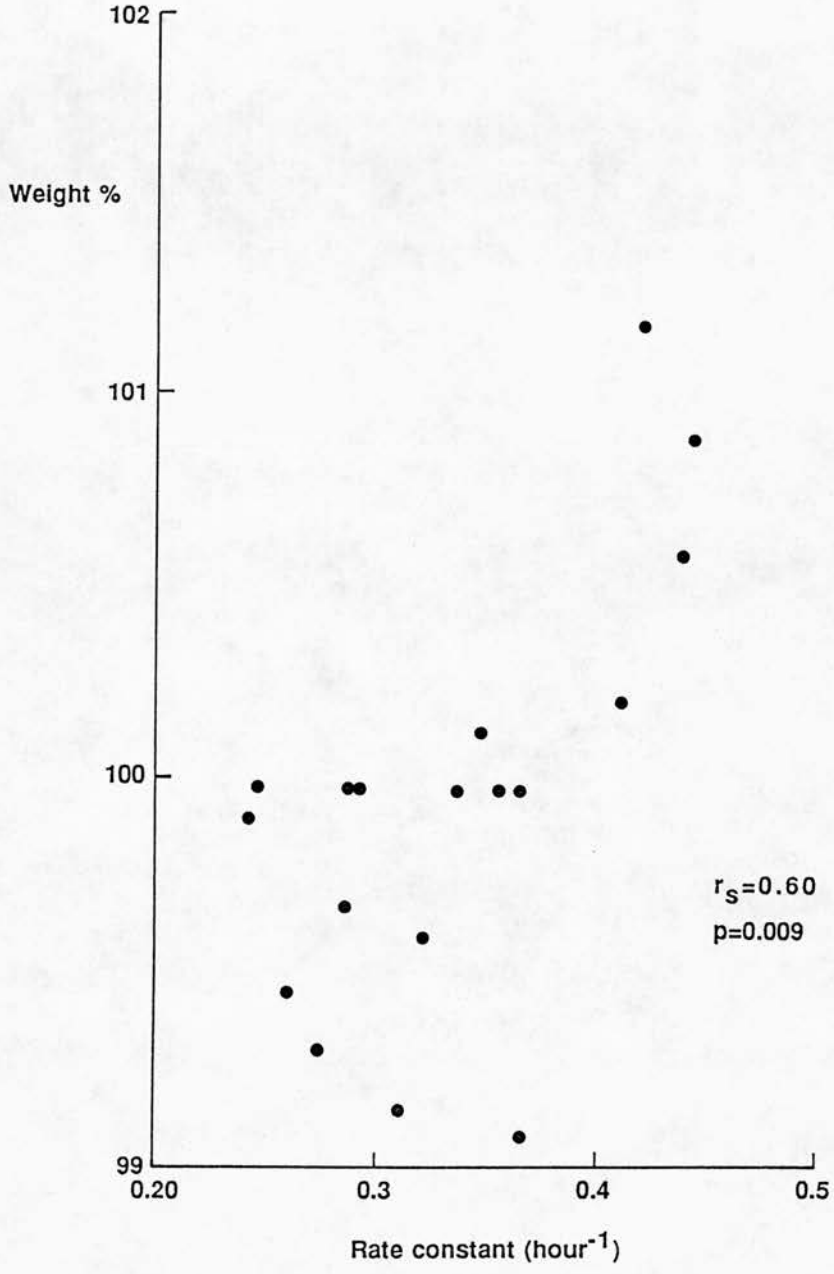

Figure S24. Erythrocyte rate constant and patient weight as a % of "dry weight", both measured after dialysis.

DISCUSSION

LEUCOCYTE SODIUM AND INCUBATION TIME

The preliminary study in undialysed uraemic patients showed that Na_{WBC} fell very sharply in the early stages of incubation. The steep fall did not persist beyond one hour, and although there were some variations after that time, the mean values obtained between 60 and 119 minutes were not significantly greater than those obtained after that. As the theoretical possibility exists that the cells would gradually recover from the effects of uraemia as the incubation progressed, it was decided to use the shortest incubation time which was likely to have allowed the cells to recover from the separation procedure, i.e. one hour. It was reassuring that no significant alterations in Na_{WBC} occurred in the main part of the study in paired samples incubated for one and three hours, as this indicated both stability and the persistence of abnormalities despite removal from the uraemic environment.

UNDIALYSED URAEMIC AND CAPD PATIENTS

Leucocytes.

This study showed that Na_{WBC} was high in a group of undialysed uraemic patients compared with healthy volunteers. The limits placed on the numbers in the study by the change in dextran prevented closer age and sex matching of the healthy volunteer group. However, within this group there was no evidence of an effect of age, sex, or the contraceptive pill on sodium content and transport, and the magnitude of changes seen in sodium content in the uraemic group make it unlikely that they were due to differences in age or sex. The slight but significant elevation of K_{WBC} in female controls concurred with

the findings of Baron and Ahmed (1969) but no such effect was found by Heagerty (1986). In their large series, Poston's group have found no effect of age or sex on leucocyte cation content or transport (L.Poston, personal communication).

Although the results in the undialysed group appeared to be in broad agreement with those of Patrick and Jones (1974) and Edmondson et al (1975a) there were some important differences. The patients in the current study were considerably less uraemic than in the two previous studies, but the difference in sodium content was more marked than previously. Furthermore, the sodium potassium ratios in the previous works were of the order of 0.27 in controls and 0.37 in patients. Possible explanations for the large differences in sodium potassium ratios in controls have been discussed in detail in the "Development of Improved Methods" section, and all those factors are likely to be relevant to cells from uraemic patients. The previously published studies measured Na_{WBC} at room temperature only, and the current work seems to be the first to have measured Na_{WBC} at 37°C in cells from uraemic patients.

In this study, no difficulties were experienced in separating leucocytes with dextran. The original workers found this method unsuitable for uraemic blood (Patrick et al, 1972) and this may again have been linked to the more severe uraemia in their patients. As previously discussed, the use of different methods to separate control and uraemic leucocytes reduced the reliability of the differences they found between controls and uraemic patients.

There was little scope in the current study to assess whether or not sodium was highest in the patients judged clinically to be sickest. Due to the great increase in availability of dialysis since the early

1970s, it is now relatively rare to come across patients with the full range of features of "uraemia". The patients in the current study were all relatively stable with renal impairment not quite severe enough to warrant the introduction of dialysis. They all had some mild symptoms of uraemia such as tiredness, nausea or itch but none were severely unwell. Almost all the patients in the previous studies had much higher plasma creatinine concentrations and presumably displayed many more of the features of uraemia. Nevertheless the undialysed patients in the current study must have had significant reductions in glomerular filtration rate, to about 5 to 20 ml/min. If, as Bricker suggested, a circulating sodium pump inhibitor is required to maintain renal sodium excretion as GFR falls, such reductions in GFR should presumably be sufficient to cause release of the pump inhibitor.

Patrick and Jones (1974) stated that their "undialysed uraemic patients, with increased leucocyte sodium content, were all hypertensive". This may have meant that all the uraemic patients were hypertensive, or that all the uraemic patients with elevated Na_{WBC} were hypertensive. The possible confounding effect of anti-hypertensive medication is mentioned and it is not clear if hypertensive in this context meant patients receiving anti-hypertensive treatment or patients whose blood pressure was elevated at the time of study. No data regarding blood pressure or treatment was provided. In the current study there were no clear cut distinctions in Na_{WBC} when patients were divided into those receiving treatment for hypertension and those not. No patient was hypertensive at the time of the study. It is not possible to exclude the possibility that the hypertensive patients would have had elevated

Na_{WBC} had they not been treated, but there were untreated, normotensive patients with clearly elevated Na_{WBC}.

As essential hypertension may be associated with raised Na_{WBC} and abnormal sodium transport (Edmondson et al, 1975b; Poston et al, 1981b), it was important to ensure, as far as possible, that the uraemic patients with elevated Na_{WBC} did not suffer from hypertensive nephropathy. The frequency with which Na_{WBC} was found to be elevated made it highly unlikely that all the cases had hypertensive nephropathy, and most of those with elevated Na_{WBC} had no evidence that they suffered from essential hypertension. Na_{WBC} was not elevated in all those patients identified as possibly having essential hypertension.

In this study, the drugs most commonly prescribed to the patients were frusemide and beta-blockers. There is no data in the literature concerning the effects of either of these groups of drugs on Na_{WBC}, and a suggestion that beta blockade has no effect on sodium efflux rate constant in essential hypertensives (Poston et al 1981a). Beta-blockers, through their actions on renin release and the sympathetic nervous system, might be expected to reduce sodium pump rate constant, and there are reports of thiazide diuretics altering Na_{WBC} (Araoye et al, 1978) or sodium pump rate constant (Milner et al, 1984). The numbers in the current study were too small to allow statistical analysis or to exclude an effect, but there were no obvious effects of either drug on cation content and transport.

This study found no evidence to support the previous finding of low K_{WBC} in uraemia (Patrick et al, 1972). Again, the patients in that study were more uraemic than those reported here.

Although Na_{WBC} was elevated in undialysed uraemic patients in the

current study, this did not appear to be due to the effect of a circulating sodium pump inhibitor which reduced sodium flux rate constant. The increase in Na_{WBC} could not be clearly attributed to either increased sodium influx or decreased pump activity. This may partly have reflected the small numbers in the study, or been because alterations in sodium content were due to combinations of changes in flux rate and rate constant. Even with the numbers studied, it was clear that the very obvious fall in rate constant found by Edmondson et al was not present in the current study.

Na_{WBC} was normal in the CAPD group and significantly lower than in the undialysed group. This was associated with a tendency for flux rate to be raised in the CAPD group, but a concomitant increase in rate constant kept Na_{WBC} close to normal. Age was similar in the two patient groups. Despite dialysis, plasma creatinine concentration was higher in the CAPD group but plasma urea, bicarbonate, calcium and phosphate concentrations were all significantly closer to normal in that group. Clinically, the patients in the CRF and CAPD groups were normovolaemic in that their blood pressures were not raised and they were neither dehydrated nor fluid overloaded. The difference in Na_{WBC} would therefore appear more likely to have been due to substances which accumulate in uraemia and are cleared by CAPD rather than substances released in response to alterations in ECF volume. As atrial pressure and atrial natriuretic peptide levels were not measured, it is impossible to be sure that clinically undetectable differences in ECF volume did not exist between the two groups.

There have been no previous reports of leucocyte sodium content and transport in CAPD patients. Although it might be expected that it would have a similar effect to haemodialysis, which appears to

normalise sodium transport, there are important differences in the two methods of treatment. In particular, the continuous nature of CAPD prevents the marked fluctuations in ECF volume, blood pressure and biochemical indices of uraemia seen in haemodialysis. Also, CAPD patients are constantly absorbing glucose from their dialysate. A further study to confirm or refute the apparent matched increase in flux rate and rate constant would be of interest. If this observation was confirmed it would underline the need to always consider active and passive sodium transport together, as it would suggest that elevated Na_{WBC} in the undialysed patients was due to the combination of an increased passive sodium influx rate and a failure of active efflux via the sodium pump to respond to this. The normal Na_{WBC} in CAPD patients appeared to not be due to a correction of the increased flux rate, but rather to an increase in sodium pump rate constant, presumably mediated through increased Na_{WBC}. Such a theory raises the possibility that, despite normal rate constants, the sodium pumps of the undialysed patients were indeed exposed to some form of inhibition and therefore could not react to increased sodium influx.

No correlation was found between the biochemical severity of uraemia in the undialysed patients and leucocyte sodium content or transport. This was also the finding in the previous studies. Despite Na_{WBC} being closer to normal in the CAPD group, significant correlations were found between it and plasma urea and creatinine concentrations. Correlation coefficients must be interpreted cautiously but possibly some substance(s) whose concentrations are determined by factors similar to those that determine plasma urea and creatinine influenced Na_{WBC}. Some other factor(s) must have been responsible for the elevation of Na_{WBC} seen in the undialysed group and it may be

that the removal of such factor(s) in the CAPD group both normalised Na_{WBC} and allowed the correlation between it and plasma biochemistry to become apparent. It was not possible to identify whether the link with Na_{WBC} was mediated through flux rate or rate constant.

The longitudinal studies, although too few in number to allow definitive conclusions to be drawn, were generally consistent. Where Na_{WBC} was high before dialysis, it fell as soon as dialysis started and in patient HC this was associated with a rise in rate constant from low to normal. No pre-dialysis measurement of rate constant was made in WG but measurements made after dialysis followed a similar pattern. Such patterns are consistent with the presence of a circulating sodium pump inhibitor, the levels of which fell after starting dialysis. There were no obvious differences clinically or biochemically between them and the other two patients who had no suggestion of raised Na_{WBC} or sodium pump inhibition. Changes in patient WG followed longitudinally were similar to the overall results in the haemodialysis study with respect to the rise in Na_{WBC} and flux rate between dialyses, but in his case rate constant fell between dialyses.

Erythrocytes.

The findings in erythrocytes from undialysed uraemic patients were consistent with previous work from this laboratory which had shown that Na_{RBC} was low in such patients (Thomas et al, 1989). As described in the theoretical background section ("Cation Content and Transport in Uraemia in Cells other than Leucocytes"), studies of erythrocytes in uraemia have shown very little consistency, with a wide range of abnormalities being described. It is therefore not surprising that the current results were in agreement with some

studies (e.g. Cumberbatch and Morgan, 1981) but not with others. Although CAPD patients have been studied less frequently than haemodialysis patients, the finding of less abnormal results in the CAPD group was in keeping with the general finding that erythrocytes from dialysed patients tend to have more normal cation content and transport than those from undialysed patients.

Comparison of leucocytes and erythrocytes.

This study clearly showed that in undialysed uraemic patients, intracellular sodium measured in the leucocyte was high, whereas in the erythrocyte it was low. The abnormalities could not be clearly linked to either abnormal sodium influx or sodium pump activity. Both cell types have been widely used as "model" cells because of the great difficulties in isolating other cell types but clearly, in uraemia at least, the findings in leucocytes or erythrocytes cannot safely be extrapolated to other cells. In theory, the leucocytes, being nucleated, are more likely to represent the generality of cells, if indeed such a concept is valid. Both cell types were consistent in that the abnormalities present in sodium content in the undialysed patients were less marked in the CAPD patients. These findings, although in keeping with the proposition that the degree of abnormality in red or white cells reflects the adequacy of dialysis (e.g. Corry et al 1986, 1987), do nothing to prove such a proposition. As the proof of this would depend on showing firstly, that patients with more normal intracellular sodium survived and experienced fewer complications of dialysis than those with abnormal sodium, and secondly, that altering dialysis regimes to normalise sodium reduced complications, a large and prolonged study would be necessary. To further show that these time consuming measurements

were more accurate indicators of adequacy of dialysis than conventional assessments based on plasma biochemistry (e.g. dialysis index) would be difficult. Furthermore, as discussed above, the results from the CAPD group suggested that Na_{WBC} was normalised, not by removing the abnormality (high flux rate) in the undialysed group, but by compensating for this abnormality with an increased rate constant.

In summary, this study showed that, in a group of undialysed uraemic patients, Na_{WBC} was higher and Na_{RBC} lower than in healthy volunteers. In a group of CAPD patients, intracellular Na in both cell types was significantly closer to normal than in the undialysed patients. Highly significant correlations were found between Na_{WBC} and plasma urea and creatinine concentrations in the CAPD group only.

HAEMODIALYSIS STUDY

This study showed that a single haemodialysis session was associated with alterations in cellular sodium content and transport, that these changes were different in red and white blood cells, and that the changes resulted in intracellular sodium content being closer to normal after dialysis.

Leucocytes

Sodium flux rate fell over dialysis in 16 of the 18 patients (Fig. S11), in association with a fall in rate constant which, although not statistically significant, was sufficient to prevent the change in sodium content reaching statistical significance. The change in rate constant would have been statistically significant but for the one patient whose rate constant was extremely high after dialysis (Fig. S12). The change over dialysis in all intracellular indices was towards the values from controls, although flux rate appeared to be as much below the normal mean after dialysis as it had been above it before. The change in flux rate correlated with the change in rate constant (Fig. S13). This apparent matching of changes in flux rate and rate constant implied a link between sodium influx and the capacity for the sodium pump to remove sodium from the cell. Such a linkage is known to occur and is mediated via intracellular sodium concentration, an increase in which stimulates utilisation of a pre-existing but inactive pool of pumps (Doucet, 1988) and new pump construction (Fambrough et al, 1987). The time course of the latter mechanism makes it unlikely to be responsible for an effect seen after four hours of dialysis, but the activation of latent pumps could conceivably occur in this timespan. Linkage of flux rate and rate constant in leucocytes has previously been described in

different circumstances by Patrick et al (1978) and in erythrocytes by Cumberbatch and Morgan (1981).

There is very little previously published information about leucocyte cation content in haemodialysis patients. What data exist tend to conflict with the current results. Patrick and Jones (1974) found Na_{WBC} to be slightly low and K_{WBC} to be considerably lower than in controls in pre-dialysis samples, and Edmondson et al (1975a) found the sodium pump rate constant to be low in haemodialysis patients compared with controls, but higher than in undialysed patients. The current study was unable to confirm any of these findings. There were potential methodological problems with those reports which have been discussed above ("Leucocyte Cation Content and Transport in Uraemia"). The most important was probably the use of different methods to isolate leucocytes in patients and controls. No details were given in those papers about the biochemical adequacy of dialysis, and in view of the changes in haemodialysis technology since that time, it is quite possible that their patients were biochemically quite different from those in the current study. In the previous studies, the patients were dialysed for 30 hours per week as opposed to 12 to 15 hours per week in the current study. It is highly likely that most of these previous dialysis patients were aged less than 50, whereas most in this study were older than that. The evidence available however would not suggest that age differences could account for the sodium content and transport differences.

There are no previous reports in the literature of leucocyte flux rates in haemodialysis patients, and no reports of changes in any aspect of leucocyte cation content and transport over a haemodialysis session. As the sodium content and rate constant were below normal in

haemodialysis patients in the report of Edmondson et al, by implication the flux rate must also have been somewhat diminished, as opposed to the findings in this study.

Erythrocytes.

Haemodialysis was associated with a significant increase in Na_{RBC} and fall in rate constant in both incubation media. The fall in flux rate was only significant in cells incubated in TC199. Although incubation in TC199 was associated with significant changes in potassium, flux rate and rate constant compared with incubation in blood, values obtained from one incubation medium correlated well with those from the other. The effect of incubation in TC199 was not similar to the effect of dialysis. This failure of removal of cells from uraemic blood to mimic the effects of dialysis could partly relate to insufficient time (two hours) in the new medium. The change in sodium and rate constant in TC199 was in the same direction but less marked than the change induced by dialysis. The change in flux rate, although in the same direction, was much greater in TC199. Of course, post-dialysis blood still contains many of the substances which accumulate in renal failure and are not present in tissue culture fluid, which is arguably more "normal" than post-dialysis plasma. As control values were not obtained for erythrocytes incubated in TC199, it is not possible to comment on how abnormal those values were. As the pre- and post-dialysis values were different, it is however clear that incubation in TC199 did not always return the cells to normal, and as the pattern of changes over dialysis were similar in both incubation media, it is likely that the post-dialysis values in TC199 were closer to normal for that medium.

The disparities in the existing literature concerning RBC cation content and transport in haemodialysis patients are enormous. In studies of undialysed uraemic patients, most of the possible combinations of abnormalities have been reported and it is therefore not surprising that the current results are at least partially in agreement with some studies. For instance, Engelhardt et al (1987) found that Na_{RBC} increased over a haemodialysis session, but there were no control values to show if this represented a change towards normal. The change was due to an increase in sodium influx, whereas in the current study the change was due to a drop in rate constant. Diez et al(1986) found Na_{RBC} to be lower than controls in haemodialysis patients, in association with a lowered flux rate. Kariya et al (1986) also found a low Na_{RBC} in haemodialysis patients, and as the rate constant was also low the flux rate must have been reduced.

There are important differences with some previous conclusions. It has been suggested on several occasions that in adequately dialysed patients erythrocyte sodium transport is normal (Corry et al, 1986; Corry et al, 1987 and Kaji and Kahn, 1987). These suggestions have been based on the failure to find abnormalities in a population of allegedly adequately dialysed patents, rather than in finding a link in individual studies between the adequacy of dialysis and the presence of sodium transport abnormalities. In the current study there was no evidence of a link between dialysis index or plasma creatinine concentration and erythrocyte sodium transport, although the spread of values for dialysis index showed that the patients in this study ranged from well dialysed to poorly dialysed.

Another important finding, if confirmed, was a link between volume

removal at dialysis and sodium pump availability, in keeping with the volume-related circulating sodium pump inhibitor theory (Izumo et al, 1984 and Quarello et al 1985). The current study found no confirmatory evidence, rate constant decreasing in leucocytes over dialysis and associated fluid removal. It is not easy to explain the wide differences in findings from different centres but one potentially confounding factor which is often not controlled is red cell age. It has been shown that red cell sodium transport changes as the cells grow older (Cheng et al, 1984), and therefore the way in which the cells are sampled is important. In the current studies, high speed centrifugation distributed the cells according to their age, and only the top third (i.e. the youngest) cells were used. Most studies do not define the way in which red cells are sampled.

As the current results showed similar trends in cells incubated in their own blood or in TC199, it seems unlikely that the use of different incubation media can explain the completely different patterns of abnormality in different studies.

Comparison of erythrocytes and leucocytes.

The pattern of changes in erythrocytes was different to that in leucocytes. In erythrocytes in both incubation media, dialysis was associated with falls in both flux rate and rate constant. Falls in both these indices also occurred in leucocytes but in erythrocytes the fall in rate constant exceeded that in flux rate, resulting in a rise in Na_{RBC}, whereas in leucocytes the balance of the alterations produced a fall in Na_{WBC}. In both cell types the sodium content was closer to values from controls after dialysis, and dialysis-associated changes in flux rate and rate constant were correlated. Although of borderline statistical significance, the direct

correlation between rate constants in the two cell types before dialysis might suggest that the sodium pumps in the two cell types were responding similarly to various external factors regulating sodium pump number and activity. The failure to find correlations after dialysis and the failure of the changes in rate constant in the two cell types to correlate may have reflected the differing capacities of the two cell types to respond to the changes which occur during dialysis.

There does not appear to be any published evidence that, when they are measured simultaneously, erythrocyte and leucocyte cation content and transport are related. There are no reports of simultaneous estimations in uraemia, but in a study of hyperthyroid patients (Khan and Baron, 1987), there were differences between RBC and WBC in intracellular sodium, Na-K-ATPase activity, active rubidium influx, and ouabain sensitive sodium flux rates and rate constants. The disparities found in the current studies are therefore not confined to uraemic and dialysis patients. It may be that the sodium pumps in both cell types are similar in their short term responses to various plasma factors which regulate pump activity. With regard to this, it is of interest that Kelly et al (1986) desribed an acute rise in levels of an endogenous digitalis-like factor after haemodialysis, a finding compatible with reduced sodium flux rate constant after haemodialysis in the current work. However, as only nucleated cells can construct new pumps, factors which lead to an alteration in sodium influx and therefore internal sodium content will acutely alter sodium pump numbers and therefore rate constant in leucocytes but not erythrocytes. Any change in erythrocytes related to increased synthesis of sodium pumps would only became apparent as new cells

appear in the circulation. Although the acute matching of rate constant and flux rate in erythrocytes cannot be mediated through construction of completely new pumps, possible mechanisms for such matching include alterations in the proportion of sodium-sodium exchange through the pump or intermittent utilisation of some pumps. As erythrocytes and leucocytes have completely different functions and metabolic requirements, their membranes are likely to have different permeabilities to external sodium and different responses in permeability to factors which affect membranes. It is hardly surprising that the two cell types appear so different in their response to uraemia and dialysis.

Although the nucleated leucocyte might in general seem to be a more typical cell, haemodialysis might be a special case. Immediately after commencing haemodialysis with a cuprophan membrane (the type used in this study) there is a marked fall in peripheral blood leucocyte count (Hoenich et al, 1986). This fall is due to clumping and adherence of the leucocytes in the pulmonary vasculature, a process which presumably involves an alteration in the energy consumption of the leucocyte. Although peripheral blood counts have returned to normal by the end of dialysis, it is not known whether the leucocyte activation influences sodium transport as measured after dialysis. Another possibility is that the populations of leucocytes sampled are different before and after dialysis. In the current study whole blood differential and total white cell counts were similar before and after dialysis, and the experimental leucocyte suspensions were always predominantly (80-95%) polymorphonuclear leucocytes. It was therefore felt unlikely that any observed alterations in sodium transport would be due to changes in

the sampled cell population. Despite the widespread acceptance of the cell suspensions obtained by using dextran, with no attempts to control the proportion of leucocytes and lymphocytes, it is possible that either changes in their relative proportions or more subtle alterations in for instance the age distribution of the leucocyte sample could have been responsible for apparent alterations in sodium content and transport. There do not appear to have been any good comparisons of sodium transport in lymphocytes and leucocytes. Such a comparison might be difficult as the findings in the method development section of this work suggest that the material used at the isolation stage can affect sodium content, and different methods are required to isolate leucocytes and lymphocytes. These difficulties were highlighted by Poston et al (1982b) when they responded to a study suggesting an absence of sodium pump activity in polymorphonuclear leucocytes (Smith and Peters, 1980). There do not appear to have been any reports of the effect of cell age on leucocyte sodium transport.

As the large number of studies of erythrocyte sodium transport in uraemia are primarily of interest only if erythrocytes to some extent reflect other cell types, evidence is urgently required that such is indeed the case. Leucocytes have not been widely used to study sodium transport in uraemia but clearly the same strictures apply as to erythrocytes.

Possible mechanisms for the dialysis induced changes in cell sodium transport.

Haemodialysis is such a complex process that the difficulties in trying to associate alterations in intracellular cation transport with either removal of uraemic "toxins" or changes in ECF volume are

very large. In experiments of this type, any associations, no matter how close, must be assumed to be casual rather than causal. As the range of patients studied had different degrees of fluid overload (weight loss over dialysis ranged from 0.3 to 3.3 kg.) and dialyses of widely differing efficiency (dialysis index ranged from 0.42 to 1.09), it was possible that the effects of these two major aspects of a dialysis session could have been separated when the results were analysed.

Plasma creatinine concentrations and dialysis index were used as indicators of the accumulation and removal of the many potentially toxic substances which accumulate in renal failure. Patient weight as a proportion of "dry weight" (weight%), mean blood pressure and ANP concentrations were measured as indicators of ECF volume. ANP is known to be released in response to changes in ECF volume (via alterations in atrial stretch), and ANP concentrations might be expected to fluctuate in the same way as those of the putative natriuretic factor/sodium pump inhibitor. Although dialysis index was not correlated with weight% before or after dialysis, it did correlate with dialysis weight loss ($r = -0.55$), indicating that the quantities of fluid removed and urea clearance were, to some extent, directly associated in this study. The expected correlations between ANP and weight% were found. Blood pressure is volume dependent in haemodialysis patients, and it was interesting that, before dialysis, ANP correlated with weight% and with blood pressure although weight% and blood pressure did not correlate. The apparently paradoxical inverse correlation between weight% and blood pressure after dialysis reflected the very small range in wt% after dialysis, and is consistent with the clinical observation that patients only remain

above their dry weight after dialysis if hypotension has prevented further fluid removal. ANP concentrations and changes did not correlate with measures of biochemical clearance, suggesting that, in this study, ANP concentrations might have been useful as an indicator of ECF volume not associated with clearances of possible molecular "toxins". As the putative natriuretic hormone/ sodium pump inhibitor has not been isolated, it is not possible to say whether or not it would have been significantly cleared by haemodialysis.

In view of these reservations it is perhaps not surprising that it proved impossible to make clear cut distinctions between the effects of volume change and the effects of molecular clearance. The only correlation found with $p<0.01$, between weight% and erythrocyte rate constant, was clearly insufficient to suggest causes for the changes in sodium transport over dialysis. It was however evidence against the presence of a sodium pump inhibitor, the levels of which should fall in response to reductions in ECF volume.

The failure to find any other associations with sodium transport does not necessarily imply that the wrong indices were chosen, but more probably that multiple factors had varying effects on active and passive sodium transport, obscuring the relationships between individual factors. There are many other factors which in theory may affect or change in parallel with intracellular sodium content and transport, including duration of dialysis, acid/base balance, and plasma concentrations of numerous substances including calcium, glucose, lipids, non-esterified fatty acids, renin, arginine-vasopressin, insulin, mineralocorticoids and glucocorticoids. It is not possible to look meaningfully at so many variables simultaneously without having a very large number of subjects, and any increase in

the number of variables considered in the current study would have seriously limited the validity of any correlations. More complicated statistical techniques such as stepwise linear regression would have had little value in a study of this size, and would not have permitted consideration of a greater number of variables. Of course, associations found with such techniques provide no further evidence of a causal relationship. Further studies in haemodialysis patients would require attempts to limit the number of factors which change, and although it would be relatively simple to look at the effects of dialysis without fluid removal or fluid removal without dialysis (i.e. ultrafiltration), both of these procedures produce multiple inter-related biochemical and physiological changes.

The three patients taking digoxin were exposed to a circulating sodium pump inhibitor, the concentration of which is not significantly reduced by dialysis. Evidence of sodium pump inhibition (raised internal sodium and decreased rate constant) was not apparent in either cell type. All these patients had been on digoxin for several months, with the dose adjusted to keep plasma digoxin concentrations in the therapeutic range. It has been shown that chronic digoxin administration stimulates the formation of new sodium pumps, and in erythrocytes, the expected pattern of raised sodium and lowered sodium pump activity is seen only in the first few days after starting treatment or in patients with features of digoxin toxicity (Ford et al, 1979). The results in this study are compatible with this, and these findings with digoxin beg the question of what changes could reasonably be expected in patients who were persistently exposed to an endogenous sodium pump inhibitor.

Another possibility that should be considered is that the observed cellular changes were coincident with but not caused by haemodialysis. All patients were from morning haemodialysis shifts and commenced dialysis at about 8 a.m.. Ng and Hockaday (1986b) found a rise in leucocyte osNERC when fasting subjects were given a glucose load. The patients in this study had all had a light breakfast prior to dialysis and most had tea and toast during dialysis. Their dietary situation was therefore not analogous to that of Ng and Hockaday's patients, whose results were disputed by another group (Poston et al, 1987). As mentioned in the introductory section, "Factors which may influence Leucocyte Cation Content and Transport in vivo", there are theoretical reasons for proposing some diurnal variation in leucocyte sodium transport. This is unlikely to be the explanation for the current findings as most controls were sampled at around 9 a.m., yet their results were closer to the post-dialysis values taken between noon and 1 p.m.. Factors related to haemodialysis seem the most likely explanation for the WBC changes.

Comparison between undialysed, CAPD and haemodialysis patients.

In leucocytes, there were interesting similarities and differences in the comparison between no dialysis and CAPD on one hand and before and after haemodialysis on the other. Unfortunately direct comparisons could not be made due to the shift in normal values between experiments. In haemodialysis patients before dialysis, the degree of elevation of Na_{WBC} appeared more marked than in the CAPD group but less marked than in the undialysed group. CAPD seemed to normalise Na_{WBC} by enabling rate constant to rise to match the elevated flux rate, whereas after haemodialysis, a drop in flux rate greater than a concurrent drop in rate constant resulted in a more

normal Na_{WBC}. As opposed to the CAPD group, no correlations were found between Na_{WBC} and the biochemical degree of uraemia in the haemodialysis group. This may have been related to the haemodialysis patients before dialysis being in a biochemical state closer to that of the undialysed patients, where no correlations were found. Although, biochemically, the haemodialysis patients after dialysis were closer to the CAPD patients, the changes produced by dialysis may not have had sufficient time to allow associations between plasma biochemistry and Na_{WBC} to become apparent. Another important consideration is the continuous cycle of increase in volume between dialyses and decrease in volume during dialysis that is occurring in the haemodialysis patients. These changes are of course absent in CAPD patients and it may be the constancy of volume in them that allows a relationship between Na_{WBC} and plasma biochemistry to be discernible.

SUMMARY OF FINDINGS AND FINAL DISCUSSION

These studies showed that sodium content in leucocytes was elevated in patients with uraemia who had never been dialysed. This increase appeared to reflect an increase in sodium flux rate rather than a reduction in sodium flux rate constant. CAPD patients had more normal Na_{WBC} and this was associated with sufficient elevation in rate constant to match flux rates similar to the undialysed group. In undialysed patients, an opposite pattern of abnormalities was seen in erythrocytes compared with leucocytes. Erythrocytes from CAPD patients had a more normal sodium content due to changes towards normal in both flux rate and rate constant. The only significant correlations between sodium transport and indices of uraemia were between leucocyte sodium content and plasma urea and creatinine concentrations in CAPD patients.

Haemodialysis was associated with falls in sodium flux rate and rate constant in both cell types. The balance of these changes was such as to result in a fall in Na_{WBC} but a rise in Na_{RBC}. It was not possible to define which of the many abnormalities in the uraemic state might have been affecting sodium transport, or to implicate altered sodium transport in the pathogenesis of any of the clinical features of uraemia.

These results provided no support for the theory that, in patients with chronic renal failure, most cells have an elevated sodium content due to the presence of a circulating sodium pump inhibitor, but rather suggested that abnormalities of sodium influx were at least as important as sodium pump activity in the production of altered intracellular sodium. The marked differences in abnormalities in red and white blood cells emphasised the danger of extrapolating

results from one cell type to another.

Much of the impetus for research into sodium transport in uraemia, including this work, has been provided by the hypothesis that sodium pump inhibition is an important pathogenetic feature of the uraemic state. Implicit in this hypothesis are the assumptions that all sodium pumps are similar in the way they respond to external factors, that the type of cell they are in does not influence the behaviour of the pumps, and that the cell cannot respond to sodium pump inhibition. Furthermore, cell sodium content is presumed to be predominantly dependent on sodium pump activity, the inhibition of which will raise sodium content. The situation is now known to be far more complex. Because sodium pump production is switched on when intracellular sodium is raised (Fambrough et al, 1987; Ford et al, 1979; Doucet, 1988), sodium influx or passive transport become inextricably linked with sodium pump activity. It becomes much more difficult firstly to accept that a sodium pump inhibitor can produce an important effect on such a responsive sytem, and secondly to convincingly demonstrate that sodium pump inhibition is occurring. Certainly, measures of sodium pump rate constant or any other measure of sodium pumping such as ouabain binding sites are unhelpful unless sodium content is known. Even when sodium content, sodium flux rate and flux rate constant are known, it is not easy to define abnormalities. Elevated sodium associated with a high flux rate and "normal" rate constant may in fact be an indication of an inability of active sodium pumping to respond. A small, undetectable rise in sodium content in association with normal flux rate and rate constant may be the anticipated pattern in cells chronically exposed to a sodium pump inhibitor. Such difficulties of interpretation seem

unavoidable in any method which attempts to reflect in vivo sodium transport.

Another increasingly evident problem is that both passive and active sodium transport are influenced by a wide range of factors. Sodium pump activity and formation reflects a combination of stimulatory and inhibitory stimuli either directly or through altered intracellular sodium. Viewed in this context, the demonstration that urine or plasma contain sodium pump inhibitory activity does not prove that such activity results in a net inhibition of cellular sodium pumping in the subject from whom the plasma or urine came. The complexity of the situation is perhaps most marked in haemodialysis patients whose biochemistry and ECF volume are never stable. Studies of sodium transport must be performed at points in this cycle, but as the mechanism of the links between plasma factors and membrane transport will often not be instantaneous, measurement of any potentially important factors at the same time point might be misleading.

A problem associated with this when using leucocytes is the time and procedures involved in isolating and then incubating the cells. The factors which were affecting sodium transport in vivo may no longer be influential when the cells are studied. The demonstration of persistence of abnormalities during prolonged incubation does not exclude the possibility that some changes have been lost in the isolation procedure. Some workers have incubated the patients' cells or even normal cells in plasma from the patients being studied, but this does not necessarily solve the problem, as concentrations of some of the constituents of plasma will fall quickly after removal from the circulation (e.g. renin substrate, adrenaline). More meaningful assessments of sodium transport may become possible when

cells can be studied in more natural conditions. Fluorescent indicators or nuclear magnetic resonance methods may prove useful in this respect.

Studies of Na-K-ATPase activity in, for instance, isolated nephron segments provide evidence of how the activity of this enzyme is regulated but these can only be performed under highly artefactual conditions. Recent studies of this type have produced further evidence to suggest that the quest for a "model" cell may indeed prove a hopeless one as several sub-types of Na-K-ATPase have been discovered with important differences in properties (Doucet, 1988). Such diversity of response in Na-K-ATPase activity, coupled with evidence from this work and elsewhere that different cells respond differently (probably not primarily because of different Na-K-ATPase, but because of differences in inward sodium transport pathways altering internal sodium concentration and therefore sodium pump formation) must cast doubt on the hypothesis that many of the clinical features of uraemia can be attributed to one factor affecting most cell types in a similar fashion. Attractive though such a unifying theory of the pathogenesis of the uraemic state is, it seems more likely that different tissues are adversely affected by different factors. Red or white blood cells have been studied because of their relative ease of isolation. All other cell types are much harder to isolate but nevertheless convincing evidence that abnormalities of sodium transport are important in a particular tissue will depend on the demonstration of such abnormalities in those particular cells. Although cell culture techniques permit the production of cells of individual type, these cells may de-differentiate in culture and it is not self-evident that the sodium

transport mechanisms of such cells will behave as they would in their natural environment.

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APPENDIX

A copy is appended of "Problems in measuring human leucocyte cation content: effects of cell preparation and handling" by J.Main, T.H.Thomas and R.Wilkinson, from Clinical Science,1989, 77, 157-60.

The Editorial Board of Clinical Science kindly gave permission for the paper to be presented here.

Problems in measuring human leucocyte cation content: effects of cell preparation and handling

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SUMMARY

1. Previous published measurements of leucocyte cation content are inconsistent, with sodium concentrations having particularly high coefficients of variation. We have measured the effects of recovery time after isolation, different types of handling, and centrifugation on leucocyte sodium and potassium content.

2. Sodium content fell markedly during the first 30 min after isolation and was stable from 1 to 3 h of incubation. There was a small but significant rise in potassium content over the same time period.

3. During incubation, occasional gentle resuspension of the cells gave optimal sodium contents, whereas mixing by inversion caused large falls in sodium and potassium content.

4. Centrifugation of stable cells at 200 *g* for 6 min caused marked increases in sodium content.

Key words: leucocytes, potassium, sodium.

INTRODUCTION

Leucocyte isolates have been widely used to study intracellular cation concentrations and sodium flux rates in many disease states including essential hypertension and uraemia [1–3]. Despite using modifications of the same method [4], different centres have obtained quite different results. Ranges for sodium concentration in normal control subjects and in patients have been extremely wide, and values for sodium efflux rate constants inconsistent [3, 5]. Calculation of sodium/potassium concentration ratios permit comparison of results expressed in different units, and the means of normal control groups vary from 0.084 to 0.31 [2, 6]. It is likely that these differences are due to methodological artefacts and further refinements of experimental technique are required to allow accurate measurement of leucocyte cation content. During our work with leucocytes we became aware that their internal ion content seemed very sensitive to handling and therefore carried out the following experiments.

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METHODS

Leucocyte isolation

Whole blood (30 ml) was taken into lithium heparin tubes, and erythrocytes were sedimented at room temperature by the addition of 5.0 ml of 6% (w/v) dextran (average mol. wt. 500 000) in TC199 solution. The leucocyte-rich supernatant was removed after 30 min and spun at 250 *g* for 3 min. The resulting cell pellet was mixed with 3 ml of distilled water for 10 s to lyse remaining erythrocytes and isotonicity was restored by the addition of hypertonic TC199. This suspension was spun at 250 *g* for 3 min to leave a pellet of leucocytes free of erythrocytes. The leucocytes were resuspended in TC199 at an osmolality of 280–290 mosmol/kg and a cell concentration of 3–8 million/ml. Aliquots (1.3 ml) of the final suspension were put into individual tubes ready for incubation at 37°C, and 0.5 ml of the original suspension was used to measure total leucocyte count on a Coulter counter model S plus IV.

Recovery from isolation procedure

Two to five (depending on the yield of cells from the isolation) serial measurements of sodium and potassium content were made in cells from nine healthy volunteers at times from 0 to 230 min after the start of the incubation. Cells were gently resuspended every 20 min during the incubation. We then compared sodium and potassium contents in 26 healthy volunteers measured after a 1 h incubation and in 18 healthy volunteers after a 3 h incubation. Sixteen of these subjects were measured at both times in the same experiment.

Cell handling

After isolation, three pairs of tubes containing leucocytes were prepared from each of 14 healthy volunteers.

One pair was left untouched, one pair was gently agitated at 15 min intervals to resuspend the cells and one pair was mixed by three inversions of the tubes at the same time intervals. This was continued for 60 min.

Centrifugation

Two pairs of tubes containing leucocytes were prepared from each of 10 healthy volunteers and the cells were kept resuspended by gentle agitation during a 60 min incubation. At the end of this time one pair was centrifuged at 200 *g* for 3 min, the cells then resuspended in the supernatant and spun again at 200 *g* for 3 min. Cation content was measured immediately in seven subjects and after a 5 min recovery period in three subjects, and the values were compared with those from the uncentrifuged tubes.

Measurement of sodium and potassium content

At the end of the incubation period the cells were spun (700 *g* for 2 min) and washed twice in ice-cold isotonic magnesium chloride. The final cell pellet was disrupted in 1 ml of 1.5 mmol/l caesium chloride and sodium and potassium concentrations were measured on an IL943 flame photometer. All measurements quoted are the mean of values from paired tubes.

Statistical analysis

The statistical significance of the differences between groups was assessed by the Friedman test, Mann–Whitney *U*-test or Wilcoxon matched-pairs signed-ranks test as appropriate.

RESULTS

Recovery from isolation procedure

The marked fall in sodium content during the first part of the incubation is shown in Fig. 1. Contents of sodium became relatively stable from 30 to 60 min after the start of the incubation. Changes in potassium content were less marked, with slight rises in a few cases during the first 30–60 min.

The mean sodium and potassium contents in the 26 subjects measured after 1 h were 3.28 (SD 0.49) and 31.4 (SD 2.3) nmol/10^6 cells, respectively. Equivalent values in the 18 subjects measured after 3 h were 3.23 (SD 0.45) and 32.4 (SD 2.1) nmol/10^6 cells. The differences at the two times were not significant. In the 16 paired samples the rise in potassium between 1 and 3 h was significant by Wilcoxon matched-pairs signed-ranks test ($P = 0.019$).

Effect of handling

The effect of handling on sodium content, potassium content and sodium/potassium ratio was statistically highly significant (Friedman test, $P < 0.001$ for all three groups). In view of this we then proceeded to direct comparisons of different types of handling, using the Mann–Whitney *U*-test. Sodium content in all 14 subjects was lower after gentle resuspension than if the cells were left undisturbed ($P < 0.001$; Fig. 2). There was no significant effect on potassium content, although there was a tendency for extreme values in the undisturbed group to revert towards the mean with gentle resuspension. The sodium/potassium ratio was also lower in the leucocytes from all 14 subjects after gentle resuspension, with a mean drop of 0.010 ($P < 0.001$).

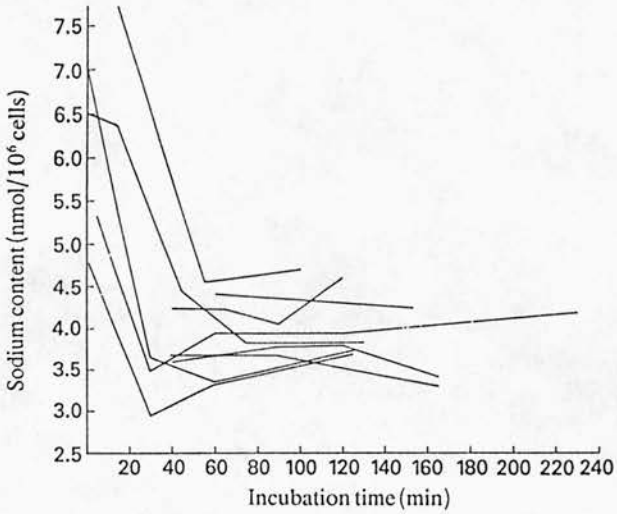

Fig. 1. Leucocyte sodium content and duration of incubation after cell isolation in nine normal control subjects.

Although sodium content also fell after repeated inversion, unlike the gently resuspended cells this was associated with a consistent fall in potassium content ($P < 0.001$) and there was no significant change in the sodium/potassium ratio.

In the gently resuspended group, mean sodium content was 3.16 nmol/10^6 cells and the coefficient of variation between subjects was 12.7%. In the same group the mean sodium/potassium ratio was 0.103 with a coefficient of variation of 12.0%.

Within-batch coefficients of variation for sodium and potassium contents in our laboratory, derived from 38 consecutive duplicate incubations, were 7.07% and 2.13%, respectively.

Effect of centrifugation

Sodium content rose in all 10 cases after centrifugation ($P < 0.001$). The difference was less marked in the three experiments where a 5 min recovery period was allowed (Fig. 3). Centrifugation had no significant effect on potassium content.

DISCUSSION

Lichtman & Weed [7] in 1969 showed a considerable change in leucocyte cation content during a 2 h incubation, but it is only relatively recently that papers on leuco-

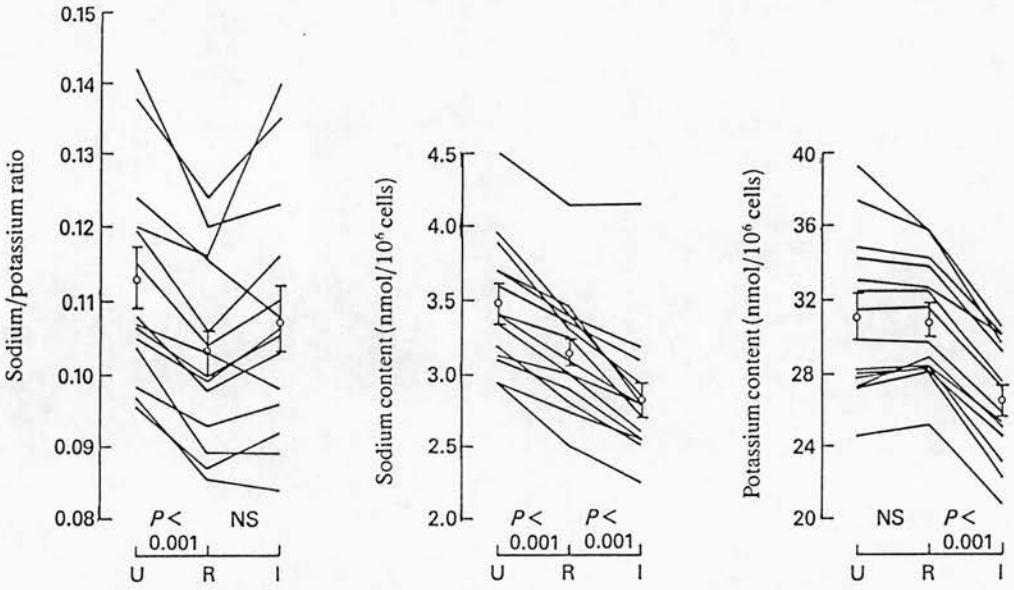

Fig. 2. Differences in sodium/potassium ratio, sodium content and potassium content in leucocytes left undisturbed (U), gently resuspended every 15 min (R) or inverted every 15 min (I) during a 60 min incubation after cell isolation. Group means (○) ± SEM (bars) are indicated. Abbreviation: NS, not significant.

cyte sodium have stated the length of incubation period used. It may be that some of the relatively high values for sodium content in the original reports concerning uraemia and essential hypertension [1, 2] were due to incomplete recovery of the cells from the isolation procedure. Although Lichtman & Weed [7] did not measure cation content between the start of the incubation and an interval of 60 min, most workers have used a 30 min period. Indeed Patrick *et al.* [8] stated that cell sodium and potassium content reaches equilibrium after this time but did not give evidence to support this. It may be that they meant that matching of measurements of influx and efflux of sodium and potassium had occurred, rather than that serial measurements of sodium and potassium content were stable. Pedersen *et al.* [9] stated that they found a high lymphocytic sodium content immediately after isolation compared with 30 min later and that stability was reached after 30–60 min, but they published no supportive data. These findings are very much in accord with ours and it is of interest that their separation procedure was carried out at 37°C. They comment on the lack of studies concerning methodological problems in measuring leucocyte sodium content, and this lack probably reflects the relatively large volumes of blood needed for each measurement when weighing is used to determine the amount of cells present. We have chosen to count our cells and this has allowed us to make many more measurements from a given volume of blood. This method does not permit estimation of intracellular water content or measurement of cation concentrations per unit dry or wet weight. However, as the changes we have found in sodium content were usually not matched by changes in potassium content, they cannot have been due to changes in cell size. The only occasion on which the changes in sodium content were not matched by a change of similar magnitude in the sodium/potassium ratio was when the cells were repeatedly inverted. This change may therefore have reflected a change in cell size, but clearly such handling must be avoided however the cell cation content is to be measured. Changes in intracellular cation content may produce a secondary osmotic change in cell volume. As sodium ions are such a small fraction of the total intracellular osmoles, the changes in sodium content we have described would have only a small effect on cell water and size. This would be difficult to measure and the need for a larger leucocyte sample would negate the advantage conferred by counting of needing considerably fewer cells and therefore less blood. Our sodium/potassium ratio in normal control subjects is similar to those of Heagerty *et al.* [3] and Ng *et al.* [6], but our SD is considerably smaller.

Our results show that sodium content is stable from 1 to 3 h, but there is a continuing small but significant rise in potassium content over this time. It is not clear if this reflects continuing recovery from the isolation procedure or an abnormality related to the conditions of incubation. These findings apply only to cells from healthy volunteers. Cells with abnormal sodium content may not develop stable sodium content over the same time span and in leucocytes from uraemic patients we have found intracellular sodium to fall for up to 3 h after incubation (J. Main, T. H. Thomas & R. Wilkinson, unpublished work).

To achieve stable and reproducible results the cell suspensions have to be consistently and gently handled during incubation. We interpret the finding of a lower sodium content and sodium/potassium ratio in the gently resuspended group as showing that these cells were better able to maintain their internal environment, possibly

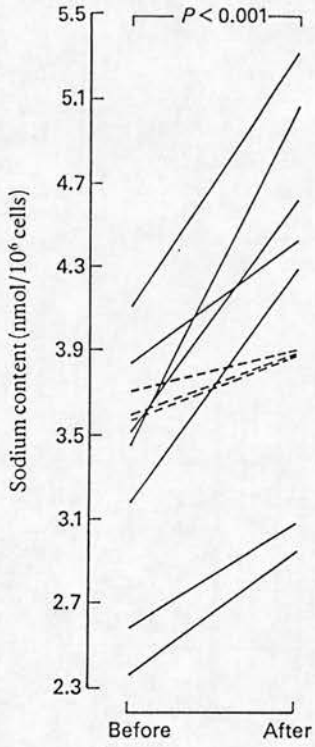

Fig. 3. Leucocyte sodium content before and after two 3 min centrifugations at 200 *g* performed after cell separation and incubation in 10 normal control subjects; No time allowed after centrifugation, ----; 5 min incubation at 37°C after centrifugation.

because regular resuspension dispersed accumulating metabolic waste products. The drop in sodium and potassium that occurred in the cells inverted periodically probably reflects severe membrane damage to a proportion of the cells, allowing free exchange of the intracellular cations with the magnesium chloride washing solution. We have found repeated cell counts to be constant over this time period in cells handled in the same fashion and therefore cell loss appears unlikely to be the reason for this finding. It is worth stressing that these were gentle inversions; more vigorous shaking of the cells produced even larger changes in content. The effects of handling might explain some of the smaller, but persistent, differences in the sodium/potassium ratio between centres, and contribute to the very large ranges seen for sodium content in some centres.

On finding the cells to be so sensitive to handling we were concerned that the method usually used to measure sodium efflux rate constant [10] would in itself alter sodium content and transport. This method involves loading the cells with ^{22}Na for 20–30 min and then washing the cells before estimating ^{22}Na disappearance from the cells over 10–20 min. Our washing and centrifugation procedure in this experiment was chosen to mimic that used to measure sodium efflux rate constant by the radioactive method, and produced marked changes in sodium content. Although sodium efflux rate constant is unchanged when leucocytes are loaded with sodium by altering flux rates, the mechanism of increased sodium content during centrifugation is unclear. If this involves interference with sodium pump activity it may alter sodium efflux rate constant. This may explain why measurements of rate constant within centres seem reasonably reproducible, presumably because the same degree of artefact is introduced each time, but measurements between centres vary widely because of uniformly different artefacts.

Despite these problems leucocytes have been widely used to investigate possible abnormalities of sodium pump activity, particularly in essential hypertension. This study has shown leucocyte cation content to be extremely sensitive to cell isolation and handling. We have demonstrated that, in cells from healthy volunteers, sodium content is stable from 1 to 3 h if the cells are handled with care, but there is a small change in potassium content. These observations apply only to cells from normal control subjects and it is quite possible that cells from patients with illness respond differently to isolation and incubation. Current methods for estimating sodium efflux rate constant may introduce some error and we are trying to develop a method which avoids the need for cell centrifugation.

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