THE INTERACTION OF CATIONS WITH THE SODIUM PUMP IN RED BLOOD CELLS

by

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This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of Leicester, entitled "The Interaction of Cations with the Sodium Pump in Red Blood Cells" , is the result of work done mainly by myself during the period of registration for the above degree.

Signed Stuart C Brand.....

Stuart C. Brand

September, 1981

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CONTENTS

ABBREVIATIONS

ADP, ATP	adenosine 5'-diphosphate, adenosine 5'-triphosphate
ATPase	adenosine triphosphatase (E.C. 3.6.1.3)
$(Na^+ + K^+)$-ATPase	the component of ATPase activity that requires Mg, Na and K and is inhibited by ouabain
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid
EDTA	ethylenediaminetetracetic acid
E-P	phosphorylated enzyme
Hb	haemoglobin
K_m	Michaelis constant
K'_K, K'_{Cs}, K'_{Na}	apparent dissociation constant of K^+- (or Cs^+-, Na^+-) site complex
PCMB	p-chloromercuribenzoic acid
PCMBS	p-chloromercuribenzene sulphonic acid
P_i	orthophosphate
S.E.M.	standard error of mean
Tris	Tris-(hydroxymethyl)-methylamine
V_{max}, M'_{max}	maximum velocity
$[K^+]_o$, $[Cs^+]_o$, $[Na^+]_o$	external potassium, caesium or sodium concentration
$[Na^+]_i$, $[K^+]_i$	intracellular sodium or potassium concentration

INTRODUCTION

It has been long established that there is an uneven distribution of the monovalent cations, potassium and sodium, between many animal cells and their extracellular environment. Red blood cells of many species present one example of this uneven distribution. The observed values of intracellular $[Na^+]$ for fresh human red cells show a range from 8.7 to 28.6 m equiv./litre cells with a mean of 18.6 m equiv./litre cells, compared with a mean plasma level of 138 m equiv./litre. Conversely, for potassium, the mean intracellular level has a range from 89 to 101 m equiv./litre cells with a mean of 95 m equiv./litre cells, compared with a mean plasma level of 4.2 m equiv./litre (Albritton, 1952).

This uneven distribution cannot be explained by any suggestion of the occurrence of some process during cellular development to cause partition, subsequently followed by a permanent impermeability of cell membranes to these cations. In fact, a substantial redistribution of ions occurs in cold-stored red cells as shown by Harris (1941). $[Na^+]_i$ levels in such cells rose from 18 to 42 m equiv./litre cells over a period of forty days, during which time the $[K^+]_i$ level fell from 90 to 53 m equiv./litre cells (Albritton, 1952). It had also been demonstrated (Harris, 1941) that subsequent elevation of temperature to 37°C caused a reversal of the ion movements seen at 2 - 5°C, with Na^+ ions leaving the cells and K^+ ions being reaccumulated against a concentration gradient. As this process was enhanced by additions of glucose before the raising of temperature to physiological levels, it was concluded that the maintenance of an uneven distribution of Na^+ and K^+ was not caused by membrane impermeability but rather depended upon some metabolic process. Support for the view that this process might be glycolysis came from later studies on human red cells (Maizels, 1949). These studies showed that not only did these cation movements not occur in the absence of glucose

but also that, if glucose was added, such movements were only seen when the pH was adjusted to appropriate levels for glycolysis to occur. Maizels also pointed out that the ion movements observed appeared to be the resultant of active transport and passive cation diffusion and further suggested that this process could not be explained simply as the active accumulation by cells of potassium with a complementary passive output of sodium ions. This was because the large amount of intracellular protein and organic phosphate, present as non-penetrating anions, would preclude this passive leak of Na^+ ions even when K^+ ions were being actively accumulated. He thus concluded that there was an active, glycolysis-dependent extrusion of sodium ions from cells at 37°C, a mechanism which protected cells from undergoing osmotic swelling and which was associated with a complementary influx of potassium which was not, of necessity, active. A later study, however (Harris and Maizels, 1952) showed that movements of K^+ ions into human erythrocytes at 37°C could not be explained as the electrostatic response to potentials set up by transport of Na^+ ions in the opposite direction, but rather suggested that the observed saturable external potassium concentration dependence implied carriage by chemical groups from the exterior to the inside of the cell. The same authors suggested the possibility of inward K^+ movements being geared to outward Na^+ movements by means of a common carrier. Studies on sodium and potassium levels in red cells of various species later confirmed that both the $[K^+]_i/[K^+]_o$ ratio and that for $[Na^+]_i/[Na^+]_o$ were dependent on the level of cell glycolytic activity (Bernstein, 1954).

Further investigations as to the nature of the connection between metabolism and active extrusion of Na^+ ions from giant axons of Sepia officinalis and Loligo forbesi showed that inhibitors of oxidative phosphorylation, such as cyanide and dinitrophenol, caused a reversible inhibition of these ion movements (Hodgkin and Keynes, 1955). The

inhibitors, at the concentrations described, were also shown to cause depletion of energy-rich phosphate esters in the axoplasm (Caldwell, 1956), suggesting that such compounds might be involved in the active transport of sodium ions.

Using a homogenate of crab leg nerves, the presence of a calcium-activated ATPase and a magnesium-activated ATPase was demonstrated (Skou, 1957). Studies were carried out on the magnesium-activated ATPase, which was shown to be similar to one previously isolated from muscle (Kielley and Meyerhof, 1948) in its response to pH changes and in the fact that it was strongly inhibited by Ca^{++} ions. The studies of Skou showed a dependence of the activity of this ATPase on the relative concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+} ions. Results suggested that magnesium was a requirement for an enzyme activity which was stimulated by sodium ions and by potassium ions in the presence of both sodium and magnesium. In a later study on the same preparation (Skou, 1960) it was shown that the enzyme had high affinity sites for Na^+ and K^+. The observed value of K_m for potassium activation (1.8 mM) was markedly lower than the K_I value for its competitive inhibition of sodium activation (9 mM), a fact taken to signify that the site of K^+-mediated stimulation was different from the site of its inhibition and therefore also different from the site of Na^+-mediated stimulation. The same studies suggested that the enzyme catalyzed on ATP-ADP exchange reaction (rather than an ATP-P_i exchange), for which the presence of magnesium was obligatory. The optimum concentration of magnesium for the ATP-ADP exchange reaction was found to be equal to the concentration of ATP present, a fact taken to suggest that magnesium was probably involved in the formation of an enzyme substrate complex.

These studies on the crab nerve homogenate led Skou to conclude that the enzyme was involved in the active transport of cations

across the nerve membrane. Several pieces of evidence were adduced to support this view. Firstly, the enzyme was thought to be located on disintegrated membrane fragments, as suggested by the study by electron microscopy of particles from nerve tissue (Hanzon and Toschi, 1959) and by the fact that previous reports of an ATPase activity from peripheral nerves (Libet, 1948; Abood and Gerard, 1954) showed that this could be isolated from the sheath but not from the axoplasm. Secondly, the enzyme had affinities for Na^+ and K^+ which would be suitable for the control of the intracellular concentration ratio of Na^+ and K^+. Finally, inhibition of the enzyme activity stimulated by $Mg^{2+} + Na^+ + K^+$, had been seen with the cardiac glycoside g-strophanthin (ouabain), which had already been shown to inhibit active cation transport in both red cells (Schatzmann, 1953) and frog skin (Koefoed-Johnsen, 1957). Confirmation that there is an association between this ATPase activity and sodium fluxes out of and potassium fluxes into cells came from several sources. The suggestion that ATP was one link in the connection between glycolysis and the active transport of potassium in human red cells had been established by the experiments of Whittam (1958). Later, it was shown that human red cell membranes, when fragmented, possessed an ATPase activity which showed several similar properties to the sodium and potassium transport system in intact red cells: not only were both processes inhibited, with similar concentration dependence, by ouabain, but also both showed a similar concentration dependence for activation by Na^+, K^+ and ammonium ion (which could substitute for K^+). Both processes also showed the phenomenon of inhibition of the activation by potassium as the $[Na^+]$ was raised (Post et al., 1960). Similar results were also obtained from studies on human red cell ghosts (Dunham and Glynn, 1961). Results from other tissues showed the presence of similar systems: in rabbit kidney cortex it was suggested that ATP hydrolysis was closely linked with the process of respiration and active cation transport (Whittam and Wheeler, 1961), whilst

two sets of workers demonstrated the presence of a Mg^{2+}-dependent ATPase activity, which could be stimulated by Na^+ ions, in brain tissue (Järnefelt, 1960; Deul and McIlwain, 1961). Studies on giant axons of Loligo (Caldwell et al., 1960) also showed that injections of certain compounds, such as ATP and arginine phosphate, which contained high-energy phosphate bonds, into nerve fibres previously poisoned with cyanide or dinitrophenol, could restore active sodium efflux. Knowledge of the involvement of these phosphate esters was extended by studies performed on squid giant axons much more recently (De Weer, 1970). These studies showed that the effect of cyanide injection was to cause a lessening of the dependence of sodium efflux on $[K^+]_o$ levels. The same effect was caused by injection of agents which raised axoplasm levels of ADP rather than of P_i. It was concluded that the sensitivity of the pump to external K^+ was governed by the ATP:ADP concentration ratio.

Studies on human red cell ghosts (Whittam, 1962) demonstrated that Na^+ ions were only effective as activators of the membrane adenosine triphosphatase when on the intracellular side of the membrane, whereas changes in the potassium concentration inside ghosts had no effect. Alterations of potassium concentration in the bathing medium, however, had a marked effect on the enzyme activity, measured as liberation of orthophosphate from ATP. In other words, the ATPase situated within red cell membranes showed the same spatial asymmetry as did the transporting mechanism for Na^+ and K^+ ions.

Finally, a close correlation was demonstrated in six tissues: human red cell membranes, frog toe muscle, squid giant axon, frog skin, toad bladder and electric eel electroplax, of sodium and potassium fluxes measured in near optimal conditions and $(Na^+ + K^+)$-ATPase activity measured in similar conditions. It was further suggested that the use of ATP by an ATPase system operating at full capacity would increase the ADP/ATP concentration ratio inside cells and consequently

accelerate ATP production from glycolysis or respiratory metabolism. A system was thus proposed whereby changes in cation levels could act as a pacemaker for energy metabolism (Bonting and Caravaggio, 1963).

The question then existed as to what was the mechanism of operation of this process for active extrusion of Na^+ from and inclusion of K^+ ions into cells. Two possibilities exist: a two-step process which would proceed by the binding of one ionic species on one side of the membrane, its transport to and release on the opposite side of the membrane, followed by a repetition of the process in the reverse direction with the other activating cation being transported. Such a process could variously be termed stepwise, consecutive or ping-pong and has the characteristic that more than one set of binding sites for monovalent cations on the enzyme is not necessarily required (in other words, the mechanism may be unitary). An alternative scheme would involve a binary mechanism (two sets of monovalent cation binding sites) with Na^+ ions and K^+ ions binding simultaneously to the enzyme and being transported in a one-step process.

The two-step (consecutive) mechanism - biochemical evidence

Much of the early work on mechanism involved studies of phosphorylation and dephosphorylation reactions, providing evidence in favour of a two-step mechanism. As early as 1963, it was proposed that a phosphorylated enzyme intermediate existed which might be used to explain the mechanism of operation of the Na^+-activated ATPase. Experiments were performed on an enzyme preparation from electric organ of Electrophorus electricus (Albers et al., 1963) which suggested that phosphorylation of the enzyme by ^{32}P-labelled ATP could be stimulated by magnesium initially and further increased in the presence of sodium. Phosphorylation was decreased by potassium. Studies on an enzyme preparation from guinea pig kidney cortex confirmed this finding and suggested that the following

scheme might apply:

$$\text{i) Enzyme (E)} + AT^{32}P \xrightarrow{xNa^+} \left[E\sim{}^{32}P\right]Na_x + ADP$$

$$\text{ii) } \left[E\sim{}^{32}P\right]Na_x \xrightarrow{yK^+} E + {}^{32}P_i + xNa^+ + yK^+$$

In this scheme, sodium ions stimulate phosphorylation, potassium ions stimulate dephosphorylation and ouabain causes inhibition of the process by blocking step (ii) and hence turnover of the intermediate (Charnock and Post, 1963).

This work was extended by Post et al. (1965), who isolated a phosphorylated intermediate which showed a saturable requirement for Na^+ ions and of which the breakdown was accelerated by K^+ ions. Further evidence linking the formation of a phosphorylated intermediate with extrusion of Na^+ ions and the subsequent dephosphorylation with entry of K^+ ions came from a study on the inhibition of the ATPase of red cells and kidney preparations by oligomycin (Whittam et al., 1964), which showed a similar effect on ^{32}P-labelling to that previously described for ouabain. Oligomycin did not affect substantially the Na^+-stimulated ^{32}P tissue labelling but did cause inhibition of the breakdown of such labelling by K^+ ions.

Studies on the formation of enzyme phosphorylated intermediate of the $(Na^+ + K^+)$-ATPase were then extended to include a proposal that two such intermediates existed. In these studies, which were performed on microsomes of electric organ from Electrophorus electricus, it was shown that there existed an ATP-ADP exchange activated by Mg^{2+} and Na^+ ions (Fahn et al., 1966a). This exchange activity was different from one which did not respond to changes in sodium ion concentration and which had been previously shown to be separate from $(Na^+ + K^+)$-ATPase activity in brain microsomes (Stahl et al., 1966). The sodium stimulation of the ATP-ADP exchange here, however, was seen at concentrations of magnesium

lower than optimum values for the ATPase activity. It was proposed that this transphosphorylation was catalyzed by the $(Na^+ + K^+)$-activated ATPase. The difference observed between optimum levels of magnesium for the two activities led to the proposal that the phosphorylated intermediate formed in the presence of low $[Mg^{2+}]$ and sodium ions, which was sensitive to ADP, was then converted at higher $[Mg^{2+}]$ to another form of phosphorylated intermediate which was insensitive to ADP. This view was supported by the fact that the ATP-ADP exchange, unlike the ATPase reaction, was not sensitive to inhibition by oligomycin. The scheme of these workers might be presented thus:

$$\text{i)}\quad E_1 + ATP \xrightleftharpoons{Na^+ \text{ low } Mg^{2+}} E_1\sim P + ADP$$

$$\text{ii)}\quad E_1\sim P \xrightarrow{\text{high } Mg^{2+}} E_2 - P$$

$$\text{iii)}\quad E_2 - P \xrightarrow{K^+} E_2 + P_i$$

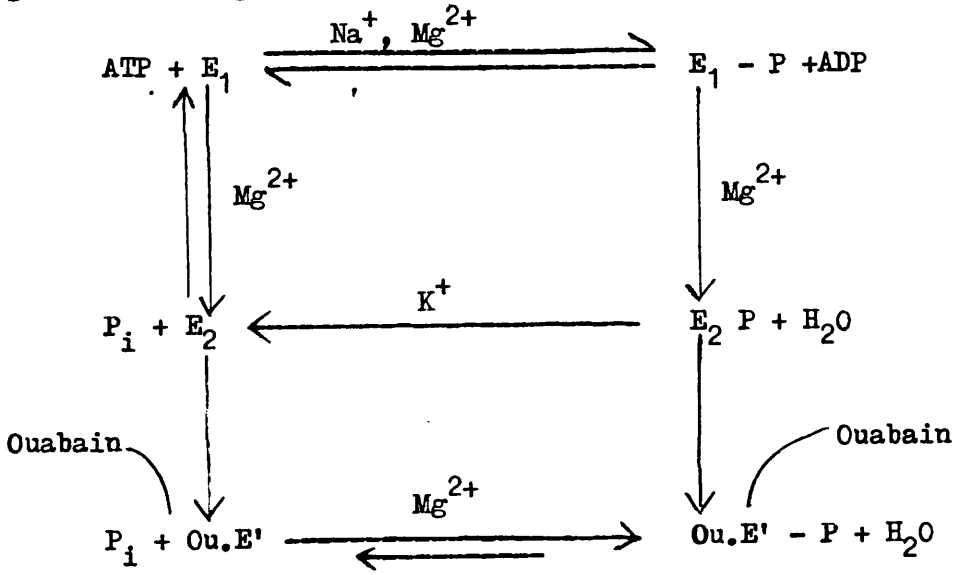
where step (ii) is inhibited by oligomycin.

A similar response of phosphorylation and dephosphorylation of a red cell membrane preparation to Na^+, K^+ and Mg^{2+} ions has also been reported (Blostein, 1968).

Further evidence which appears to favour a two-step mechanism involving phosphorylated intermediates, came from studies on binding of cardiac glycosides to the enzyme prepared from cardiac muscle. Two binding pathways were observed: one stimulated by $(Na^+ + ATP + Mg^{2+})$, the other by Mg^{2+} + inorganic phosphate. This second ouabain binding pathway was inhibited by sodium (Schwartz et al., 1968). The first ouabain binding pathway described (that stimulated by $Na^+ + Mg^{2+} + ATP$) was inhibited in the presence of K^+ - consequently this was taken as being consistent with the E_2P form of the enzyme already described.

A later and influential series of experiments, performed on membrane preparations obtained by differential centrifugation of kidney

homogenates, further examined the sensitivity of the two proposed phosphorylated intermediates to dephosphorylation by K^+ or ADP (Post et al., 1969). The preparation was allowed to equilibrate with γ^{32}P-labelled ATP and sodium only, to achieve binding of ATP without phosphorylation. Then a chase of non-radioactive ATP and Mg^{2+} ions was applied, so that only the bound, labelled ATP could phosphorylate. Results showed that, for a preparation treated in this manner, potassium did not affect the initial amount of phosphorylation although it did increase the rate of dephosphorylation. In a complementary experiment, the preliminary equilibration with labelled ATP was performed in the presence of magnesium but in the absence of Na^+ and K^+ ions. The chase solution applied in this case contained non-radioactive ATP + Na^+ ions + varying amounts of ADP. In this case, although ADP did produce a decreased initial amount of phosphorylation (explained as a rapid reversal of phosphorylation), it did not cause any effect on the dephosphorylation rate. Since it did not increase this dephosphorylation rate, it was concluded that the remaining phosphorylated enzyme must have been an ADP-insensitive form which had arisen from that which was ADP-sensitive. The same authors (Post et al., 1969) explained the previously reported dual pathway for ouabain binding to the enzyme by suggesting the following reaction scheme:



where E_1P and E_2P were the two previously described phosphorylated inter-

mediates and E' was a form of the enzyme inhibited by ouabain. It should be noted, however, that the role of Mg^{2+} in such a scheme was later subject to dispute (see later section) and the results obtained in these experiments (where chelating agents such as EDTA were used to alter $[Mg^{2+}]$) were retracted (Fukushima and Post, 1978).

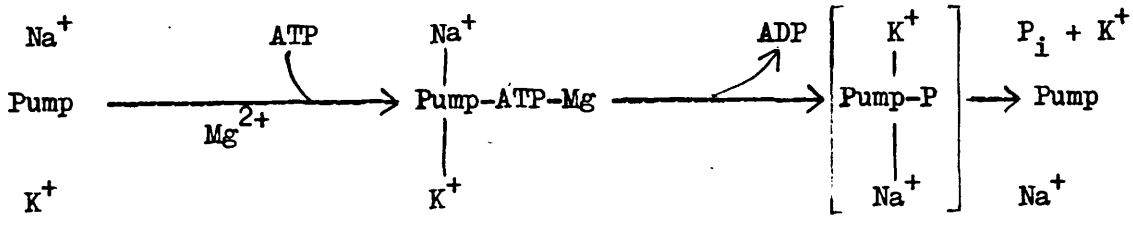
A large mass of biochemical evidence has thus been presented to suggest the existence of two forms of phosphorylated intermediate of the enzyme and to propose sequential reaction schemes involving these intermediates. Not all the biochemical evidence, however, is consistent with the scheme described above. In studies performed on an enzyme preparation from bovine brain, Kanazawa and co-workers demonstrated an effect of potassium ions on phosphoprotein formation, and thus concluded that a scheme where potassium-enzyme interactions only occurred after the formation of a phosphorylated intermediate was not plausible (Kanazawa et al., 1970). Further evidence inconsistent with the original proposed two-step mechanism came from the finding that K^+ ions stimulated the Na^+-dependent ATP-ADP exchange activity in an enzyme preparation from guinea pig kidney cortex (Banerjee and Wong, 1972) and from a study which demonstrated that Mg^{2+}, Na^+ and K^+ were required for the first intermediary reaction with the enzyme (Shamoo and Brodsky, 1972). This ATP-ADP exchange reaction corresponds to step (i) in the scheme of Fahn et al. (1966a) described previously. This step would occur in the presence of a low $[Mg^{2+}]/[ATP]$ ratio before the K^+-enzyme interaction in step (iii). In addition, a study on the time course and order of formation of two different kinds of phosphoprotein (Fukushima and Tonomura, 1973) revealed that the ratio of EP (phosphorylated enzyme) sensitive to dephosphorylation by ADP was low in the earlier stages of reaction and increased later, suggesting that an ADP-insensitive phosphorylated intermediate was formed before the ADP-sensitive form, a result also inconsistent with the previously proposed mechanism.

The Transition-State Mechanism

A requirement of the cyclical scheme of Post et al. (1969) is that ouabain should only bind to the E_2 conformation of the enzyme (the K^+-sensitive form). Results from experiments performed on the binding of ^3H-labelled ouabain to an ox brain enzyme preparation were presented in 1973 and showed that ouabain binding was obtained both when experimental conditions were designed to favour E_2 formation (in the presence of a high $[Na^+]$ and a level of Mg^{2+} (5 mM) greater than the [ATP] of 3 mM) and also when a control was set up with minimal sodium levels and no ATP present, but a $[Mg^{2+}]$ as in the first experiment. This second ouabain binding pathway (stimulated by magnesium) was inhibited by Na^+ ions, confirming the previous findings of Tobin and Sen (1970). A surprising finding, however, was that it was the binding pathway supported by Mg^{2+} alone, rather than that supported by $Na^+ + Mg^{2+}$ + ATP, which showed the same dependence on pH as did the hydrolysis of ATP brought about by the enzyme preparation (Whittam and Chipperfield, 1973).

As a result of these studies an alternative mechanism was proposed. This scheme involved the simultaneous transport of Na^+ ions out of and K^+ ions into cells in a single step process. A requirement of this process was that there would exist both inward facing sites for Na^+ and outward facing sites for K^+ and that these ions could bind simultaneously. In other words, the mechanism was both binary and simultaneous. It may be represented thus (Whittam and Chipperfield, 1975):

INSIDE OF CELL



OUTSIDE OF CELL

This mechanism involves a non-isolatable unstable transition-state complex

which is that species shown in square brackets above. The mechanism does not require the formation of a complex of the pump with Mg.ATP before binding of Na^+ and K^+ can occur and further actually requires the presence of K^+ (externally) before the formation of the complex.

Other Experimental Approaches to Test Mechanism

Much of the evidence presented in early studies designed to elucidate the mechanism of operation of the sodium pump was based upon biochemical studies of the enzyme functioning in some form of isolated preparation. An alternative approach, however, to distinguish between possible simultaneous and consecutive mechanisms is the use of kinetic analysis. The earliest attempt at such a treatment for the $(Na^+ + K^+)$-ATPase was a study of the activation of a rabbit kidney microsomal preparation of the enzyme by Na^+ and K^+ ions (Green and Taylor, 1964). This study was unable to distinguish between a one-step and a two-step process. A theoretical study of kinetics by Baker and Stone (1966) excluded the early suggestion of a cyclical scheme by Shaw (for report of this scheme, see Glynn, 1957) unless certain steps were made reversible. This analysis was, however, unable to draw a satisfactory distinction between the simultaneous and consecutive modes of operation and pointed out the importance of finding an answer to the question as to whether or not the concentration of one substrate (intracellular Na^+) affected the affinity for the other (external K^+). This point is one upon which the studies presented in this thesis are based. A critical difference between the two-step mechanism described earlier and the simultaneous mechanism proposed by Whittam and Chipperfield (1973) is that in the case of the former Na^+ ions bind to a different conformation of the pump at a time before K^+ ions bind. The single-step mechanism, however, is based on an assumption of simultaneous attachment of both ions.

Early kinetic studies of pump mechanism have generally been based upon the adaptation of a substrate-modifier mechanism to fit the pump operation - see Frieden (1964). As the pump actually utilises three substrates: Na^+, K^+ and Mg.ATP, such studies are based in practice on measurements of pump activation by one of these substrates at various fixed levels of a second, with the third substrate held at constant levels throughout the experiments. Such a procedure has been adopted in two sets of experiments on fragmented membrane preparations. In the first such study, performed on a rat brain enzyme preparation, it was suggested that the order of attachment of Na^+, K^+ and Mg.ATP was random, a result inconsistent with a two-step mechanism in which K^+ binds after both Na^+ and ATP (Hexum et al., 1970). A second study, performed on a human erythrocyte membrane preparation, also excluded the possibility of K^+ always binding after ATP, but showed a discrepancy from the work mentioned above, in that the results did not suggest a random order of binding, but rather that K^+ always bound to the enzyme first (Peter and Wolf, 1972).

As already stated, much evidence as to the existence of phosphorylated intermediate forms of the pump enzyme has been put forward during the last twenty years. Such evidence is not here and has not in the past been subject to any dispute. What remains under discussion, however, is the question as to whether or not such intermediates exist in the order originally proposed, or even whether they represent part of the normal mode of operation of the sodium pump. If they do not, then is the pump mechanism a one-step process, as described previously, which does not include phosphorylated intermediates? Such a mechanism is based on an assumption that there is simultaneous attachment of Na^+ and K^+ ions to the enzyme, a state of affairs clearly in contrast with the proposed two-step mechanism in which K^+ acts with the second form of the phosphorylated intermediate (E_2P) and Na^+, earlier, with first intermediate (E_1P).

If a kinetic study is to be designed to attempt to discover which of these two options is correct and the pump is to be considered as an enzyme mechanism with two substrates, then, of necessity, [Mg. ATP] levels must be held constant throughout. This then permits the application of a kinetic analysis such as that described by Frieden (1964) for a single-substrate enzyme system with modifier. Such an analysis, however, is based upon the need to assume that there is no marked competition between the substrate and modifier (or in this case, second substrate). That competition between Na^+ and K^+ does occur is, however, well established - see Garrahan and Glynn (1967), Priestland and Whittam (1968). Consequently, a system must be used which enables the concentration of one substrate (for example, Na^+) with access to its activating binding sites to be varied, whilst its concentration with access to binding sites activated by K^+ ions must be minimised. Obviously, such a requirement cannot be met by a fragmented membrane preparation - a cellular system with its inherent asymmetry is needed.

Several earlier studies have attempted to address this problem. Flux measurements made on giant axons of squid (Baker et al., 1969) suggested that there was no dependence of the shape of the activation curve for potassium influx on the intracellular (axoplasm) composition. A more definite result was obtained by two sets of workers in experiments on human red cells which had been modified by means of a cation loading procedure (see Methods section for a description of the PCMBS loading procedure) to have various $[Na^+]_i$ levels (Garay and Garrahan, 1973; Chipperfield and Whittam, 1976). The results of Garay and Garrahan showed that fits of experimental flux data to a linear plot of an activation equation which takes account of the fact that there is more than one K^+-site externally, produced lines intersecting on the abscissa. This meant that there was no dependence of the affinity for external cation - in the case of these experiments, rubidium - on the intracellular sodium

concentration. The same authors later published a further study on this subject (Garrahan and Garay, 1974) confirming their view that sites on opposite sides of the membrane were independent, though such a result was disputed in discussion appended to the paper by another author. These three studies on red cells, however, have one thing in common. In all cases, influx measurements were made from Ringer solutions in which the main cation was Na^+. It should consequently be noted that kinetic treatment of such results may be suspect due to the competitive effects at the external K^+-binding sites (Frieden, 1964; Garrahan and Glynn, 1967). Similarly, if competition at intracellular sites is to be eliminated, it is required that the intracellular potassium concentration be minimal. A result from experiments on low-potassium and high-potassium sheep red cells also suggested independence of sites on opposite sides of the membrane (Hoffman and Tosteson, 1971). Although the flux measurements were made with the cells suspended in sodium-free Ringer solutions, all cells contained significant amounts of potassium. If a non-ordered mechanism applies, the competitive effects with either product (in this case intracellular K^+ or external Na^+) may be seen (Cleland, 1970). Such competitive effects are seen as abscissa intercept effects and consequently false K_A values for affinity constants are obtained, rendering any conclusion about independence or interdependence of binding sites on opposite sides of the membrane suspect.

Two studies have subsequently appeared in which such competitive effects were eliminated. Using human red cell ghosts, and measuring pump activity as the ouabain-sensitive liberation of $^{32}P_i$ from γ^{32}P-ATP, Chipperfield and Whittam (1974) demonstrated a dependence of the affinity of external binding sites for K^+ on the $[Na^+]$ within the ghosts. Throughout these experiments, the external $[Na^+]$ was less than 1 mM, and $[K^+]_i$ minimised - in both environments choline was the major cation. A

subsequent paper (Chipperfield and Whittam, 1976) presented results of a similar type from work performed on intact human red cells modified internally (to vary $[Na^+]_i$ and replace intracellular K^+ with choline), by the PCMBS cation loading procedure.

The Scope of the Present Work

It is the purpose of the studies presented in this thesis to extend the previous investigations as to whether or not sodium and potassium binding sites are interdependent. Although, as stated above, some evidence has already been presented in this area, there is a clear need to extend these studies in several ways. Firstly, the investigations published deal with the effects on binding of external potassium, of substantial changes in $[Na^+]_i$ level. Such changes were generally from a non-saturating to a saturating level. It would be interesting, therefore, to see if the induced changes in external cation affinity applied over a wider part of the activation curve for intracellular Na^+. As stated earlier, the interaction of K^+ with the pump is crucial with regard to solution of mechanism. An approach which might shed light on this problem would be to investigate whether or not there is a connection between cation binding sites operating in the reverse direction: in other words, does the $[K^+]$ externally influence the binding of intracellular sodium? In order to answer these questions experiments have been performed measuring pump activation by one substrate at several fixed concentrations of the other. This entails, for the first part of the work, construction of activation curves for external potassium in cells with minimal $[K^+]_i$ and various fixed $[Na^+]_i$ levels. For the second part of the work, it is necessary to prepare cells of five or six $[Na^+]_i$ levels in order to construct activation curves at various fixed $[K^+]_o$ levels. A choice exists as to how to measure the level of pump activation. Some workers have utilised γ^{32}P-labelled ATP and measured the liberation of labelled inorganic phosphate as a measure

of enzyme activity. Such techniques are particularly valuable when working with particulate enzyme preparations.

As already stated, a system must be chosen for such studies which has the asymmetry of normal cells still intact, but which also allows the possibility of performing manipulations of the intracellular ionic composition. One example of such a system is the dialysed giant axon of the squid, but clearly in terms of viability and availability alone, the system of choice is the red blood cell. It is established that red cells may be cold-stored for substantial periods of time (several weeks) yet still induced to synthesize ATP from such substrates as adenine and inosine (Whittam and Wiley, 1967). Further, during incubation of such cells at physiological temperatures, there is not the problem of maintenance of an adequate oxygen supply. Another advantage is that the ability to manipulate intracellular ionic composition is already established in this system (Garrahan and Rega, 1967). This procedure, which utilises the mercurial substance PCMBS, has been described and further characterized as part of this work - see Methods section. The use of PCMBS-modified red cells has the advantage that, when compared to ghost preparations, leakiness to cations is generally less prevalent. As such leaks would lead to distortion of results by the ensuing competitive inhibitory effects, this is a consideration of some importance. Two types of red cell have been used for these experiments: human and pig. This allows the extension of the work to an additional system - the choice of pigs as the additional source of blood being based upon the fact that the red cells of this species are free of the complications involved with, for example, sheep red cells: here cells may be of two types, low or high intracellular potassium, depending on the genotype of the animal. Pig red cells have the additional advantage that they do not show the high levels of passive permeability to other cations seen in ox red cells - in fact, serum levels of Na^+ and

K^+ in the pig are similar to those of man.

As it had been decided to use these cellular systems, the method of choice for pump activity measurements was the determination of ouabain-sensitive ion fluxes. Although some experiments were performed on cells equilibrated with ^{24}NaCl-containing solutions by measurement of $^{24}Na^+$ efflux, these fluxes were normally obtained as influx measurements due to the technical superiority of such experiments. Radio-labelled ^{42}KCl solutions were used for potassium influx measurements. The work has also been extended to include the use of radioactive caesium solutions. As early as 1953, the use of $^{134}Cs^+$ isotope solutions as an alternative to $^{42}K^+$ for cation flux studies was the subject of investigation (Love and Burch, 1953). $^{134}Cs^+$ was found to be a particularly good substitute for the $^{42}K^+$ isotope in view of the great difference in half-lives of their radioactive decay processes. The value of 2.05 years for the Cs^+ isotope makes experimental possibilities much more satisfactory than the 12.5 hours found with $^{42}K^+$. A further advantage for the studies being performed here was the fact that the $^{134}Cs^+$ isotope could be obtained readily as a $^{134}Cs^+$ solution of low concentration and high specific activity, whereas ^{42}KCl was supplied as an isotonic solution, making large dilutions necessary for experiments including the use of low $[K^+]_o$ levels. In addition, it has been reported that the affinity of the pump for caesium is markedly lower than that for potassium (Whittam and Ager, 1964; Baker et al., 1969) and consequently, there is not the need to work at such low concentrations when constructing Cs^+-activation curves as when working with potassium.

The experiments performed here have also included a study of the effects of variation of intracellular magnesium levels on pump activation externally by potassium. Referring back to the earlier description of two-step mechanisms, it will be seen that a dual function for magnesium was proposed. The function of Mg^{2+} as a co-factor for ATP

is well documented: it was discovered that over a range of $[Mg^{2+}]$ and $[ATP]$ levels tested the optimum concentration for activation was 1:1 both in human red cells (Dunham and Glynn, 1961) and in rabbit kidney preparation (Wheeler and Whittam, 1962). Further supporting evidence came from an indirect source: it was shown that competitive inhibition of the ATPase observed with calcium was in fact seen with a Ca-ATP complex rather than ionic Ca^{2+}, suggesting that Mg.ATP, rather than ATP alone was the true substrate (Epstein and Whittam, 1966). The secondary role - that for free Mg^{2+} - in the proposals of Fahn et al. (1966a, b) was to facilitate conversion from the ADP-sensitive phosphorylated intermediate, E_1P, to the K^+-sensitive form, E_2P. This role has been questioned in view of more recent results showing that effects previously attributed to Mg^{2+} could have been caused by the chelating agent, EDTA, used to control magnesium concentration (Klodos and Skou, 1975). In view of the crucial relevance of K^+ binding discussed earlier and of this lack of clarity over the function of Mg^{2+}, experiments were carried out to investigate further the influence of Mg^{2+} on cation influx. These are presented as Chapter III of the Results section.

METHODS

BLOOD

Human blood was kindly supplied by National Blood Transfusion Service, Regional Headquarters, Sheffield, and also by the Leicester Royal Infirmary, Department of Haematology. Blood was stored at 4°C in 'acid-citrate-dextrose' anticoagulant and was generally used for experiments when less than four weeks old. In order to avoid the differences in affinity for external potassium between different human blood groups, previously encountered by Chipperfield and Whittam (1976), group A blood was avoided. Most experiments were performed on group O blood cells.

Pig blood (kindly supplied by Leicester City Council, Environmental Health Department, Meat Inspector's Office) was obtained from freshly killed pigs and was also collected into 'acid-citrate-dextrose' anticoagulant. This was then stored at 4°C and generally used within two weeks of collection.

CATION LOADING OF CELLS

A modification of the procedure as described by Garrahan and Rega (1967) and later modified by Sachs (1970) was used. Red cells were first separated from plasma and buffy coat by centrifugation for 5 minutes at 2100 g. in an MSE Minor centrifuge (MSE Instruments, Crawley, Sussex), followed by aspiration of supernatants. Cells were then washed at least three times in isosmotic (150 mM) choline chloride solution prior to suspension in the cation loading solutions, at approximately 5% haematocrit.

Cation loading solutions were made up to an osmolarity of 360 mosmoles/ litre, of the following composition: NaCl/KCl/choline chloride (175 mM); $MgCl_2$ (2 mM); p-chloromericuribenzene sulphonic acid (PCMBS) (0.2 mM). The suspensions were incubated in a shaking water bath for 5 - 6 hours at 4°C, after which time they were centrifuged for 10 minutes at 1500 g. in an MSE Mistral 4L centrifuge (MSE Instruments, Crawley, Sussex), supernatants aspirated and cells resuspended in identical solutions for further incubation for 16 - 18 hours at 4°C. At the completion of this second loading period, the suspensions were again separated, supernatants discarded and the cells suspended, at approximately 10% haematocrit, in resealing solutions. The resealing solutions were of the same composition as the cation loading solutions except that the PCMBS was replaced by 5 mM cysteine (L-cysteine, free base), which penetrates the membrane to the 'permeability sites' to which PCMBS binds (Sutherland et al., 1967). Adenine (5 mM) and inosine (10 mM) were also included to facilitate the synthesis of ATP (Whittam and Wiley, 1967). The resealing suspensions were incubated in a shaking water bath for one hour at 37°C. At the end of this period the suspensions were again separated, the cells being retained and washed at least three times with about eight volumes of ice-cold isosmotic choline chloride buffered to pH 7.4 with 10 mM Tris-HCl.

CHARACTERIZATION OF CELLS LOADED BY THE PCMBS PROCEDURE

It has been previously reported (Jacob and Jandl, 1962) that the sulphydryl inhibitor p-chloromercuribenzoic acid (PCMB) causes disruption of cation gradients, spherocytosis and osmotic haemolysis, without inhibition of glycolysis. Further, the more hydrophilic compound

PCMBS, was found to penetrate red cell membranes much less readily than PCMB. However, later work (Sutherland et al., 1967) showed that some PCMBS penetrates the membrane, is bound inside the cell, possibly to haemoglobin, and is not desorbed on recovery. Furthermore, an inhibition of ouabain-sensitive Rb^+ influx into red cells had been seen in the presence of PCMBS (Rega et al., 1967), as well as a previous observation on inhibition of $(Na^+ + K^+)$ activated ATPase by PCMB (Skou, 1963).

Although the influence on ATPase activity is mediated by membrane bound PCMBS, as is the case with haemolytic effects (Tanaka and Nakai, 1977), it was decided, in view of the remaining intracellular portion, to test both cell fragility and the ability of cells to synthesize ATP following treatment by the PCMBS procedure. First, however, it was necessary to investigate the intracellular ionic conditions produced by such treatments.

Resultant monovalent cation concentrations

The PCMBS procedure enables almost any intracellular sodium and potassium content to be obtained (Garrahan and Rega, 1967). Under particular investigation here, however, was the ability to produce conditions of low (non-saturating) $[Na^+]_i$, at the same time maintaining the minimal intracellular potassium levels necessary to avoid inhibition at the intracellular sodium pump binding sites. Values are presented of resultant $[Na^+]_i$ over a low range of loading solution sodium concentrations (1 - 15 mM), for both human and pig blood experiments (Tables 1 and 2). The $[K^+]_i$ levels resulting from these loading procedures (carried out with potassium-free solutions) are also presented.

TABLE 1. Intracellular $[Na^+]$ and $[K^+]$ in human red blood cells following loading by the PCMBS procedure in K^+-free solutions of varying low (1 - 15 mM) $[Na^+]$.
Initial normal values for cold-stored human red cells: $[K^+]_i$ = 97.82 ± 2.97 mmoles/litre cells. $[Na^+]_i$ = 23.12 ± 0.82 mmoles/litre cells. MEAN ± S.E. (n = 6)

LOADING SOLUTION $[Na^+]$ mM	RESULTANT $[Na^+]_i$ mmoles/litre cells		RESULTANT $[K^+]_i$ mmoles/litre cells	
	RANGE OF VALUES	MEAN ± S.E. (n=8)	RANGE OF VALUES	MEAN ± S.E. (n=8)
1	0.79 - 1.52	1.10 ± 0.09	0.27 - 0.58	0.45 ± 0.04
2	1.35 - 1.90	1.66 ± 0.06	0.28 - 0.62	0.45 ± 0.04
4	2.20 - 3.82	2.98 ± 0.17	0.22 - 0.73	0.46 ± 0.06
6	3.33 - 4.06	3.82 ± 0.09	0.20 - 1.52	0.88 ± 0.19
8	3.35 - 5.94	4.67 ± 0.29	0.25 - 1.47	0.78 ± 0.13
15	5.57 -11.49	8.01 ± 0.62	0.22 - 1.10	0.69 ± 0.09

TABLE 2 Intracellular $[Na^+]$ and $[K^+]$ in pig red blood cells following loading by the PCMBS procedure in K^+-free solutions of varying low (1 - 15 mM) $[Na^+]$

Initial normal values for fresh pig red blood cells: $[K^+]_i$ = 113.73 mmoles/litre cells

$[Na^+]_i$ = 7.05 mmoles/litre cells

(Values mean of 3 estimations)

LOADING SOLUTION $[Na^+]$ mM	RESULTANT $[Na^+]_i$ mmoles/litre cells		RESULTANT $[K^+]_i$ mmoles/litre cells	
	RANGE OF VALUES	MEAN $\pm$ S.E. (n=7)	RANGE OF VALUES	MEAN $\pm$ S.E. (n=7)
2	2.00 - 3.62	2.81 $\pm$ 0.23	2.77 - 5.92	4.58 $\pm$ 0.43
4	3.78 - 5.50	4.62 $\pm$ 0.23	2.71 - 6.33	4.49 $\pm$ 0.50
6	5.75 - 6.32	5.93 $\pm$ 0.08	2.56 - 7.62	5.10 $\pm$ 0.69
8	6.42 - 8.33	7.73 $\pm$ 0.26	3.63 - 7.57	4.88 $\pm$ 0.50
15	11.64 -15.54	13.52 $\pm$ 0.54	3.84 - 7.74	4.97 $\pm$ 0.54

The two conclusions from these results were firstly that there is a considerable variation in the resultant $[Na^+]_i$ and $[K^+]_i$ produced from treatment of cells by the PCMBS procedure with loading solutions of these compositions and secondly that intracellular potassium could not be voided as easily from pig red cells as was the case with human red cells. It had been previously shown (Sutherland et al., 1967) that the extent of PCMBS interaction with red blood cells was dependent upon the amount of PCMBS present, temperature and duration of exposure. Consequently, when pig cells of minimal $[K^+]_i$ were required, this was achieved in some cases by extending the loading period from approximately 24 hours to approximately 40 hours. In these experiments, loading solutions were changed both after 15 and 24 hours. Resealing was as described in procedure above. A comparison of the resultant intracellular ionic compositions in pig red blood cells loaded with a 24 hour and with a 40 hour incubation is also presented (Figure 1), showing that, in addition to the more effective voiding of cellular potassium, there is an additional loss of cellular sodium from cells loaded for 40 hours.

In addition to the variation in resultant cellular cation levels produced in different experiments with different batches of blood, evidence is also presented (Figure 2) to show that there is some variation between separate loadings performed on the same blood.

The main conclusion for monovalent cation loading of pig or human red blood cells by the PCMBS procedure is that, although a graded response to alterations in loading solution cation concentration can be reproduced, variation as to where on the resultant internal ionic composition scale this occurs is unavoidable. Consequently, internal conditions resulting from any given loading procedure cannot be predicted accurately.

Figure 1. Comparison of resultant $[Na^+]_i$ and $[K^+]_i$ levels in pig red cells treated by the PCMBS cation loading procedure with a 24 hour (closed symbols) or 40 hour (open symbols) loading period at 4^oC.
(●) and (○) $[K^+]_i$ mmoles/litre cells
(▲) and (△) $[Na^+]_i$ mmoles/litre cells
Values were obtained from simultaneous treatments on the same batch of blood.

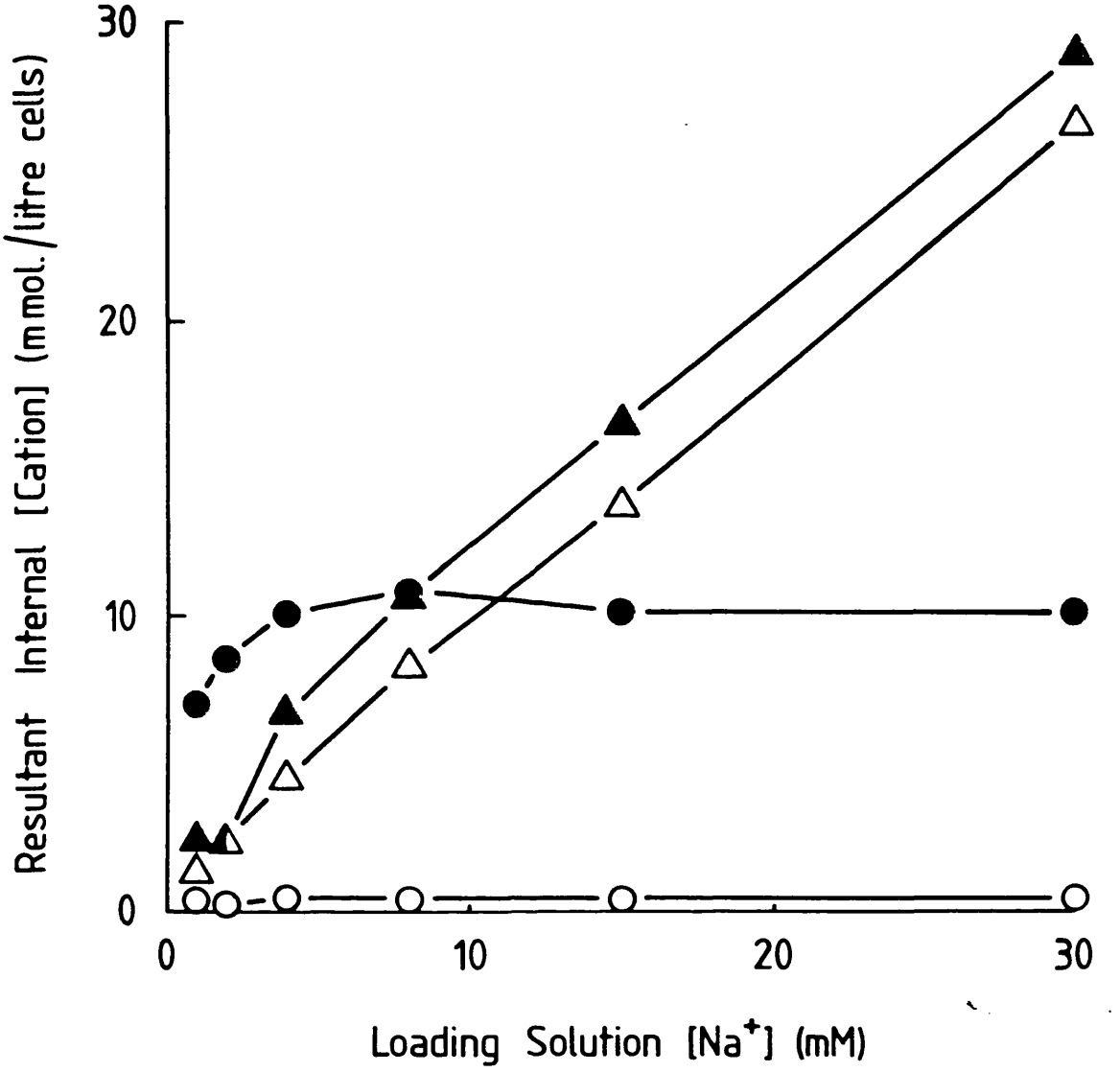
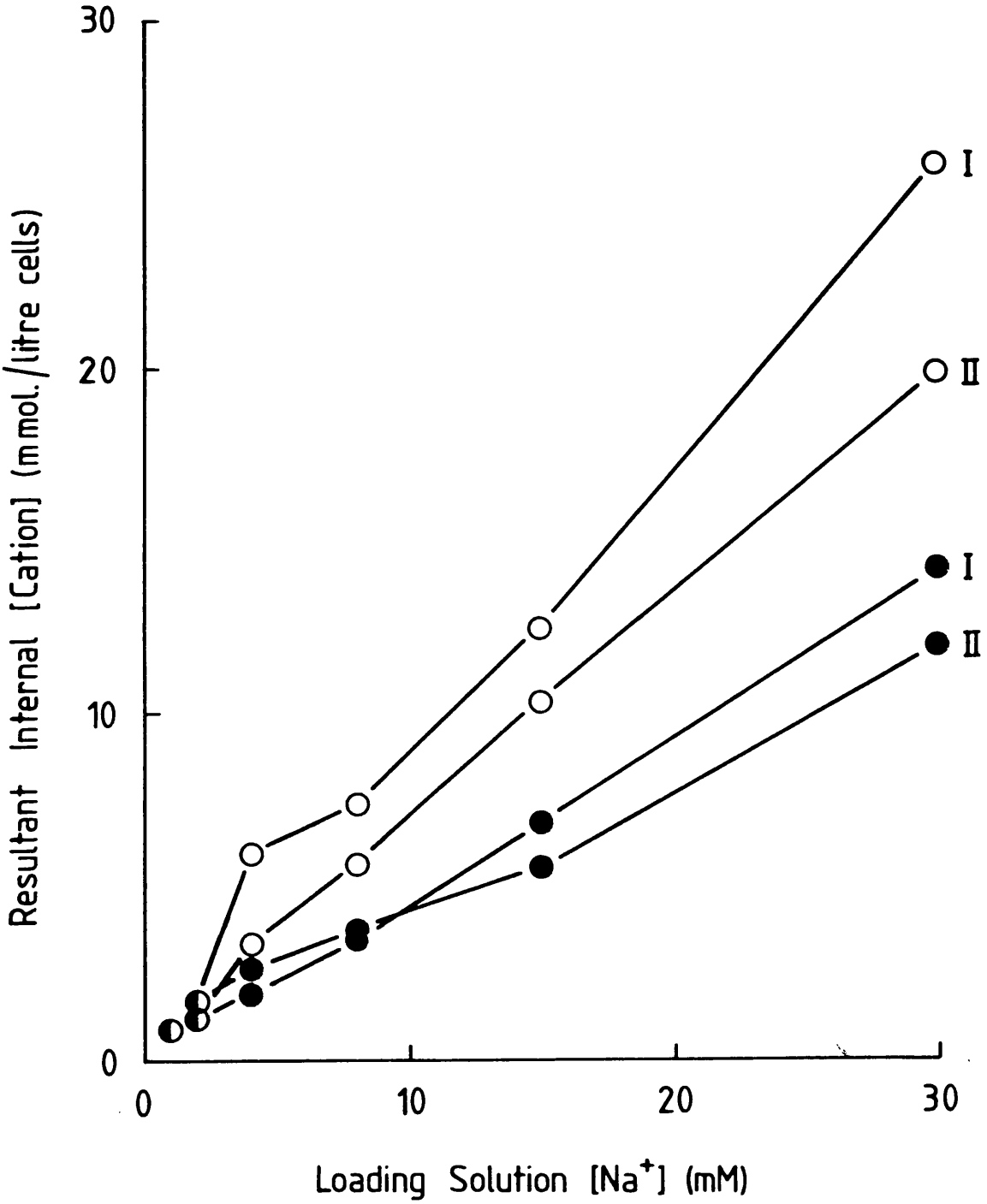

30
20
10
0
0
10
20
30
Resultant Internal [Cation] (mmol./litre cells)
Loading Solution [Na+] (mM)

Figure 2. Comparison of resultant $[Na^+]_i$ levels after two separate treatments, carried out simultaneously on the same batch of human blood (●) or pig blood (○), by the PCMBS cation loading procedure.
Resultant mean $[K^+]_i$ levels:

Human blood:	I	0.60 mmoles/litre cells
	II	0.53 mmoles/litre cells
Pig blood:	I	1.47 mmoles/litre cells
	II	1.32 mmoles/litre cells


30
20
10
0
0
10
20
30
Resultant Internal [Cation] (mmol./litre cells)
Loading Solution [Na+] (mM)
I
II
I
II

Magnesium loading

The use of the PCMBS procedure for magnesium loading has been described previously (Dunn, 1974; Wade, 1977). The procedure used was as described previously for monovalent cation loading, with $MgCl_2$ substituted for NaCl/KCl/choline chloride to the required level. Resultant $[Mg^{++}]_i$ values from various loading solution concentrations are presented, for human red blood cells (Table 3) and pig red blood cells (Table 4). As was the case with monovalent cation loading, a substantial variation in the resultant concentration values occurs, again making accurate prediction of final intracellular composition impossible.

The influence of alterations in loading solution magnesium concentration on the sodium loading process in human red cells was also investigated (Table 5). Resultant $[Na^+]_i$ levels appeared to be reduced at higher loading solution magnesium levels. Consequently, in experiments where a constant $[Na^+]_i$ was required whilst varying $[Mg^{++}]_i$, an elevated loading solution $[Na^+]$ was used in the loading solutions of higher $[Mg^{++}]$.

Ability of cells to synthesize ATP following treatment by the PCMBS procedure

Investigations were performed on both human and pig red cells loaded by the PCMBS procedure to yield low intracellular sodium levels. The relationship between $[K^+]_i$ and the final $[ATP]_i$ following resealing of cells was investigated. Intracellular ATP estimations were performed on supernatants from cells deproteinized with four volumes of 0.6 M perchloric acid and centrifuged at 2100 g. for 5 minutes. The assay was performed enzymically using Boehringer Mannheim test kits (BCL, London), based on

TABLE 3. Intracellular $[Mg^{++}]$ in human red blood cells following loading by the PCMBS procedure in K^+-free solutions of fixed 30 mM [NaCl].

LOADING SOLUTION $[Mg^{++}]$ mM	RESULTANT $[Mg^{++}]_i$ mmoles/litre cells	
	RANGE OF VALUES	MEAN ± S.E. (n=5)
2	2.67 - 4.18	3.14 ± 0.27
5	3.46 - 4.82	4.02 ± 0.25
10	5.68 - 10.03	6.87 ± 0.80
15	7.22 - 8.73	7.99 ± 0.24
20	6.68 - 11.22	9.08 ± 0.81
30	10.54 - 15.62	12.67 ± 0.94
40	10.95 - 21.69	15.84 ± 1.84
60	17.20 - 20.83	18.83 ± 0.65

TABLE 4. Intracellular $[Mg^{++}]$ in pig red blood cells following loading by the PCMBS procedure in K^+-free solutions of fixed 30 mM [NaCl].

All loadings were of a 24 hour duration.

LOADING SOLUTION $[Mg^{++}]$ mM	RESULTANT $[Mg^{++}]_i$ mmoles/litre cells	
	RANGE OF VALUES	MEAN ± S.E. (n=6)
2	3.46 - 5.79	4.84 ± 0.32
5	4.94 - 6.52	5.88 ± 0.25
10	6.91 - 8.28	7.60 ± 0.27
15	7.49 - 11.19	9.69 ± 0.61
30	11.57 - 18.29	14.81 ± 1.38

TABLE 5. Intracellular $[Na^+]$ in human red blood cells following loading by the PCMBS procedure in K^+-free solutions of varying $[Mg^{++}]$. All loading solutions contained 40 mM NaCl.

LOADING SOLUTION $[Mg^{++}]$ mM	RESULTANT $[Mg^{++}]_i$ mmoles/litre cells MEAN ± S.E. (n=6)	RESULTANT $[Na^+]_i$ mmoles/litre cells	
		RANGE OF VALUES	MEAN ± S.E. (n=6)
2	3.24 ± 0.30	18.36 - 25.48	20.95 ± 0.98
5	3.94 ± 0.19	16.75 - 21.23	19.38 ± 0.69
10	7.96 ± 1.19	15.24 - 23.32	18.44 ± 1.16
15	8.38 ± 0.18	19.66 - 21.27	20.47 ± 0.22
30	12.53 ± 0.92	10.07 - 19.43	14.88 ± 1.67
60	18.45 ± 0.54	11.35 - 13.75	12.56 ± 0.51
90	20.42 ± 0.36	9.85 - 11.63	10.71 ± 0.32

the method of Bücher (1947).

The ability of human red cells to synthesize ATP from adenine and inosine has been previously reported (Whittam and Wiley, 1967). The same authors reported that the $[ATP]_i$ for half-maximal stimulation of potassium transport was 0.4 - 0.5 μmoles/ml cells, with a normal $[ATP]_i$ of 1 μmole/ml cells being sufficient to avoid ATP being rate-limiting for active potassium transport. It was necessary, therefore, to ensure that $[ATP]_i$ could be maintained at this value in order to study the influence of the other ligands (Na^+, K^+ and Mg^{++}) on pump rates.

The effect of the PCMBS procedure, with its incorporated resealing in solutions containing adenine (5 mM) plus inosine (10 mM), was first investigated (Table 6). A plot of resultant $[ATP]_i$ levels against resultant $[K^+]_i$ is also presented (Figure 3). It can be seen that, in human red cells, the $[ATP]_i$ is increased as efficiently by cells subjected to the PCMBS procedure and resealed in the presence of adenine and inosine, as by cells simply incubated in a Ringer solution containing these substrates. In addition, the voiding of most of the normal intracellular potassium content does not impair this capacity for ATP synthesis.

It has been demonstrated that pig red cells, although not normally showing glycolytic activity <u>in vivo</u>, are capable of a significant amount of such activity <u>in vitro</u>, this being limited by their low permeability to glucose (Kim and McManus, 1971a). Further, the same authors (1971b) showed that the pentose sugar ribose could be metabolised by pig red cells to form lactate and could maintain intracellular ATP levels, the optimum concentration for this effect being 3 mM.

An investigation was performed into the effects of various substrates on the ability of pig red cells to maintain $[ATP]_i$ during a

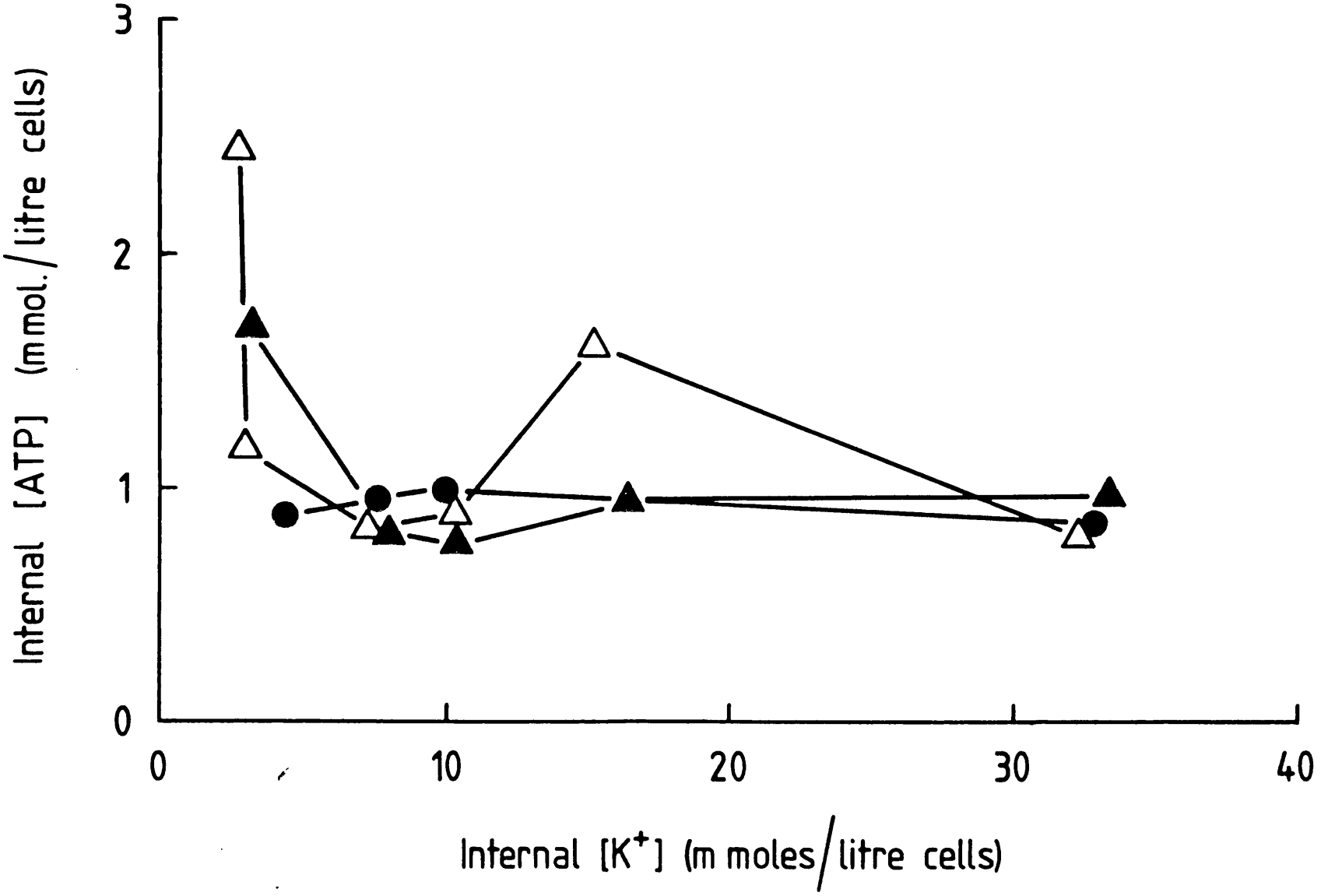
TABLE 6. Comparison of ATP levels in human red blood cells before and after the PCMBS procedure. Loading and resealing solutions contained 100 mM KCl; 30 mM NaCl; 45 mM choline chloride. Loading procedure was as described previously. Values are means of three similar experiments.

	$[ATP]_i$ mmoles/litre cells
Untreated, cold stored cells (age about 5 weeks)	0.20
Cold-stored cells incubated at 10% haematocrit in choline Ringer containing 5 mM adenine plus 10 mM inosine for 1 hour at 37°C	0.78
PCMBS treated cells, resealed in the absence of adenine and inosine	0.35
PCMBS treated cells, resealed in the presence of 5 mM adenine plus 10 mM inosine	0.75

Figure 3. $[ATP]_i$ levels in human red cells following loading by the PCMBS procedure to yield different intracellular potassium levels.

All resealing solutions contained adenine (5 mM) and inosine (10 mM).

Values from three separate experiments are presented.


3
2
1
0
0
10
20
30
40
Internal [ATP] (mmol./litre cells)
Internal [K+] (m moles/litre cells)

period of maximal pump activity - an incubation for one hour at 37°C in a Ringer solution containing 10 mM KCl. The substrates compared were adenine plus inosine, ribose and glucose. It was found (Table 7) that fresh pig red cells appear capable of maintaining $[ATP]_i$ in the presence or absence of either glucose or ribose, but that, in the presence of adenine plus inosine, the $[ATP]_i$ actually increases. Results from intracellular phosphate estimations, carried out by the method of Fiske and Subbarow (1925), show clearly an adequate presence of inorganic phosphate in the cells for this synthesis of ATP. It was also shown that the inclusion of phosphate in the resealing medium was not necessary for ATP synthesis (Figure 4). Recently, this finding has been confirmed and extended to show that in pig blood an extracellular inosine concentration of as low as 40 nM could support the cells' entire energy requirements. This fact and the additional evidence that physiological blood inosine levels in the pig may be up to 40 times that mentioned above, has led to the suggestion that inosine could be the physiological energy source of pig red cells (Jarvis *et al.*, 1980).

Finally, an investigation was carried out into the depletion of cellular ATP during a typical flux measurement incubation. Both human and pig red cells were examined. Cells were loaded to give $[Na^+]_i$ of approximately 10 mM, and various $[K^+]_i$ levels. They were then incubated for one hour at 37°C, at 10% haematocrit in a Ringer solution of composition: choline chloride (125 mM); $MgCl_2$ (10 mM); KCl (10 mM); Tris-HCl, pH 7.4 at 37°C (10 mM); adenine (5 mM) and inosine (10 mM). $[ATP]_i$ levels were measured before and after incubation in the presence and absence of ouabain (10^{-4}M) (Table 8). The investigation revealed that

TABLE 7. The ability of pig red blood cells to synthesize ATP from various substrates. Cells were incubated at 10% haematocrit in Ringer of composition: choline chloride (150 mM); Tris-HCl, pH 7.4 at 37°C (10 mM); substrate as specified.
Values from two experiments are quoted.
All values are mean of duplicate estimations.

SUBSTRATE	EXPERIMENT	$[ATP]_i$ mmoles/litre cells	$[Phosphate]_i$ mmoles/litre cells
Fresh cells (not incubated)	I	0.42	6.65
	II	0.65	7.68
Incubated without substrate	II	0.63	4.45
+ 5 mM glucose	I	0.49	3.43
	II	0.58	4.68
+ 5 mM ribose	I	0.48	3.08
	II	0.59	3.96
+ 5 mM adenine, 10 mM inosine	I	0.71	1.60
	II	1.15	0.78

Figure 4. Influence of the presence or absence of phosphate in resealing solutions for the PCMBS cation loading procedure - effect on $[ATP]_i$ of cells loaded to produce different intracellular sodium levels.

(●) Resealing solutions buffered with Tris-PO_4 (10 mM)

Mean $[K^+]_i$ following resealing: 1.32 mmoles/litre cells

Mean $[ATP]_i$ following resealing: 1.08 mmoles/litre cells

(○) Resealing solutions buffered with Tris-HCl (10 mM)

Mean $[K^+]_i$ following resealing: 1.47 mmoles/litre cells

Mean $[ATP]_i$ following resealing: 1.04 mmoles/litre cells

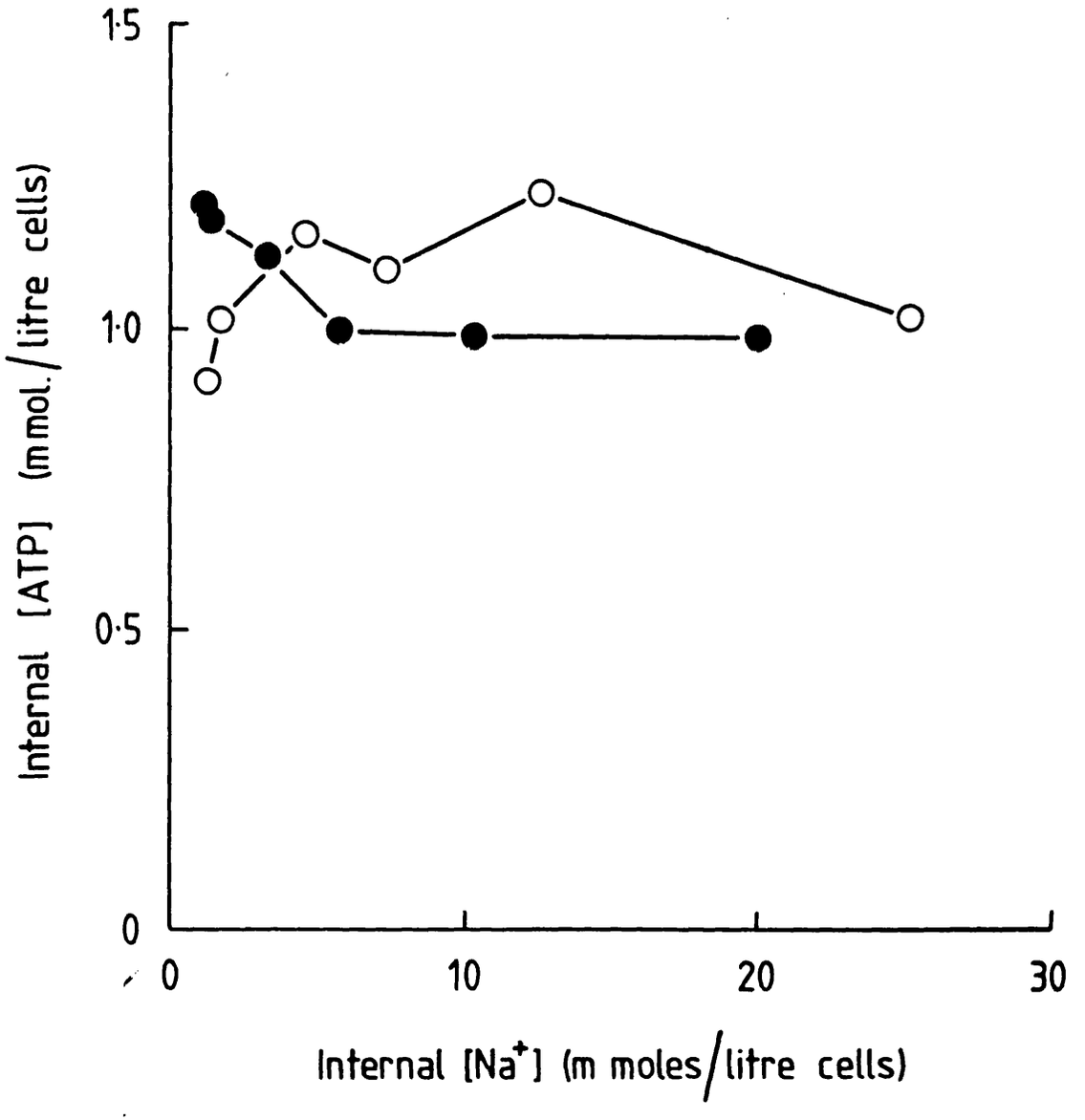

1·5
1·0
0·5
0
0
10
20
30
Internal [ATP] (mmol./litre cells)
Internal [Na+] (m moles/litre cells)

TABLE 8 Monitoring of $[ATP]_i$ in human and pig red blood cells during incubation for one hour at 37°C in Ringer solution containing KCl (10mM). All values are means of duplicate estimations.

TYPE OF BLOOD	INTRACELLULAR COMPOSITION mmoles/litre cells $[Na^+]_i$	$[K^+]_i$	$[ATP]_i$ mmoles/litre cells BEFORE INCUBATION	AFTER INCUBATION (plus ouabain 10^{-4}M)	AFTER INCUBATION IN ABSENCE OF OUABAIN
Human		1.20	1.06	1.26	1.12
	6.32	11.61	1.09	1.40	1.12
		78.66	1.17	1.26	1.13
Pig		3.09	1.05	1.01	0.96
		7.38	1.16	1.11	0.88
		12.40	1.18	0.87	0.94
	9.79	15.80	1.13	1.37	0.81
		20.26	1.22	0.89	0.95
		94.66	1.45	1.13	1.17
		108.26	1.45	1.67	1.44

$[ATP]_i$ did not fall, after one hour of pump activity, to a level below 80% of its initial value. In addition, the pig cells showed the same independence of $[K^+]_i$ as seen previously in human red cells (Figure 3).

The conclusion reached from these investigations into synthesis of ATP by human and pig red cells is that, in all cases, inclusion of adenine (5 mM) and inosine (10 mM) in both resealing solutions when loading cells and in Ringer solutions for flux measurement incubations would facilitate maintenance of $[ATP]_i$ at a level of approximately 1 mmole/litre cells, during the one hour period of these flux incubations.

Cell fragility

Among the effects of PCMBS reported previously (Sutherland et al., 1967), is a tendency for uncontrolled increases in membrane permeability to lead to irreversible changes in cell integrity, such as haemolysis. As some haemolysis was often observed during the loading process, it was decided to examine whether or not the fragility of cells following treatment by the PCMBS procedure and resealing, was markedly different to that of a washed sample of cells. The procedure was as detailed below.

Cells were washed in the normal manner, and then suspended at approximately 15% haematocrit in a choline Ringer solution of composition: choline chloride (150 mM); adenine (5 mM); inosine (10 mM); Tris-HCl, pH 7.4 at 20°C (10 mM). 0.2 ml aliquots of this suspension were then added to test tubes containing standard volumes of NaCl solutions of various

concentrations (20 - 150 mM). These were shaken, allowed to stand for 2 - 3 minutes, and then separated by centrifugation at 2100 g. for two minutes. Optical densities of the resultant supernatants were then measured at 540 nm on a Pye-Unicam SP 600 spectrophotometer (Pye-Unicam Instruments Limited, Cambridge), against a standard which consisted of a 0.2 ml aliquot of the cell suspension haemolysed in standard volume of 7 mM NH_4OH solution (Wooton, 1964). Further cell suspension samples were taken for intracellular ion estimation. Fragility curves could then be constructed for a series of cells with different intracellular ionic composition.

An investigation was first carried out into the loading, by the PCMBS procedure, of several alkali metal cations - Na^+, K^+, Li^+, Rb^+ and Cs^+. Results are presented as a series of fragility curves for the sodium and potassium loading experiments (Figures 5 and 6) and also as a table of values for the 50% haemolysis point obtained with cells loaded varying each of the cations (Table 9). The range of loading solution cation concentrations tested was from 20 - 145 mM. These experiments showed that, for all the cations tested except Li^+, the tendency for haemolysis increased markedly as the loading solution, and consequently the intracellular concentration of any one cation was increased. The shift in fragility curves when moving from a low (20 mM) to a high (145 mM) loading solution concentration was consistently of the order of 100 mosmoles/litre. Further, at low cation concentrations fragility never exceeded, and frequently was lower than in a group of cells not treated by the PCMBS procedure, but simply washed in isotonic choline chloride before testing.

In view of the flux experiments to be conducted, however, these results posed another question. For flux studies designed to

Figure 5. Fragility of human red cells loaded, to vary $[Na^+]_i$, by the PCMBS cation loading procedure. Loading solutions contained NaCl as specified, KCl (30 mM) and were made up to 350 mosmoles/litre with choline chloride.

(●) Loading solution 145 mM NaCl; resultant $[Na^+]_i$: 84.42 mmoles/litre cells

(▲) Loading solution 100 mM NaCl; resultant $[Na^+]_i$: 55.71 mmoles/litre cells

(■) Loading solution 60 mM NaCl; resultant $[Na^+]_i$: 33.14 mmoles/litre cells

(▼) Loading solution 20 mM NaCl; resultant $[Na^+]_i$: 18.47 mmoles/litre cells

(○) Washed cells, not subjected to PCMBS procedure.

These results, together with those from further experiments, are included in Table 9.

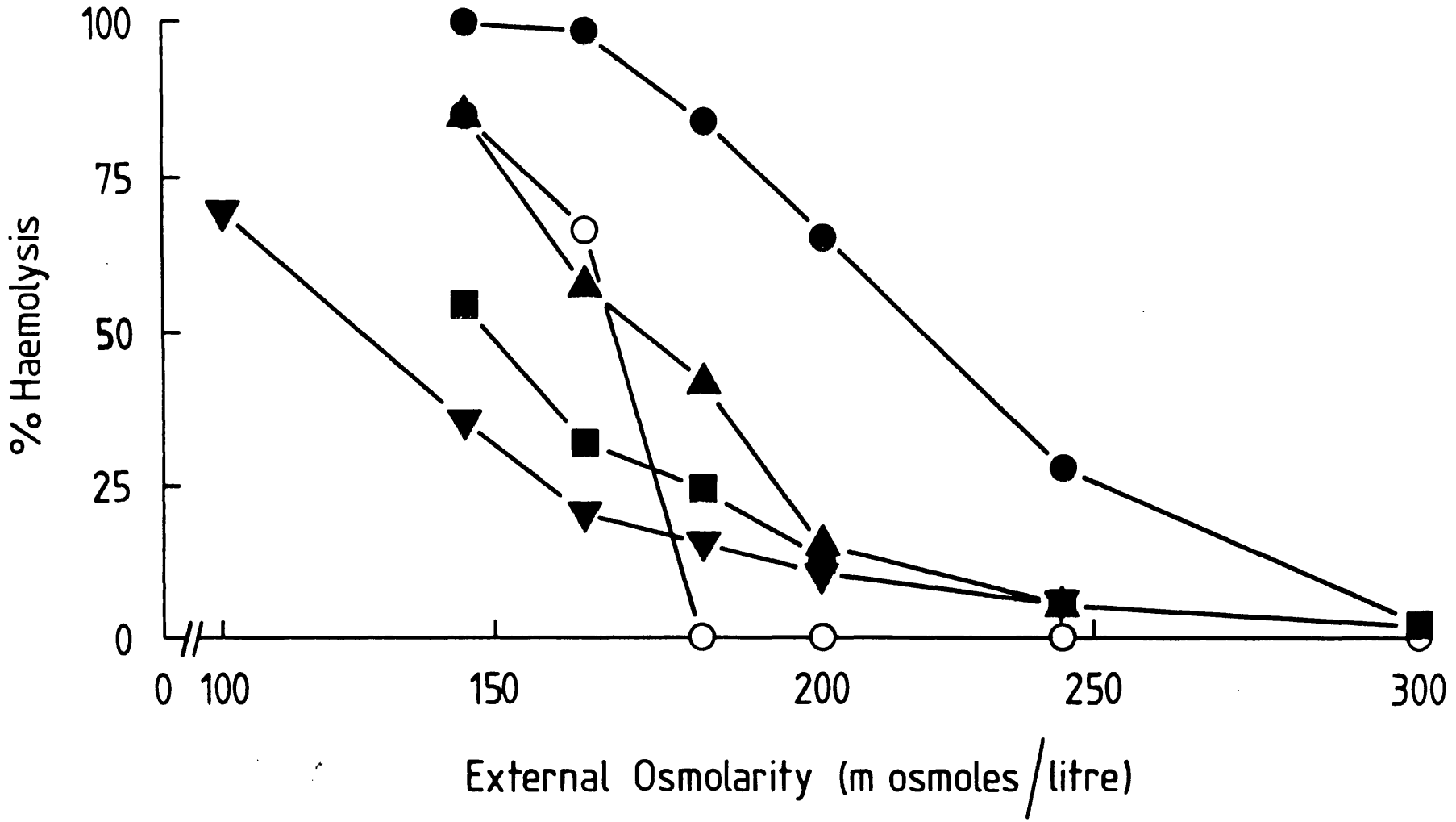

100
75
50
25
0
% Haemolysis
0
100
150
200
250
300
External Osmolarity (m osmoles/litre)

Figure 6. Fragility of human red cells loaded, to vary $[K^+]_i$, by the PCMBS cation loading procedure. Loading solutions contained KCl as specified, NaCl (30 mM) and were made up to 350 mosmoles/litre with choline chloride.

(●) Loading solution 145 mM KCl; resultant $[K^+]_i$: 53.54 mmoles/litre cells

(▲) Loading solution 100 mM KCl; resultant $[K^+]_i$: 35.72 mmoles/litre cells

(■) Loading solution 60 mM KCl; resultant $[K^+]_i$: 19.72 mmoles/litre cells

(▼) Loading solution 20 mM KCl; resultant $[K^+]_i$: 7.14 mmoles/litre cells

(○) Washed cells, not subjected to PCMBS procedure.

These results, together with those from further experiments, are included in Table 9.

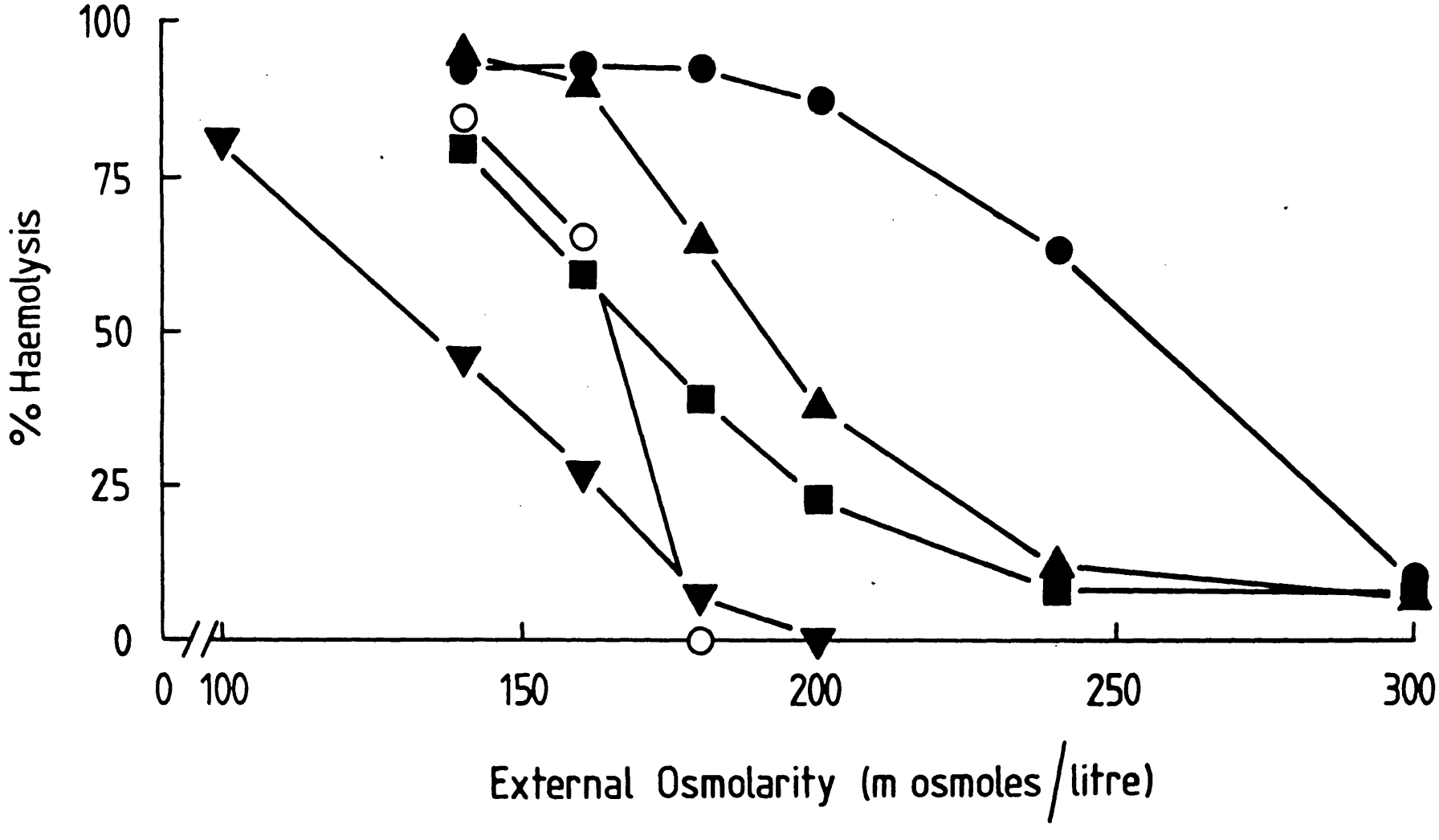

100
75
50
25
0
% Haemolysis
0
100
150
200
250
300
External Osmolarity (m osmoles/litre)

TABLE 9. Cation loading (by PCMBS procedure) and cell fragility. Loading solutions include 30 mM NaCl (or KCl for $[Na^+]$ variation experiment) and choline chloride as required.

All values from mean of duplicate estimations.

CATION	LOADING SOLUTION CONCENTRATION mM	RESULTANT INTRACELLULAR CONCENTRATION mmoles/litre cells	50% HAEMOLYSIS POINT mosmoles/litre
Li^+	145	45.87	155
	100	41.70	144
	75	28.03	126
	50	19.20	114
	25	8.98	124
Na^+	145	84.42	216
	125	67.81	193
	100	55.71	170
	80	46.32	156
	60	33.14	144
	40	21.19	137
	20	18.47	122
K^+	145	53.54	256
	125	51.88	227
	100	35.72	192
	80	27.60	181
	60	19.72	170
	40	13.14	142
	20	7.14	134
Rb^+	145	cells unstable - almost total haemolysis at 300 mosmoles/litre	
	125	64.73	177
	100	39.14	132
	75	25.41	128
	50	22.18	113
	25	7.72	113
Cs^+	145	70.89	266
	125	61.99	254
	100	44.26	200
	75	31.14	186
	50	18.53	158
	25	10.38	131

The value obtained from washed cells was 50% haemolysis at 165 mosmoles/litre.

observe affinity changes at sodium pump binding sites, it is necessary to prepare cells of minimal $[K^+]_i$, and various, but low $[Na^+]_i$. These cells would have, therefore, to be loaded with the substitute ion choline$^+$. Were cells of a high $[\text{choline}^+]_i$ likely to show a similar substantial increase in fragility? This question was investigated in both human and pig red blood cells. Results are presented from two experiments on human cells (Table 10) which show that over the range of $[Na^+]_i$ values normally used in these flux experiments (approximately 1 - 20 mM), fragility of cells with minimal $[K^+]_i$ levels is consistently not greater than that of a sample of washed cells. In most cases the cells are actually less fragile than such a washed sample. Results from pig red cells loaded for similar internal ionic compositions (Table 11) also include a comparison of cells loaded for the normal 20 - 24 hour period, and those loaded for the previously described 40 hour period - incorporating an extra loading solution change. In both cases the cell fragility was consistently lower than that of a sample of washed cells. Cells loaded for the longer period to void more effectively their internal K^+ content were, however, considerably more fragile than those loaded for the normal 20 - 24 hour period, presumably because of an increased intracellular component of PCMBS binding during the loading, which could lead to more of the mercurial remaining present, with access to membrane sites, even after resealing. It should be stressed, however, that even in these cases, cell fragility did not exceed that of a washed sample of cells not treated with PCMBS.

It was concluded that, for both pig and human red cells, intact cell preparations of internal ionic compositions as specified could be achieved, which showed no signs of increased fragility due to the loading process.

TABLE 10. Fragility of human red blood cells loaded in K^+-free PCMBS solutions to yield varying low $[Na^+]_i$ levels.

Data from two experiments are presented separately.

All values of 50% haemolysis points are from fragility curves constructed as described previously.

EXPERIMENT NUMBER	LOADING SOLUTION $[Na^+]$ mM	RESULTANT $[Na^+]_i$ mmoles/litre cells	50% HAEMOLYSIS POINT mosmoles/litre
I	1	1.17	176
	2	1.65	173
	4	3.34	178
	8	5.94	138
	15	11.49	166
	30	19.29	150
	Washed cells	(23.12)	170
Mean $[K^+]_i$ in loaded cells = 0.70 mmoles/litre cells			
II	1	1.15	106
	2	1.88	118
	4	3.10	122
	8	5.70	112
	15	9.15	112
	30	16.59	124
	Washed cells	(22.77)	160
Mean $[K^+]_i$ in loaded cells = 1.23 mmoles/litre cells			

TABLE 11. Fragility of pig red blood cells loaded in K^+-free PCMBS solutions to yield varying low $[Na^+]_i$ levels.

All values of 50% haemolysis points are from fragility curves constructed as described previously.

LOADING PERIOD	LOADING SOLUTION $[Na^+]$ mM	RESULTANT $[Na^+]_i$ mmoles/litre cells	50% HAEMOLYSIS POINT mosmoles/litre
24 hours	1	2.38	108
	2	2.27	136
	4	6.61	111
	8	10.79	112
	15	16.73	106
	30	29.19	96
	Washed cells	(17.53)	212
Mean $[K^+]_i$ in loaded cells = 9.38 mmoles/litre cells			
40 hours	1	1.40	178
	2	2.42	203
	4	4.63	158
	8	8.27	144
	15	13.98	150
	30	26.97	152
	Washed cells	(17.53)	212
Mean $[K^+]_i$ in loaded cells = 0.32 mmoles/litre cells			

Cell fragility and magnesium loading

Consideration of both cell shape and size, and passive permeability to magnesium after loading by the PCMBS procedure has been given previously (Wade, 1977). Cells loaded with varying amounts of magnesium did not appear to show a substantial increase in passive permeability.

The investigation carried out here into fragility of both human and pig red cells after magnesium loading, showed no increase compared to a washed sample of cells, except in some high $\left[Mg^{++}\right]_i$ cells (Table 12).

As with sodium and potassium loading, there appears to be no basis for an assertion that a more fragile cell type is produced by the loading procedure.

FLUX MEASUREMENTS

In all experiments the cation flux due to the sodium pump was taken as being the difference between the mean of duplicate estimations of total flux and the mean of duplicate estimations of flux in the presence of ouabain (10^{-4}M). The concentration of ouabain (10^{-4}M) was as used previously by other workers (Chipperfield and Whittam, 1976; Garay and Garrahan, 1973) and is well in excess of the documented value for the half-maximal inhibition of active transport in human red cells: 3 - 7 x 10^{-8}M (Skou, 1965).

An investigation has been carried out into the need for a period of preincubation of cells in ouabain. It had been previously discovered that magnesium in the external solution would stimulate ouabain

TABLE 12. Cell fragility and Mg^{++} loading by the PCMBS procedure. All 50% haemolysis point values are obtained from fragility curves constructed as described previously.

EXPERIMENT	LOADING SOLUTION $[Mg^{++}]$ mM	RESULTANT $[Mg^{++}]_i$ mmoles/litre cells	50% HAEMOLYSIS POINT mosmoles/litre
I Human blood	2	2.54	151
	5	4.21	156
	15	8.73	166
	30	15.62	162
	60	18.58	164
	90	20.70	129
	Washed cells	(1.93)	170
Mean $[Na^+]_i$ = 17.75 mmoles/litre cells			
II Human blood	2	2.38	158
	5	3.46	154
	15	8.13	158
	30	11.23	174
	60	17.20	177
	90	20.45	194
	Washed cells	(1.92)	192
Mean $[Na^+]_i$ = 15.40 mmoles/litre cells			
III Pig blood	2	4.93	160
	5	6.24	167
	10	8.21	168
	15	10.94	149
	30	17.76	156
	Washed cells	(4.62)	212
Mean $[Na^+]_i$ = 22.57 mmoles/litre cells			

binding to human red cells, only when sodium was absent (Whittam, unpublished observations); consequently a Ringer solution of the following composition has been used for flux measurements: choline chloride (135 mM); $MgCl_2$ (10 mM); Tris-HCl, pH 7.4 at 37°C (10 mM); adenine (5 mM); inosine (10 mM). Human red cells were washed and then suspended at 10% haematocrit in such a Ringer solution, and $^{42}K^+$ influx from an external medium containing 10 mM. $^{42}K^+$ was measured. Variations in the time of preincubation reveal no increased inhibition of the total $^{42}K^+$ influx with periods of preincubation up to 30 minutes (Figure 7). Consequently, in all flux experiments no preincubation of cells in ouabain has been carried out.

$^{24}Na^+$ efflux

This was measured by the method described by Priestland and Whittam (1968). Cells were first loaded, at approximately 50% haematocrit, with tracer $^{24}NaCl$ by incubating in the presence of the isotope for 3 hours at 37°C. Cells were then washed at least five times in buffered ice-cold isosmotic choline chloride. This washing process was continued until such time as a supernatant sample showed radioactivity levels not significantly higher than background values. Duplicate samples of cells were also subjected to this incubation and washing procedure in the absence of $^{24}NaCl$. These were then haemolysed in 7 mM NH_4OH and used for intracellular ion estimation. The cells loaded with tracer were then suspended at 10% haematocrit in a choline Ringer, as described above. 10 ml aliquots of the cell suspension were placed in flasks in a shaking water bath at 37°C, ouabain (10^{-4}M) was added where required, and the flux incubation was commenced by the addition of the required amount of external potassium or caesium.

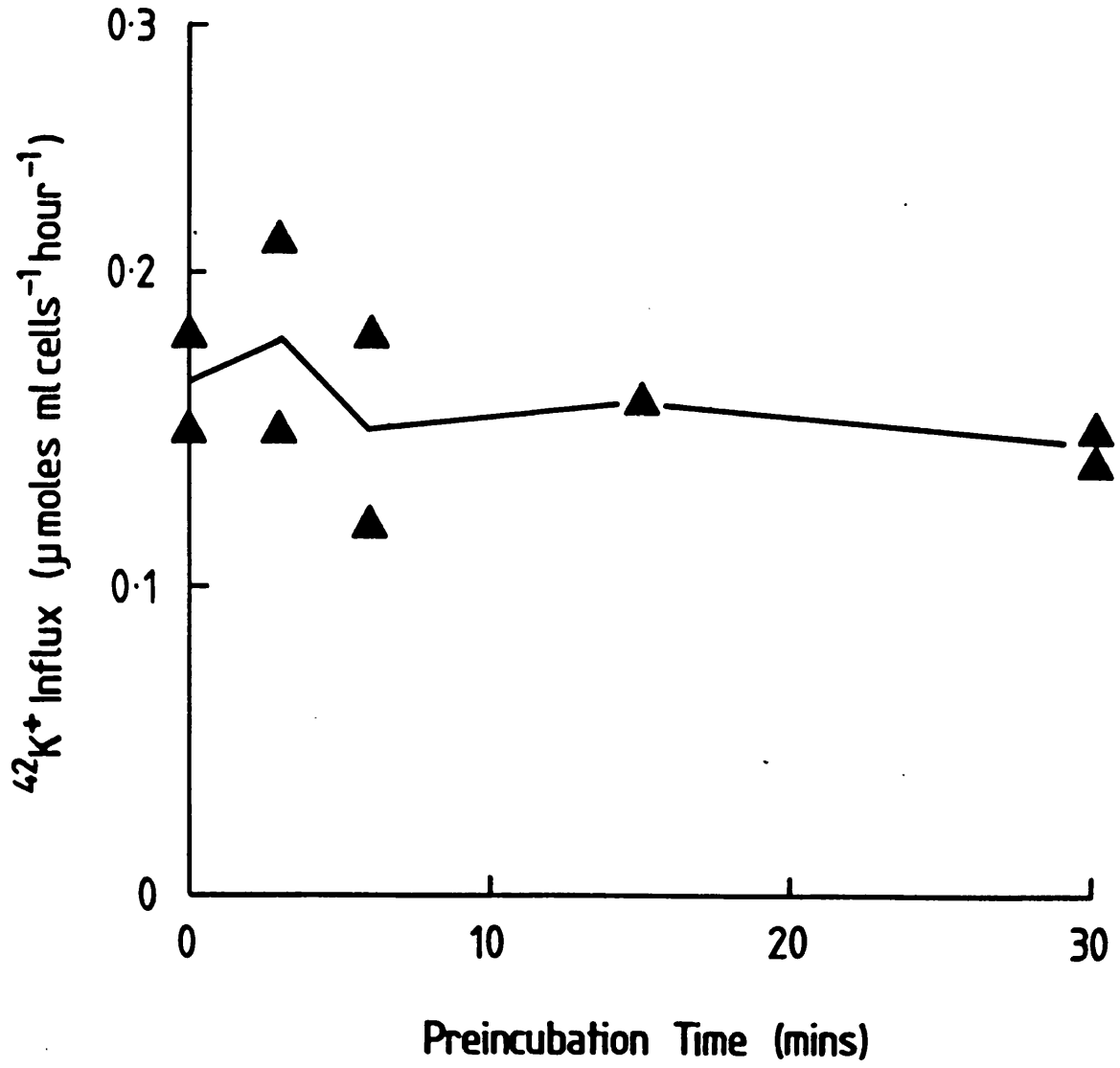
Figure 7. Influence of preincubation time on ouabain inhibition of $^{42}K^{+}$ influx into washed human red cells.

$[Ouabain]_o = 10^{-4}M$

$[K^{+}]_o = 10$ mM

Total $^{42}K^{+}$ influx in absence of ouabain = 4.05 μ moles. ml cells^{-1} hr^{-1}

Values presented are means of duplicate estimations.


0·3
0·2
0·1
0
$^{42}K^+$ Influx (μmoles ml cells^{-1} hour^{-1})
0
10
20
30
Preincubation Time (mins)

Further samples of cell suspensions were centrifuged (2100 g., 2 minutes) and supernatants discarded, to facilitate determination of cell counts per minute. In addition, samples of the cell suspension were placed in haematocrit tubes and centrifuged for 30 minutes at 2100 g.

As the rate of appearance of extracellular counts does not appear to be linear within the first 15 minutes of an incubation of this type, samples of flask contents were removed 15 minutes and 75 minutes after the start of the incubation, and the hour between these two samplings used as that for the flux rate determination. The samples were centrifuged (2100 g., 2 minutes) and 2 ml volumes of supernatant taken for counting.

Calculation of $^{24}Na^{+}$ efflux

Let N_t^c = cell counts per minute at time t minutes after the start of the incubation,

and N_t^s = supernatant counts per minute at time t minutes after the start of the incubation

$$N_t^c = N_o^c e^{-kt}$$

$$\frac{N_t^c}{N_o^c} = e^{-kt} \quad \text{and } N_t^c = N_o^c - N_t^s$$

$$\text{so, } \frac{N_o^c - N_t^s}{N_o^c} = e^{-kt}, \text{ or } \ln\left(1 - \frac{N_t^s}{N_o^c}\right) = -kt \quad \text{(Equation A)}$$

$$N_o^c - N_{15}^s = N_{15}^c$$

Substituting in equation A above for N_o^c, to give cell counts from time t = 15 minutes, onwards.

$$\ln\left(1 - \frac{N_{75}^s - N_{15}^s}{N_o^c - N_{15}^s}\right) = -k$$

$$\text{or } \ln \frac{N_o^c - N_{15}^s - N_{75}^s + N_{15}^s}{N_o^c - N_{15}^s} = -k$$

$$\text{(i.e.) } k = \ln \frac{N_o^c - N_{15}^s}{N_o^c - N_{75}^s}$$

and, rate of sodium efflux = $k.\ (Na^+)_i$ μmoles ml cells^{-1} hour^{-1}

$^{42}K^+$ influx

The technique used was a modification of that described by Glynn (1956), which makes use of the fact that $^{42}K^+$ uptake is known to be linear with time for at least 2 hours, both in the presence and absence of external sodium (Garrahan and Glynn, 1967).

Cells, having been loaded by the PCMBS procedure, were suspended at 10% haematocrit in choline Ringer solution. Flasks were preparedto contain the required amount of ^{42}KCl and sufficient ouabain to give a final concentration of 10^{-4}M where required, and experiments were started by addition of 10 ml cell suspension to each flask, followed by immediate transfer of the flask to a shaking water bath at 37°C. The dispensing of tracer ^{42}KCl to flasks before incubation was by means of an 'Agla' micrometer syringe outfit (Burroughs Wellcome and Co., London). At the completion of one hour, the reaction was terminated by pouring the contents of the flasks into test tubes in an ice bath. Suspensions were centrifuged (2100 g., 1½ minutes) and samples of supernatant removed for radioactivity counting. Cells were then washed twice in ice-cold buffered isosmotic choline chloride solution and haemolysed in 7 mM NH_4OH solution prior to counting of radioactivity. Samples of haemolysate were taken for Hb estimation.

$^{134}Cs^+$ influx

$^{134}Cs^+$ influx was measured by essentially the same method as $^{42}K^+$ influx. Again, in all experiments, a stock solution containing $^{134}CsCl$ was dispensed into flasks prior to the addition of a red cell suspension to start the reaction. As when measuring $^{42}K^+$ influx, no preincubation with ouabain ($10^{-4}M$) was used. The only modification involved with $^{134}Cs^+$ influx measurements was that the cells were counted prior to being haemolysed in 7 mM NH_4OH for Hb estimation.

Haemoglobin (Hb) estimation

Haemoglobin estimation was as oxyhaemoglobin, by measuring optical density at 540 nm against that of packed cell standards haemolysed in 7 mM NH_4OH solution (Wooton, 1964). Optical density measurements were made on a Pye-Unicam SP600 spectrophotometer.

The absorption spectra of pig and human haemoglobin standard solutions were compared (Figure 8), showing that there is no spectrum difference and that pig blood Hb levels may also be measured satisfactorily at a wavelength of 540 nm.

ION ESTIMATION

Intracellular ion estimation

Samples of cells were taken from the Ringer suspension, prior to the flux incubation, washed once more in buffered isosmotic choline chloride and then haemolysed in 7 mM NH_4OH. A sample of haemolysate

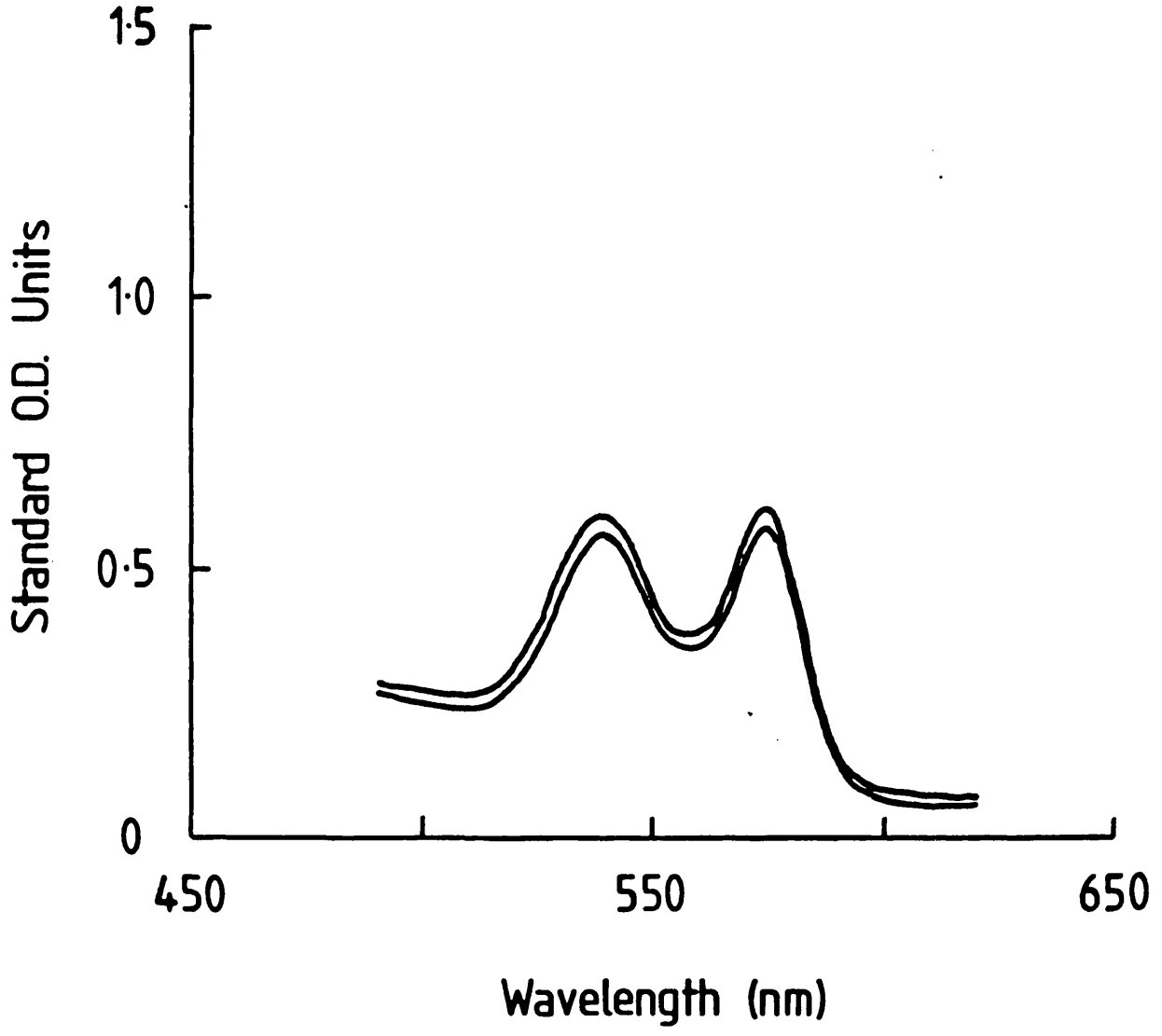
Figure 8. Comparison of pig and human haemoglobin standards.

Standards were packed red cells lysed in 7 mM NH_4OH (Wooton, 1964).

Spectra obtained on a SP800 Pye-Unicam ultraviolet spectrophotometer (Pye-Unicam Instruments Ltd., Cambridge).

Upper trace - pig haemoglobin

Lower trace - human haemoglobin


1·5
1·0
0·5
0
Standard O.D. Units
450
550
650
Wavelength (nm)

was removed for Hb estimation. $[K^+]_i$ and $[Na^+]_i$ were measured by flame emission, $[Mg^{++}]_i$ by flame absorption. All flame photometry was carried out on a Pye-Unicam SP90 atomic absorption spectrophotometer (Pye-Unicam Instruments Limited, Cambridge).

Extracellular potassium estimation

It was essential that $[K^+]_o$ should be determined accurately, as, at the low concentrations used in many of the experiments, significant changes in this concentration were likely to occur during the hour of incubation. The external concentration used to calculate the influx value was taken as that midway between the known initial concentration and the determined final concentration. The known initial concentration had been previously determined as accurate, by dispensing, from the Agla micrometer syringe apparatus, duplicate aliquots of tracer into standard volumes of water and, after allowing one week for decay of $^{42}K^+$, estimating resultant concentrations by flame photometry. The final concentrations were also determined by flame photometry on suitable dilutions of supernatant removed at the end of the flux incubation.

Extracellular caesium estimation

Extracellular caesium estimation could not be performed on radioactive solutions by flame photometry, because of the half-life time of the isotope (2.05 years). The assumption was made that there was a zero intracellular caesium concentration at the start of the incubation and that consequently no change in specific activity of the tracer $^{134}CsCl$

would occur. Therefore, concentration could be directly calculated from an estimate of radioactivity present. As with potassium solutions, duplicate aliquots of the $^{134}CsCl$ stock solution were dispensed into standard volumes of water, and specific activity was calculated from the counting of these standards. The final external $[Cs^+]$ could then be estimated directly from the supernatant counts per minute at the end of the flux incubation.

MATERIALS

Radioactive isotopes

$^{24}Na^+$, $^{42}K^+$ and $^{134}Cs^+$ were obtained from the Radiochemical Centre, Amersham, Bucks. $^{24}NaCl$ and ^{42}KCl were obtained as sterile isotonic solutions, $^{134}CsCl$ as an aqueous solution of concentration 0.43 mM and specific activity 13.138 mCi/mg Cs. All isotopes were counted directly with no scintillant present. ^{42}K was counted in haemolysates of red cells or diluted supernatants, by use of a Geiger-Müller tube connected to either a Panax scaler/timer (Panax Limited, Redhill, Surrey) or a Philips XL1101 single channel spectrometer + XL1150 scaler/timer (Philips Medical Systems Division, Eindhoven, Netherlands). $^{24}Na^+$ and $^{134}Cs^+$ were counted either as a pellet of red cells or as an undiluted aliquot of supernatant in a well type scintillation chamber connected to the Philips system described above.

Chemicals

All chemicals were of Analar grade when possible. p-Chloromercuribenzenesulphonic acid (PCMBS) was obtained from Sigma Chemicals Inc., St. Louis, Missouri, U.S.A.

RESULTS

I. THE INFLUENCE OF INTRACELLULAR SODIUM ON EXTERNAL CATION AFFINITY

Kinetic studies allow a distinction to be made between consecutive and simultaneous transport sequences on the one hand and unitary and binary mechanisms on the other. It should, however, be remembered that evidence from such studies cannot be used as a source of proof of the validity of a particular mechanism, but rather to either discount or show consistency with one (Segal et al., 1952).

The question as to whether the binding sites for external potassium and for intracellular sodium are independent or show some connection, is clearly of great importance in the distinction between unitary and binary mechanisms. Experiments conducted on squid giant axons (Baker et al., 1969) and on human red cells (Garay and Garrahan, 1973) found no connection and suggested that the shape of the pump activation curve for external potassium is independent of intracellular sodium concentration. A similar independence of binding sites was found in low potassium and high potassium sheep red cells (Hoffman and Tosteson, 1971). Experiments conducted on human red cell ghosts (Chipperfield and Whittam, 1974) and on intact human red cells modified by the PCMBS loading procedure (Chipperfield and Whittam, 1976) suggested that there was, however, a connection between the potassium sites and the sodium sites on opposite sides of the membrane. The discrepancy between these results and those previously obtained by other workers which suggested independence of sites was explained by the fact that such a connection could only be observed when experimental conditions were so arranged that no inhibition from either external sodium or intracellular potassium was permitted. Such inhibition had not been eliminated on both sides of the membrane in the previous experiments. In the case of the earlier work on squid giant axons (Baker et al., 1969) and on human red cells (Garay and Garrahan,

1973) external sodium was present. Similarly, in the experiments on sheep red cells which suggested independence of sites (Hoffman and Tosteson, 1971) the intracellular potassium level had not been minimised.

The purpose of the experiments presented here was thus to extend the previous studies and to investigate the nature of attachment of external cations (potassium or caesium) and of internal sodium to the sodium pump in red blood cells. The question under consideration was whether or not the affinity for external potassium or caesium is dependent on the intracellular sodium concentration, in conditions where the other requirements for pump activation, namely Mg^{++} and ATP, are kept at a constant level sufficient to be not rate limiting. Caesium has been used as an alternative to potassium as the external activating cation in some experiments. In addition, the work has been extended to include experiments on pig red cells, which present an alternative system of cells of similar ionic composition to that found in human red cells.

Experiments have been conducted measuring pump activity as ouabain-sensitive $^{42}K^{+}$ or $^{134}Cs^{+}$ influx into cells previously modified using the PCMBS loading procedure. These modifications allowed both adjustment of intracellular sodium levels as required and also voiding of as much as possible of the normal high level of intracellular potassium. All influx measurements were made from sodium-free Ringer solutions in which the major cation was choline. The measures described above were taken to avoid the inhibitory effects caused by the presence of products (intracellular potassium and external sodium) of bisubstrate reactions (Cleland, 1970). It has been shown, for instance, that the presence of either intracellular potassium or external sodium modifies the pattern of activation by external potassium.

If results amenable to kinetic analysis for mechanistic considerations are to be obtained, it is, therefore, vital that

experimental design is such as to allow elimination of these products. The previously stated requirement (Cleland, 1963a), that kinetic distinction between the ping-pong (consecutive) and simultaneous mechanisms for bisubstrate reactions requires that the apparent Michaelis constant and the maximum velocity for one of the substrates be determined at several fixed concentrations of the other substrate in the absence of reaction products, can thus be met.

I i) a) HUMAN BLOOD EXPERIMENTS - POTASSIUM AS EXTERNAL CATION

The previous work on human red cells investigating the influence of $[Na^+]_i$ on external potassium binding involved experiments comparing cells of two conditions (Chipperfield and Whittam, 1976). These conditions, achieved by modification of intracellular ionic composition using the PCMBS loading procedure, were one of low (5 - 12 mmoles/litre cells) $[Na^+]_i$ and another of high, saturating (25 - 41 mmoles/litre cells) $[Na^+]_i$.

The first evidence presented (Table 13; Figure 9) is from experiments designed to attempt to confirm the previous work on human red cells. Cells of two groups have been compared: one of low $[Na^+]_i$ (2.94 - 7.76 mmoles/litre cells) and the other of high, saturating $[Na^+]_i$ (19.48 - 34.06 mmoles/litre cells). Blood of three different groups was used for these experiments (A, B and AB) and in each case a similar result was obtained. Elevation of $[Na^+]_i$ from a value within the low range described above to one within the high range caused a substantial increase in $^{42}K^+$ influx. In each experiment the value of V_{max} for $^{42}K^+$ influx was increased by at least fifty per cent, and in three experiments out of four it was more than doubled. This shows a response of increased pump activity as $[Na^+]_i$ is raised. In addition, in each case elevation of the

$[Na^+]_i$ level from the low to the high range of values produced a substantial increase in the K_m for $^{42}K^+$ influx, representing a decrease in the affinity for potassium. These results confirm and extend those of Chipperfield and Whittam (1976) and further show that, although increasing $[Na^+]_i$ produces an increase in V_{max} and K_m for $^{42}K^+$ influx, in all cases, the absolute values for these kinetic constants are different from group to group. In particular, both constants are shown to be higher in group AB blood. This question of variation of kinetic constants for $^{42}K^+$ influx between different blood groups has been mentioned elsewhere (Chipperfield and Whittam, 1976) and would be worthy of further investigation, although such experiments could not be performed here.

Values for the kinetic constants V_{max} and K_m were obtained using the computational method of Wilkinson (1961). This method involves the fitting of a bilinear regression on to provisional values for a fit to the Michaelis-Menten equation and its first derivative. The method has the advantage that it allows the weighting errors which are introduced from even linear regression fits of double reciprocal plots to be eliminated. A computer programme to yield both values of the provisional estimates and their fine adjustments with errors was formulated (Figure 10). It was found that when hyperbolae were constructed from the Michaelis-Menten equation using the values of V_{max} and K_m obtained by this computational method, good fits to the original experimental data were always obtained.

It has already been stressed that the presence of external sodium and its consequent inhibition of $^{42}K^+$ influx may exclude the possibility of observing any dependence between binding sites on opposite sides of the membrane. Although influx measurements were routinely performed from sodium-free Ringer solutions, some sodium leakage from

TABLE 13 The influence of $[Na^+]_i$ on kinetic constants for ouabain-sensitive $^{42}K^+$ influx into human red blood cells. Experiments comparing cells of low (non-saturating) $[Na^+]_i$ and high (saturating) $[Na^+]_i$ levels. All cells were prepared to have minimal $[K^+]_i$ - less than 0.8 mmoles/litre cells. Values obtained by computational method of Wilkinson (1961) (see text and Figure 10). Corrected values are corrected for interference by $[Na^+]_o$ of 1 mM. (see text).

EXPERIMENT NUMBER	BLOOD GROUP	$[Na^+]_i$ (mmoles/litre cells)	V_{max} ($\pm$ S.E. of Mean) (μmoles ml cells^{-1} hr^{-1})	K_m ($\pm$ S.E. of Mean) (μM)	Corrected K_m (μM)	V_{max}/corrected K_m ratio
I	A	5.03	1.61 $\pm$ 0.06	13 $\pm$ 5	11	146
		21.83	3.36 $\pm$ 0.29	113 $\pm$ 25	94	36
II	AB	7.76	2.61 $\pm$ 0.13	206 $\pm$ 31	172	15
		34.06	5.45 $\pm$ 1.01	617 $\pm$ 250	514	11
III	B	5.64	1.46 $\pm$ 0.04	79 $\pm$ 11	66	22
		22.55	2.32 $\pm$ 0.13	140 $\pm$ 27	117	20
IV	B	2.94	0.65 $\pm$ 0.05	62 $\pm$ 16	52	13
		19.48	1.55 $\pm$ 0.27	321 $\pm$ 88	268	6

Data from Experiment I are also presented in Figure 9.

Figure 9. Activation of $^{42}K^+$ influx into human red cells by external potassium.

Open symbols represent ouabain-insensitive $^{42}K^+$ fluxes, measured as the mean of two flux determinations in the presence of 10^{-4} M ouabain.

Closed symbols represent ouabain-sensitive $^{42}K^+$ fluxes, calculated as the difference between the means of two flux determinations in the absence of ouabain and two determinations (as shown in open symbols) in the presence of 10^{-4} M ouabain.

(△)(▲) $[Na^+]_i$ = 5.03 mmoles/litre cells

V_{max} = 1.61 ± 0.06 μmoles ml cells^{-1} hr^{-1}

K_m = 13 ± 5 μM

(□)(■) $[Na^+]_i$ = 21.83 mmoles/litre cells

V_{max} = 3.36 ± 0.29 μmoles ml cells^{-1} hr^{-1}

K_m = 113 ± 25 μM

The hyperbolic curves for ouabain-sensitive fluxes have been obtained from fits of the Michaelis-Menten equation to the values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.

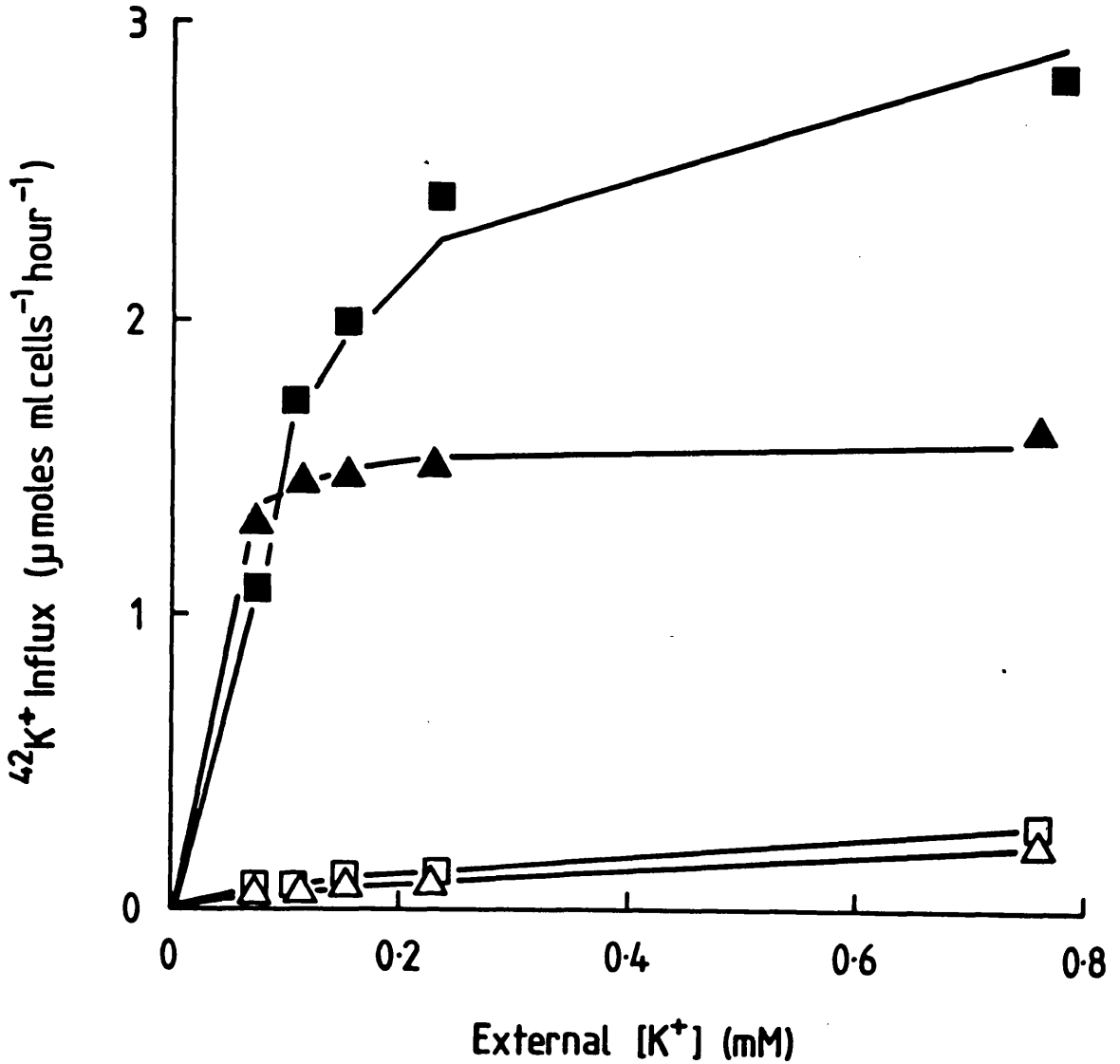

3
2
1
0
0
0·2
0·4
0·6
0·8
$^{42}K^+$ Influx (μmoles ml cells^{-1} hour^{-1})
External [K^+] (mM)

Figure 10. Computer programme used to calculate provisional estimates and final values, with standard errors, of the kinetic constants V_{max} and K_m from the Michaelis-Menten equation, using the statistical method of Wilkinson (1961). (With acknowledgement for programme preparation to Dr. S.A. Petersen).

```
list
      PROGRAM MICMEN(INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
        DIMENSION S(10),V(10),SKM(10),F(10),FPRIM(10)
3       WRITE(6,601)
        N=1
601     FORMAT(10X,*TYPE IN SUBSTRATE CONC AND VELOCITY PAIRS*)
2        READ(5,*) S(N),V(N)
        IF(S(N).EQ.-1)GOTO1
        N=N+1
        GOTO2
1        SIGVX=0
        SIGX2=0
        SIGVY=0
        SIGXY=0
        SIGY2=0
        SIGF2=0
        SIGFFP=0
        SIGVF=0
       SIGFP2=0
        SIGVFP=0
        SIGVV=0
        SIGFF=0
         N=N-1
        DO 101 I=1,N
        SIGVX=SIGVX+V(I)**3
        SIGX2=SIGX2+V(I)**4
        SIGVY=SIGVY+V(I)**3/S(I)
        SIGXY=SIGXY+V(I)**4/S(I)
        SIGY2=SIGY2+V(I)**4/S(I)**2
101     CONTINUE
        DEL=SIGVX*SIGY2-SIGVY*SIGXY
        AKM=(SIGX2*SIGVY-SIGVX*SIGXY)/DEL
        VMAX=(SIGX2*SIGY2-SIGXY**2)/DEL
        WRITE(6,602)AKM,VMAX
602     FORMAT(/,/,/,/,*PROVISIONAL KM=*,F8.6,*      VMAX=*,F8.6).
        DO 102 I=1,N
        SKM(I)=S(I)+AKM
        F(I)=VMAX*S(I)/SKM(I)
        FPRIM(I)=-VMAX*S(I)/SKM(I)**2
        SIGF2=SIGF2+F(I)**2
        SIGFFP=SIGFFP+F(I)*FPRIM(I)
        SIGVF=SIGVF+V(I)*F(I)
        SIGFP2=SIGFP2+FPRIM(I)**2
        SIGVFP=SIGVFP+V(I)*FPRIM(I)
       SIGVV=SIGVV+V(I)**2
102     CONTINUE
        DEL=SIGF2*SIGFP2-SIGFFP**2
        B1=(SIGFP2*SIGVF-SIGFFP*SIGVFP)/DEL
        B2=(SIGF2*SIGVFP-SIGFFP*SIGVF)/DEL
        VOMAK=VMAX
        VMAX=VMAX*B1
        AKM=AKM+B2/B1
        S2=(SIGVV-B1*SIGVF-B2*SIGVFP)/(N-2)
         S=SQRT(S2)
        SEKM=(S/B1)*SQRT(SIGF2/DEL)
        SEVMAX=VOMAK*S*SQRT(SIGFP2/DEL)
       WRITE(6,603)AKM,SEKM
603     FORMAT(/,/,/,10X,*FINAL KM=*,F8.6,*+/-*,F6.5)
        WRITE(6,604)VMAX,SEVMAX
604     FORMAT(/,10X,*FINAL VMAX=*,F8.6,*+/-*,F6.5)
        WRITE(6,605)
605     FORMAT(/,/,/,10X,*TYPE 1 TO CONTINUE*)
        READ(5,*)IANS
       IF(IANS.EQ.1)GOTO3
        STOP
       END
```

cells during the flux incubations was inevitable. A correction is therefore necessary. For the purposes of this correction, the least advantageous position was assumed: that of the lowest inhibition constant for external sodium and the highest $[Na^+]_o$ observed. The inhibition constant for inhibition of $^{42}K^+$ influx by external sodium in human red cells has been previously determined as 5 - 10 mM (Priestland and Whittam, 1968) and observation of the $[Na^+]_o$ level following flux incubations revealed a value not exceeding 1 mM, even from cells with $[Na^+]_i$ levels greater than 30 mmoles/litre cells. These observations were made by flame photometry on supernatant samples which had been kept for sufficient time for most of the radioactivity ($^{42}K^+$ half-life = 12.5 hours) to have been removed. To assume the least advantageous position thus involved using an inhibition constant of 5 mM and an $[Na^+]_o$ level of 1 mM. The correction applied was:

$$\text{Apparent } K_m = \text{True } K_m \ (1 + [Na^+]_o/I)$$

where I is the inhibition constant. (Cleland, 1963b).

In all cases this correction decreases the K_m value from that computed and slightly lessens the dependence of K_m on the $[Na^+]_i$ level. Despite these effects the response to an increase in $[Na^+]_i$ is consistently to produce an increase in V_{max} for $^{42}K^+$ influx and a decrease in affinity for external potassium.

During the course of this work further evidence appeared in the literature suggesting dependence of external cation affinity on internal sodium concentration. This evidence (Sachs, 1977) involved investigations over a series of $[Na^+]_i$ values all within a low range (1 - 8 mmoles/litre cells) in human red cells modified by the PCMBS loading procedure to be nominally K^+-free. There is, however, difficulty in reproducing pre-determined intracellular ionic compositions,

particularly those of low $[Na^+]_i$ level as required for these experiments, when using the PCMBS loading procedure (see Methods). In view of this problem and the fact that the evidence presented (Sachs, 1977) from work on a low range of $[Na^+]_i$ levels appears to be drawn from a single experiment, it was decided to conduct a series of experiments to extend these studies. Six experiments have been carried out, each on a separate batch of blood. In all cases cells were prepared to have a minimal $[K^+]_i$ level, and this never exceeded 0.8 mmoles/litre cells. The range of $[Na^+]_i$ levels used was from 1.19 to 5.69 mmoles/litre cells. In each experiment two (in one experiment three) conditions were compared by duplicate measurements of $^{42}K^+$ influx in the presence and absence of ouabain (10^{-4}M). Results are presented as Table 14 and Figure 11.

Although, because of the difficulty in reproducing predetermined conditions of $[Na^+]_i$ and $[K^+]_i$ level, experiments cannot be readily compared, within each individual experiment results are consistent with those of Sachs (1977) and with those observed in the earlier experiments (Table 13) over a wider range of $[Na^+]_i$ levels. Increases in $[Na^+]_i$ again caused substantial increases in $^{42}K^+$ influx - in one experiment a 29% increase in $[Na^+]_i$ caused V_{max} for influx to rise from 0.22 to 0.41 µmoles ml cells^{-1} hr^{-1}, in another a four-fold increase in $[Na^+]_i$ caused a three-fold rise in V_{max}. Similar changes in $[Na^+]_i$ level caused two to sixteen-fold increases in K_m value, representing a marked decrease in external potassium affinity (Table 14). It should also be added that the cell conditions under investigation show that the influence of intracellular sodium level on external cation binding is reproducible in every experiment, even where flux values and V_{max} values are considerably lower than those encountered in earlier experiments (Table 13). With one exception, the V_{max} values here are found to be less than 1 µmole ml cells^{-1} hr^{-1}.

Kinetic constants were again computed using the statistical method of Wilkinson (1961), and hyperbolae constructed from application of the Michaelis-Menten equation to calculated values of V_{max} and K_m. Such hyperbolae again yielded very good fits to experimental data. A similar correction for interference by external sodium leaking from the cells during flux incubation was again applied - the $[Na^+]_o$ level was assumed to be 1 mM and the value of the inhibition constant 5 mM.

Values are also presented (Table 14) of the V_{max}/K_m ratio for $^{42}K^+$ influx. A decrease in the value of this ratio was seen within each experiment as $[Na^+]_i$ was raised. Alterations in this ratio introduced by changes in the concentration of $[Na^+]_i$ have been used elsewhere as a source of mechanistic information. The results obtained here are not consistent with the information previously published (Sachs, 1977) and this will be referred to again later in this chapter (see Section I (vi)).

It should also be noted that there was marked variation in values of V_{max} and K_m obtained in different experiments performed on different batches of blood but with similar $[Na^+]_i$ levels. For example (see Table 14), in one experiment using cells of $[Na^+]_i$ 2.31 mmoles/litre cells a value of 0.41 μmoles ml cells^{-1} hr^{-1} for V_{max} was calculated together with a value of 81 μM of K_m for $^{42}K^+$ influx. In another experiment where $[Na^+]_i$ was 2.23 mmoles/litre cells, very different values for these kinetic constants were obtained: 0.16 μmoles ml cells^{-1} hr^{-1} for V_{max} and 35 μM for K_m. Nevertheless, there was consistency within each of the six experiments as to the effect of changes of $[Na^+]_i$ on both V_{max} and K_m for $^{42}K^+$ influx.

TABLE 14. The influence of $[Na^+]_i$ on kinetic constants for ouabain-sensitive $^{42}K^+$ into human red blood cells.
Experiments comparing cells of different, low (non-saturating $[Na^+]_i$ levels).
All cells were prepared to have minimal $[K^+]_i$ - less than 0.8 mmoles/litre cells.
Values of V_{max} and K_m obtained by computational method of Wilkinson (1961) - see text and Figure 10.
Corrected values are corrected for interference by $[Na^+]_o$ of 1 mM - see text.

EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/litre cells)	V_{max} ($\pm$ S.E.M.) (μmoles ml cells^{-1} hr^{-1})	K_m ($\pm$ S.E.M.) (μm)	Corrected K_m (μm)	V_{max}/K_m ratio
I	1.19	0.28 $\pm$ 0.02	11 $\pm$ 1	9	31.1
	4.76	0.77 $\pm$ 0.08	173 $\pm$ 44	144	5.4
II	2.75	0.57 $\pm$ 0.01	157 $\pm$ 8	131	4.4
	3.98	0.86 $\pm$ 0.17	303 $\pm$ 111	253	3.4
	5.69	1.85 $\pm$ 0.45	806 $\pm$ 272	672	2.8
III	2.00	0.44 $\pm$ 0.02	42 $\pm$ 10	35	12.6
	3.37	0.89 $\pm$ 0.06	164 $\pm$ 29	137	6.5
IV	1.68	0.26 $\pm$ 0.02	33 $\pm$ 15	28	9.3
	3.33	0.75 $\pm$ 0.10	153 $\pm$ 51	128	5.9
V	1.78	0.22 $\pm$ 0.03	19 $\pm$ 3	16	13.8
	2.31	0.41 $\pm$ 0.01	81 $\pm$ 7	68	6.0
VI	2.23	0.16 $\pm$ 0.01	35 $\pm$ 7	29	5.5
	3.76	0.56 $\pm$ 0.02	211 $\pm$ 22	176	3.2

Data from Experiment III also presented in Figure 11.

Figure 11. Activation of $^{42}K^+$ influx into human red cells by external potassium.

Open symbols represent ouabain-insensitive $^{42}K^+$ fluxes, measured as the mean of two flux determinations in the presence of 10^{-4} M. ouabain.

Closed symbols represent ouabain-sensitive $^{42}K^+$ fluxes, calculated as the difference between the means of two flux determinations in the absence of ouabain and two determinations (as shown in open symbols) in the presence of 10^{-4} M. ouabain.

(△) (▲) $[Na^+]_i$ = 2.00 mmoles/litre cells

V_{max} = 0.44 ± 0.02 μmoles ml cells^{-1} hr^{-1}

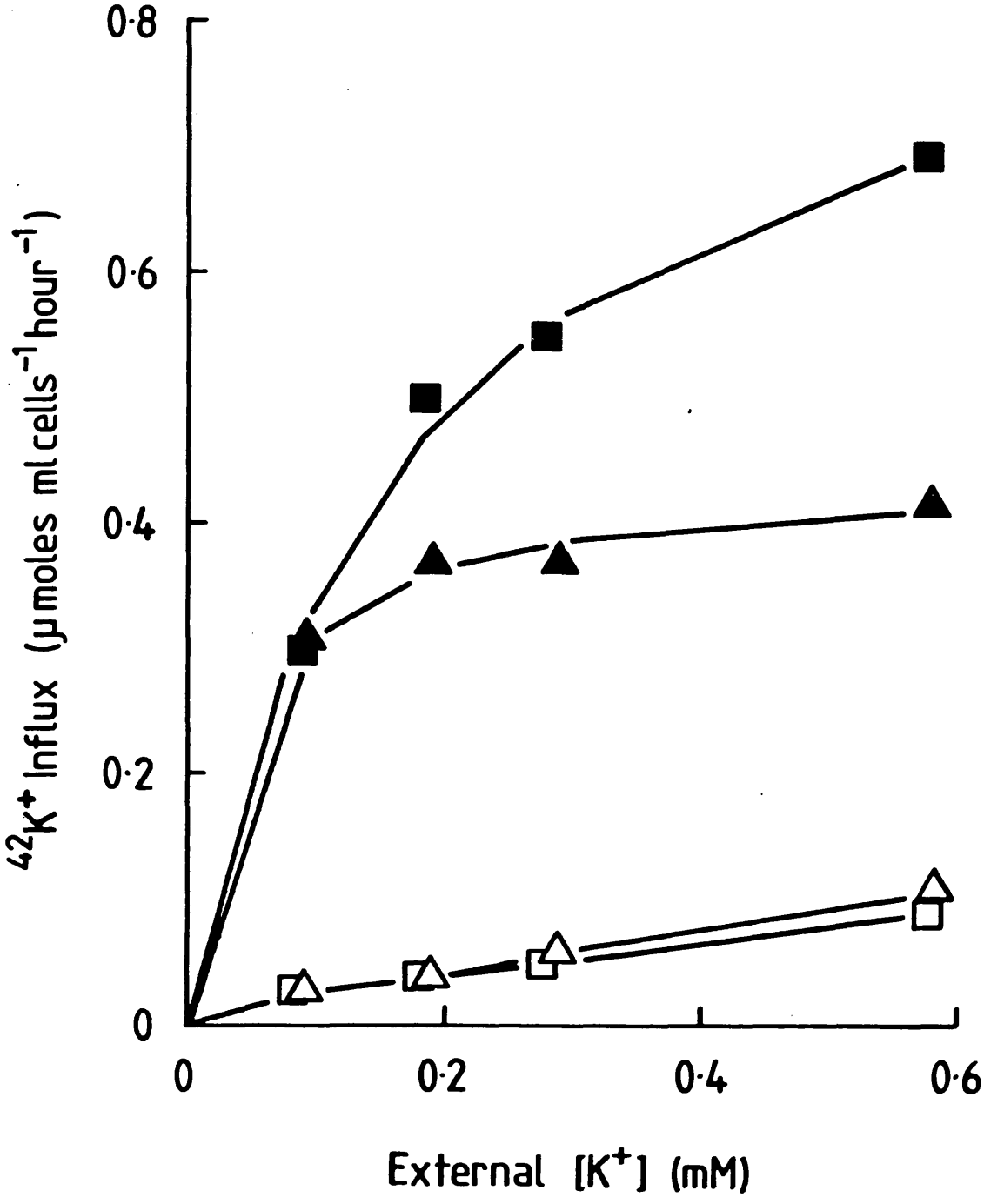
K_m = 42 ± 10 μM

(□) (■) $[Na^+]_i$ = 3.37 mmoles/litre cells

V_{max} = 0.89 ± 0.06 μmoles ml cells^{-1} hr^{-1}

K_m = 164 ± 29 μM

The hyperbolic curves for ouabain-sensitive fluxes have been obtained from fits of the Michaelis-Menten equation to the values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.


0·8
0·6
0·4
0·2
0
0
0·2
0·4
0·6
$^{42}K^+$ Influx (μmoles ml cells^{-1} hour^{-1})
External [K^+] (mM)

I. i) b) INFLUENCE OF INTERNAL SODIUM ON INHIBITION OF $^{42}K^+$ INFLUX BY EXTERNAL SODIUM

It has already been demonstrated using human red cell ghosts that activation of the $(Na^+ + K^+)$-ATPase (as measured by production of inorganic phosphate) by external K^+ is inhibited by the presence of external Na^+ (Whittam and Ager, 1964; Schatzmann, 1965). This inhibition was further illustrated (Priestland and Whittam, 1968) as a decrease in $^{42}K^+$ influx into intact red cells. There is, therefore, a clearly established interaction between $\left[Na^+\right]_o$ and potassium binding on the same (outer) side of the membrane. In addition, results already presented in a previous section of this work (Tables 13 and 14) show a "transmembrane" interaction between $\left[Na^+\right]_i$ and external potassium binding.

It was decided, therefore, to test whether this influence exerted by intracellular sodium and mediated on the opposite side of the membrane was extended to affect the inhibition of potassium binding by external sodium. Experiments of the type described previously for the determination of the inhibition constant were performed (Priestland and Whittam, 1968). These experiments involved duplicate measurements of $^{42}K^+$ influx into two groups of red cells modified by the PCMBS procedure to have low and high $\left[Na^+\right]_i$ levels, and minimal (less than 0.5 mmoles/litre cells) $\left[K^+\right]_i$. Influx measurements were made at four external sodium levels and inhibition constant values were derived from Dixon plots of resultant data.

Results of these experiments show that a two to four-fold reduction in the value of the inhibition constant occurred when $\left[Na^+\right]_i$ was raised from one low (non-saturating) to another high (saturating) level (Figure 12). It should be added, however, that this could be explained in one of two ways. Firstly, a decrease in inhibition constant could be

Figure 12. Inhibition of $^{42}K^+$ influx by external Na^+, in human red cells.

Cells were prepared of high, saturating and low, non-saturating $[Na^+]_i$ levels by the PCMBS cation loading procedure. All cells were prepared to have minimal $[K^+]_i$ (<0.5 mmoles/litre cells). Ouabain-sensitive $^{42}K^+$ influxes were determined at two external $[K^+]$ levels - 0.2 mM and 0.4 mM, and at four $[Na^+]_o$ levels - 0, 5 mM, 10 mM, 15 mM.

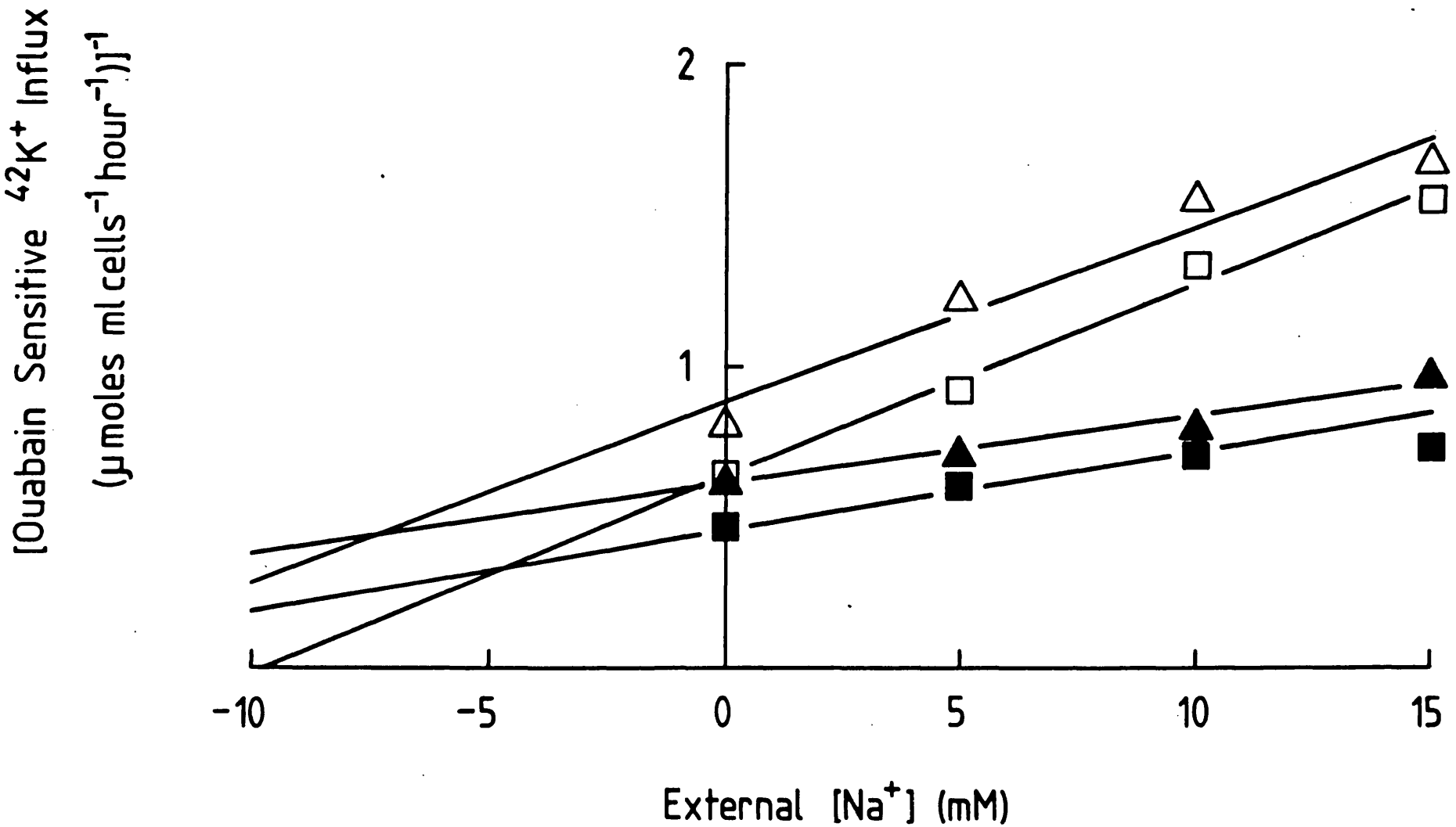
All points are calculated as the difference between means of duplicate flux determinations in the presence and absence of ouabain (10^{-4}M).

Lines fitted by least squares fit programme.

Inhibition constants given by intercept of two lines.

	$[Na^+]_i$ (mmoles/litre cells)	$[K^+]_o$ (mM)	INHIBITION CONSTANT (mM)
(△)	2.33	0.2	7.78
(▲)	2.33	0.4	
(□)	21.85	0.2	3.73
(■)	21.85	0.4	

In another experiment a similar result was produced: the inhibition constant for external sodium was measured as 10.60 mM using cells with $[Na^+]_i$ of 4.59 mmoles/litre cells. This value was reduced to 2.61 mM when $[Na^+]_i$ was raised to 18.94 mmoles/litre cells.


[Ouabain Sensitive $^{42}K^+$ Influx
(μ moles ml cells^{-1} hour^{-1})]$^{-1}$
2
1
-10
-5
0
5
10
15
External [Na^+] (mM)

caused simply by the previously reported fall in potassium binding as $[Na^+]_i$ is raised, with no concomitant change in external sodium binding. A second possibility would involve changes in affinity for both external potassium and external sodium. What is clear, however, is that there is not a proportionate decrease in sodium binding at external sites as potassium binding is reduced by elevation of $[Na^+]_i$ levels. Consequently, the value of the inhibition constant for inhibition of $^{42}K^+$ influx by external sodium is dependent on the $[Na^+]_i$ level: as $[Na^+]_i$ is raised, this "transmembrane" effect is seen as an increased effectiveness of external sodium as an inhibitor of pump activity.

HUMAN BLOOD EXPERIMENTS - CAESIUM AS EXTERNAL CATION

I. ii) a) Comparison with activation by potassium and inhibition by external Na^+.

The advantages in the use of radioactive caesium ($^{134}Cs^+$) as an alternative external activating cation for the sodium pump, include the very long half-life of the radioactive decay of the isotope (2.05 years) when compared with $^{42}K^+$ (12.5 hours). The fact that $^{134}CsCl$ could be readily obtained (Radiochemical Centre, Amersham, Bucks.) as a solution of high specific activity and low concentration, eliminated the difficulties involved in the use of ^{42}KCl (which was supplied as isotonic solution) when attempting to measure influx from external solutions of low cation concentration.

Comparisons of caesium and potassium as external activating cations for the $(Na^+ + K^+)$-ATPase have been made previously. Using human red cell ghosts, Whittam and Ager (1964) showed that although a poor substitute cation for sodium within ghosts, caesium could substitute at external sites for potassium, although the concentration for half-maximal

activation was about four times greater than with potassium. Further it has been demonstrated in squid giant axons, that not only the affinity of the pump but also the maximum velocity was lower when the pump was activated by Cs^+ rather than K^+ ions (Baker et al., 1969). This result, which was obtained by flux measurements shows one difference from that previously reported by Whittam and Ager (1964) who had measured pump activity in human red cell ghosts as liberation of orthophosphate: they had observed no difference in ATPase activity at saturating $[K^+]_o$ or $[Cs^+]_o$. This difference will be referred to again later (Chapter II (ii)).

Further information as to any difference between pump activation by K^+ or Cs^+ externally was obtained from an experiment performed using both isotopes to activate the pump and measuring ion influxes on identical human red blood cell samples. The results (Figure 13) confirm the finding previously seen using flux measurements in squid giant axons (Baker et al., 1969). When ouabain-sensitive $^{42}K^+$ and $^{134}Cs^+$ influxes are measured as the difference between means of duplicate estimations in the presence and absence of ouabain (10^{-4}M), not only is the amount of ion influx much greater with K^+ (V_{max} 5.35 $\pm$ 0.58 μmoles ml cells^{-1} hr^{-1} compared with 3.11 $\pm$ 0.16 μmoles ml cells^{-1} hr^{-1} with Cs^+), but also there is a two-fold affinity difference. The affinity for K^+ is higher (K_m = 0.93 mM as opposed to 1.81 mM with Cs^+).

As explained previously, it is necessary to correct values of K_m calculated from experiments of this type, in order to allow for the effect of the small amount of external sodium present during flux incubations, owing to leakage from the cells. In the case of the inhibition of $^{42}K^+$ influx by external sodium, the inhibition constant had been previously determined (Priestland and Whittam, 1968). It was thus necessary to carry out a preliminary experiment to determine the inhibition constant

Figure 13. Activation of $^{42}K^+$ and $^{134}Cs^+$ influxes into human red cells.

Cells were washed three times in ice-cold isotonic choline chloride and then influx measurements made as described in Methods.

Open symbols represent ouabain-insensitive fluxes, measured in the presence of 10^{-4}M ouabain. Points are means from duplicate estimations.

Closed symbols represent ouabain-sensitive fluxes, calculated as the difference between the means of two flux determinations in the absence of ouabain and two in the presence of ouabain (10^{-4}M).

All fluxes measured from sodium-free Ringer solutions.

(△)(▲) - $^{134}Cs^+$ influx

V_{max} = 3.11 ± 0.16 μmoles ml cells^{-1} hr^{-1}

K_m = 1.81 ± 0.30 mM

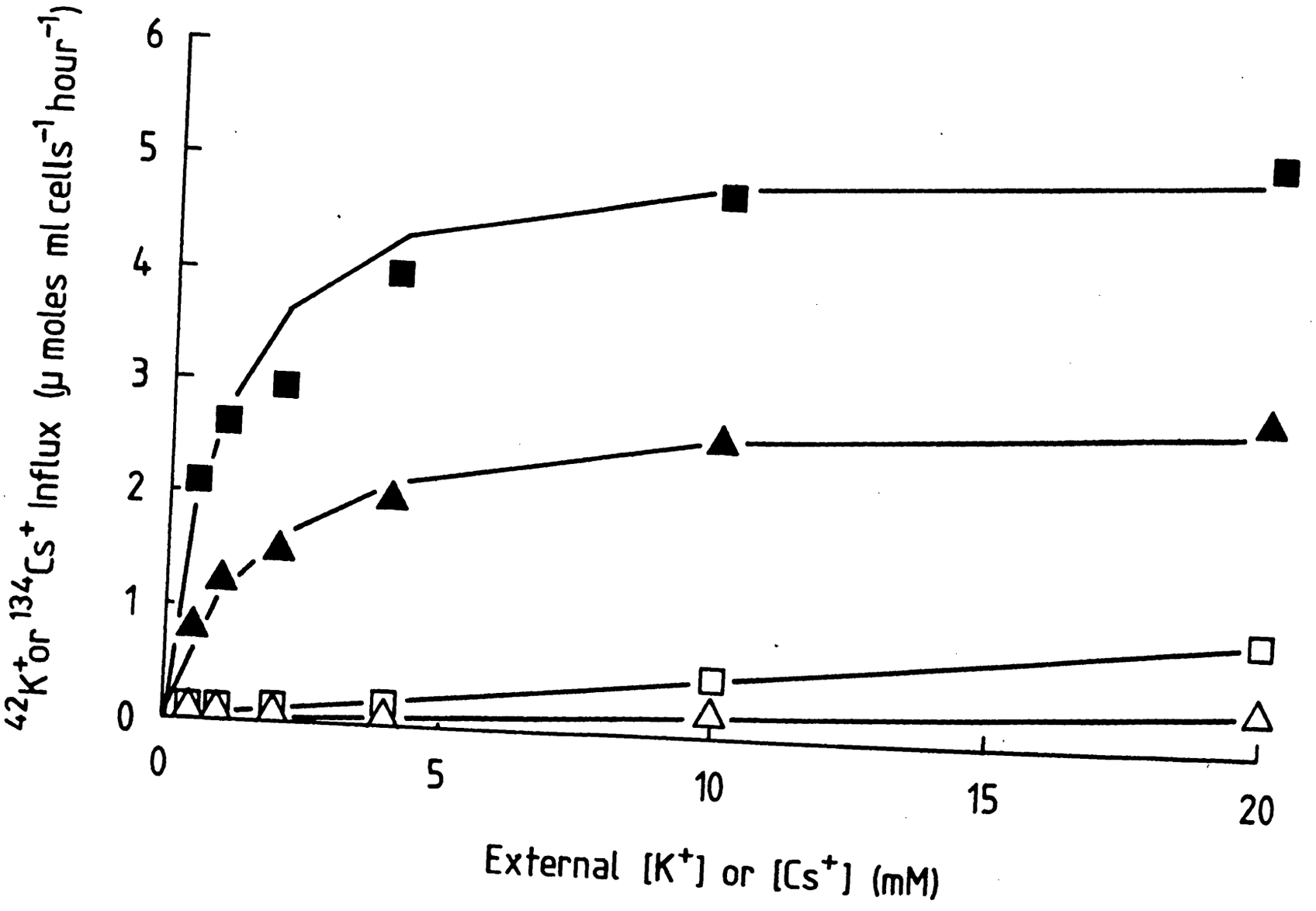
(□)(■) - $^{42}K^+$ influx

V_{max} = 5.35 ± 0.58 μmoles ml cells^{-1} hr^{-1}

K_m = 0.93 ± 0.39 mM

Values for V_{max} and K_m were obtained by the statistical method of Wilkinson (1961).

Hyperbolic curves for ouabain-sensitive fluxes are from fits to the above values of V_{max} and K_m of the Michaelis-Menten equation.


$^{42}K^+$ or $^{134}Cs^+$ Influx (μ moles ml cells^{-1} hour^{-1})
6
5
4
3
2
1
0
0
5
10
15
20
External [K^+] or [Cs^+] (mM)

for the inhibition of $^{134}Cs^+$ influx by external sodium. This experiment was performed before a radioactive caesium solution had been obtained - pump activity was measured as efflux of $^{24}NaCl$. As shown in experiments considering $^{42}K^+$ influx, there is also dependence of the inhibition constant for inhibition of $^{134}Cs^+$ influx by external sodium on the intracellular sodium concentration. As was found in the previous experiments on potassium activation of the pump, this is seen as a marked fall in inhibition constant as $[Na^+]_i$ is raised (in this case an increase of $[Na^+]_i$ from 3.05 to 21.66 mmoles/litre cells causes a fifty per cent decrease in inhibition constant from 12.19 mM to 6.69 mM - see Figure 14).

I ii) b) <u>THE INFLUENCE OF INTRACELLULAR SODIUM ON EXTERNAL CAESIUM AFFINITY</u>

Experiments were performed to investigate the relationship between $[Na^+]_i$ and the concentration dependence of $^{134}Cs^+$ influx. The experiments investigate a range of $[Na^+]_i$ levels designed to include both types of experiment described in the last section considering the K_m for $^{42}K^+$ influx. The range of $[Na^+]_i$ levels used was from 1.37 - 41.02 mmoles/litre cells. Again, values of ouabain-sensitive $^{134}Cs^+$ influx were calculated as the difference between means of duplicate flux measurements with and without ouabain ($10^{-4}M$).

The cells for these experiments were prepared using the same PCMBS loading procedure as was used for experiments in the previous section. This involved voiding as much as possible of the intracellular potassium. Routine observation of $[K^+]_i$ levels by flame photometry of haemolysates showed values not exceeding 0.8 mmoles/litre cells. There is, as stated previously, difficulty in achieving exactly pre-determined conditions - therefore each experiment should only be considered individually as, for example, $[K^+]_i$ levels may vary from one batch of

Figure 14. Inhibition of $^{134}Cs^+$ influx by external Na^+, in human red cells.

Cells were prepared of high, saturating and low, non-saturating $[Na^+]_i$ levels by the PCMBS cation loading procedure. All cells were of minimal $[K^+]_i$ level (<0.5 mmoles/litre cells).

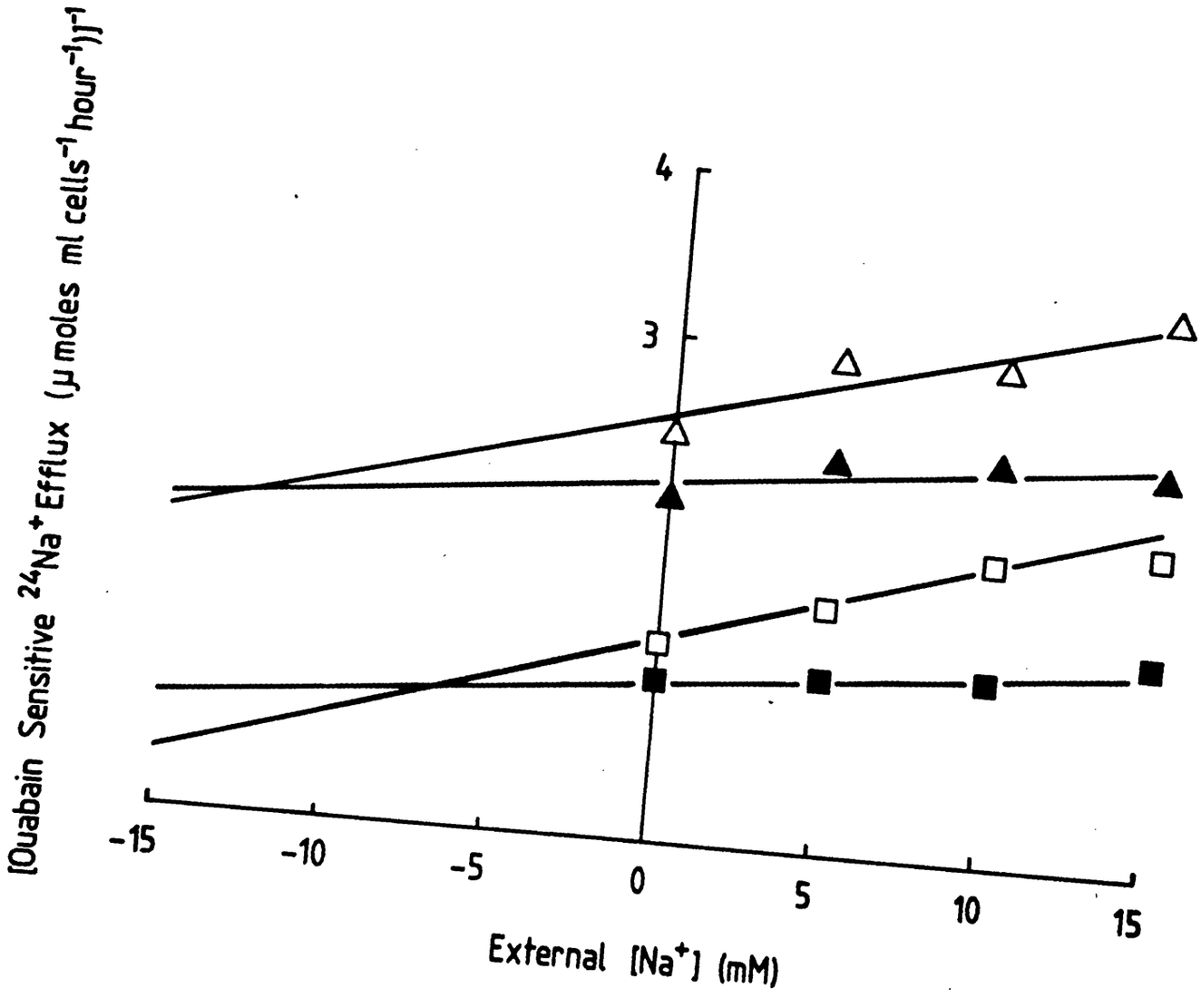
Pump activity was measured as $^{24}Na^+$ efflux, at two $[Cs^+]_o$ levels - 2 mM and 4 mM and at four $[Na^+]_o$ levels - 0, 5 mM, 10 mM, 15 mM.

All points are calculated as the difference between means of duplicate efflux determinations in the presence and absence of ouabain (10^{-4} M).

Lines are fitted by least squares fit programme.

Inhibition constants given by intercept of two lines.

	$[Na^+]_i$ (mmoles/litre cells)	$[Cs^+]_o$ (mM)	INHIBITION CONSTANT (mM)
(△)	3.05	2	12.19
(▲)	3.05	4	
(□)	21.66	2	6.69
(■)	21.66	4	


[Ouabain Sensitive $^{24}Na^+$ Efflux (μ moles ml cells^{-1} hour^{-1})]$^{-1}$
4
3
-15
-10
-5
0
5
10
15
External [Na^+] (mM)

blood to another treated in the same way. Thus, although there is a graded response within each experiment to an increase in $[Na^+]_i$ the magnitude of the changes in V_{max} varies considerably: in one experiment a ten-fold increase in V_{max} occurs as $[Na^+]_i$ is raised from 1.37 to 14.72 mmoles/litre cells, but in another only a three-fold increase in V_{max} as $[Na^+]_i$ is raised from 2.76 - 41.02 mmoles/litre cells (Table 15).

There is also a clear dependence of affinity for external caesium on $[Na^+]_i$ level. In each experiment an increase in the value of K_m for external caesium is seen as $[Na^+]_i$ is raised and in three experiments our of four there is at least a three-fold increase (Table 15).

The inhibition constant for external sodium had been determined as being greater than 6 mM even when $[Na^+]_i$ was saturating (see Figure 14) and, as in experiments considering $^{42}K^+$ influx measurements, a level of external sodium of less than 1 mM was routinely observed. Consequently these two values have been used for a correction of K_m values (Table 15) to allow for interference by external sodium. Again the effect of the correction is to slightly lessen the dependence of K_m on $[Na^+]_i$ level.

The results presented here (Table 15, Figure 15) again demonstrate that hyperbolae, constructed from fits of the Michaelis-Menten equation to the values of V_{max} and K_m calculated, yield very good fits to the experimental data. Further, the double advantage in the use of $^{134}Cs^+$ as the external activating cation is illustrated by these results. Comparison of data in Tables 14 and 15 shows that (as suggested by the experiment presented earlier - Figure 13) the pump has a considerably lower affinity for external Cs^+ than for external K^+: thus, not only are low concentrations of sufficiently high specific activity more readily achieved, but also the external cation concentrations required for the construction of activation curves are not as low, when using caesium.

TABLE 15 The influence of $[Na^+]_i$ on kinetic constants for ouabain-sensitive $^{134}Cs^+$ influx into human red blood cells.

All cells were prepared to have minimal $[K^+]_i$ - less than 0.8 mmoles.litre cells^{-1}.

Values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.

Values of K_m have been corrected for inhibition by external Na^+: $[Na^+]_o$ = 1 mM and inhibition constant 6 mM.

EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/litre cells)	V_{max} ($\pm$ S.E.M.) (μmoles ml cells^{-1} hr^{-1})	K_m ($\pm$ S.E.M.) (μm)	Corrected K_m (μm)	V_{max}/K_m ratio
I	1.48	0.32 $\pm$ 0.02	312 $\pm$ 39	267	1.2
	2.39	0.66 $\pm$ 0.06	640 $\pm$ 88	549	1.2
	3.94	1.03 $\pm$ 0.08	1019 $\pm$ 113	873	1.2
II	1.37	0.05 $\pm$ 0.01	222 $\pm$ 98	190	0.3
	4.03	0.37 $\pm$ 0.04	530 $\pm$ 85	454	0.8
	14.72	0.51 $\pm$ 0.05	808 $\pm$ 110	693	0.7
III	2.76	0.25 $\pm$ 0.02	291 $\pm$ 73	249	1.0
	3.62	0.31 $\pm$ 0.02	354 $\pm$ 75	303	1.0
	7.76	0.43 $\pm$ 0.01	430 $\pm$ 38	369	1.2
	21.74	0.72 $\pm$ 0.04	778 $\pm$ 99	667	1.1
	41.02	0.75 $\pm$ 0.04	991 $\pm$ 133	849	0.9
IV ≠	2.20	0.45 $\pm$ 0.04	562 $\pm$ 98	482	0.9
	3.79	0.70 $\pm$ 0.01	770 $\pm$ 10	660	1.1

≠ Pump activity measured as $^{24}Na^+$ efflux in this experiment; all other experiments $^{134}Cs^+$ influx. Data from Experiment I are also presented as Figure 15.

Figure 15. Activation of $^{134}Cs^+$ influx into human red cells by external caesium.

Open symbols represent ouabain-insensitive $^{134}Cs^+$ fluxes, measured as the mean of two flux determinations in the presence of 10^{-4} M. ouabain.

Closed symbols represent ouabain-sensitive $^{134}Cs^+$ fluxes, calculated as the difference between the means of two flux determinations in the absence of ouabain and two determinations (as shown in open symbols) in the presence of 10^{-4}M. ouabain.

(△) (▲) $[Na^+]_i$ = 1.48 mmoles/litre cells

V_{max} = 0.32 ± 0.02 μmoles ml cells^{-1} hr^{-1}

K_m = 312 ± 39 μM

(□) (■) $[Na^+]_i$ = 2.39 mmoles/litre cells

V_{max} = 0.66 ± 0.06 μmoles ml cells^{-1} hr^{-1}

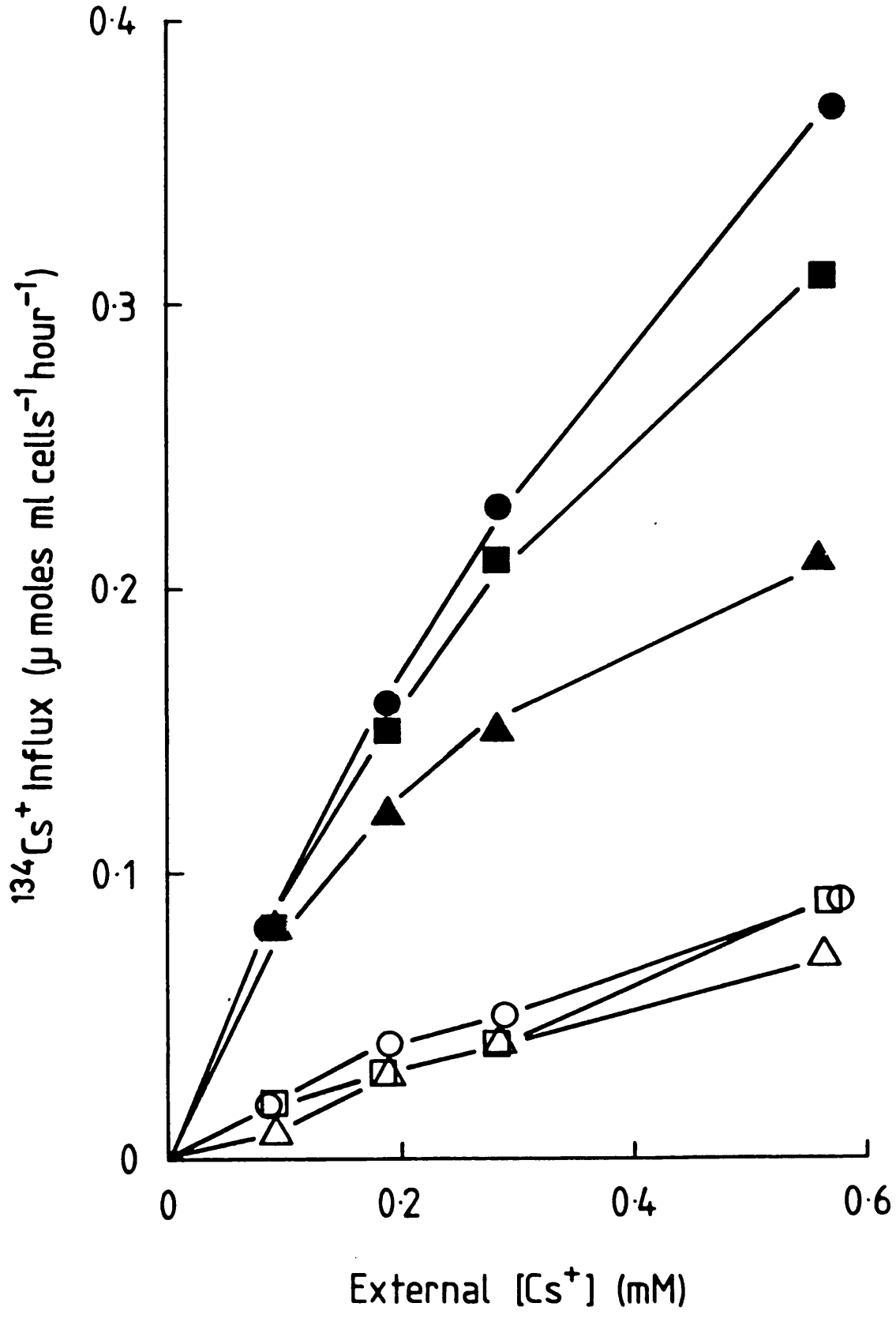
K_m = 640 ± 88 μM

(○) (●) $[Na^+]_i$ = 3.94 mmoles/litre cells

V_{max} = 1.03 ± 0.08 μmoles ml cells^{-1} hr^{-1}

K_m = 1019 ± 113 μM

The hyperbolic curves for ouabain-sensitive fluxes have been obtained from fits of the Michaelis-Menten equation to the values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.


0·4
0·3
0·2
0·1
0
0
0·2
0·4
0·6
$^{134}Cs^+$ Influx (μ moles ml cells^{-1} hour^{-1})
External [Cs^+] (mM)

I iii) ALTERNATIVE TREATMENT OF RESULTS. STOICHIOMETRY

Previous authors (Chipperfield and Whittam, 1976) have stressed the importance of experimental conditions when conducting experiments designed to investigate the influence of ion binding on one side of the membrane on the binding of ions on the opposite side. It was shown that the ability to see changes in affinity for external potassium brought about by changes in internal sodium concentration was diminished in experiments where inhibition by external sodium was permitted. Further, it was shown that hyperbolic activation did not occur in the presence of external sodium.

Consideration of the activation of sodium efflux from red cells, especially the fact that there appears to be a fixed number of Na^+ and K^+ ions transported per pump cycle (Whittam and Ager, 1965), led to the formulation of a rate equation to explain the slightly sigmoid nature of the activation (Garay and Garrahan, 1973). The equation is:

$$M = M'_{max} / (1 + K'_{Na} / [Na^+]_i)^n$$

where K'_{Na} = apparent dissociation constant of the Na-site complex

M = flux at given sodium concentration $[Na^+]_i$

M'_{max} = flux at non-limiting $[Na^+]_i$

n = number of sites occupied before transport can occur

Linear plots could thus be obtained of

$[Na^+]_i / M^{-1/n}$ vs $[Na^+]_i$

with gradient = $(M'_{max})^{-1/n}$

and abscissa intercept of $- K'_{Na}$

This form of plot may also be used for cation influx studies. Best fits are obtained for influx with n = 2. Fits to these plots are very good even when inhibition at external sites is minimal ($[Na^+]_o$ less than 1 mM).

Plots of this type have thus been constructed for the experiments presented previously (Table 14) investigating $^{42}K^+$ influx into human red cells. The kinetic constants obtained from such plots are presented (Table 16) together with the data from one experiment in plotted form (Figure 16).

The significance of such results is two-fold. Firstly, in just the same way as an increase in $[Na^+]_i$ level always causes an increase in K_m for external potassium, so an increase in the apparent dissociation constant K'_K is also seen. In other words, the apparent dissociation constant may be used as an alternative measure of affinity, a rise in the value of this constant representing a decrease in affinity. Secondly, the fact that best fits for $^{42}K^+$ influx are consistently obtained with n = 2, and as will be seen later (Chapter II) the fact that best fits for activation by intracellular sodium are obtained with n = 3, is in agreement with the suggested stoichiometry for human red cells (Whittam and Ager, 1965). In addition, except at higher $[Na^+]_i$ levels (e.g. Experiment II - 5.69 mmoles/litre cells $[Na^+]_i$) there is good agreement between V_{max} values obtained by both methods.

Results from the experiments involving $^{134}Cs^+$ influx into human red cells may be treated in the same manner. The data from these experiments (see Table 15, Figure 15) have been fitted to the equation of Garay and Garrahan (1973). Again best fits are obtained with n = 2 (Table 17) and to demonstrate this r values for lines fitted by least squares fit linear regression are included. Agreement on the V_{max} values obtained by the two methods is again reasonable except at high $[Na^+]_i$ levels.

TABLE 16 Kinetic constants for ouabain-sensitive $^{42}K^{+}$ influx into human red blood cells. Dependence on $[Na^{+}]_i$ Experiments I - VI as described in Table 14.

EXPERIMENT NUMBER	$[Na^{+}]_i$ mmoles/litre cells	V_{max} (μmoles ml cells^{-1} hr^{-1})		K_m (μM)	K'_K (μM)
		Value derived from the statistical method of Wilkinson	Value from rate equation Garay and Garrahan (n = 2)	Value derived from the statistical method of Wilkinson	Value from rate equation Garay and Garrahan (n = 2)
I	1.19	0.28	0.30	11	16
	4.76	0.77	0.71	173	61
II	2.75	0.57	0.54	157	59
	3.98	0.86	0.76	303	103
	5.69	1.85	1.26	806	168
III	2.00	0.44	0.45	42	22
	3.37	0.89	0.84	164	61
IV	1.68	0.26	0.28	33	24
	3.33	0.75	0.68	153	52
V	1.78	0.22	0.27	19	22
	2.31	0.41	0.41	81	36
VI	2.23	0.16	0.16	35	12
	3.76	0.56	0.52	211	73

Figure 16. Activation of ouabain-sensitive $^{42}K^+$ influx into human red cells.

Plot of data previously presented as Figure 11, fitted to the rate equation of Garay and Garrahan (1973) - see text - n, number of sites = 2.

(△) $[Na^+]_i$ = 2.00 mmoles/litre cells

V_{max} = 0.45 μmoles ml cells^{-1} hr^{-1}

K'_K = 22 μM

(■) $[Na^+]_i$ = 3.37 mmoles/litre cells

V_{max} = 0.84 μmoles ml cells^{-1} hr^{-1}

K'_K = 61 μM

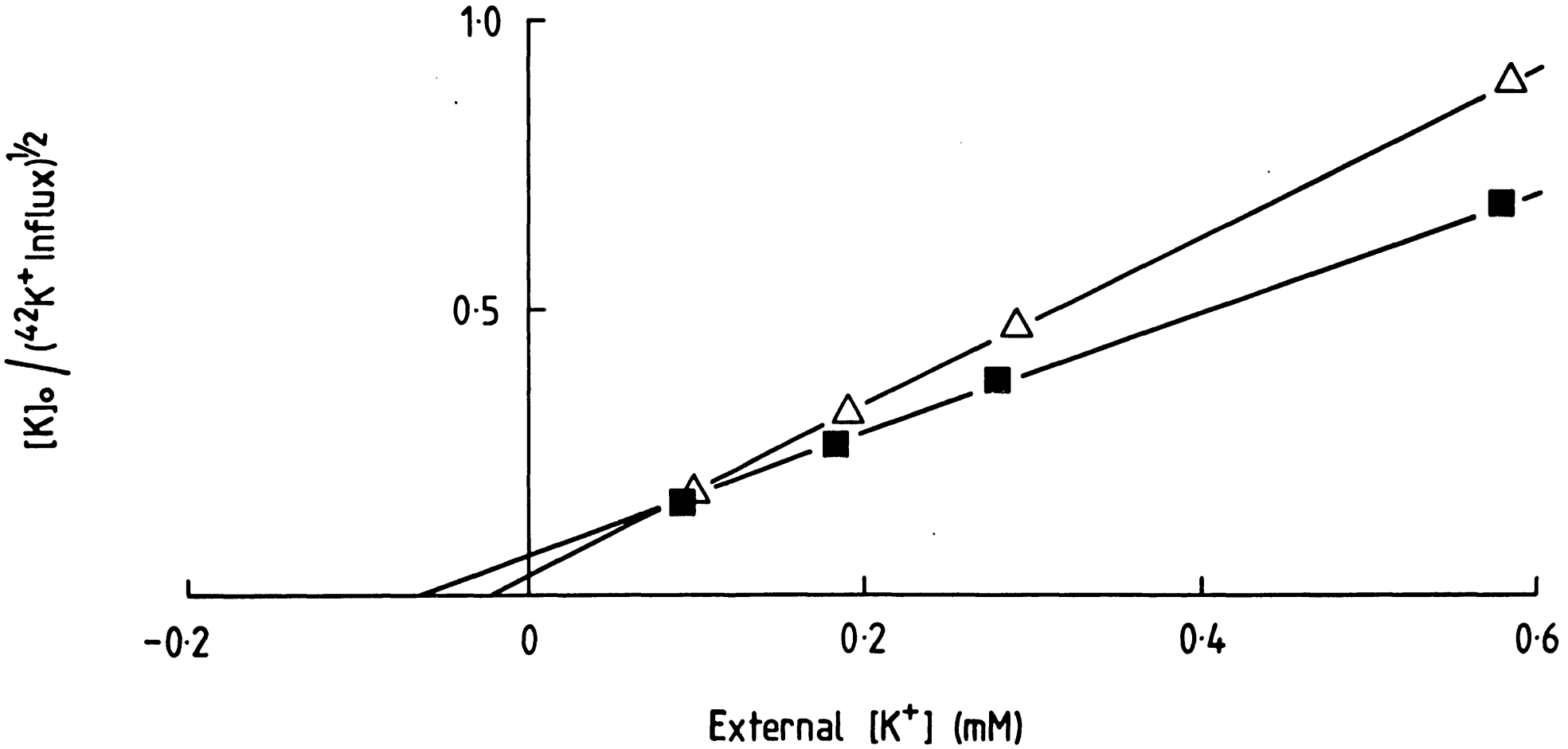

1·0
0·5
[K]o / (42K+ Influx)1/2
-0·2
0
0·2
0·4
0·6
External [K+] (mM)

TABLE 17 Kinetic constants for ouabain-sensitive $^{134}Cs^+$ influx into human red blood cells - dependence on $[Na^+]_i$.
Experiments I - IV as presented in Table 15.

EXPERIMENT NUMBER	$[Na^+]_i$ mmoles/litre cells	V_{max} (μmoles ml cells^{-1} hr^{-1})		K_m (μM)	K'_{Cs} (μM)
		Value derived from the statistical method of Wilkinson	Value from fit to rate equation of Garay and Garrahan	Value derived from the statistical method of Wilkinson	Value from rate equation of Garay and Garrahan (r for line fit)
I	1.48	0.32	0.30	312	102 (r = 1)
	2.39	0.66	0.49	640	146 (r = 1)
	3.94	1.03	0.62	1019	175 (r = 1)
II	1.37	0.05	0.05	222	86 (r = 0.99)
	4.03	0.37	0.29	530	134 (r = 1)
	14.72	0.51	0.34	808	158 (r = 1)
III	2.76	0.25	0.25	291	131 (r = 1)
	3.62	0.31	0.31	354	149 (r = 1)
	7.76	0.43	0.41	430	149 (r = 1)
	21.74	0.72	0.66	778	230 (r = 1)
	41.02	0.75	0.66	991	273 (r = 1)
IV	2.20	0.45	0.39	562	170 (r = 1)
	3.79	0.70	0.57	770	205 (r = 1)

The results presented confirm that K'_{Cs} may be used as an alternative measure of caesium affinity - again there is an increase in K'_{Cs} as $[Na^+]_i$ is raised. Although the increase in K'_{Cs} is not more than 60% of the increase seen in K_m in any one experiment, it should be stressed that there is, nevertheless, consistency within each experiment.

I iv) PIG BLOOD EXPERIMENTS - POTASSIUM AS EXTERNAL CATION

The reasons for the use of pig red blood cells as an alternative system for these studies have been mentioned previously (see Introduction). It was necessary, in the first instance, to establish what experimental conditions would be required to facilitate an investigation similar to that described in the previous section on human red cells. It is clear that one requirement again would be the reduction of intracellular potassium levels to avoid inhibition at intracellular sites. The preparation of cells by the PCMBS procedure using cation loading solutions which were potassium-free achieved this, although, as discussed previously (see Methods), it was not as easy to void potassium from pig red cells as from human red cells. The cells used in these experiments consequently had $[K^+]_i$ levels of the range 0.5 - 4.5 mmoles/litre cells.

The activation of the sodium pump by intracellular sodium has been established (Figure 17) in two experiments on PCMBS modified cells. Consequently cells were prepared to investigate the nature of attachment of external potassium at various $[Na^+]_i$ levels which were non-saturating. The range of $[Na^+]_i$ levels used was 1.68 - 9.69 mmoles/litre cells. As previously seen in human red cells, there exists a clear dependence of $^{42}K^+$ influx on $[Na^+]_i$ level (Table 18, Figure 18). In three experiments at least a fifty per cent increase in the V_{max} value for $^{42}K^+$ influx is observed as $[Na^+]_i$ is raised by a factor of two and in all cases

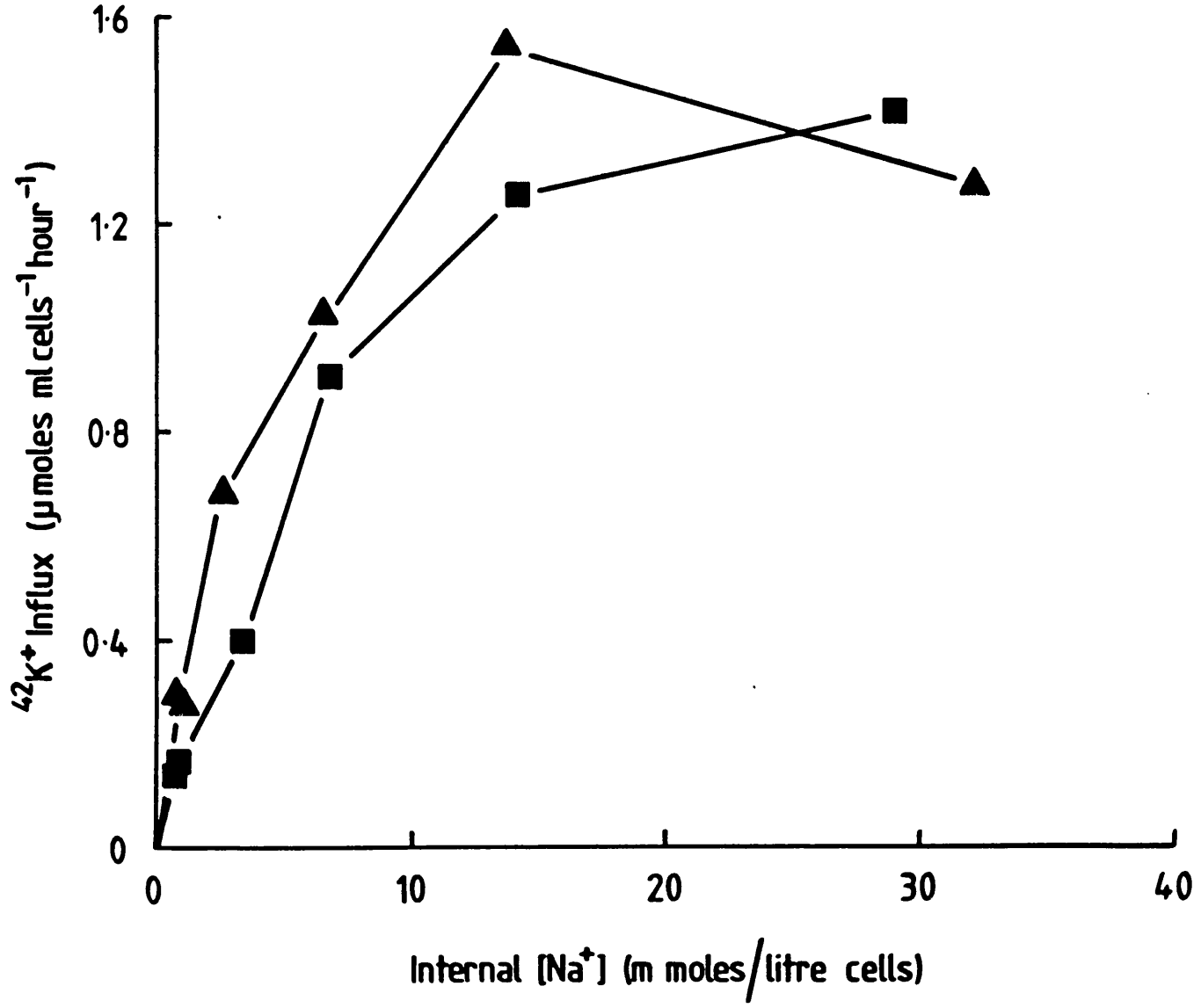
Figure 17. Activation of the sodium pump by intracellular sodium in pig red cells.

Six groups of cells were prepared by the PCMBS cation loading procedure to have different $[Na^+]_i$ levels and low $[K^+]_i$ levels. In all cells $[K^+]_i$ < 8 mmoles/litre cells.

Ouabain-sensitive $^{42}K^+$ influx was measured as the difference between duplicate estimations in the presence or absence of ouabain (10^{-4}M).

External $[K^+]$ was 5 mM for all influx measurements.

Results are presented from two separate experiments on different batches of blood.


1·6
1·2
0·8
0·4
0
$^{42}K^+$ Influx (μ moles ml cells^{-1} hour^{-1})
0
10
20
30
40
Internal [Na^+] (m moles/litre cells)

an increase in $[Na^+]_i$ produces a rise in the value of V_{max}. Values of V_{max} have been determined by both of the methods described previously: the statistical method of Wilkinson (1961), and secondly by the fitting in linear form of the multiple-site activation equation of Garay and Garrahan (1973) - see Section I (iii) (Figure 19). There is again good agreement of the values for this kinetic constant derived by either method (Table 18).

It is noticeable that if both pig and human red cells are prepared to have various $[Na^+]_i$ levels which are non-saturating, then the values of V_{max} for $^{42}K^+$ influx obtained are all of a similar range: 0.39 - 1.47 μmoles ml cells^{-1} hr^{-1} for pig red cells of $[Na^+]_i$ of 1.68 - 9.69 mmoles/litre cells and 0.16 - 1.85 μmoles ml cells^{-1} hr^{-1} for human red cells of 1.19 - 5.69 mmoles/litre cells (Tables 14, 18). At saturating $[Na^+]_i$ levels, however, values of V_{max} for $^{42}K^+$ influx appear higher in human red cells (greater than 2 μmoles ml cells^{-1} hr^{-1} in three experiments out of four - Table 13) than seen in the two experiments on pig red cells presented in Figure 17 (approximately 1.5 μmoles ml cells^{-1} hr^{-1}).

In pig red cells, as in human red cells, there is also a response of external potassium affinity (whether measured by K_m or apparent dissociation constant K'_K) to changes in $[Na^+]_i$ level. As the presence of either intracellular potassium or extracellular sodium leads to deviation from hyperbolic activation, it was considered necessary to present values of both K_m and K'_K, particularly as the residual $[K^+]_i$ levels at the commencement of these flux measurements was unavoidably a little higher than that in the human red cell experiments. When the method of fitting the multiple-site equation (Garay and Garrahan, 1973) is used, then, as in human red cells, best fits are obtained with n = 2. In all cases, values of r for fits to the linear form of the equation were greater than or equal to 0.999 (for example, see Figure 19). Results are presented from three experiments (Table 18) which demonstrate that

TABLE 18 The influence of $[Na^+]_i$ on kinetic constants for ouabain-sensitive $^{42}K^+$ influx into pig red cells.

Cells prepared of various $[Na^+]_i$ levels by the PCMBS loading procedure, and influx measurements were made, in the presence and absence of 10^{-4}M ouabain, at four different $[K^+]_o$ levels. All $^{42}K^+$ influx measurements were made in duplicate.

EXPERIMENT NUMBER	$[Na^+]_i$ mmoles/litre cells	V_{max} (μmoles ml cells^{-1} hr^{-1})		K_m (μM)	K'_K (μM)
		Value (± S.E.M.) from the statistical method of Wilkinson (1961)-see text	Value from fit of rate equation of Garay and Garrahan (n = 2)	Value (± S.E.M.) from the statistical method of Wilkinson	Value from fit of rate equation of Garay and Garrahan (n = 2)
I	1.68	0.39 ± 0.03	0.37	86 ± 21	33
	4.82	1.00 ± 0.17	0.90	241 ± 81	83
	5.84	1.47 ± 0.23	1.29	300 ± 90	96
II	3.11	0.46 ± 0.03	0.46	35 ± 15	14
	6.13	0.72 ± 0.11	0.67	152 ± 55	57
III	2.14	0.66 ± 0.04	0.64	129 ± 25	52
	6.96	0.89 ± 0.14	0.82	191 ± 66	69
	9.69	0.99 ± 0.17	0.90	258 ± 89	92

Data from experiment I are also presented as Figures 18 and 19.

Figure 18. Activation of $^{42}K^+$ influx into pig red cells by external potassium.

Open symbols represent ouabain-insensitive $^{42}K^+$ fluxes, measured as the mean of duplicate estimations in the presence of ouabain (10^{-4}M).

Closed symbols represent ouabain-sensitive $^{42}K^+$ fluxes, calculated as the difference between the means of two flux determinations in the absence of ouabain and two determinations (as shown in open symbols) in the presence of ouabain (10^{-4}M).

(△) (▲) $[Na^+]_i$ = 1.68 mmoles/litre cells

V_{max} = 0.39 ± 0.03 μmoles ml cells^{-1} hr^{-1}

K_m = 86 ± 21 μM

(□) (■) $[Na^+]_i$ = 4.82 mmoles/litre cells

V_{max} = 1.00 ± 0.17 μmoles ml cells^{-1} hr^{-1}

K_m = 241 ± 81 μM

(○) (●) $[Na^+]_i$ = 5.84 mmoles/litre cells

V_{max} = 1.47 ± 0.23 μmoles ml cells^{-1} hr^{-1}

K_m = 300 ± 90 μM

The hyperbolic curves for ouabain-sensitive fluxes have been obtained from fits of the Michaelis-Menten equation to the values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.

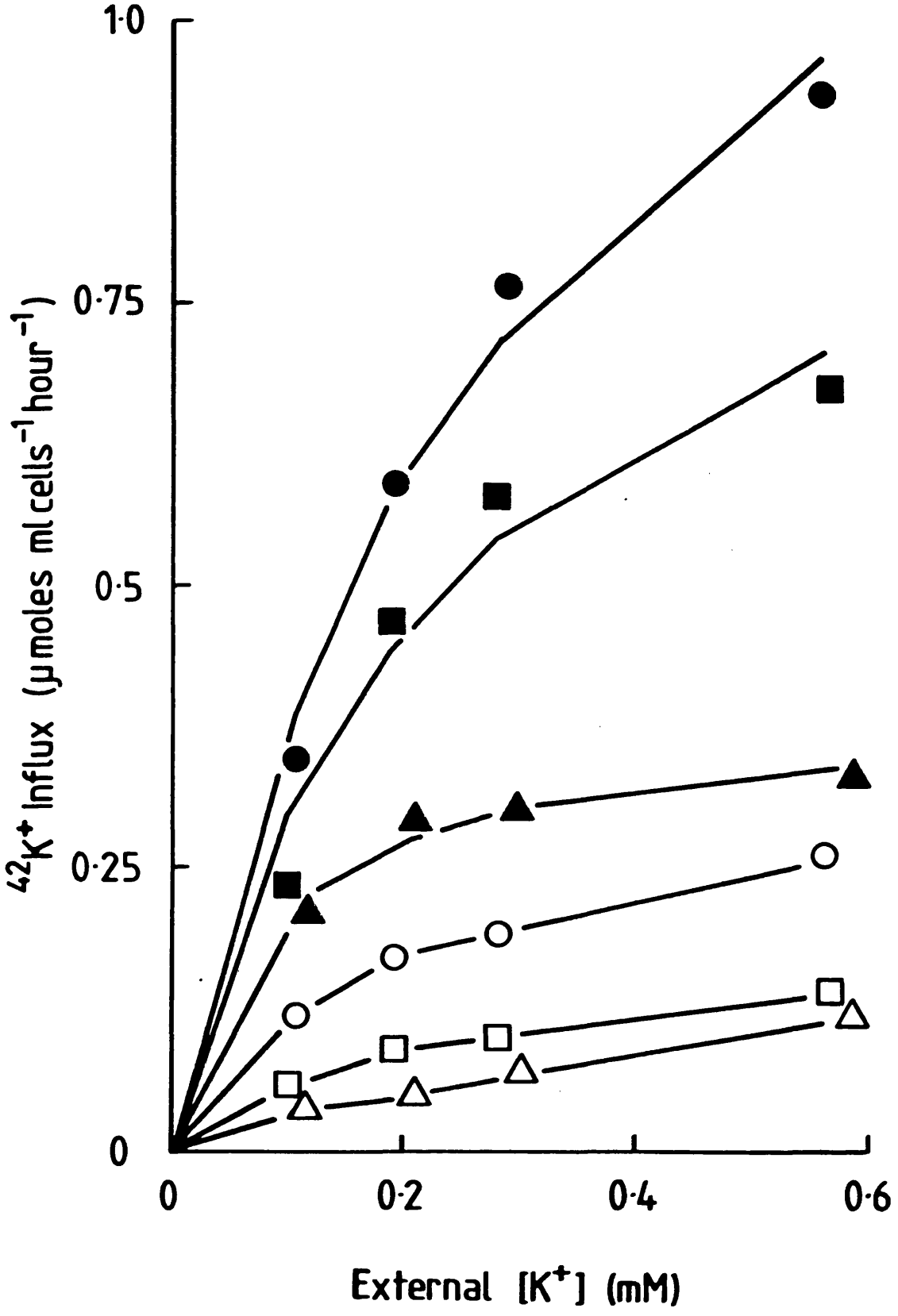

1·0
0·75
0·5
0·25
0
0
0·2
0·4
0·6
$^{42}K^+$ Influx (μ moles ml cells^{-1} hour^{-1})
External [K^+] (mM)

Figure 19. Activation of ouabain-sensitive $^{42}K^{+}$ influx into pig red cells.

Plot of data previously presented (Figure 18), fitted to the rate equation of Garay and Garrahan (1973) - see text - n, number of sites = 2.

(△) $[Na^{+}]_i$ = 1.68 mmoles/litre cells

V_{max} = 0.37 μmoles ml cells^{-1} hr^{-1}

K_m = 33 μM

(■) $[Na^{+}]_i$ = 4.82 mmoles/litre cells

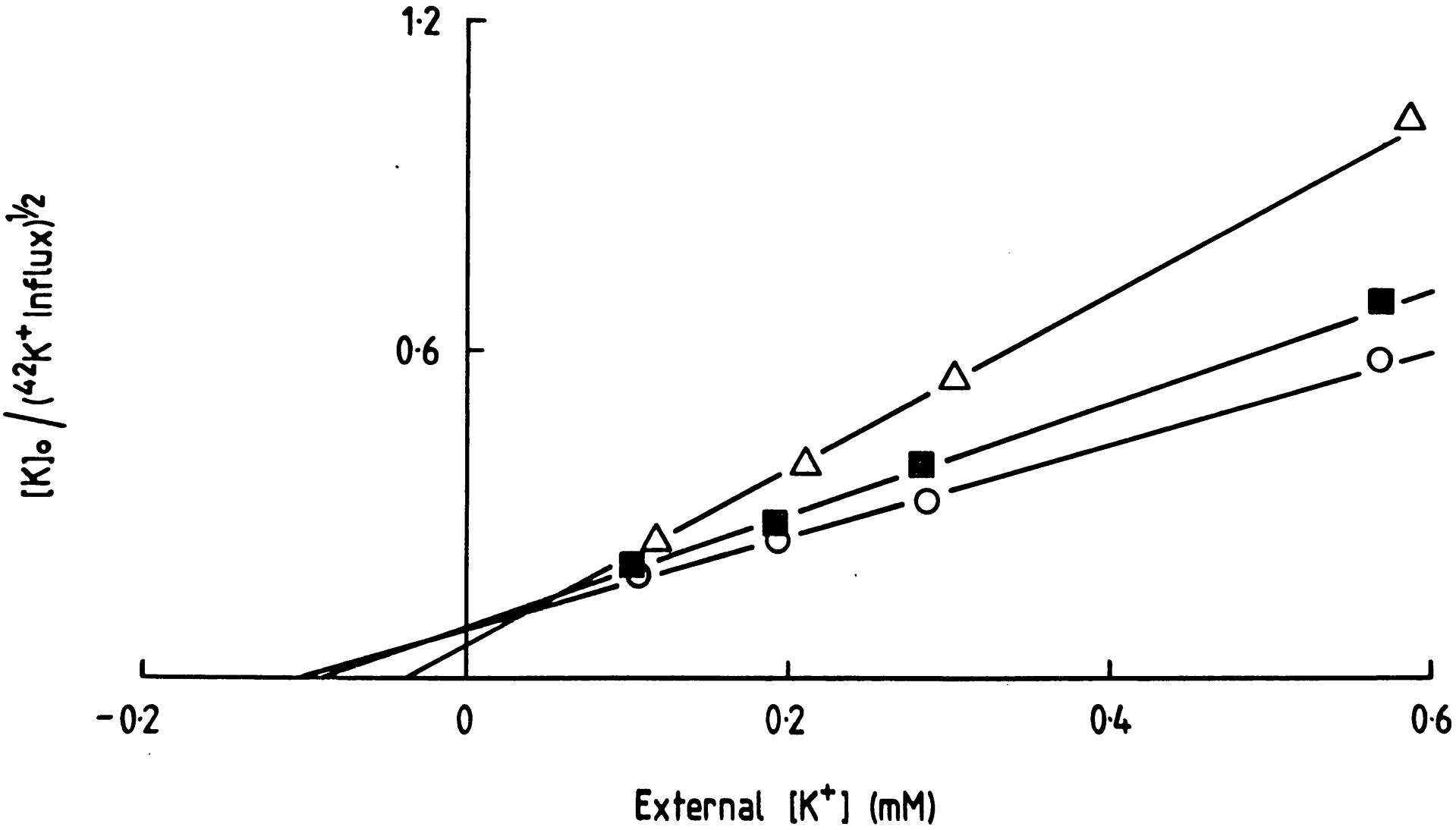
V_{max} = 0.90 μmoles ml cells^{-1} hr^{-1}

K_m = 83 μM

(○) $[Na^{+}]_i$ = 5.84 mmoles/litre cells

V_{max} = 1.29 μmoles ml cells^{-1} hr^{-1}

K_m = 96 μM


1·2
0·6
[K]o / (42K+ influx)1/2
-0·2
0
0·2
0·4
0·6
External [K+] (mM)

at least a two-fold increase in both K_m and K'_K is seen within each experiment as $[Na^+]_i$ is raised within the range 1.68 - 9.69 mmoles/litre cells, and in one experiment doubling the value of $[Na^+]_i$ causes a rise in K_m from 35 - 152 μM and in K'_K from 14 - 57 μM. Comparison of results with human blood experiments (Tables 16, 18) shows that K_m and K'_K are of similar magnitude in pig cells to values calculated for human red cells.

It has thus been demonstrated that raising $[Na^+]_i$ level, within the range that causes a rise in V_{max} for $^{42}K^+$ influx into pig red cells, causes a marked decrease in the affinity of the sodium pump in such cells for external potassium.

I v) PIG BLOOD EXPERIMENTS - CAESIUM AS EXTERNAL CATION

The advantages of the use of $^{134}Cs^+$ as an external activating cation compared to $^{42}K^+$ can again be applied to experiments with pig red cells (see Section I (ii) (a)).

Experiments were designed to expand the information already acquired on the transmembrane influence of intracellular sodium on external cation attachment to the pump. Three experiments have been performed in each case comparing two non-saturating $[Na^+]_i$ levels and constructing activation curves for external caesium. In addition, in one experiment, two further $[Na^+]_i$ conditions were included, both at high saturating $[Na^+]_i$ levels. The cells used for these experiments were again prepared by the PCMBS loading procedure to have $[K^+]_i$ levels as low as possible (always less than 4 mmoles/litre cells).

Although, when hyperbolae were constructed by fitting the Michaelis-Menten equation to values of V_{max} and K_m for $^{134}Cs^+$ influx calculated by the statistical method of Wilkinson (1961), reasonable

fits to the experimental results were obtained, the alternative method of fitting results to the multiple-site rate equation of Garay and Garrahan (1973) has also been used (Table 19, Figure 21). In this case the equation is of the linear form:

$$[Cs^+]_o/M^{-1/n} = K'_{Cs}/M'_{max}{}^{-1/n} + [Cs^+]_o/M'_{max}{}^{1/n}$$

Again, best fits were obtained with n (number of sites) = 2. When a least squares linear regression programme was used, r was always found to be greater than or equal to 0.99, compared with values in the range 0.87 - 0.96 for n = 1 or 3.

There is, as with $^{42}K^+$ influx into pig red cells, a graded response to elevation of $[Na^+]_i$ of the rate of $^{134}Cs^+$ influx (for example, see Figure 20). This is further reflected by increases in the V_{max} values calculated. In one experiment raising $[Na^+]_i$ from 1.15 to 4.22 mmoles/litre cells causes a four-fold increase in V_{max} for $^{134}Cs^+$ influx from 356 to 1469 μM and in another elevation from non-saturating (2.21 mmoles/litre cells) $[Na^+]_i$ to saturating (46.58 mmoles/litre cells) causes in excess of a seven-fold increase in V_{max}. Good agreement is obtained between V_{max} values obtained by both methods described above, except at the highest $[Na^+]_i$ levels.

It had been shown in earlier experiments that, using human red cells as the experimental system, the amount of $^{134}Cs^+$ influx observed at a given $[Na^+]_i$ level was markedly less than $^{42}K^+$ influx in the same conditions (Figure 13). This did not simply reflect the lower affinity of the pump for external caesium, but was also seen at saturating external potassium and caesium levels: that is, the V_{max} value for $^{134}Cs^+$ influx was lower (about 60% of the value for $^{42}K^+$ influx). Such a difference is also noticeable in pig red cells: V_{max} values from these cells of $[Na^+]_i$

within the range 1.15 - 5.50 mmoles/litre cells were 0.11 - 0.79 μmoles ml cells^{-1} hr^{-1} compared with a range of 0.39 - 1.47 μmoles ml cells^{-1} hr^{-1} when measuring $^{42}K^+$ influx into cells of $[Na^+]_i$ 1.68 - 9.69 mmoles/litre cells.

This difference between K^+ pump activation and Cs^+ pump activation poses the question as to whether there is an altered stoichiometry of the pump when activated by external Cs^+. An alternative explanation, if the stoichiometry was the same (3 Na^+ ions pumped out: 2 K^+ or Cs^+ ions pumped in), would be that there is also a lower maximum rate of sodium efflux at saturating $[Na^+]_i$ levels when caesium is the external cation. Evidence will be presented later to support this second view (see Chapter II (ii) and (iv)).

The rates of $^{134}Cs^+$ influx observed are thus lower than would be observed with $^{42}K^+$ influx in similar conditions. This also reflects the lower affinity for external Cs^+ exhibited by the pump in pig red cells. Over the same ranges of $[Na^+]_i$ described above K_m values calculated from these results (Table 19) were within the range 255 - 2043 μM compared with a range of 35 - 300 μM for external potassium (Table 18). There is a relationship between both K_m and the apparent dissociation constant for the caesium-site complex (K'_{Cs}) and the intracellular sodium level. At least a 50% increase in K_m value is seen within each of three experiments and, in one case, raising $[Na^+]_i$ from 2.21 to 5.50 mmoles/litre cells causes the K_m value to rise from 255 μM to 1048 μM. Similarly, although the increases in K'_{Cs} value are proportionally smaller, they do occur on every occasion within an individual experiment that $[Na^+]_i$ is raised. This represents, as seen previously in human red cells and with $^{42}K^+$ influx studies in pig red cells, a decreased affinity for external caesium at higher $[Na^+]_i$ levels.

TABLE 19 The influence of $[Na^+]_i$ on kinetic constants for ouabain-sensitive $^{134}Cs^+$ influx into pig red cells. Cells were prepared of various low $[Na^+]_i$ levels by the PCMBS loading procedure, and influx measurements were made, in the presence and absence of 10^{-4}M ouabain at four different $[Cs^+]_o$ levels. All $^{134}Cs^+$ influx measurements were made in duplicate.

Values of V_{max} and K_m have been calculated by the method of Wilkinson (1961).

Values of M'_{max} and K'_{Cs} are obtained by linear fits to the rate equation:

$M = M'_{max}/(1 + K'_{Cs}/[Cs^+]_o)^n$ (Garay and Garrahan, 1973).

EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/litre cells)	V_{max} (μmoles ml cells^{-1} hr^{-1})		K_m (μM)	K'_{Cs} (μM)
		Value from statistical method (± S.E.M.)	Value from fit to rate equation of Garay and Garrahan (n = 2)	Value from statistical method (± S.E.M.)	Value from fit to rate equation of Garay and Garrahan (n = 2)
I	1.15	0.12 ± 0.01	0.12	356 ± 74	127
	4.22	0.61 ± 0.01	0.40	1469 ± 11	281
II	4.13	0.61 ± 0.11	0.46	1432 ± 460	321
	5.35	0.79 ± 0.19	0.54	2043 ± 303	412
III	2.21	0.11 ± 0.02	0.10	255 ± 78	127
	5.50	0.42 ± 0.04	0.40	1048 ± 234	290
	27.55	0.90 ± 0.09	0.55	1623 ± 68	354
	46.58	0.98 ± 0.09	0.53	1867 ± 323	385

Results from Experiment I are also presented as Figures 20, 21.

Figure 20. Activation of $^{134}Cs^+$ influx into pig red cells by external caesium.

Open symbols represent ouabain-insensitive $^{134}Cs^+$ fluxes, measured as the mean of duplicate estimations in the presence of ouabain (10^{-4}M).

Closed symbols represent ouabain-sensitive $^{134}Cs^+$ fluxes, calculated as the difference between the means of duplicate estimations in the absence of ouabain and (as shown in open symbols) in the presence of ouabain (10^{-4}M).

(△) (▲) $[Na^+]_i$ = 1.15 mmoles/litre cells

V_{max} = 0.12 ± 0.01 μmoles ml cells^{-1} hr^{-1}

K_m = 356 ± 74 μM

(□) (■) $[Na^+]_i$ = 4.22 mmoles/litre cells

V_{max} = 0.61 ± 0.01 μmoles ml cells^{-1} hr^{-1}

K_m = 1469 ± 11 μM

The hyperbolic curves for ouabain-sensitive fluxes have been obtained from fits of the Michaelis-Menten equation to the values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961) - see text and Figure 10.

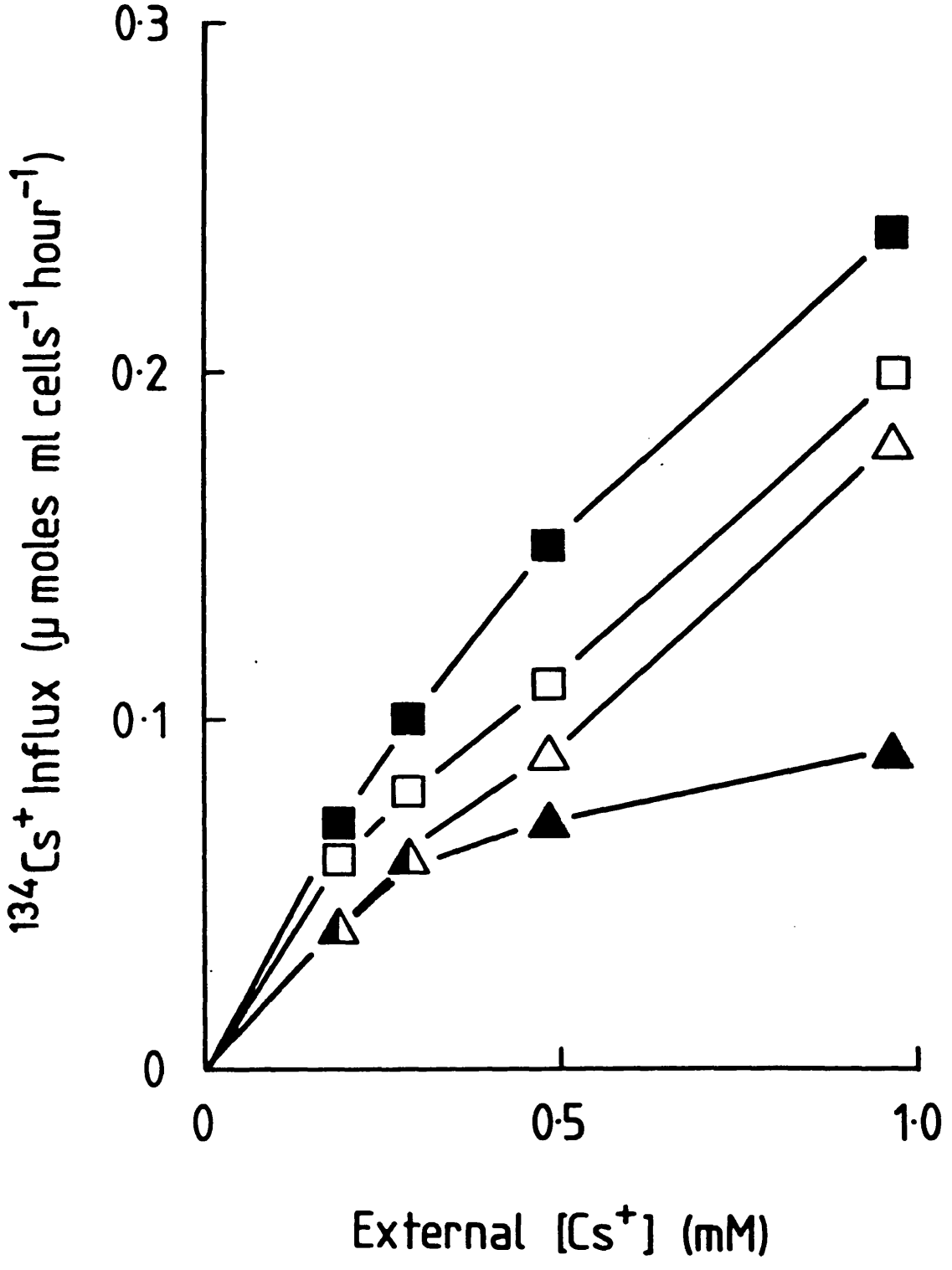

0·3
0·2
0·1
0
0
0·5
1·0
$^{134}Cs^+$ Influx (μ moles ml cells^{-1} hour^{-1})
External [Cs^+] (mM)

Figure 21. Activation of ouabain-sensitive $^{134}Cs^{+}$ influx into pig red cells.

Plot of data previously presented as Figure 20, fitted to the rate equation of Garay and Garrahan (1973) - see text - n, number of sites = 2.

(Δ) $[Na^{+}]_i$ = 1.15 mmoles/litre cells

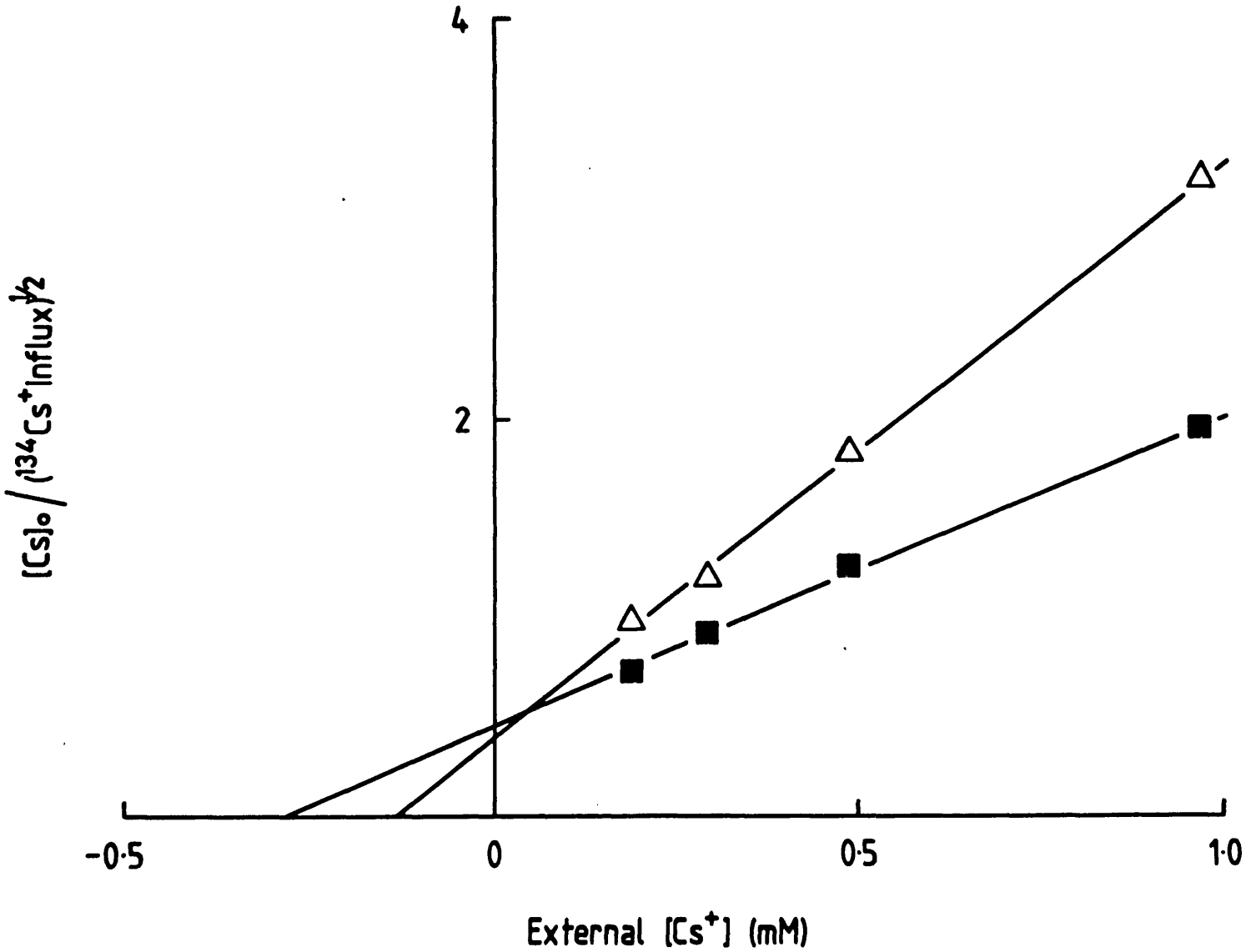
V_{max} = 0.12 μmoles ml cells^{-1} hr^{-1}

K'_{Cs} = 127 μM

(■) $[Na^{+}]_i$ = 4.22 mmoles/litre cells

V_{max} = 0.40 μmoles ml cells^{-1} hr^{-1}

K'_{Cs} = 281 μM


4
2
-0·5
0
0·5
1·0
$[Cs]_o / (^{134}Cs^+ Influx)^{1/2}$
External $[Cs^+]$ (mM)

I vi) RESULTS ON THE VARIATION OF THE V_{max}/K_m RATIO FOR EXTERNAL CATION INFLUX WITH INTRACELLULAR SODIUM CONCENTRATION

Results presented in sections I (ii), (iii), (iv) and (v) consistently demonstrate a dependence of the affinity for external potassium or caesium on $[Na^+]_i$ levels. These results confirm the previous work of Chipperfield and Whittam (1974, 1976) and the more recent studies of Sachs (1977). If the $(Na^+ + K^+)$-activated ATPase is considered as a bisubstrate enzyme reaction, taking external potassium and intracellular sodium as the two substrates at constant magnesium and ATP levels, then the ratio of V_{max}/K_m for $^{42}K^+$ (or $^{134}Cs^+$) influx may be calculated and mechanistic significance drawn from how, if at all, the value of this ratio alters with changes in $[Na^+]_i$ (Laidler and Bunting, 1973; Sachs, 1977).

When $[Na^+]_i$ was raised from one, low, non-saturating to another, high, saturating level then the V_{max} and the K_m for $^{42}K^+$ influx were both increased but the V_{max}/K_m ratio decreased - results from experiments on human red cells (Chipperfield and Whittam, 1976). This finding was confirmed in each of four experiments of this type presented in these studies (Table 13).

There is, however, another type of result to consider in relation to the V_{max}/K_m ratio. This is the change which occurs when $[Na^+]_i$ is altered at less than saturating levels. Evidence will now be presented from experiments previously described (Tables 14, 15, 18 and 19) involving respectively investigations in human and pig red cells of $^{42}K^+$ and $^{134}Cs^+$ influx. In all these experiments, the changes in $[Na^+]_i$ considered are <u>within</u> a non-saturating range. Results are presented of the values of V_{max}/K_m for each cation influx at each $[Na^+]_i$ level (Tables 20, 21 and Figure 22). It can be seen that in only one set of experiments -

those considering $^{42}K^+$ influx into human red cells (Tables 14, 20) is there consistency within each individual experiment. Here, results from six experiments fail to confirm the effect on V_{max}/K_m ratio recently reported (Sachs, 1977). In the results presented from investigations within a single experiment, of eight $[Na^+]_i$ levels, he reports an increase in V_{max}/K_m ratio as $[Na^+]_i$ is raised. In this case, however, in each of six experiments, a decrease in the value of the V_{max}/K_m ratio for $^{42}K^+$ influx is seen as $[Na^+]_i$ is raised from one value to another within the range 1.19 - 5.69 mmoles/litre cells. The decrease is at least thirty per cent in each case and is as much as a six-fold reduction in V_{max}/K_m ratio in one experiment. A decrease in V_{max}/K_m ratio is seen graphically as intersection of double reciprocal plots to the right of the ordinate and above the abscissa. As an example of this results have been presented (Figure 23) as a Lineweaver-Burk plot from one experiment previously described (Expt. III in Table 14 and Figure 11). Results from experiments considering $^{42}K^+$ influx into pig red cells, however, are not consistent - although in two experiments out of three a decrease in V_{max}/K_m is observed as $[Na^+]_i$ is raised, in another the result is inconclusive (see Table 20). Further, if experiments where the external activating cation was caesium are considered (Table 21) then no definite conclusion can be drawn. In three experiments out of four conducted on human red cells, an increase in $[Na^+]_i$ causes not only an increase in the V_{max} and K_m for $^{134}Cs^+$ influx, but also an increase in the V_{max}/K_m ratio. The fourth experiment shows little change in the V_{max}/K_m ratio, although the values for V_{max} and K_m were each raised by a factor of three as $[Na^+]_i$ was increased from 1.48 to 3.94 mmoles/litre cells. The three experiments presented from pig red cell investigations are again inconclusive as to the effect on the V_{max}/K_m ratio for $^{134}Cs^+$ influx of increases in $[Na^+]_i$

which cause increases in both V_{max} and K_m values: in two experiments no marked change is seen and in the other an increase from 0.34 to 0.42 is seen as $[Na^+]_i$ is raised from 1.15 to 4.22 mmoles/litre cells. Results from this experiment have also been presented as double reciprocal plots (Figure 24), demonstrating that an increase in V_{max}/K_m ratio as $[Na^+]_i$ is raised is seen as intersection of such plots to the left of the ordinate and below the abscissa.

These investigations thus show that, considering results from a number of experiments using two systems and two external activating cations, no firm conclusion can be drawn as to the effect of changes in $[Na^+]_i$ which cause elevation of V_{max} and K_m values for influx on the value of the V_{max}/K_m ratio. It is also shown that it would be dangerous to draw any conclusion as to the nature of any effect of $[Na^+]_i$ on this ratio without considering results from a substantial number of experiments. This point, and any significance of such results for considerations of mechanism will be further discussed later (see Discussion).

TABLE 20. The influence of $[Na^+]_i$ on the V_{max}/K_m ratio for $^{42}K^+$ influx into human or pig red blood cells of minimal $[K^+]_i$.

For experimental details, see relevant tables and associated text.

HUMAN RED CELLS (Data from Table 14)			PIG RED CELLS (Data from Table 18)		
EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/l.cells)	V_{max}/K_m ratio	EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/l.cells)	V_{max}/K_m ratio
I	1.19	31.1	I	1.68	4.5
	4.76	5.4		4.82	4.1
				5.84	4.9
II	2.75	4.4	II	3.11	13.1
	3.98	3.4		6.13	4.7
	5.69	2.8			
III	2.00	12.6	III	2.14	5.1
	3.37	6.5		6.96	4.6
				9.69	3.8
IV	1.68	9.3			
	3.33	5.9			
V	1.78	13.8			
	2.31	6.0			
VI	2.23	5.5			
	3.76	3.2			

TABLE 21. The influence of $[Na^+]_i$ on the V_{max}/K_m ratio for $^{134}Cs^+$ influx into human or pig red blood cells of minimal $[K^+]_i$. For experimental details, see relevant tables and associated text.

HUMAN RED CELLS (Data from Table 15)			PIG RED CELLS (Data from Table 19)		
EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/l.cells)	V_{max}/K_m ratio	EXPERIMENT NUMBER	$[Na^+]_i$ (mmoles/l.cells)	V_{max}/K_m ratio
I	1.48	1.20	I	1.15	0.34
	2.39	1.20		4.22	0.42
	3.94	1.18			
II	1.37	0.26	II	4.13	0.43
	4.03	0.81		5.35	0.39
	14.72	0.74			
III	2.76	1.00	III	2.21	0.43
	3.62	1.02		5.50	0.40
	7.76	1.17			
IV	2.20	0.93			
	3.79	1.06			

In experiment IV pump activity was measured as $^{24}Na^+$ efflux.

Figure 22. Plots of V_{max}/K_m ratio for ouabain-sensitive $^{42}K^+$ or $^{134}Cs^+$ influx into human or pig red cells.

Dependence on $[Na^+]_i$ level.

Values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961), applied to ouabain-sensitive fluxes calculated as the difference between means of duplicate estimations in the presence and absence of ouabain (10^{-4}M).

Results presented in Tables 20 and 21.

A - Human Blood - $^{42}K^+$ influx experiments.

B - Pig Blood - $^{42}K^+$ influx experiments.

C - Human Blood - $^{134}Cs^+$ influx experiments.

D - Pig Blood - $^{134}Cs^+$ influx experiments.

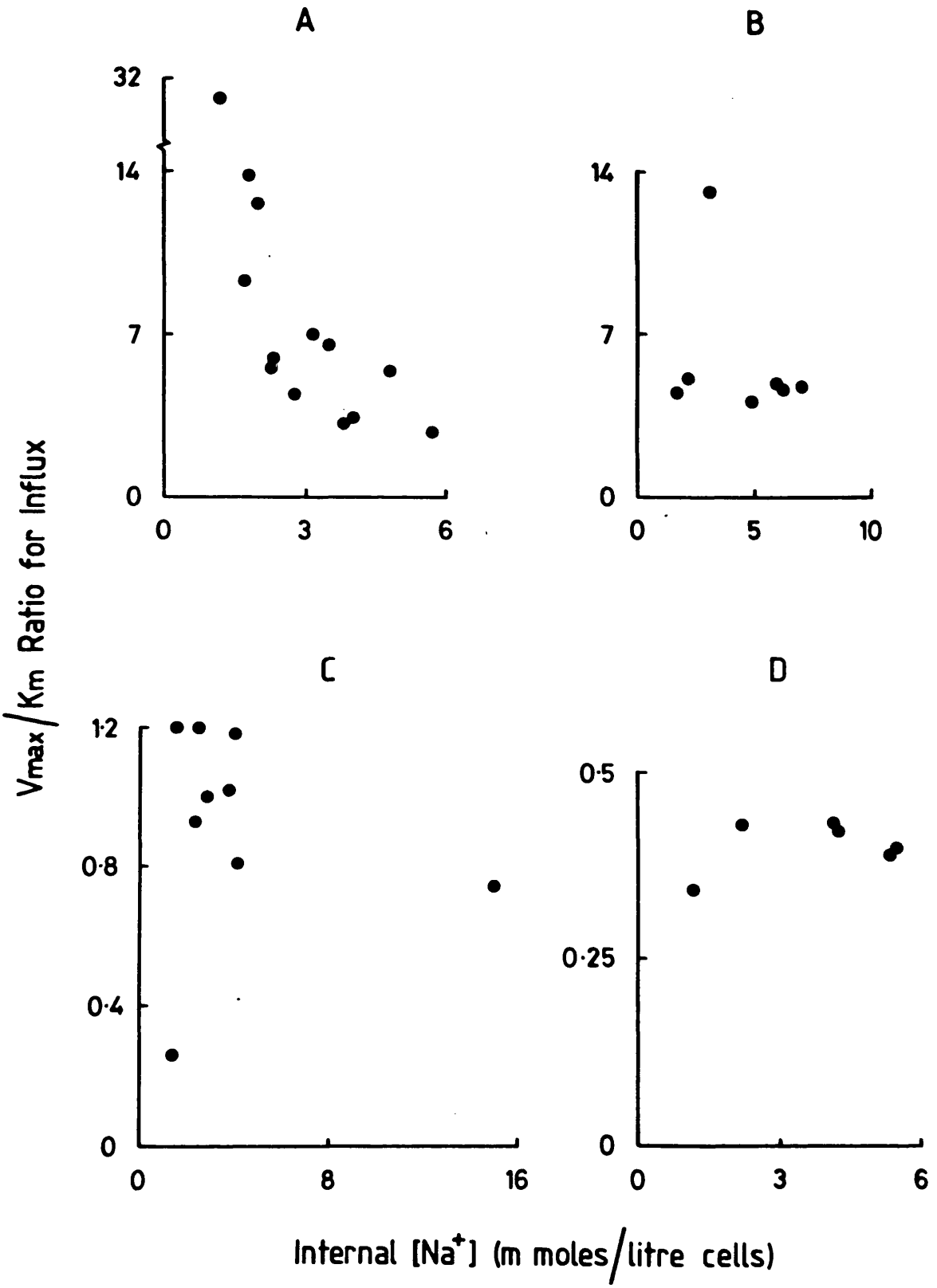

A
B
C
D
32
14
7
0
0
3
6
14
7
0
0
5
10
1·2
0·8
0·4
0
0
8
16
0·5
0·25
0
0
3
6
Vmax/Km Ratio for Influx
Internal [Na+] (m moles/litre cells)

Figure 23. Dependence of ouabain-sensitive $^{42}K^+$ influx into human red cells on intracellular sodium concentration.

Lineweaver-Burk plots constructed from fits of the Michaelis-Menten equation to values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961).

Results showing a decrease in V_{max}/K_m ratio for influx as $[Na^+]_i$ is raised, and consequent intersection of plots to the right of the ordinate and above the abscissa.

(△) $[Na^+]_i$ = 2.00 mmoles/litre cells

V_{max} = 0.44 ± 0.02 μmoles. ml cells^{-1} hr^{-1}

K_m = 42 ± 10 μM

V_{max}/K_m ratio = 10.5 (12.6 following correction for inhibition by external sodium)

(■) $[Na^+]_i$ = 3.37 mmoles/litre cells

V_{max} = 0.89 ± 0.06 μmoles. ml cells^{-1} hr^{-1}

K_m = 164 ± 29 μM

V_{max}/K_m ratio = 5.4 (6.5 following correction for inhibition by external sodium)

This experiment was previously presented as Experiment III in Table 14 and as Figure 11.

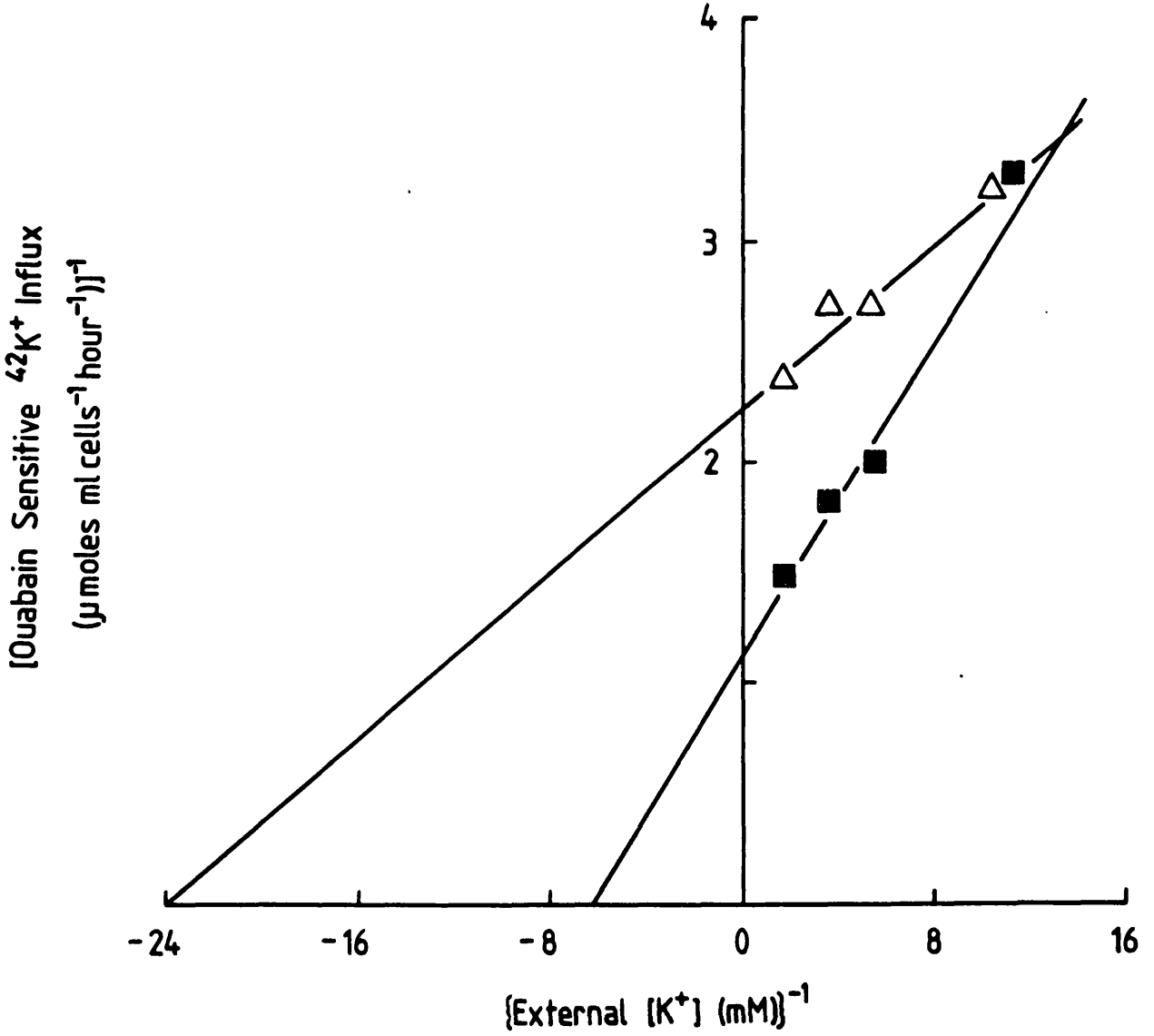

4
3
2
-24
-16
-8
0
8
16
[Ouabain Sensitive $^{42}K^+$ Influx
(μ moles ml cells^{-1} hour^{-1})]$^{-1}$
{External [K^+] (mM)}$^{-1}$

Figure 24. Dependence of ouabain-sensitive $^{134}Cs^+$ influx into pig red cells on intracellular sodium concentration.

Lineweaver-Burk plots constructed from fits of the Michaelis-Menten equation to values of V_{max} and K_m obtained by the statistical method of Wilkinson (1961).

Results showing an increase in V_{max}/K_m ratio for influx as $[Na^+]_i$ is raised, and consequent intersection of plots to the left of the ordinate and below the abscissa.

(△) $[Na^+]_i$ = 1.15 mmoles/litre cells

V_{max} = 0.12 ± 0.01 μmoles ml cells^{-1} hr^{-1}

K_m = 356 ± 74 μM

V_{max}/K_m ratio = 0.34

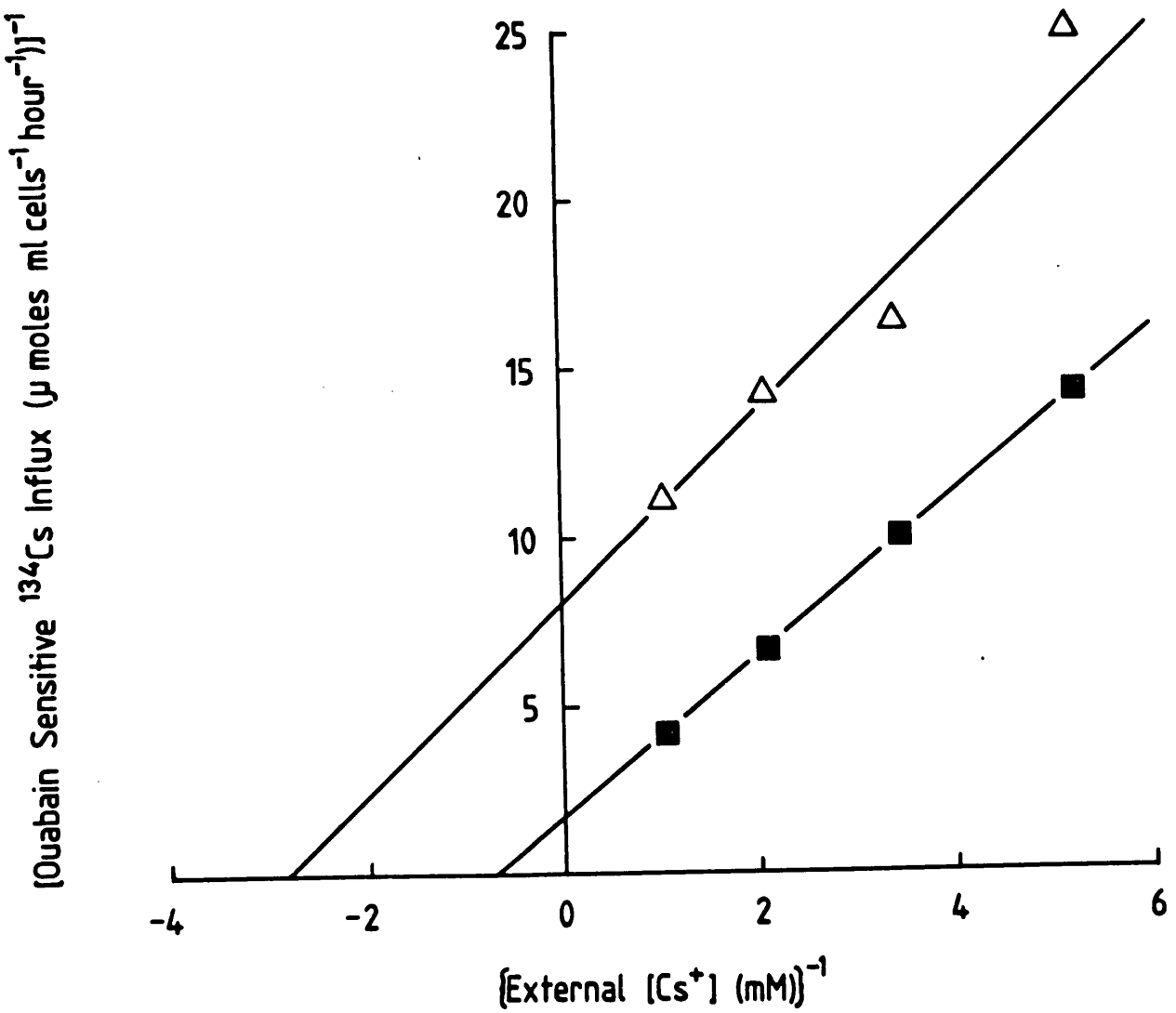
(■) $[Na^+]_i$ = 4.22 mmoles/litre cells

V_{max} = 0.61 ± 0.01 μmoles ml cells^{-1} hr^{-1}

K_m = 1469 ± 11 μM

V_{max}/K_m ratio = 0.42

This experiment was previously presented as Experiment I in Table 19 and as Figure 20.


{Ouabain Sensitive ^{134}Cs Influx (μ moles ml cells^{-1} hour^{-1})}$^{-1}$
25
20
15
10
5
-4
-2
0
2
4
6
{External [Cs^+] (mM)}$^{-1}$

II. THE INFLUENCE OF EXTERNAL ACTIVATING CATIONS ON INTERNAL SODIUM BINDING

The results already presented (Chapter I) show that in four experimental systems, involving respectively human and pig red cells and using K^+ and Cs^+ as alternative external activating cations, an influence is exerted by the $[Na^+]_i$ level on the binding of external cations. In other words, the ease of attachment of, for example, potassium externally is determined by the occupancy of sodium sites on the opposite side of the membrane. As such a transmembrane influence has been established in one direction, the question may logically be asked as to whether there is a similar influence operating in the opposite direction: that is, does the concentration of external activating cation exert any influence on sodium attachment to intracellular sites? As will be further discussed later (see Discussion) there is substantial significance as to what mechanisms of pump operation are feasible, implicit from the results of such studies.

Although the evidence presented in Chapter I is inconsistent with a unitary mechanism: that is, one which only involves one set of binding sites, it sheds no light on the question as to whether or not the order of binding of intracellular sodium and external potassium or caesium is obligatorily fixed. An investigation of whether or not there is a transmembrane influence operating in the opposite direction does, however, address this problem.

Brief mention of the existence of an influence of external potassium concentration on internal sodium binding in human red cells has been made previously by one set of workers (Chipperfield and Whittam, 1976). Results from a substantial number of experiments have not, however, been reported. It was thus decided to conduct experiments using the four

systems mentioned above. Such experiments were conducted measuring pump activity as either $^{42}K^+$ influx or $^{134}Cs^+$ influx as opposed to $^{24}Na^+$ efflux, because of the fact that there are less technical problems associated with influx measurements.

It has already been stressed that there is difficulty, when working with cells modified by the PCMBS procedure, in achieving exact reproducibility of conditions. Further it has been shown (see Chapter I (vi)) that a clear position cannot be established on kinetic investigations of this type, without several experiments being conducted. Consequently, the results presented in this section involve a substantial number of similar experiments being performed on each system.

II i) HUMAN BLOOD EXPERIMENTS - POTASSIUM AS EXTERNAL CATION

In order to establish whether or not there exists any influence of external cation concentration on intracellular sodium binding, it is very important to meet the experimental requirements set out for the extraction of such kinetic information. Firstly, the assumption that it is a bisubstrate enzyme reaction under consideration here, will only be acceptable if the system has constant and adequate levels of magnesium and ATP. It is then required to measure the maximum velocity and apparent Michaelis constant for one of the substrates at several fixed concentrations of the other substrate in the absence of products of the reaction (Cleland, 1963a). In this case, the products are taken to be intracellular potassium or caesium and external sodium. Thus, all flux measurements must be made from sodium-free Ringer solutions: this was achieved by using choline as the major external cation. Next, the cells must have minimal $[K^+]_i$ levels: this was achieved by the modification

of the intracellular ionic composition by means of the PCMBS loading procedure, to produce not only various $[Na^+]_i$ levels as required, but also to void as much intracellular potassium as possible and to substitute with, again, choline.

The activation of the sodium pump by intracellular sodium in human red cells has been previously demonstrated to be non-hyperbolic - it is, in fact, slightly sigmoid at low $[Na^+]_i$ levels (Garay and Garrahan, 1973). It is, therefore, not possible to use the Michaelis-Menten form of kinetics for studies of such activations and an alternative indicator of affinity for intracellular sodium must be used. This is the apparent dissociation constant (K'_{Na}) of the Na^+-site complex. The activation equation proposed was one which takes account of the fact that this is a multiple site system and is of the form:

$$M = M'_{max}/(1 + K'_{Na}/[Na^+]_i)^n$$

where M = flux at the given sodium concentration $[Na^+]_i$

M'_{max} = flux at non-limiting intracellular sodium concentration

K'_{Na} = apparent dissociation constant of the Na^+-site complex

and n = number of Na^+ sites

The assumptions needed to produce such a rate equation were that the sodium sites on the intracellular membrane face were of the same affinity for Na^+ and that there was no interaction between them. It had already been suggested (Whittam and Ager, 1965) that the number of Na^+ and K^+ ions transported per pump cycle in human red cells was fixed. Their findings, subsequently confirmed by the fitting of flux data (Garay and Garrahan, 1973; Chipperfield and Whittam, 1976) suggested that the number of Na^+ sites was three. This stoichiometry was confirmed by results

presented here - in all cases fits to the linear form of the rate equation described above were best when n = 3.

Experiments were conducted by preparing batches of human red cells to have $[Na^+]_i$ values up to approximately 20 mmoles/litre cells. Five or six such conditions were used. Flux measurements using $^{42}K^+$ labelled solutions, were then made in duplicate in the presence and absence of ouabain (10^{-4}M). Routine observation of $[K^+]_i$ levels showed these to be never in excess of 0.4 mmoles/litre cells. Two extracellular potassium concentrations were used and were chosen to be within the portion of the activation curve for external K^+ that shows the greatest response to changes in $[K^+]_o$ (see Figure 13), that is, less than 2 mM.

Results are presented from six experiments using potassium as the external cation (Table 22). In each experiment the same response to an increase in $[K^+]_o$ is seen: there is an increased flux at each intracellular sodium level (see Figure 25). This is further reflected as an increase in the maximum velocity (M'_{max}) value obtained by fits to the linear form of the rate equation above. The increase in M'_{max} observed varied from a 50% increase in one experiment where $[K^+]_o$ was raised from 0.19 to 0.57 mM, to an increase from 0.53 to 1.58 μmoles ml cells^{-1} hr^{-1} for a similar three-fold increase in $[Na^+]_i$ levels. As a measure of the goodness of fit to the data of the linear form of the rate equation when the number of Na^+ sites is taken as three, r values are quoted for fits by least squares linear regression.

As shown in the previous chapter, the apparent dissociation constant is a satisfactory measure of affinity - an increase in the value of this constant representing a decrease in affinity. Values are presented of K'_{Na} in Table 22. In each of the six experiments an increase is seen as $[K^+]_o$ is raised: from a 15 per cent increase in K'_{Na} as $[K^+]_o$

TABLE 22. Kinetic constants for sodium pump activation by intracellular Na^+ in human red blood cells.

Dependence on $[K^+]_o$ levels.

Values are based on fits to the linear form of the rate equation of Garay and Garrahan (1973) (see text) of data from duplicate estimations of $^{42}K^+$ influx in the presence and absence of ouabain (10^{-4}M). Five or six intracellular sodium concentrations were used in each experiment.

EXPERIMENT NUMBER	$[K^+]_o$ (mM)	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_{Na} (μM)	r for linear regression fit (n,no. of sites=3)
I	0.28	0.80	435	1
	1.88	1.58	816	0.99
II	0.27	0.74	379	1
	1.91	1.92	1206	1
III	0.27	1.27	1186	1
	0.94	3.38	2182	1
IV	0.29	0.82	1048	1
	1.86	1.48	1330	1
V	0.20	0.53	541	1
	0.60	1.58	1557	1
VI	0.19	1.08	1227	0.99
	0.57	1.59	1423	1

Results from Experiment I are also presented as Figures 25 and 26.

Figure 25. Activation of the sodium pump by intracellular sodium in human red cells.

(Pump activity measured as ouabain-sensitive $^{42}K^{+}$ influx).

Ouabain-sensitive $^{42}K^{+}$ influx, calculated as the difference between the means of duplicate flux estimations in the absence of ouabain and in the presence of 10^{-4}M ouabain.

(▲) $[K^{+}]_{o}$ = 0.28 mM

(■) $[K^{+}]_{o}$ = 1.88 mM

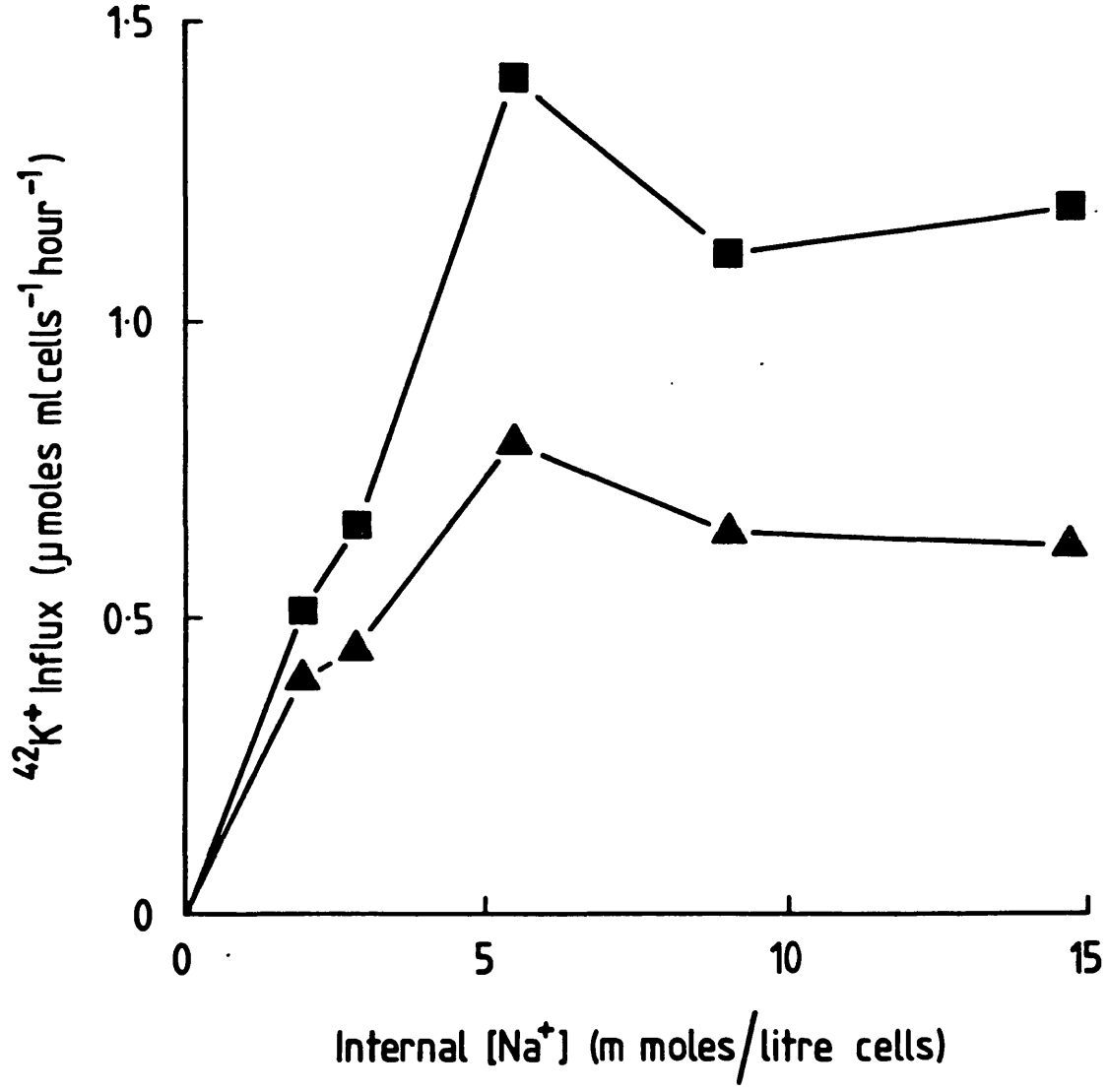

1·5
1·0
0·5
0
0
5
10
15
$^{42}K^+$ Influx (μ moles ml cells^{-1} hour^{-1})
Internal [Na^+] (m moles/litre cells)

Figure 26. Activation of ouabain-sensitive $^{42}K^+$ influx into human red cells by intracellular sodium.

Plots of results presented in Figure 25, fitted to the linear form of the activation equation of Garay and Garrahan (1973) - see text.

n, number of Na^+-sites = 3.

(▲) $[K^+]_o$ = 0.28 mM

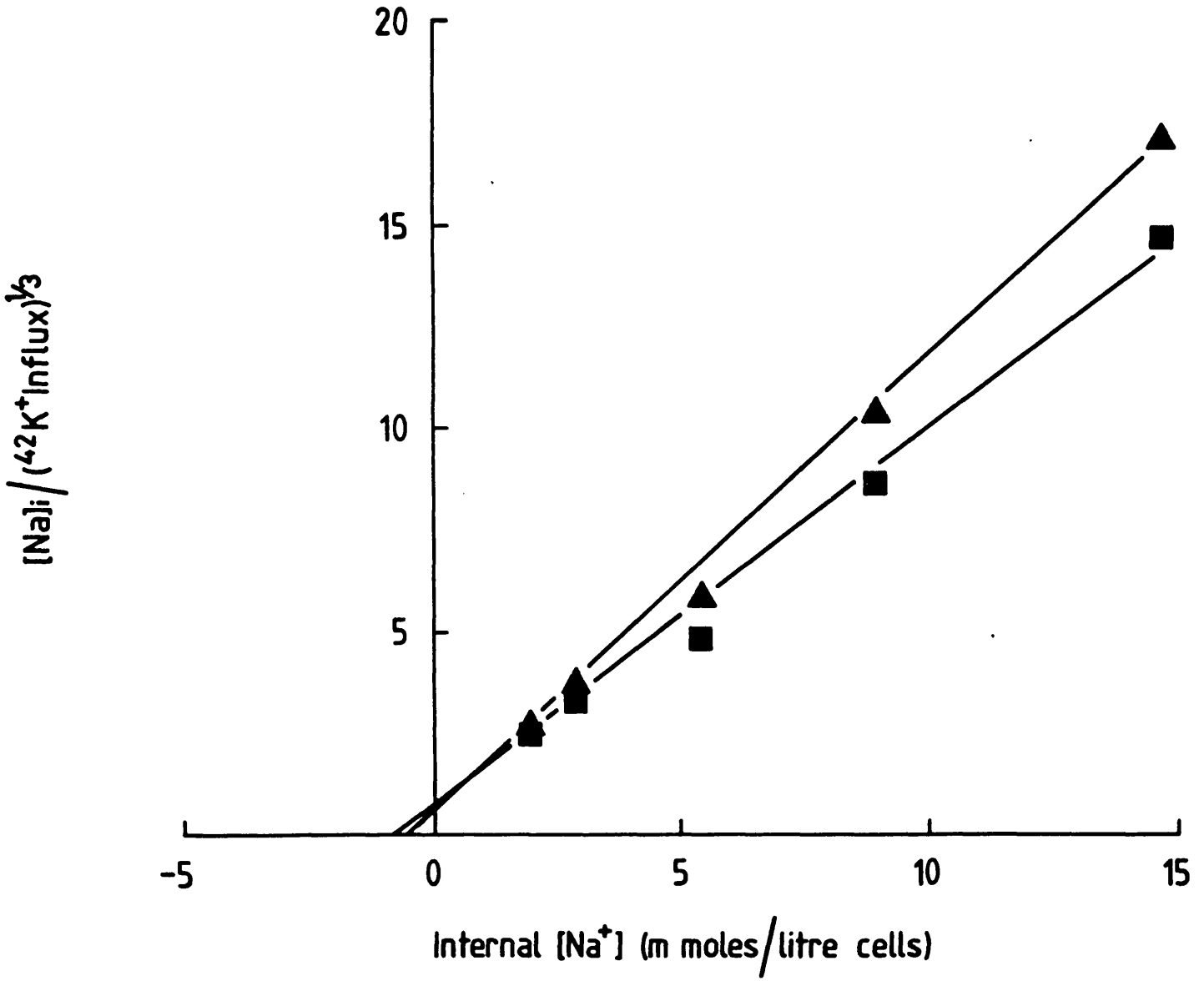
M'_{max} = 0.80 μmoles ml cells^{-1} hr^{-1}

K'_{Na} = 435 μM

(■) $[K^+]_o$ = 1.88 mM

M'_{max} = 1.58 μmoles ml cells^{-1} hr^{-1}

K'_{Na} = 816 μM


20
15
10
5
-5
0
5
10
15
[Nali]/(42K+Influx)1/3
Internal [Na+] (m moles/litre cells)

increases from 0.19 to 0.57 mM, to an increase of K'_{Na} from 379 to 1206 μM for a seven-fold increase in $[K^+]_o$.

In addition to the data presented in Table 22, results from a single experiment are presented as activation curves (Figure 25) and as the fitted linear forms of the rate equation (Figure 26).

II ii) HUMAN BLOOD EXPERIMENTS - CAESIUM AS EXTERNAL CATION

The earlier work which described (see Chapter I) an influence of intracellular sodium levels on external potassium binding, had been extended to show similar effects when caesium was substituted for potassium as the external activating cation. With both external cations a graded response to increases in $[Na^+]_i$ had been seen, both of the flux at any given $[Na^+]_i$ level and of V_{max} and K_m values. Further, caesium has the practical advantage that solutions of low concentration with comparatively high specific activity are readily achieved using the $^{134}Cs^+$ isotope. It was only whencomparing the effect of $[Na^+]_i$ on the V_{max}/K_m ratio for influx that any difference between potassium and caesium was seen (see Tables 20 and 21), though this is not, because of the inconclusive nature of the results, necessarily indicative of any mechanistic difference when the pump is activated externally by potassium or caesium.

The question thus follows as to whether or not the effect of occupancy of external cation sites on intracellular sodium binding, already established with external potassium, can be extended to the situation where caesium is substituted for potassium. This has been investigated by conducting seven experiments, measuring $^{134}Cs^+$ influx into cells prepared to have five or six different $[Na^+]_i$ levels. The $[Cs^+]_o$ levels used, though of a wider range than for the external potassium experiments, were maintained within the range of maximal response to

changes in concentration (see Figure 13) - in this case not greater than 5 mM. Flux measurements were again fitted to the cube form of the activation equation of Garay and Garrahan (see Section II (i)). This form (n = 3) again gives best fits when using a least squares linear regression programme, confirming the suggestion that in human red cells the stoichiometry of 3 Na^+ ions out: 2 K^+ or Cs^+ ions in is not altered by substitution of potassium by caesium externally. Values of r for the line fits are again presented (Table 23).

In each experiment the response to an increase in $[Cs^+]_o$ is an increased flux at each $[Na^+]_i$ level (e.g. see Figure 27). This is confirmed by the fact that in each case a substantial (up to four-fold) increase in maximum velocity (M'_{max}) is seen as the $[Cs^+]_o$ level is increased. As observed in previous experiments, however, the range of maximum velocity values is lower with caesium than with potassium externally: with a range of $[K^+]_o$ from 0.19 to 1.91 mM the maximum velocity values calculated were from 0.53 - 1.92 (in one experiment a high value of 3.38) μmoles ml cells^{-1} hr^{-1}, but with $[Cs^+]_o$ levels from 0.19 - 5 mM the calculated maximum velocity values were from 0.16 - 1.09 μmoles ml cells^{-1} hr^{-1}. This confirms the previously expressed view (see Chapter I (v)), that the lower rate of pump activity seen when Cs^+ is substituted for K^+ as the external activating cation does not represent an altered stoichiometry, merely a decreased number of Na^+ ions extruded from and Cs^+ ions transported into cells, whilst maintaining the 3 Na^+ ions out to 2 Cs^+ ions in ratio.

The results obtained (Table 23) also show a dependence of the apparent dissociation constant (K'_{Na}) on external caesium concentration. In each of seven experiments, at least a 15% increase in K'_{Na} is seen as $[Cs^+]_o$ is increased and in one experiment a 130% rise in K'_{Na} (from 233 μM to 535 μM) is seen as $[Cs^+]_o$ rises from 0.19 to 0.57 mM. This represents

TABLE 23. Kinetic constants for sodium pump activation by intracellular Na^+ in human red cells.

Dependence on $[Cs^+]_o$ levels.

Values are based on fits to the linear form of the rate equation of Garay and Garrahan (1973) (see text) of data from duplicate estimations of $^{134}Cs^+$ influx in the presence and absence of ouabain (10^{-4}M).

Five or six intracellular sodium concentrations were used in each experiment.

EXPERIMENT NUMBER	$[Cs^+]_o$ (mM)	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_{Na} (μM)	r for linear regression fit (n, number of sites = 3)
I	0.29	0.19	785	1
	5.00	0.80	1186	1
II	0.29	0.29	1250	1
	5.00	1.09	1460	0.99
III	0.29	0.21	685	1
	5.00	0.58	933	1
IV	0.29	0.20	593	1
	0.58	0.35	871	1
	1.43	0.51	1154	1
	2.85	0.67	1210	1
V	0.29	0.26	704	1
	5.00	0.95	1080	1
VI	0.19	0.19	233	1
	0.28	0.30	364	1
	0.57	0.55	535	1
VII	0.29	0.16	780	1
	0.57	0.26	925	1

Results from Experiment I are also presented as Figures 27 and 28.

Figure 27. Activation of the sodium pump by intracellular sodium in human red cells.
(Pump activity measured as ouabain-sensitive $^{134}Cs^+$ influx).
Ouabain-sensitive $^{134}Cs^+$ influx calculated as the difference between the means of duplicate flux determinations in the absence of ouabain and in the presence of 10^{-4}M ouabain.

(▲) $[Cs^+]_o$ = 0.29 mM

(■) $[Cs^+]_o$ = 5.00 mM

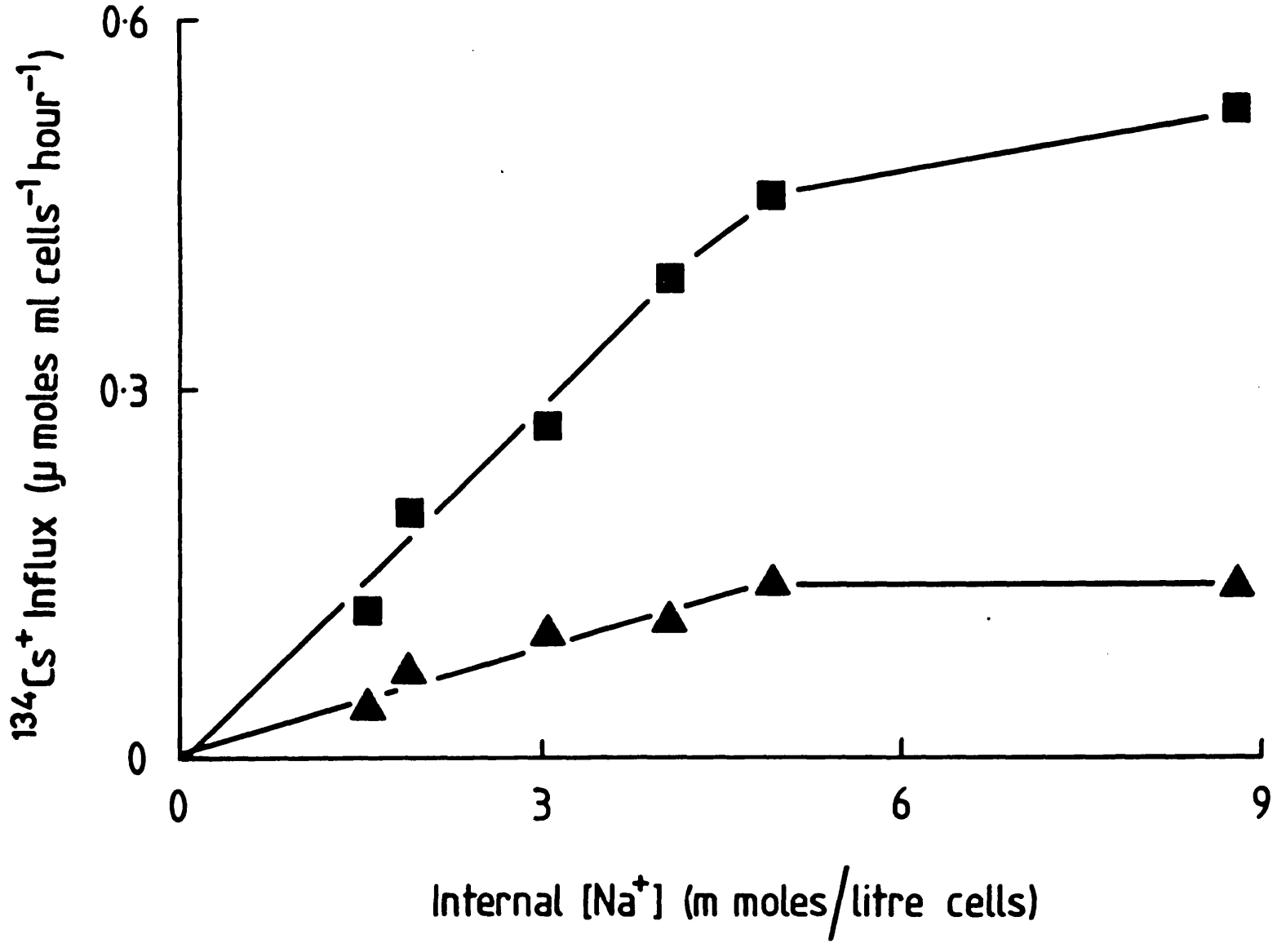

0·6
0·3
0
0
3
6
9
$^{134}Cs^{+}$ Influx (μ moles ml cells^{-1} hour^{-1})
Internal [Na^{+}] (m moles/litre cells)

Figure 28. Activation of ouabain-sensitive $^{134}Cs^+$ influx into human red cells by intracellular sodium.

Plots of results presented in Figure 27, fitted to the linear form of the activation equation of Garay and Garrahan (1973) - see text.

n, number of Na^+ sites = 3.

(△) $[Cs^+]_o$ = 0.29 mM

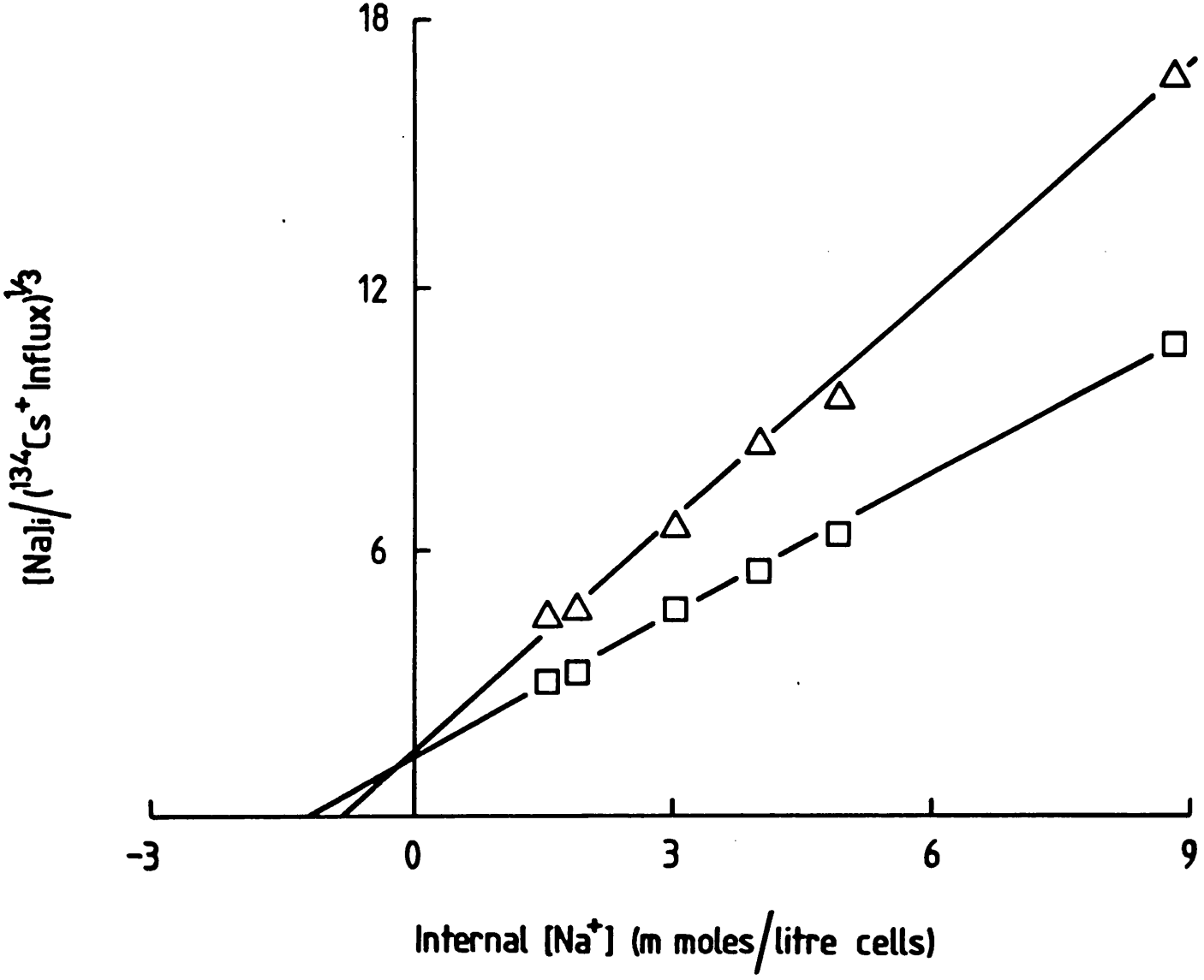
M'_{max} = 0.19 μmoles ml cells^{-1} hr^{-1}

K'_{Cs} = 785 μM

(□) $[Cs^+]_o$ = 5.00 mM

M'_{max} = 0.80 μmoles ml cells^{-1} hr^{-1}

K'_{Cs} = 1186 μM


18
12
6
-3
0
3
6
9
[Na]i/(134Cs+ Influx)1/3
Internal [Na+] (m moles/litre cells)

a decreased affinity for intracellular sodium as $[Cs^+]_o$ is raised - an effect which is seen on each occasion within the seven experiments that $[Cs^+]_o$ is raised.

In addition to the results presented in Table 23, results from a single experiment are presented as activation curves (Figure 27) and as the fitted linear forms of the rate equation (Figure 28).

II iii) PIG BLOOD EXPERIMENTS - POTASSIUM AS EXTERNAL CATION

Although in the course of these studies it has been demonstrated that there is a transmembrane influence of intracellular $[Na^+]$ on external cation binding exhibited in pig red cells in the same way as seen in human red cells, it is not an established fact that the sodium pump operates by the same mechanism in both systems. It is, therefore, necessary to establish for this system also whether there is a mirror-image effect, that is, the effect described previously for human red cells, of occupancy of external cation sites on the nature of attachment of intracellular Na^+.

First the activation of the pump in pig red cells by both external K^+ and external Cs^+ was determined in separate experiments. Activation curves are presented (Figure 29) which show that, as seen previously in human red cells (Figure 13) the amount of ion influx is considerably greater, both at low and saturating concentrations, when using potassium rather than caesium as the activating cation on the external side. The activation curves further reveal the levels of $[K^+]_o$ and $[Cs^+]_o$ required for the subsequent affinity experiments.

Four experiments are presented using external $[K^+]$levels of less than 2 mM - i.e. within the range of greatest response to concentration changes. These experiments all exhibit a graded increase in flux at

any given low $[Na^+]_i$ level and maximum velocity (M'_{max}) at saturating $[Na^+]_i$ as the $[K^+]_o$ level is raised (Table 24). The increase in maximum velocity seen ranges from a 30% increase (0.54 to 0.71 μmoles ml cells^{-1} hr^{-1}) for an increase in $[K^+]_o$ from 0.22 to 0.59 mM, to a four-fold increase in another experiment as $[K^+]_o$ is raised from 0.11 to 0.57 mM. It is noticeable also that the range of maximum velocity values is similar to that seen in human red cells over a similar range of $[K^+]_o$ levels: a range of 0.34 - 1.84 μmoles ml cells^{-1} hr^{-1} for $[K^+]_o$ levels of 0.11 - 1.91 mM in pig red cells and 0.53 - 1.92 μmoles ml cells^{-1} hr^{-1} (with one exception) for $[K^+]_o$ levels of 0.19 - 1.92 mM in human red cells (Tables 22 and 24).

Results presented in Table 24 also show a dependence of K'_{Na}, the apparent Na^+-site dissociation constant, on $[K^+]_o$, illustrating the same affinity effect (a decreased affinity as $[K^+]_o$ is raised) as seen previously in human red cells. Values of K'_{Na} have been obtained from least squares linear regression analysis of the linear form of the multiple site rate equation of Garay and Garrahan (1973). Again, best fits were obtained with number of sites = 3, confirming the suggestion that the stoichiometry of the pump is the same in pig red cells as previously confirmed in human red cells. A 45 - 200% increase in K'_{Na} values is observed in four experiments investigating a 2.5 - 7-fold increase in external potassium concentration. One of the four experiments presented (Table 24) has also been presented on the form of activation curves (Figure 30) and linear plots of the three site rate equation (Figure 31).

Figure 29. Activation of $^{42}K^+$ and $^{134}Cs^+$ influxes into pig red cells, by external potassium and caesium respectively.

Cells were washed three times in ice-cold isotonic choline chloride solution and then influx measurements made as described in Methods.

Open symbols represent ouabain-insensitive fluxes, measured in the presence of 10^{-4}M ouabain. Values are means of duplicate determinations.

Closed symbols represent ouabain-sensitive fluxes, calculated as the difference between means of two flux determinations in the absence of ouabain and two in the presence of 10^{-4}M ouabain.

All flux measurements were made from sodium-free Ringer solutions.

(△)(▲) $^{134}Cs^+$ INFLUX

(□)(■) $^{42}K^+$ INFLUX

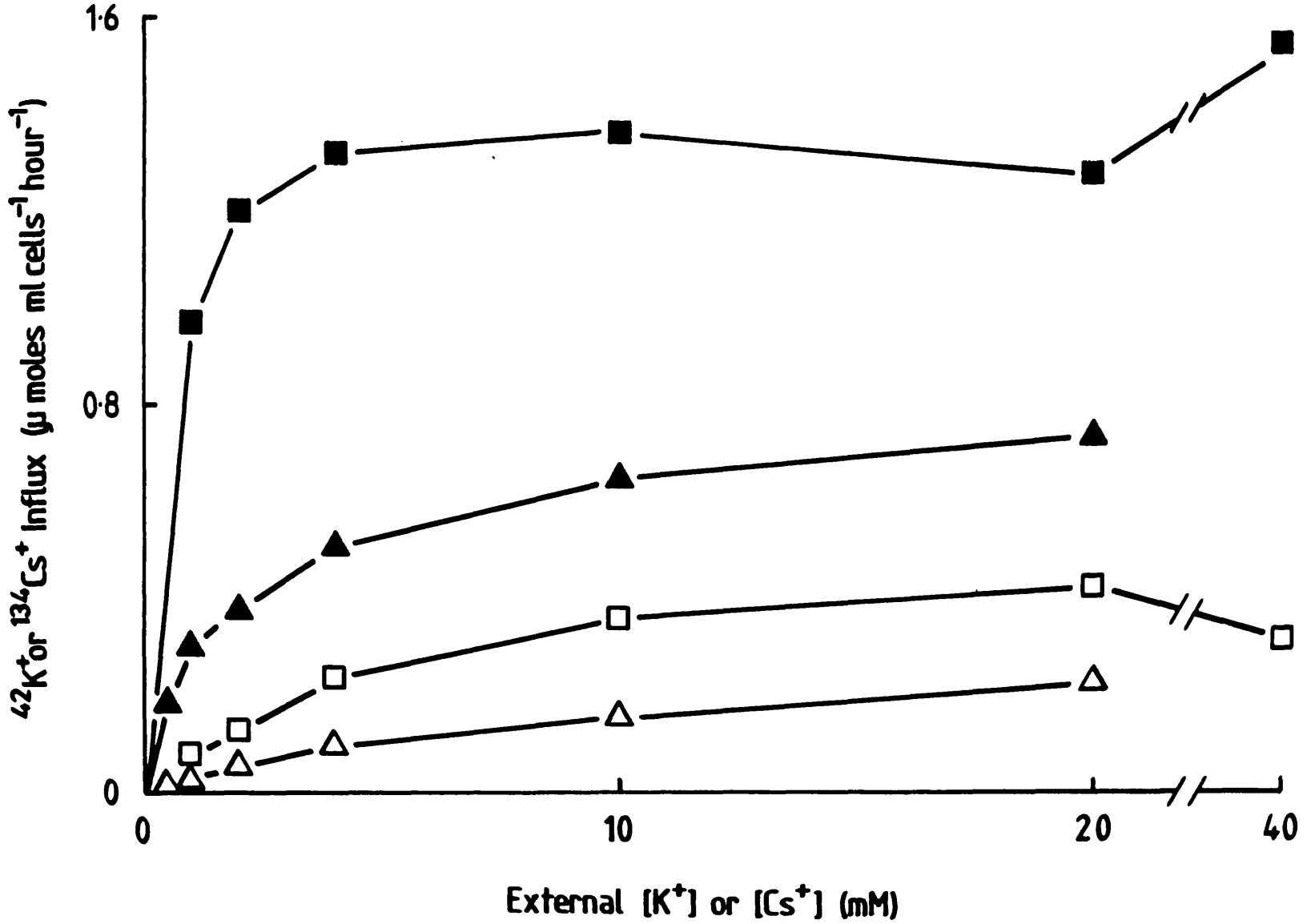

1·6
0·8
0
0
10
20
40
$^{42}K^+$ or $^{134}Cs^+$ Influx (μ moles ml cells^{-1} hour^{-1})
External [K^+] or [Cs^+] (mM)

TABLE 24. Kinetic constants for sodium pump activation by intracellular Na^+ in pig red cells.

Dependence on $[K^+]_o$ levels.

Values are based on fits to the linear form of the multiple-site rate equation of Garay and Garrahan (1973) (see text) of data from duplicate estimations of $^{42}K^+$ influx with and without ouabain (10^{-4}M).

At least five $[Na^+]_i$ levels were used in each experiment.

EXPERIMENT NUMBER	$[K^+]_o$ (mM)	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_{Na} (μM)	r for linear regression fit (n, number of sites = 3)
I	0.28	0.76	317	1
	1.91	1.84	834	1
II	0.22	0.54	146	1
	0.59	0.71	213	1
III	0.30	0.64	410	1
	0.98	1.10	703	1
IV	0.11	0.34	378	0.99
	0.20	0.78	698	1
	0.29	1.15	1008	1
	0.57	1.45	1138	0.99

Data from Experiment I are also presented as Figures 30 and 31.

Figure 30. Activation of the sodium pump by intracellular sodium in pig red cells.
(Pump activity measured as ouabain-sensitive $^{42}K^+$ influx).
Ouabain-sensitive $^{42}K^+$ influx calculated as the difference between the means of duplicate flux determinations in the absence of ouabain and in the presence of 10^{-4}M ouabain.

(▲) $[K^+]_o$ = 0.28 mM

(■) $[K^+]_o$ = 1.91 mM

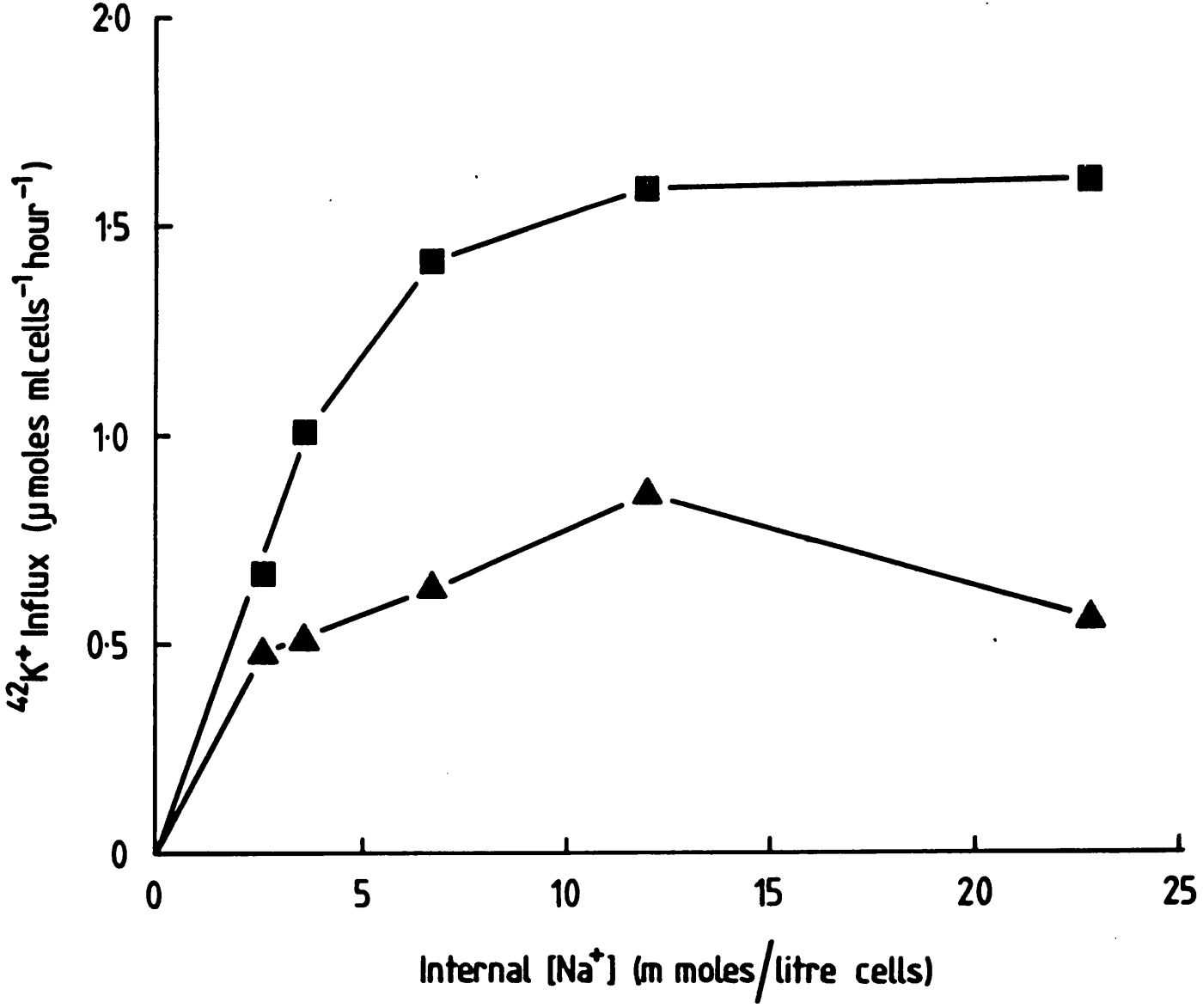

2·0
1·5
1·0
0·5
0
$^{42}K^+$ Influx (μ moles ml cells^{-1} hour^{-1})
0
5
10
15
20
25
Internal [Na^+] (m moles/litre cells)

Figure 31. Activation of ouabain-sensitive $^{42}K^+$ influx into pig red cells by intracellular sodium.

Plots of results presented in Figure 30, fitted to the linear from of the activation equation of Garay and Garrahan (1973) - see text.

n, number of sites = 3.

(△) $[K^+]_o$ = 0.28 mM

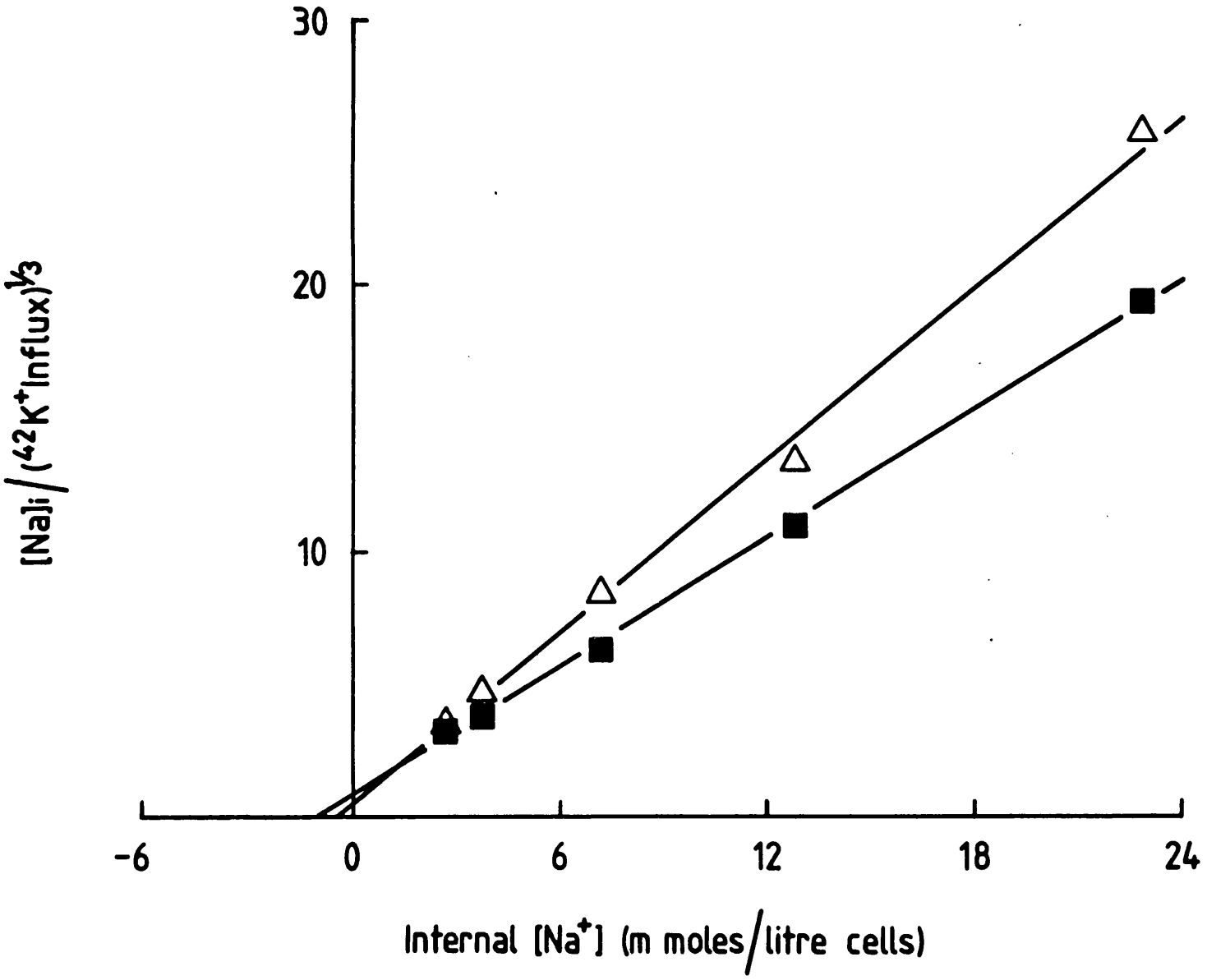
M'_{max} = 0.76 μmoles ml cells^{-1} hr^{-1}

K'_K = 317 μM

(■) $[K^+]_o$ = 1.91 mM

M'_{max} = 1.84 μmoles ml cells^{-1} hr^{-1}

K'_K = 834 μM


30
20
10
-6
0
6
12
18
24
[Na]i/(42K+influx)1/3
Internal [Na+] (m moles/litre cells)

II iv) PIG BLOOD EXPERIMENTS - CAESIUM AS EXTERNAL CATION

As in human cells, despite the lower flux values observed for any given cation concentration (including saturating levels) - see Figure 29 - caesium presents a very satisfactory alternative to potassium at the external side of the membrane. A transmembrane effect on the binding of ions on one side of the membrane by the concentration of activating cation on the other side has now been demonstrated to exist for both pig and human red cells with potassium at the outer face. This transmembrane effect operates in both directions, and has also been shown using caesium on the outside of human red cells. Further evidence as to the similarity of the two systems could thus come from studies of pig red cells using external Cs^+. In such a system it has already been shown (Chapter I (v)) that $[Na^+]_i$ levels influence both V_{max} and K_m values for $^{134}Cs^+$ influx and consequently it is necessary to investigate the influence of caesium on intracellular sodium binding.

This has been carried out by way of five experiments on pig cells modified by the PCMBS procedure to have minimal $[K^+]_i$ levels (less than 2 mmoles/litre cells) and up to six different $[Na^+]_i$ levels. Influx measurements from $^{134}Cs^+$ labelled, sodium-free Ringer solutions were then made and the consequent activation curve results fitted to the linear form of the multiple-site rate equation of Garay and Garrahan (1973). No discrepancy from the stoichiometry suggested in the last section was observed - again, best fits were obtained with n, number of Na^+ sites = 3.

As stated previously for human red cells (see Section II (ii)) it appears that the decreased pump activity seen, even at saturating concentrations, when Cs^+ rather than K^+ is used as the external activating cation, is reflected as both a decreased movement of Cs^+ ions in and of Na^+

ions out of cells. It does not represent an altered pump stoichiometry, a fact confirmed by the fitting of flux data to linear form of the multiple-site rate equation of Garay and Garrahan (1973).

Elevation of $[Cs^+]_o$ level causes an increase in pump activity at any given $[Na^+]_i$ condition as seen previously in experiments when K^+ was the external cation. This is further seen in an increase in the maximum velocity (M'_{max}): a three to seven-fold increase in M'_{max} was seen in each experiment as $[Cs^+]_o$ was raised from a lower to a higher value within the range 0.28 - 5 mM. This range of external caesium levels had been chosen to be in the non-saturating range of the previously determined activation curves (Figure 29). As seen previously in human red cells, the values of maximum velocity were lower than those with potassium as the external cation: the highest value seen here is 1.26 μmoles ml cells^{-1} hr^{-1} at an external $[Cs^+]$ of 5mM, compared with a value of 1.84 μmoles ml cells^{-1} hr^{-1} at 1.91 mM $[K^+]_o$ in one experiment, and 1.45 μmoles ml cells^{-1} hr^{-1} at 0.37 mM $[K^+]_o$ in another.

It can also be seen (Table 25) that there is, in the same way as shown with external potassium in this system, a dependence of the intracellular sodium binding on external cation concentration. This is seen as a 45 - 100% increase in the value of the apparent dissociation constant of the Na^+-site complex in each of five experiments as $[Cs^+]_o$ is raised (e.g. in one experiment K'_{Na} increases from 244 to 623 μM as $[Cs^+]_o$ is raised from 0.29 to 5 mM). This again represents a decrease in the affinity for intracellular sodium observed on each occasion that the external caesium concentration is raised, an effect now established in a substantial number of experiments on each of the four systems used. Results from one experiment on this system, apart from being shown in Table 25, are also presented as activation curves (Figure 32) and as linear plots of the three-site rate equation (Figure 33).

TABLE 25. Kinetic constants for pump activation by intracellular Na^+ in pig red cells.

Dependence on $[Cs^+]_o$ levels.

Values are based on fits to to the linear form of the multiple-site (n = 3) rate equation of Garay and Garrahan (1973) (see text) of data from duplicate $^{134}Cs^+$ influx determinations with and without ouabain (10^{-4}M).

At least five $[Na^+]_i$ levels were used in each experiment.

EXPERIMENT NUMBER	$[Cs^+]_o$ (mM)	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_{Na} (μM)
I	0.28	0.21	929
	2.50	0.96	1398
II	0.29	0.22	696
	5.00	1.26	1010
III	0.29	0.10	244
	5.00	0.65	623
IV	0.29	0.11	480
	5.00	0.58	998
V	0.29	0.15	876
	0.58	0.27	1239
	1.41	0.49	1722

Data from Experiment I are also presented as Figures 32 and 33.

Values of r for fit to data were 1.0 in every case.

Figure 32. Activation of the sodium pump by intracellular sodium in pig red cells.
(Pump activity measured as ouabain-sensitive $^{134}Cs^{+}$ influx).
Ouabain-sensitive $^{134}Cs^{+}$ influx calculated as the difference between the means of duplicate flux determinations in the absence of ouabain and in the presence of 10^{-4}M ouabain.

(▲) $[Cs^{+}]_{o}$ = 0.28 mM

(■) $[Cs^{+}]_{o}$ = 2.50 mM

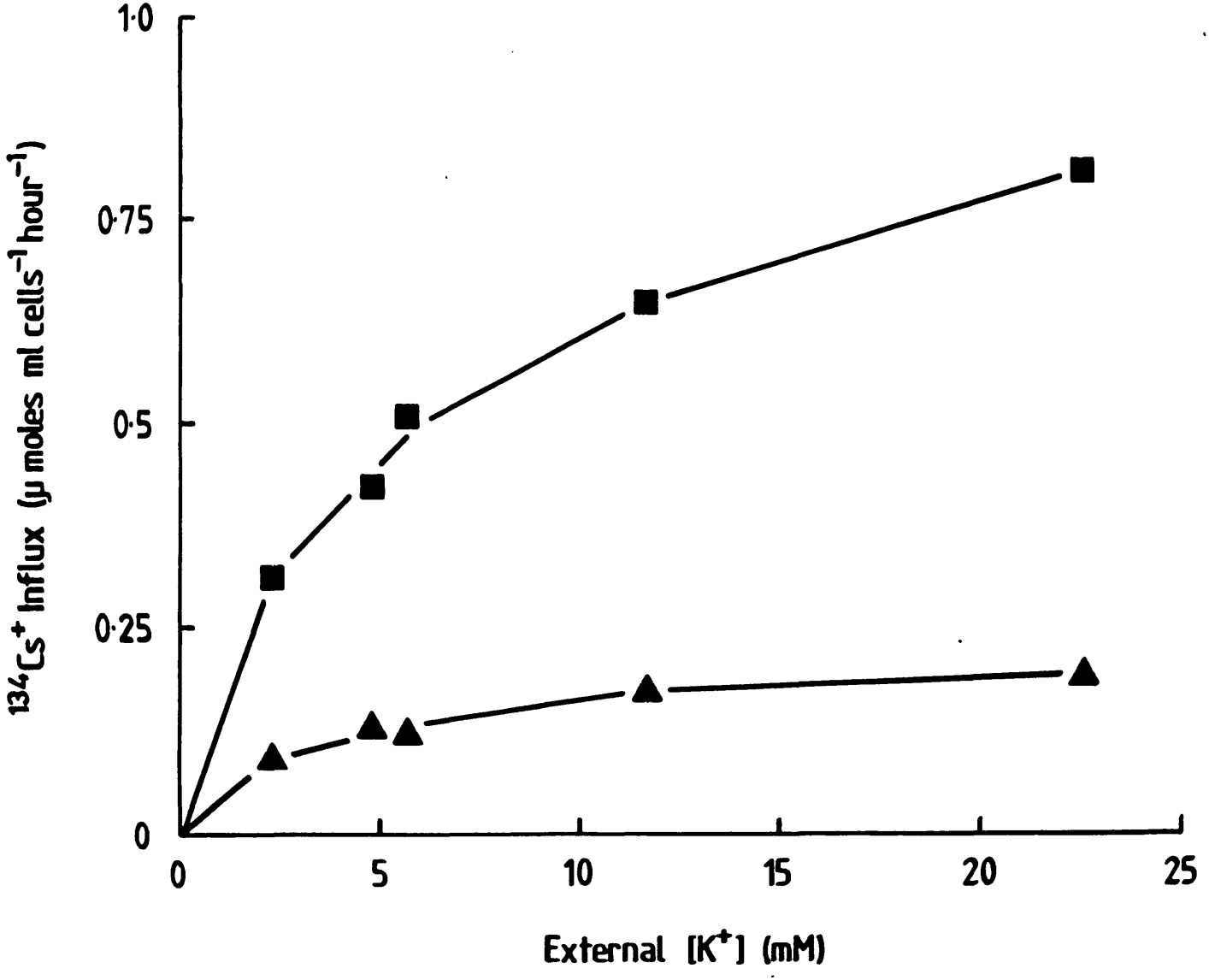

1·0
0·75
0·5
0·25
0
0
5
10
15
20
25
$^{134}Cs^{+}$ Influx (μ moles ml cells^{-1} hour^{-1})
External [K^{+}] (mM)

Figure 33. Activation of ouabain-sensitive $^{134}Cs^+$ influx into pig red cells by intracellular sodium.

Plots of results presented in Figure 32, fitted to the linear form of the activation equation of Garay and Garrahan (1973) - see text.

n, number of sites = 3.

(△) $[Cs^+]_o$ = 0.28 mM

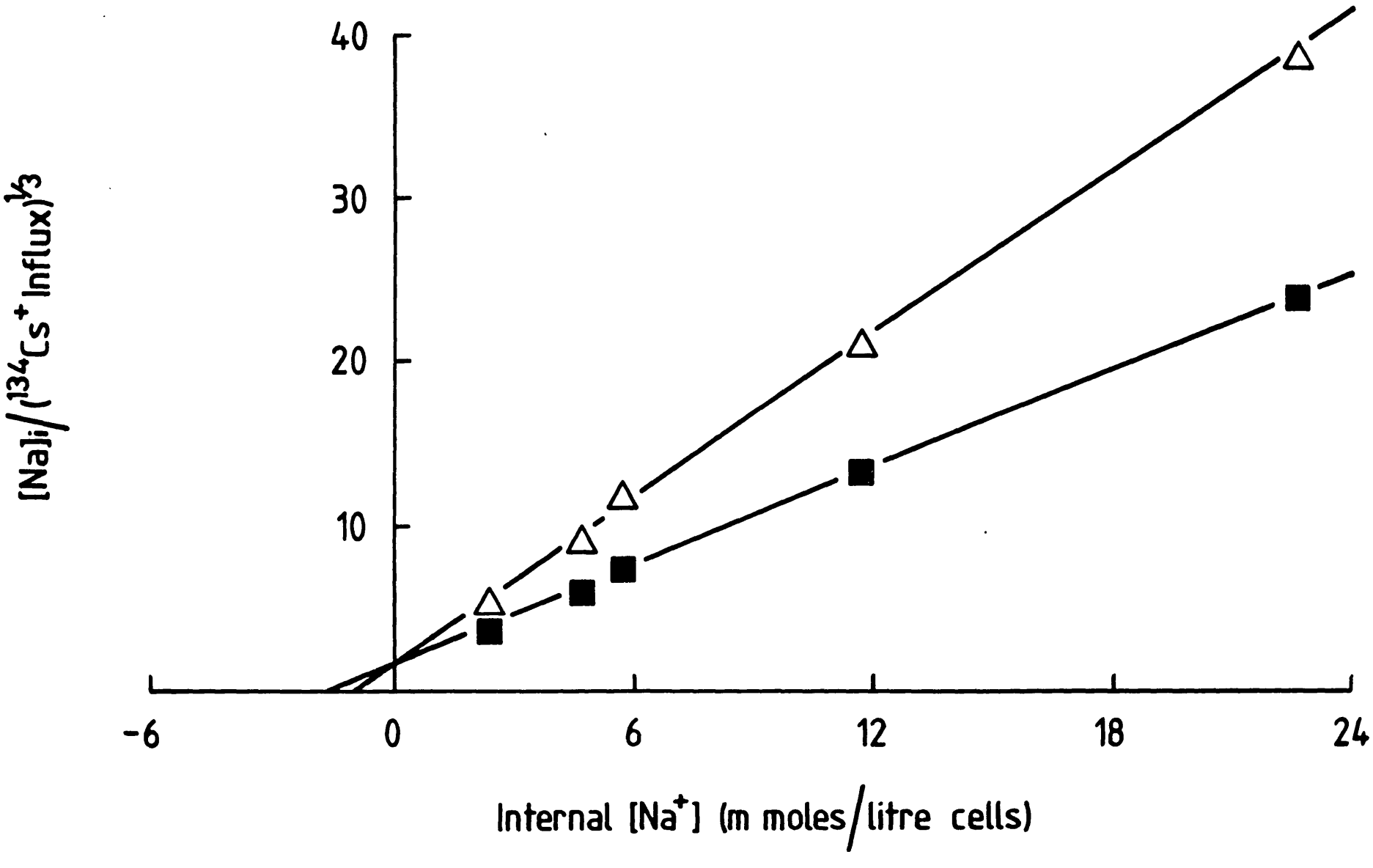
M'_{max} = 0.21 μmoles ml cells^{-1} hr^{-1}

K'_{Na} = 929 μM

(■) $[Cs^+]_o$ = 2.50 mM

M'_{max} = 0.96 μmoles ml cells^{-1} hr^{-1}

K'_{Na} = 1398 μM


40
30
20
10
-6
0
6
12
18
24
[Nali/(134Cs+ Influx)1/3
Internal [Na+] (m moles/litre cells)

III. INVESTIGATIONS AS TO THE EFFECT OF INTRACELLULAR MAGNESIUM LEVELS ON PUMP ACTIVITY

One of the problems associated with the consideration of the $(Na^+ + K^+)$-activated ATPase as a simple bisubstrate enzyme reaction, is that magnesium and ATP, in addition to intracellular Na^+ and external K^+ are also requirements for pump activation. The studies presented in Chapter I and II on the subject of Na^+:K^+ and Na^+:Cs^+ interactions with the pump are based on the maintenance of $[Mg.ATP]_i$ at adequate constant levels. Changes in $[ATP]_i$ are known to cause alterations in pump activity in human red cells (Whittam and Wiley, 1967). It has been further demonstrated, more recently, that a change in $[ATP]_i$ in squid axons from 50 μM to 5 mM causes an eight-fold increase in the apparent affinity for external potassium (Beaugé and Dipolo, 1978). This confirms the importance of the maintenance of ATP at adequate levels as previously described (see Methods - Figure 3).

Changes in $[Mg^{++}]_i$ are also known to cause alterations in pump activity confirming the need to maintain this cation at adequate intracellular levels. Following the loading of cells by the PCMBS loading procedure, routine observation of intracellular $[Mg^{++}]$ showed values within the range 1.5 - 3.5 mmoles/litre cells for human red cells. Results presented later in this section (Tables 26 and 27) suggest that levels of magnesium elevated above these cause pump inhibition. This suggests that adequate levels to act as a co-factor for ATP have been achieved.

Using reconstituted human red cell ghosts, Bodemann and Hoffman (1976b) have shown an effect of $[Mg^{++}]_i$ on ATP-promoted ouabain binding. It was proposed that lowering of $[Mg^{++}]_i$ level removed the

dependence of this ouabain binding on the combined presence of intracellular sodium and external potassium and created a situation in which external potassium could inhibit the rate of such binding in the absence of intracellular sodium. This was interpreted as indicating that relative affinities at cation sites on the external side of the membrane for sodium and potassium had been altered - either or both of an increase in external Na^+ affinity and a decrease in external K^+ affinity. This concept was further supported by evidence that at low $[Mg^{++}]_i$ levels the rate of Na:K exchange was more sensitive to changes in $[Mg^{++}]_i$ than was the rate of Na:Na exchange.

Further, work on PCMBS modified human red cells has shown (Wade, 1977) that magnesium internally was a non-competitive inhibitor of $^{24}Na^+$ efflux - that is, the maximum velocity was reduced as $[Mg^{++}]_i$ was raised, but the affinity for intracellular sodium remained unaltered.

The position thus stood that a transmembrane influence of $[Mg^{++}]_i$ on affinity for external cations had been suggested as a result of studies on ouabain binding to human red cell ghosts and a transmembrane influence of $[ATP]_i$ on K^+ binding externally, from studies using squid axons. Evidence has not appeared on this subject as a result of flux studies using modified red cells and it was therefore decided, particularly because of the importance assigned to the role of free magnesium in the original proposals for a consecutive mechanism (Fahn et al., 1966b) to perform investigations into the effect of $[Mg^{++}]_i$ levels on $^{42}K^+$ and $^{134}Cs^+$ influxes into human red cells modified by the PCMBS procedure.

III i) THE INFLUENCE OF $[Mg^{++}]_i$ ON EXTERNAL POTASSIUM BINDING

The PCMBS loading procedure, which was originally described (Garrahan and Rega, 1967) as a means of adjusting alkali metal cation

levels in red cells, has been adapted and subsequently characterized for modification of intracellular magnesium levels (see Methods). In order to test whether or not the $[Mg^{++}]_i$ level has any effect on external potassium binding it is necessary to have conditions of different $[Mg^{++}]_i$ but with $[Na^+]_i$ constant, because of the fact that it has already been established that, irrespective of $[Mg^{++}]_i$, a change in $[Na^+]_i$ will alter external potassium affinity (see Chapter I).

Results are presented from four experiments in which human cells were prepared of varying $[Mg^{++}]_i$ levels and saturating (in all cases > 12.3 mmoles/litre cells) $[Na^+]_i$. Duplicate $^{42}K^+$ influx measurements were then made into these cells both in the absence and presence of ouabain (10^{-4}M) and at least four $[K^+]_o$ levels. Resultant data suggested that activations at higher $[Mg^{++}]_i$ levels were not perfectly hyperbolic - values of V_{max} and K_m calculated, as described previously, by the statistical method (Wilkinson, 1961) had larger associated standard errors than seen previously. Consequently, results have been fitted to the linear form of the multiple-site rate equation of Garay and Garrahan:

$$M = M'_{max}/(1 + K'_K/[K^+]_o)^n$$

where M = flux at given potassium concentration $[K^+]_o$

M'_{max} = flux at non-limiting $[K^+]_o$

K'_K = apparent dissociation constant of the K^+-site complex

n = number of K^+ sites

Again best fits to the linear form occur with n = 2, confirming previous evidence on stoichiometry. Values of r for fits of such lines by least squares linear regression programme are quoted (Table 26).

Results show that in each of four experiments, elevation

TABLE 26. Values of kinetic constants for ouabain-sensitive $^{42}K^+$ influx into human red cells of varying $[Mg^{++}]_i$ level.

Kinetic constants were calculated from fits of the rate equation of Garay and Garrahan (1973) (see text) to data from duplicate estimations of $^{42}K^+$ influx in the presence and absence of ouabain (10^{-4}M).

Such flux measurements were made for at least four $[K^+]_o$ levels.

All cells were prepared to have $[Na^+]_i > 12$ mmoles/litre cells

$[K^+]_i < 0.5$ mmoles/litre cells

EXPERIMENT NUMBER	$[Mg^{++}]_i$ (μequiv. ml cells^{-1})	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_K (μM)	r for line fit (n, number of sites = 2)
I	4.07	2.78	145	1
	6.14	1.43	115	1
	11.94	0.49	66	0.99
	17.82	0.30	57	1
II	7.89	2.03	66	0.99
	10.54	1.27	54	1
III	10.38	2.01	84	0.99
	17.94	1.21	70	1
IV	2.38	1.53	42	1
	4.72	1.00	38	1
	19.20	0.32	45	1

Results from Experiment I are also presented as Figure 34.

Figure 34. Activation of ouabain-sensitive $^{42}K^{+}$ influx into human red cells by external potassium. Dependence on intracellular magnesium concentration.

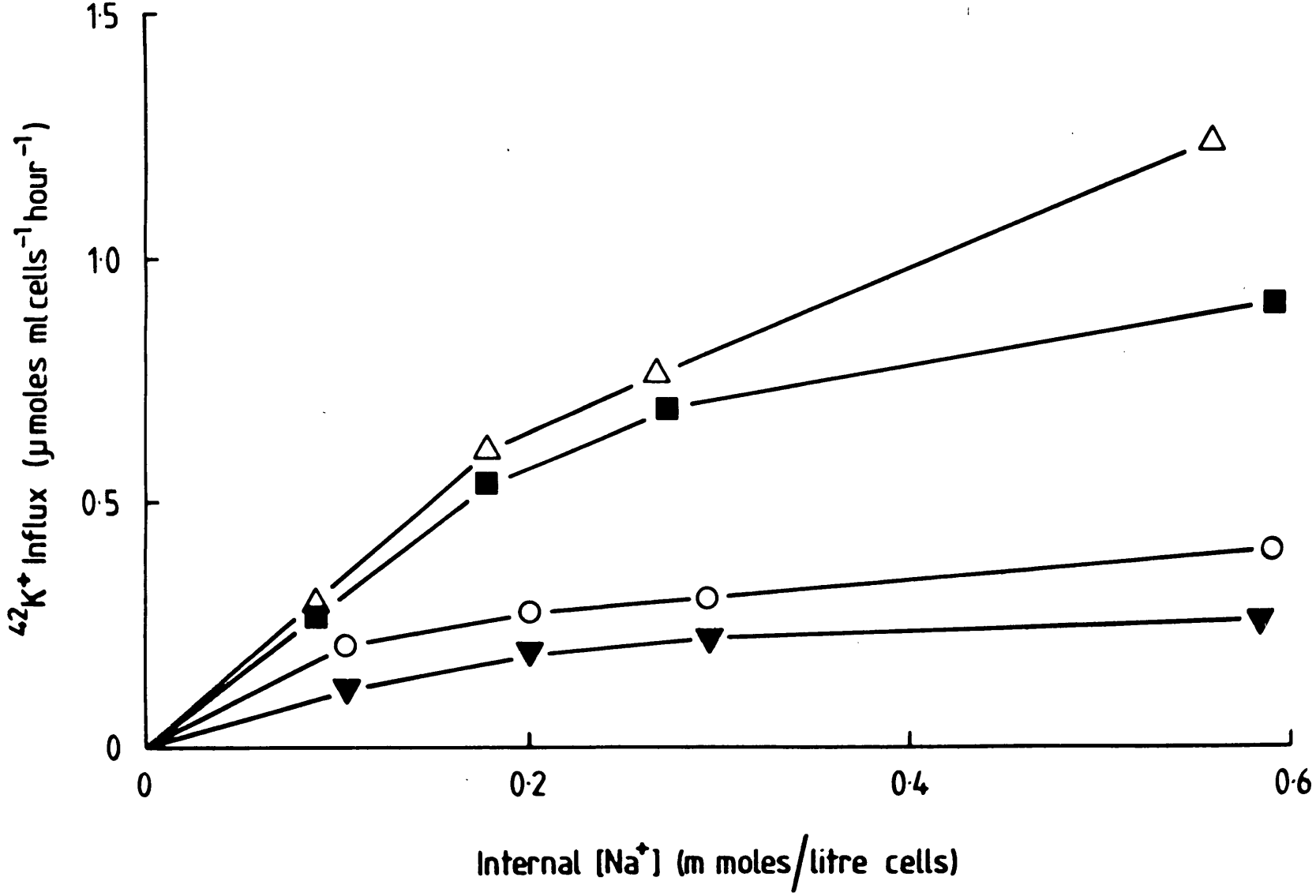
All points are calculated as the difference between the means of duplicate estimations in the presence and absence of ouabain (10^{-4}M).

(△) $[Mg^{2+}]_i$ = 4.07 mmoles/litre cells

(■) $[Mg^{2+}]_i$ = 6.14 mmoles/litre cells

(○) $[Mg^{2+}]_i$ = 11.94 mmoles/litre cells

(▼) $[Mg^{2+}]_i$ = 17.82 mmoles/litre cells


1·5
1·0
0·5
0
0
0·2
0·4
0·6
$^{42}K^+$ Influx (μmoles ml cells^{-1} hour^{-1})
Internal [Na^+] (m moles/litre cells)

of $[Mg^{++}]_i$ level causes a decrease in pump activity measured as $^{42}K^+$ influx (Figure 34) seen at each external potassium concentration. This is further noted as a decrease in calculated values of maximum velocity (M'_{max}). The decrease in M'_{max} seen varies from a 35% reduction as $[Mg^{++}]$ is raised from 2.38 to 4.72 mmoles/litre cells in one experiment, to a nine-fold reduction (2.78 to 0.30 μmoles ml cells^{-1} hr^{-1}) in another experiment where $[Mg^{++}]_i$ was raised by a factor of four (Table 26).

The inhibition of $^{42}K^+$ influx does not, however, appear to be a simple non-competitive type, as was that previously shown for $^{24}Na^+$ efflux. In three experiments out of four a simultaneous decrease in the value of the apparent dissociation constant (K'_K) was observed, representing an increased affinity for external K^+ at higher $[Mg^{++}]_i$ levels. Although in one experiment little change in K'_K value is seen (Expt. IV - Table 26), in three others a decrease was observed, which in one experiment was a 60% fall from 145 to 57 μM for a four-fold increase in $[Mg^{++}]_i$ from 4.07 to 17.82 mmoles/litre cells.

III ii) THE INFLUENCE OF $[Mg^{++}]_i$ ON EXTERNAL CAESIUM BINDING

Previous work presented in this Results section has shown a similar reponse of external Cs^+ binding, to modifications of intracellular conditions, to that seen when external K^+ binding was under investigation. Results from three experiments are presented (Table 27) which indicate a similar phenomenon here.

Cells were again prepared, by means of the PCMBS loading procedure, to have various $[Mg^{++}]_i$ levels, minimal (less than 0.5 mmoles/litre cells) $[K^+]_i$ and saturating (greater than 15 mmoles/litre cells) $[Na^+]_i$ levels. Duplicate $^{134}Cs^+$ influx measurements, using at least four

$[Cs^+]_o$ levels, were then made in the absence and presence of ouabain (10^{-4}M). Ouabain-sensitive fluxes were calculated as the difference between the mean of such paired flux estimations. Finally, values of kinetic constants were obtained from linear fits to the two site rate equation of Garay and Garrahan - see previous section. As seen in the previous section, values of maximum velocity (M'_{max}) decrease with rising intracellular magnesium levels. A two-fold reduction in M'_{max} is seen within each of three experiments. In addition, the inhibition of $^{134}Cs^+$ influx into human red cells by $[Mg^{++}]_i$ increases cannot be demonstrated to be a simple non-competitive type. In one experiment from three a 50% reduction in K'_{Cs} is observed, and in another a 30% reduction. Although further results are necessary to elucidate further the nature of the connection between intracellular magnesium levels and external caesium binding, the experiments presented here suggest that affinity for external Cs^+ increases at higher $[Mg^{++}]_i$ levels (Table 27).

TABLE 27. Values of kinetic constants for ouabain-sensitive $^{134}Cs^+$ influx into human red cells of varying $[Mg^{++}]_i$ levels. Kinetic constants were calculated from fits of the rate equation of Garay and Garrahan (1973) (see text) to data from duplicate estimations of $^{134}Cs^+$ influx in the presence or absence of ouabain (10^{-4}M).

Such flux measurements were made for at least four $[K^+]_o$ levels. All cells were prepared to have $[Na^+]_i > 15$ mmoles/litre cells

$[K^+]_i < 0.5$ mmoles/litre cells

EXPERIMENT NUMBER	$[Mg^{++}]_i$ (μequiv. ml cells^{-1})	M'_{max} (μmoles ml cells^{-1} hr^{-1})	K'_{Cs} (μM)	r for line fit (n, number of sites = 2)
I	3.51	0.43	181	0.99
	6.21	0.23	93	1
II	3.26	0.79	168	0.99
	7.93	0.72	160	0.99
	21.69	0.39	161	1
III	6.15	0.61	211	1
	20.83	0.23	145	1

DISCUSSION

The question which this thesis attempts to answer is whether the sodium pump operates by a one-step or a two-step mechanism. The approach used has been to discover whether the ions activating the pump (Na^+ and K^+ or Cs^+) bind simultaneously or at different times in the pump cycle, by investigating whether or not there exists an influence of one activating cation on the affinity for the other. As described earlier (see Introduction), a one-step mechanism would require simultaneous binding of Na^+ ions internally, K^+ ions externally and the Mg.ATP complex (Whittam and Chipperfield, 1973). A two-step (consecutive or ping-pong) mechanism, however, would be characterized by some product releasing step or steps occurring before another substrate binding step or steps.

At the time of commencement of these studies (1976), the evidence for simultaneous attachment of ions at opposite sides of the membrane had not been conclusive, probably because of the difficult nature of the experimental design involved. Although biochemical evidence had been put forward favouring a simultaneous transport mechanism (Lindenmayer et al., 1974) it should be noted both that these authors had assumed independence of sites and that a simultaneous transport mechanism had yet to be satisfactorily reconciled with the evidence on phosphorylated intermediates.

Choice and design of experimental systems

As stated in the Introduction, one approach to the elucidation of function of the ($Na^+ + K^+$)-activated ATPase is to obtain biochemical information from particulate enzyme preparations. For studies with possible kinetic significance, however, it is necessary to

use a property of the enzyme *in situ* in a cellular membrane - that is the asymmetry seen as selection of K^+ ions from a medium, where $[K^+] < [Na^+]$, for inward transport and of Na^+ ions from an intracellular environment, where $[Na^+] < [K^+]$, for outward transport (Whittam, 1962). The giant axon of the squid presents one possibility for such studies and is a system which has been used considerably in the past. A need exists, if the activation curves for cations acting at sites facing the cell interior are to be constructed, to be able to adjust the chemical composition of this intracellular environment. This was established for the squid giant axon by a method involving dialysis of an internal solution through a glass capillary tube (Brinley and Mullins, 1967) and was subsequently used for studies into effects, on the influx and efflux of sodium, of various high-energy phosphate compounds and of changes to the $[Na^+]_i$ level (Brinley and Mullins, 1968). The use of such a preparation to investigate the sidedness of ligand interactions with the sodium pump has been demonstrated in recent publications showing a trans-membrane influence of ATP concentration on external potassium affinity (Beaugé and Dipolo, 1978, 1979, 1981). The availability of squid giant axons remains, however, a major obstacle to their use for the studies being carried out here. No such problems of availability exist when using the red blood cells of either man or pigs. The justification for the use of such systems can only be achieved by an adequate characterization of the method to be used to alter intracellular ionic composition. Fragility of cells following resealing, ability to synthesize and maintain intracellular ATP levels and consistency of ionic conditions achieved have all been investigated (see Methods). The fragility experiments described suggest that membrane integrity and leakiness to cations are not impaired by the loading process. Further, ATP levels within cells are maintained for periods of at least one hour, during

incubations in K^+-rich solutions which would support a high rate of pump activity. Thus, although there is the disadvantage that the cellular system obviously has a much lower level of $(Na^+ + K^+)$-activated ATPase activity than does a purified preparation, such a disadvantage is not of great significance when metabolic conditions can be satisfactorily maintained over the period of flux measurement, particularly as there is no need for an oxygen supply to the system.

The area of greatest concern with the use of the PCMBS-modified red cell remains the reproducibility of intracellular ionic conditions. Clearly this system does not have the defined accuracy of a dialysis or perfusion technique in which an internal solution of the required concentrations is produced. The experiments to be conducted, however, required that conditions inside the cell be substantially altered from the normal ones - physiological conditions of high $[Na^+]_o$ and $[K^+]_i$, through the competitive effects of these ions (which are products of pump function) actually preclude investigations into the influence of one substrate on activation by the other. As most cellular systems possess a high intracellular potassium level, this is not a problem peculiar to the red cell. The results presented in the Methods section show that the manipulation of such intracellular ionic conditions by the PCMBS procedure, whilst achieving effective voiding of most K^+ from cells, does produce varying responses in different batches of blood as to the $[Na^+]_i$ levels produced. This problem of reproducibility has been circumvented by carrying out a substantial number of experiments and considering the response to changes in $[Na^+]_i$ within each. The results presented demonstrate that responses to such changes within each individual experiment are generally consistent, even though comparison of results from cells with similar $[Na^+]_i$ levels from different experiments may show different values for the kinetic constants for fluxes measured.

Section I.

Simultaneous attachment of ions - implications for mechanism.

Kinetic studies

In an early important theoretical kinetic analysis (Baker and Stone, 1966) it was suggested that results from studies on red cells and crab nerve could favour an ordered reaction mechanism, one component of which was the reaction of K^+ at a single step after which Na^+ did not act. Referring to previous studies on frog muscle (Keynes, 1965) it was noted that elevation of $[K^+]_o$ from 2.5 mM to 20 mM did not produce a detectable change in the internal sodium affinity. It should be noted that the major external cation in these experiments was Li^+, an unfortunate choice as this cation had already been demonstrated, in experiments on red cell ghosts, to act in the same way as Na^+ at internal sites and to stimulate at the exterior with a half-maximal concentration of 20 mM (Whittam and Ager, 1964). As the [LiCl] in efflux solutions was greater than 100 mM, some competition between this cation and potassium at the external levels being investigated might have precluded possibilities of observing any affinity changes - the presence of such a competitive agent causes abscissa intercept effects on double reciprocal plots and leads to false affinity values being obtained (Cleland, 1970). Baker and Stone also referred to the fact that results from two studies on red cells suggested that there might be an affinity effect operating across the membrane and stressed the importance of experiments designed to investigate whether or not such a connection exists.

It has already been stressed that there is considerable importance attached to the question of experimental design. There is evidence from several sources of apparent independence of sites on

opposite sides of the membrane. In each case, however, the experimental conditions had not been designed to eliminate the competitive effects introduced by either external Na^+ or intracellular K^+ or both (Hoffman and Tosteson, 1971; Garay and Garrahan, 1973; Garrahan and Garay, 1974; Chipperfield and Whittam, 1976). The competitive effects exerted by any product present would mask any observed dependence of external cation affinity on intracellular sodium concentration, as seen in results of experiments on human red cell ghosts (Chipperfield and Whittam, 1974). Despite this, a substantial amount of evidence now exists to suggest that there is simultaneous attachment of ions on opposite sides of the membrane. Such evidence comes from experiments where competitive effects on either side of the membrane have been minimised. The results presented in Section I of Results here demonstrate a dependence of external cation (potassium or caesium) affinity on $[Na^+]_i$ levels. These results have consistently applied in all experiments performed on either pig or human red cells. Further, during the course of these studies, a similar result has appeared in the literature from a study carried out elsewhere on human red blood cells (Sachs, 1977). The results published here and in the work of Sachs confirm that this affinity effect does not only apply to a comparison of the pump in a saturated and a non-saturated mode of operation, but also is observed when two non-saturating $[Na^+]_i$ levels are compared. In each case elevation of $[Na^+]_i$ causes not only an increase in the amount of K^+ or Cs^+ ions transported (at all levels - there is also an increase in the V_{max} value) but also an increase in the K_m value and the K'_K or K'_{Cs} value, representing a decrease in external cation affinity. These results answer the previous suggestion (Garrahan and Garay, 1974) that such affinity changes would be best seen at non-saturating levels of cations on either side of the membrane. Still more recently, a set of results has been published from experiments

on a porcine kidney enzyme preparation. These results (Yamaguchi and Tonomura, 1980) support the view that there is simultaneous binding of $3Na^+$ and $2K^+$ or $2Rb^+$ ions to the enzyme. These studies use a more direct method to establish binding levels - a membrane filtration method (Yamaguchi and Tonomura, 1979) involving exposure of the enzyme to radio-labelled solutions containing ^{3}H-D-glucose and cations, at 0^oC, for 30 minutes followed by filtration and comparison of radioactivity ratios of $^{22}Na^+/^3H$ or $^{42}K^+/^3H$ from experiments with and without enzyme. This allows the calculation of the amounts of $^{22}Na^+$ or $^{42}K^+$ bound. Results demonstrated the simultaneous existence of two types of site characterized by markedly different relative affinities for Na^+ and K^+.

The implications from results of this type for mechanism are clear: any unitary mechanism (that is, one based upon the existence of only one set of sites) is excluded. The question is thus formed as to whether this evidence can be reconciled with the pump mechanisms previously proposed, involving phosphorylated intermediates.

Occluded sites - 'flip-flop' mechanisms.

Earlier biochemical studies on a purified enzyme preparation of guinea pig kidney had revealed that various K^+ congeners, such as Rb^+, could form a relatively stable complex with a dephosphorylated form of the enzyme (Post et al., 1972). The formation of this complex was taken as being of mechanistic significance: it is suggested that there was an occluded or closed form of the enzyme's site for K^+ ion transport. Two groups of workers have postulated a mechanism which could involve such occluded enzyme forms (Stein et al., 1973; Repke and Schön, 1973; Repke et al., 1974).

The model of Stein and co-workers was based upon a tetrameric protein structure with both low and high affinity Na^+-sites existing at the same time on both sides of the membrane. In this scheme, the transport of cations would involve two energetically similar conformations of the the enzyme: simultaneous binding of Na^+ and K^+ would occur, followed by a "flipping" of the enzyme bringing the ions to the interior cavity of the enzyme structure. This process would involve both a change in the position of the sub-units of the tetramer and a change in their affinity from 'favouring Na^+' to 'favouring K^+' or vice versa. The ions would then redistribute as governed by this affinity change and a subsequent 'flop' of the enzyme back to the original conformation would facilitate their release on the opposite sides of the membrane. A further feature of this system was that each pair of sub-units would undergo the same process, but 180^o out of phase. This feature, known as 'half-of-the-sites reactivity', was used to explain the observation that only one phosphate from ATP could be retained by the tetramer at any time - one of the sub-units would be phosphorylating as the other was undergoing dephosphorylation.

It should be stressed, however, that although at first sight a model of this type may appear to provide a satisfactory means of reconciliation between the biochemical evidence described earlier and the evidence for simultaneous attachment of ions on opposite sides of the membrane, this is by no means a universally accepted position. Studies on an enzyme preparation from kidney, using a fluorescence technique involving the fluorescent analogue of ATP, formycin triphosphate (Karlish et al., 1978a) have led to the suggestion that the occluded form of the enzyme containing K^+ was in fact formed by the binding of K^+ at low affinity sites, which could be the internal sites at which K^+-inhibition of ATP binding is mediated (Karlish et al., 1978b). This

suggestion contrasts with the model of Stein et al., in that the occluded K^+ in that model arises from binding at high K^+-affinity external sites. Further, important recent studies on the phosphorylation capacity of an enzyme preparation have shown that each of the two sub-units which can be phosphorylated can undergo this process simultaneously - a fact which rules out the prospect of a 'half-of-the-sites' mechanism (Peters et al., 1981).

Reconciliation of biochemical and kinetic evidence.

Although the evidence from flux studies presented in Sections I and II of the Results section of this thesis strongly supports a simultaneous attachment of ions, the problem remains as to how such evidence could be combined with biochemical results elsewhere. A 'half-of-the-sites' mechanism has the advantage that it allows for both the co-existence of Na^+ and K^+ sites and the formation of a phosphorylated intermediate which undergoes conformational changes. A further attempt at combination of these ideas has arisen from a study on ouabain binding in erythrocytes of man and sheep (Joiner and Lauf, 1978). These authors have shown that the rate of ^{3}H-ouabain binding to red cells was directly related to K^+ pump activation, a result which contrasts with that of some previous workers (Bodemann and Hoffman, 1976a). This result has been taken to imply a conformational change during the pump cycle and represents the first evidence from an intact cellular system on this point. To explain that this binding is stimulated by intracellular Na^+, inhibited by external K^+ and that its rate is dependent on the level of pump activity, Joiner and Lauf propose that external K^+ binding must occur after the binding of ouabain initiated by Na^+. Further, in a recent review published at the time that these experiments were being

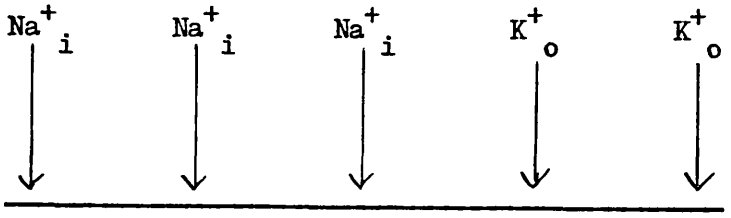
completed (Robinson and Flashner, 1979), it was stated that one possibility which might allow the fitting of data to a consecutive type of model would be a later suggestion of Sachs (1979) that a cyclical pump operation about the first form of phosphorylated intermediate, allowing the uncoupled efflux of sodium mode, might apply during normal pump operation. Consequently, Robinson and Flashner (1979) have suggested that the fitting of flux data may not facilitate a distinction between unitary and binary mechanisms. The problem remains that biochemical evidence cannot always be readily accounted for by the observed behaviour of ion movements in an intact cellular system. As recently as 1975, a study on an isolated enzyme system from guinea pig kidney has suggested that a reversed pump mode via K^+-sensitive then ADP-sensitive phosphorylated intermediates is possible - this has been taken as further evidence that there is a separation of the steps at which the two ions act (Taniguchi and Post, 1975).

These considerations lead to what is clearly the critical question remaining open: the timing of K^+ binding to the pump.

Section II. Random and fixed order mechanisms

A distinction between simultaneous and consecutive transport processes or between unitary and binary mechanisms does not provide a complete answer to the question of how the sodium pump operates. A further question can now be asked. Do Na^+ and K^+ ions bind to the pump in a fixed order or randomly?

The conclusion of Joiner and Lauf described above is that, although a simultaneous transport process can occur, the evidence favours a fixed order of attachment, thus: -



Application of bisubstrate kinetics

Support for the fixed order of attachment has come from the work of Sachs (1977) on human red cells. This author demonstrates an effect of $[Na^+]_i$ at low levels, on external K^+ and Cs^+ affinity - consequently a simultaneous mechanism is favoured. A kinetic analysis involving consideration of the effect of changes to $[Na^+]_i$ on the V_{max}/K_m ratio for influx is included and is used to suggest that there is a fixed order of addition. Before discussing these results in relation to those presented here, it is probably wise to mention the limitations of the kinetic approach. Apart from the important premise that kinetic information should be used to discount possible mechanisms and cannot be used to prove them (Segal et al., 1952), there is the problem posed by the fact that multiple-site activation equations have had to be applied to data obtained (see Sections I, II of Results; also, Garay and Garrahan, 1973) rather than simple Michaelis-Menten kinetics, when considering activation by intracellular Na^+. Further, application to this enzyme system of enzyme-substrate-modifier kinetics as presented previously (Whittam and Chipperfield, 1975) should carry the precaution that it is only possible, not certain, that such an analysis is valid for a two or three substrate system (Frieden, 1964).

The kinetic approach used by Sachs (1977) should now be discussed: it is based upon consideration of the $(Na^+ + K^+)$-activated ATPase as a bisubstrate reaction - for which it is necessary to assume and achieve constant $[Mg.ATP]$ levels. The analysis presented in 1977 was based on data from a $^{42}K^+$ influx experiment on human red cells. These cells had been modified by a PCMBS loading procedure to produce eight different $[Na^+]_i$ levels within the range 1.07 - 7.96 mmoles/litre cells. A single experiment varying $[Na^+]_i$ over six values within the range 1.20 - 12.81 mmoles/litre cells and using caesium externally (measurement of $^{137}Cs^+$ influx) was also presented. In each case the results show the same effects as do those presented in Section I here: increases in $[Na^+]_i$ produce both an increased number of K^+ or Cs^+ ions transported and also a decreased affinity for these ions externally. Sachs also proposes that on each occasion that $[Na^+]_i$ is raised, not only do the values of V_{max} and K_m for cation influx both increase, but also the value of the V_{max}/K_m ratio is raised. Results obtained in these studies do not concur (see Section I (vi)). It was decided, because of the previously discussed problems in reproducing pre-determined $[Na^+]_i$ levels when using the PCMBS cell loading procedure, to conduct a substantial number of experiments. The results obtained, at lower $[Na^+]_i$ levels, from four types of experiment involving human and pig red cells with K^+ and Cs^+ as external activating cations, did not produce any consistent trend with regard to the behaviour of the V_{max}/K_m ratio for influx at different $[Na^+]_i$ levels (Tables 20, 21; Figure 22). A total of sixteen experiments are involved in this consideration, suggesting that there is some danger in the formation of conclusions as to any significance of a trend seen in one single experiment. Finally, a previous result of Chipperfield and Whittam (1976) has been confirmed here: that for changes of $[Na^+]_i$ from non-saturating to saturating

levels, the ratio actually deereases in value as $[Na^+]_i$ is raised (see Table 13).

Sachs has pointed out that there is a problem with the application of simple bisubstrate kinetics to this enzyme system, because of the fact that there is a stoichiometry (confirmed by fits of the flux data obtained here to multiple-site activation equations) of $3Na^+$ out: $2K^+$ or Cs^+ in. The analysis presented, however, is based upon the assumption that the pump may transport inwards when only loaded with one K^+ or Cs^+ ion externally - yet data here suggest that better fits to the influx data are obtained with n (number of sites) = 2 than n = 1. Further, the distinction between simultaneous and ping-pong mechanisms is based upon assumptions, that are said to be unlikely, concerning the equality of rate constants of certain partial reactions and the constants for binding for the two K^+ sites. Nevertheless, if the analysis presented is justified, the data of Sachs clearly fits a simultaneous model more readily than a consecutive one. It is also argued that the evidence favours an ordered reaction with Na^+ binding before K^+, based upon an assessment of the shape of activation curves, at various $[Na^+]_i$ levels, for external K^+ - a further extension of the application of bisubstrate kinetics.

How valid then, is the application of such a kinetic treatment? Analysis of the effects of alteration of the concentration of one substrate (Na^+) on the V_{max}/K_m ratio for activation by the other (K^+), suggests the following (Laidler and Bunting, 1973): -

(1) If V_{max}/K_m ratio remains constant - that is, if double reciprocal plots are parallel, then a special case exists where the ping-pong mechanism is possible.

(2) If V_{max}/K_m ratio for substrate A (K^+) increases as $[B]$ (Na^+) is raised, then an intersection of double reciprocal plots is observed below the abscissa and to the left

of the ordinate. Such a phenomenon is consistent with a simultaneous model.

It should be noted that in their treatment of bisubstrate kinetics, the same authors state that consideration of activation patterns alone is fraught with sufficient complications to make the use of other information, such as that from inhibition studies, almost essential for the formation of any conclusions. In addition, during another treatment of bisubstrate enzyme kinetics it has been noted that if a third substrate could add between the two under consideration, then the kinetic characteristics observed could be falsely changed from those of a simultaneous enzyme mechanism to those of a ping-pong one (Segel, 1975). Clearly, if a random order mechanism applies, Mg.ATP could, in theory, add between Na^+ and K^+ creating precisely this problem. Further, no technical reason for the difference between the results of Sachs described above and those presented in Section I (vi) is, as yet, clear. Consequently it seems safest to state that a kinetic analysis of this type, based upon the application of bisubstrate kinetics, may be unsafe for mechanistic considerations.

An alternative, experimental approach.

For a system which operates such that the binding of Na^+ ions internally and K^+ ions externally is not of a fixed order, but rather random, then just as an influence of intracellular sodium level on the affinity for external potassium has been observed, it would be possible that a reverse influence might apply - that is, an influence of external potassium (or caesium) level on the affinity for intracellular sodium. This reverse transmembrane influence seems unlikely, however, if the order of binding is fixed with sodium being exclusively bound before external cations.

When experimental conditions are designed so as to investigate non-saturating levels of external K^+ and Cs^+ and the influence, if any, that changes in these concentrations have on the attachment of sodium ions on the opposite (intracellular) side of the membrane, then the following conclusions can be drawn: -

(1) In each of the four systems tested, for any given intracellular sodium level there was an increase in pump activity as $[K^+]_o$ or $[Cs^+]_o$ was raised. This applies even in conditions of pump saturation internally.

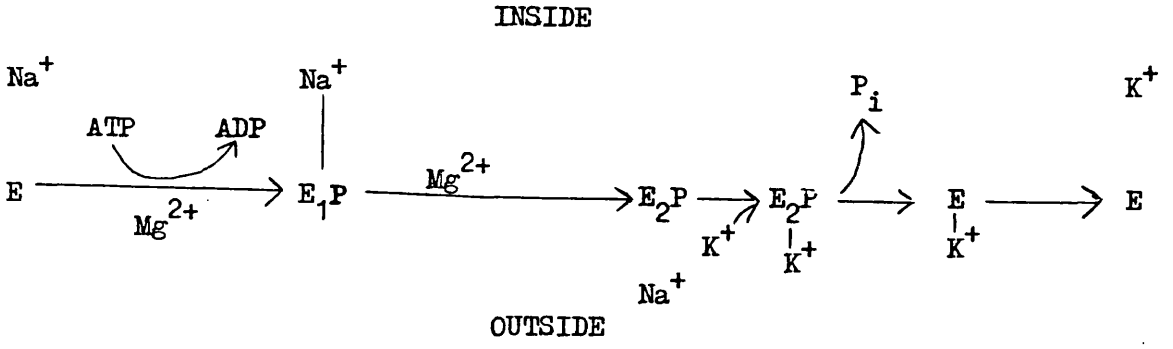
(2) The affinity for intracellular Na^+ is raised as the external cation concentration is reduced - this effect is seen as a decrease in the value of the apparent dissociation constant for the Na^+-site complex (K'_{Na}).

(3) Activation curves for intracellular sodium do <u>not</u> give good fits to the hyperbolic activation of the Michaelis-Menten equation, but give very good fits to a three-site rate equation (Garay and Garrahan, 1973).

In other words, the interdependence of sites implied by consideration of results presented earlier concerning the influence of $[Na^+]_i$ on external cation binding, has been clearly shown not to be a "one-way" process. If this is so, then bearing in mind the problems involved with the application of a bisubstrate kinetic analysis to this system, the conclusion from this section of the work is that any ordered process, by which either Na^+ or K^+ is bound to the pump exclusively before the other, is not compatible with these results, simply because it appears impossible for the level of attachment of an ion at the outside of the membrane to influence the affinity of intracellular sites for Na^+, an ion which in such ordered mechanisms would be bound <u>exclusively before</u> K^+ attachment.

Section III. The role of magnesium

In the two-step (ping-pong) mechanism as proposed by Fahn *et al.* (1966a):



magnesium has a dual role. Firstly it is required as a co-factor for ATP (see Introduction) and secondly to stimulate the conversion of the ADP-sensitive conformation of the phosphorylated intermediate (E_1P) to the K^+-sensitive form (E_2P).

This early proposal as to the second role of magnesium (that of free Mg^{2+}) has been subjected to some question. Firstly, in studies on a brain ATPase preparation, using ^{32}P-labelled ATP to follow phosphoenzyme formation, it was discovered that an ADP-insensitive phosphoenzyme was formed before the ADP-sensitive form (Fukushima and Tonomura, 1973). Secondly, in later experiments on a brain enzyme preparation, it has been shown that the two forms of phosphoenzyme described above have a different sensitivity to ADP and to K^+ - but not, as proposed previously, due to the different Mg^{2+} levels used, rather because of an effect of the chelating agent EDTA (Klodos and Skou, 1975). Further, the alternative chelator CDTA, as used in the experiments of Post *et al.* (1969), was demonstrated to be unsatisfactory as its reaction with Mg^{2+} is too slow - consequently a fast removal of Mg^{2+} could not be achieved and there would be a transient free Mg^{2+} component present. This would aid phosphorylation of the enzyme and consequently produce

false values of dephosphorylation rates (Klodos and Skou, 1977). Another source of evidence to question this Mg^{2+} role in the pump mechanism was the discovery that N-ethylmaleimide (NEM) blocked all partial reactions and the overall reaction of the ATPase of a purified preparation from rabbit kidney outer medulla (Schoot et al., 1977). This contrasts with the previously reported view that NEM simply blocked the conversion of an ADP-sensitive phosphorylated intermediate to a K^+-sensitive one - the conversion of E_1P to E_2P, which was stimulated by free Mg^{2+} (Fahn et al., 1966b).

As with the investigations of interactions between monovalent cations and the pump, an alternative approach to the biochemical investigations discussed above is the use of flux studies. Previous studies from this laboratory demonstrated an inhibition of ouabain-sensitive sodium efflux at raised free $[Mg^{2+}]$ levels inside red cells (Wade, 1977), a result which was also confirmed in biochemical studies of an ATPase preparation of ox brain. It has also been subsequently reported that the elevation of $[Mg^{2+}]_i$ above levels of approximately 10^{-3}M causes progressive inhibition of Na:K and Na:Na exchange in red cells (Flatman and Lew, 1979). These authors have extended their studies recently (Flatman and Lew, 1981) and include discussion of the possibility that, at higher levels, magnesium may cause the formation of a non-reactive form of the second phosphorylated intermediate. This proposal had previously received support from two other authors (Wade, 1977; Forgac, 1980).

The investigations reported here (Results III) show two effects. Firstly, it is confirmed that elevation of $[Mg^{2+}]$ inside the human red cell produces an inhibition of pump activity measured as ouabain-sensitive $^{42}K^+$ or $^{134}Cs^+$ influx. This inhibition is seen at each $[K^+]_o$ or $[Cs^+]_o$ level tested, including saturating concentrations

- in other words, the V_{max} value for influx is also reduced. Secondly, the pattern of inhibition seen is different from that reported for the inhibition of sodium efflux at elevated $[Mg^{2+}]_i$ levels (Wade, 1977). In those studies the inhibition reported was of a non-competitive type: changes in $[Mg^{2+}]_i$ levels did not produce any change in intracellular sodium affinity. The inhibition pattern here was clearly mixed - both V_{max} and K_m values for $^{42}K^+$ and $^{134}Cs^+$ influx were altered by changes in the $[Mg^{2+}]_i$ level. This result is consistent with one reported previously (Bodemann and Hoffman, 1976b) suggesting that the different sensitivities of Na:Na and Na:K exchange to changes in $[Mg^{2+}]_i$ could reflect a change in the relative affinity for these two ions externally.

Although it should be stressed that the results presented do not provide for an assessment of the components of free and bound magnesium within cells, a problem which has been discussed elsewhere (Flatman and Lew, 1980), they do indicate that changes in $[Mg^{2+}]_i$ levels, induced simultaneously on the same batch of human blood, can lead to changes in the affinity for <u>external</u> cations. It is felt that this result suggests that free magnesium must be bound to the enzyme at the time that potassium and caesium are bound externally. As stated previously, the original results favouring a role for magnesium in the conversion of E_1P (ADP-sensitive phosphorylated intermediate) to E_2P (K^+-sensitive phosphorylated intermediate) has been called into question. In a further investigation into the effects of divalent cations on pump phosphoenzyme, Fukushima and Post (1978) have proposed that magnesium may, in fact, rather than being responsible for an $E_1P \longrightarrow E_2P$ conversion step, be bound to all forms of the phosphoenzyme. The results presented here would be permitted by such a scheme.

CONCLUSION

The conclusion from the studies presented here is that evidence is presented to indicate that ligands binding to the $(Na^+ + K^+)$-activated adenosine triphosphatase (E.C. 3.6.1.3.), the enzyme which has been established to be responsible for active extrusion of Na^+ ions from and inclusion of K^+ ions into cells, can influence the affinity for attachment of other ligands on the opposite side of the membrane. This type of transmembrane influence, a further example of which is the influence of $[ATP]$ internally on external potassium affinity recently demonstrated by Beaugé and Dipolo (1978, 1979, 1981), has been demonstrated in three ways during the course of the studies presented here:

(1) An influence of intracellular $[Na^+]$ on external cation affinity. This was demonstrated for both K^+ and Cs^+ externally, in experiments on both human and pig red cells.

(2) An influence of $[K^+]$ and $[Cs^+]$ externally on intracellular sodium affinity in both human and pig red cells.

(3) An influence of $[Mg^{2+}]_i$ on external K^+ and Cs^+ affinity in the human red cell.

This evidence is taken to signify that the attachment of Na^+ and Mg^{2+} internally cannot occur exclusively before the attachment of external K^+ or Cs^+. Equally, K^+ or Cs^+ cannot attach exclusively before either Na^+ or Mg^{2+}. These two requirements, deduced from experiments carried out in the intact cell systems described, when taken together with the evidence of Whittam *et al.* (1976), which demonstrates that enzyme preparations from fragmented membranes which are prepared in different ways may lead to inconsistency of results obtained in relation to pump reactions and mechanism, lead to the

conclusion that a mechanism which is based on biochemical evidence from enzyme preparations alone should be only considered satisfactory if able to account for data obtained in the intact cell. In this case the data from intact cell studies favour a mechanism where the attachment of intracellular Na^+ ions and of external K^+ or Cs^+ ions is simultaneous, as is that of the Mg.ATP complex. The evidence is further consistent with the order of such attachments to the pump being random.

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ABSTRACT

THE INTERACTION OF CATIONS WITH THE SODIUM PUMP IN RED BLOOD CELLS by

S.C. BRAND

An investigation has been carried out into the interactions of some cations with the sodium pump of red blood cell membranes. Two mechanisms for this system have been proposed previously: one is a two-step (consecutive) mechanism in which sodium ions are transported in one step and then potassium ions in a separate, second step. An alternative mechanism involves simultaneous attachment and transport of both ions.

The experiments presented involve studies of pump activation by either reactant at various concentrations of the other. A requirement for these studies is that products of the reaction be absent. This indicates the use of a cellular system with its inherent asymmetry and the modification of such a system to allow changes in intracellular ionic composition. This has been achieved with human and pig red blood cells modified by a cation loading procedure involving the sulphydryl inhibitor p-chloromercuribenzene sulphonic acid. Pump activity has been measured mainly as the ouabain-sensitive influx of $^{42}K^{+}$-labelled potassium and $^{134}Cs^{+}$-labelled caesium, which is a congener for potassium.

The results presented in the first section of the thesis confirm and extend a previous result showing that the affinity for external potassium or caesium is dependent on the intracellular sodium concentration. The second section of results demonstrates that the reverse trans-membrane influence applies - that is, a dependence of intracellular sodium affinity on the extracellular cation concentration.

These results, together with those in a third section which demonstrate an influence of intracellular magnesium levels on external cation binding, are consistent with and explicable in terms of not only simultaneous binding of sodium, potassium or caesium and magnesium to the pump, but also a random order of attachment.