SODIUM TRANSPORT IN PREMENSTRUAL SYNDROME:

LEUCOCYTES AND PLATELETS AS MODEL CELLS

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ABSTRACT

The aim of this project was to study the sodium pump in premenstrual syndrome (PMS). As most women with PMS complain of "bloatedness" and water retention, researchers have tried, without much success, to establish scientific proof for this phenomenon especially since changes in the sodium pump activity have been reported in diseases where water retention occurs. Also there is evidence that ovarian hormones influence the sodium pump and cyclical changes in these hormones occur in PMS. Thus, there are several links between PMS and the sodium pump.

An ex vivo study was carried out using leucocytes to assess sodium transport during the follicular and luteal phases of the menstrual cycle in women with PMS and normal controls. The results showed a significantly decreased pump activity and increased percentage of intracellular water in the luteal phase of women with PMS.

Since ovarian hormones may play a role in PMS we attempted to investigate their effect on the sodium pump, in vitro. Ethanol, required to dissolve the steroid hormones, altered Na^+,K^+-ATPase activity. Ouabain, essential for assessing Na^+,K^+-ATPase activity, appears to react with these hormones. Therefore, technical difficulties made this work impossible.

Leucocytes are difficult to use, requiring lengthy, complicated procedures and large volumes of blood. Hence, human platelets were chosen as an alternative. A method was

devised to assess sodium transport in platelets and the effects of various stimulators and inhibitors of sodium influx were studied. The pattern of Na^+,K^+-ATPase activity was similar to that previously described for leucocytes and to that reported with rubidium influx in human platelets. However, there were subtle differences between sodium and potassium transport that should be considered in future studies. The advantages of this new methodology were applied for the assessment of a cardiotonic drug, milrinone.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ANP	α-atrial natriuretic peptide
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ions
CV	Coefficient of variation
DHT	Dihydrotestosterone
DLIS	Digoxin-like immunoreactive substance
ECF	Extracellular fluid
H^+	Hydrogen ions
ICF	Intracellular fluid
K^+	Potassium ions
Mg^{2+}	Magnesium ions
Na^+	Sodium ions
Na^+,K^+-ATPase	Sodium,potassium-adenosine triphosphatase
OIERC	Ouabain insensitive efflux rate constant
O/C	Oral contraceptive
OSERC	Ouabain sensitive efflux rate constant
Pi	Inorganic phosphate
PMS	Premenstrual syndrome
Rb^+	Rubidium ions
TBW	Total body water
TERC	Total efflux rate constant

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CHAPTER 1: INTRODUCTION

This chapter may be considered as three separate sections. Section one describes some of the relevant features of premenstrual syndrome (PMS). An understanding of this condition is necessary because this project investigates the sodium pump in women with PMS and compares the results to those of asymptomatic controls.

Section two describes the transport of sodium and potassium across the cell plasma membrane, mainly concentrating on the sodium pump. The cell model and method are considered with a view to developing an improved technique for studying the sodium pump.

Section three describes the topics covered by this project and the reasons why they have been included

I. Premenstrual Syndrome

Premenstrual syndrome (PMS) has been known to exist for centuries and one aphorism by Hippocrates appears to describe premenstrual changes. However, it was not until 1931 that Frank used the term of premenstrual tension (O'Brien 1987).

PMS may be defined as a condition of psychological and/or somatic symptoms that occur in the luteal phase of the menstrual cycle and are relieved at the onset of menstruation (Massil & O'Brien, 1986). The symptoms that may occur are listed later in the text.

An extensive study in New Zealand showed that 85% of women have some premenstrual symptoms at some time (Pullon et al, 1989) and a summary of studies from the UK, Holland, USA,

Sweden and France involving women from all social classes and races, suggests that PMS has a prevalence of 87%. However, of the women studied only 40% had PMS that affects normal functioning in daily life (Massil & O'Brien, 1986) and only 3% had PMS that severely debilitates, causing a major disruption to social, occupational and family life (Johnson et al, 1988). There is no difference in the prevalence or severity of premenstrual symptoms reported by black and white women (Stout et al, 1986).

The physical symptoms are cramps, breast swelling and tenderness, weight increase, abdominal bloating, oedema (of the face, hands and legs), headaches, migraine, vertigo, nausea, abdominal and pelvic pain, constipation, change in skin condition (acne, eczema and herpetic eruption), increase in thirst and appetite and craving for sweet or salty foods.

The psychological symptoms are tension, fatigue, depression, irritability, anxiety, aggression, lethargy, change in sleep patterns and libido, loss of concentration, clumsiness and weepiness (Sharma, 1982; Reid & Yen, 1983).

As can be seen above, the symptoms are varied and diagnosis must be made on their timing and not their character. Therefore, the most important factor in distinguishing PMS from other conditions is the appearance of symptoms before menstruation and their disappearance at the onset of menstruation (Massil & O'Brien, 1986). The psychological symptoms may cause PMS to be blamed for underlying psychological and psychiatric problems. Only by having previous knowledge of the woman's personality can such

errors be eliminated. PMS is often confused with the menopause and dysmenorrhoea, hence a knowledge of the age and history of the patients is necessary for an accurate diagnosis. Bloatedness is a common symptom in PMS but it may also be due to obesity or in rare cases ascites or ovarian cysts. Other symptoms that can occur in PMS, such as lethargy may in fact be due to anaemia or hypothyroidism, breast pain to non-cyclical mastalgia or even breast cancer and irritability and anxiety to thyrotoxicosis (Massil & O'Brien, 1986). Therefore, diagnosis is a difficult process and is usually achieved using a daily self assessment using linear visual analogue scales for symptom assessment (Fig 1.1).

Fig 1.1: Visual Analogue Scale (O'Brien, 1987)

Please complete every day at the same time. Make a vertical mark on each line below to record your feelings at the moment. Do not look at the previous day's chart.

Any bleeding today mark B or S...
B=Bleeding   S=Spotting
Date————————— Leave blank if no bleeding

Depressed   ———————————————————— Elated

Irritable   ———————————————————— Calm

Bloated   ———————————————————— Thin

Clumsy   ———————————————————— Coordinated

Severe breast ——————————————————— No breast
discomfort   discomfort

Severe   ———————————————————— No headache
headache

Any comments

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General health questionnaires were used to exclude women with underlying psychiatric illness (Hussain et al, 1990). Self- assessment causes bias because the patients expect to have symptoms premenstrually. A study showed that women who are led to believe that they are premenstrual report a higher degree of symptoms than those led to believe they are intermenstrual (Ruble, 1977). Hence, this factor must be considered during the diagnosis and study of such women.

The aetiology of PMS, although much studied, remains uncertain (Reid & Yen, 1981; Sharma, 1982). Many theories have been proposed including oestrogen excess or progesterone deficiency, vitamin deficiency (A, B, B complex, B6 and E), hypoglycaemia, alterations in prostaglandins and fluid retention (Sharma, 1982).

Fluid retention has not been proven because there is no significant weight increase in the luteal phase of the menstrual cycle of women with PMS. We therefore, suggest that there is a redistribution of fluid so that an increase in intracellular water occurs accompanied by a decrease in the sodium pump activity. These changes may indirectly cause the symptoms of the disease.

Factors affecting PMS
a) <u>Hormones</u>:

Since ovarian hormones are cyclical and have many effects on the body that are considered as links to premenstrual symptoms, progesterone and oestrogen have for many years been assumed to play a role in the pathogenesis of PMS (O'Brien,

1987; Strickler, 1987).

Studies thus far have suggested theories of oestrogen excess, oestrogen/progesterone imbalance and progesterone deficiency (Strickler, 1987). Hammarback et al (1989) suggest that oestrogen and progesterone excess are related to the severity of symptoms whereas Rubinow et al (1988) failed to show significant differences in levels of these hormones in PMS or due to the stage of the menstrual cycle (Varma, 1984). The hormone imbalance and progesterone deficiency theories are also discredited by this work and the work of others (O'Brien et al, 1980; Rubinow et al, 1988). Administering progesterone does alleviate certain symptoms which may result from its anaesthetic and diuretic properties (Strickler, 1987). However, the successful treatment of a condition with an agent does not imply that this agent plays a causal role in the symptoms.

b)   <u>Vitamin B6</u>:

Vitamin B6 is a cofactor in the synthesis of serotonin and dopamine. There has been speculation that oestrogen, taken in the form of oral contraceptive pills (O/C), leads to a deficiency of vitamin B6. This would cause depression due to diminished synthesis of serotonin and tryptophan. Deficiency of dopamine would lead to decreased prolactin and may result in the breast symptoms and fluid balance changes (Sharma, 1982; Strickler, 1987). The treatment of PMS with vitamin B6, although widely used, has not been proven to be effective and its role in the cause of PMS has not been shown (Reid & Yen,

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1983).

c)   Hypoglycaemia:

Hypoglycaemia has some symptoms in common with PMS. These include fatigue, food cravings, altered mental state and vague gastrointestinal symptoms (Sharma, 1982; Strickler, 1987). However, there is no support for any alteration in glucose metabolism due to PMS.

d)   Prostaglandins:

Prostaglandins may contribute to PMS because excessive localised $PGF_{2\alpha}$ causes functional dysmenorrhoea and uterine bleeding results from increased prostacyclin (Strickler, 1987). Endometrial PGE_2 and $PGF_{2\alpha}$ concentrations are higher in the luteal compared to the follicular phase of the menstrual cycle, where the symptoms occur (Wood & Jacubowicz, 1980). Mefenamic acid, a prostaglandin synthesis inhibitor, does not improve the breast symptoms as expected but does improve mental and menstrual symptoms (Wood & Jacubowicz, 1980). It has been suggested that the symptoms of PMS can all be linked to prostaglandins. PGE_1 is synthesised from dietary linoleic acid. Women with PMS have above normal levels of linoleic acid but reduced levels of its metabolites suggesting its metabolism is defective (Brush et al, 1984; Horrobin & Manku, 1989). Administering oil of primrose (72% linoleic acid) improves PMS symptoms (Sharma, 1982; Strickler, 1987).

e)   <u>Fluid retention</u>:

Although most women complain of bloatedness and water retention (O'Brien, 1987), weight gain has not been observed in the premenstrual phase (Andersch et al, 1978). Oestrogen excess, progesterone deficiency, renin-angiotensin overactivity and aldosterone excess have all been suggested as the cause of this fluid retention. However, the scientific research that has so far been carried out does not support the hypothesis that PMS symptoms are caused by fluid retention (Reid & Yen, 1981).

The subjective symptoms of swelling and bloating are best reconciled by fluid shifts without sodium and water retention (Strickler, 1987). Capillary permeability to proteins appears to be increased in the luteal phase suggesting that fluid shifts do occur (Jones et al, 1966). Subjects have been studied in the highly controlled situation of a metabolic ward and have shown a slight but significant gain in weight and retention of water and sodium (Bruce & Russell, 1962). The same magnitude of change has been observed mid-cycle without the occurrence of symptoms. Thus, PMS is probably not related to this weight gain. Total body water (TBW) is not increased premenstrually (Preece et al, 1975) in women with and without breast pain. This supports the theory of a fluid shift rather than water retention. Exchangeable sodium is increased in depression and a cyclical variation of electrolytes and the fluid state of neurones may be related to the mood changes seen in PMS (O'Brien et al, 1980).

f)   <u>α-atrial natriuretic peptide (ANP)</u>:

ANP, which may reflect fluid and electrolyte changes in PMS, was lower in the luteal phase of a group of PMS patients compared to controls (Hussain et al, 1990). All the data points to no alteration in TBW (Preece et al, 1975) or slight gain in weight (Bruce & Russell, 1962; Bancroft & Backstrom, 1985) putting serious doubt that water retention occurs in PMS. There could possibly be water redistribution (Bancroft & Backstrom, 1985; O'Brien, 1987), a hypothesis which is supported by the findings of the present project.

g)   <u>Serotonin</u>:

Serotonin is linked to the regulation of body water (Batmanghelidj, 1987) and alterations in mood. Changes in cellular water may alter tryptophan (a precursor of serotonin) metabolism as "free" water creates the energy required for a conformational change to occur (Batmanghelidj, 1987). Changes in tryptophan synthesis and metabolism have for a long time been associated with mood (Wurtman, 1988).

There is considerable evidence of a relationship between serotonin and depression, serotonin and carbohydrate craving and between carbohydrate craving and mood (Wurtman, 1988). Carbohydrate intake may be regulated by brain tryptophan levels which in turn, regulates the level of serotonin.

In PMS, depression and appetite changes are closely linked and occur in the luteal phase. Women with PMS increase their carbohydrate intake in the late luteal phase which improves depression and other mood-related symptoms (Wurtman,

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1988). A carbohydrate meal will increase brain synthesis of serotonin and hence PMS patients may consume carbohydrates to improve their mood (Wurtman et al, 1989).

It has been shown that whole blood serotonin levels are decreased in the luteal phase of women with PMS (Rapkin et al, 1987). Also the serotonin content of platelets obtained from PMS patients was significantly decreased premenstrually (Ashby et al, 1988). Platelet rich plasma and platelet poor plasma both have less serotonin in the premenstrual phase (Taylor et al, 1984).

The majority of women are aware of menstrual cycle-related changes in mood. The alterations can be extremely severe, such that in two cases, PMS was accepted by British courts as reason enough for reducing charges of murder to manslaughter due to diminished responsibility (Brahams, 1981; Dennerstein et al, 1984). In the USA, there is some controversy over the use of PMS as a valid defence for criminal behaviour (Casper, 1989), whereas in France, PMS is recognised as a cause of temporary insanity (Sharma, 1982).

II. The Sodium Pump

The human body may be considered as a number of different compartments (water, dissolved and undissolved solids). The water compartment can be divided into the intracellular and extracellular fluid. The fluid compartments of the body have different compositions (Baron, 1972). Sodium ions are largely

found in the extracellular fluid and potassium inside the cells (Morgan, 1984). This means the intracellular sodium concentration is much lower than the extracellular concentration and the potassium concentration is much higher in the intracellular fluid than in the extracellular fluid. (eg in leucocytes the intracellular concentration of sodium is approximately 17 $mmol.l^{-1}$ compared to the plasma concentration of 139 $mmol.l^{-1}$. The intracellular potassium is 150 $mmol.l^{-1}$ and the plasma concentration is 4 $mmol.l^{-1}$.) Therefore, a mechanism called the sodium pump is required to maintain this electrochemical gradient (Sweadner & Goldin, 1980).

Historical aspects

As early as 1902, Overton, concluded that there must be a mechanism to oppose the influx of sodium and the efflux of potassium that occurs during the contraction of muscle as the intracellular concentrations of these ions remained the same in youth and in old age (Glynn, 1989). The term the "sodium pump" was coined by Dean (1941). It is now accepted that most mammalian cells have sodium pumps in their membranes which pump sodium out of and potassium into the cell against concentration gradients (Skou, 1986). This process requires energy in the form of adenosine triphosphate (ATP) which is broken down by an ATPase. The sodium pump has a specific ATPase, the sodium, potassium transporting adenosine triphosphatase (Na^+,K^+-ATPase EC 3.6.1.37) which is membrane bound (Sweadner & Goldin, 1980).

Evolution of the pump

The sodium pump probably evolved from an ATP-driven pump that expelled hydrogen from anaerobic unicellular organisms to combat the fall in intracellular pH caused by fermentation processes. This evolved into a new type of pump that involved phosphorylation and dephosphorylation of the pump during each cycle (Glynn, 1989). Whichever way the pump evolved, it has many important functions that make it essential to the survival of nearly all animal cells.

The Enzyme

a) Location and occurrence:

The enzyme that drives the pump catalyses the reaction for the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate. The energy released by this reaction is used to transport ions across the cell membrane, against the electrochemical gradient. Na^+,K^+-ATPase is found throughout the animal kingdom in all cells. By 1975, Na^+,K^+-ATPase was known to be present in erythrocytes and said to be localised only on the plasma membrane. This was concluded because erythrocytes have no other membranes. In other tissues, such as the heart, kidney, epithelial cells, liver and brain, the localisation of Na^+,K^+-ATPase was proving more difficult (Schwartz et al, 1975). More recently, it has been stated that its occurrence is limited to the plasma membrane and this enzyme system is now considered to be a marker for the plasma membrane (Schuurmans Stekhoven & Bonting, 1981). Na^+,K^+-ATPase activity varies from one tissue to another. It is most active

in excitatory and secretory tissues where it is required to cause a membrane potential from which an action potential can be achieved. Three sodium ions (Na^+) are transported out of and two potassium ions (K^+) into the cell, so there is a net movement of a positive charge to the outside of the cell (Skou, 1986). Therefore, the sodium pump is electrogenic (Kaplan, 1983).

b)   Structure:

The enzyme consists of two subunits, an α-subunit and a β-subunit. The α-subunit is a polypeptide chain (molecular weight: 112,000) that probably spans the membrane. The β-subunit is a glycopeptide (molecular weight: 35,000) that probably loops through the membrane with the carbohydrate portion on the extracellular surface (Skou, 1986). As the molecular weight of the enzyme is about 250,000 it was initially thought that there were two α-subunits and one or two β-subunits (Schuurmans Stekhoven & Bonting, 1981) with most accepting the $(\alpha,\beta)_2$ structure. There is still some controversy about the exact structure (Glynn, 1989).

c)   The mechanism of action:

In a normally working pump, there are four substrates, (ATP, H_2O, intracellular sodium and extracellular potassium) and four products (ADP, inorganic phosphate, extracellular sodium and intracellular potassium) (Glynn, 1989). In 1965, Post et al, showed that ATP can phosphorylate the pump when sodium ions are present. If potassium ions are then added

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inorganic phosphate is released. This proved the reversibility of the phosphorylation.

Fahn et al (1966) suggested that there were two forms of the phosphoenzyme. E_1P which can easily revert back to the enzyme releasing its phosphate group to ADP and E_2P which cannot be easily reversed but is readily hydrolysed in the presence of K^+. E_1P is converted to E_2P during the normal working of the pump.

It was suggested that the ions (K^+ and Na^+), that will dephosphorylate the enzyme, become trapped within it when hydrolysis occurs. These ions are later released after a slow conformational change. An increase in the ATP concentration will make the enzyme available for phosphorylation sooner. Hence, ATP must catalyse the normally slow conformational change (Post et al, 1972).

When the presence of an uncoupled efflux was found, there had to be a modification in the scheme, so a slow return pathway was added (Sachs, 1986).

The model below has been suggested from these findings:

Fig 1.2: Model of modes of transport of the sodium pump
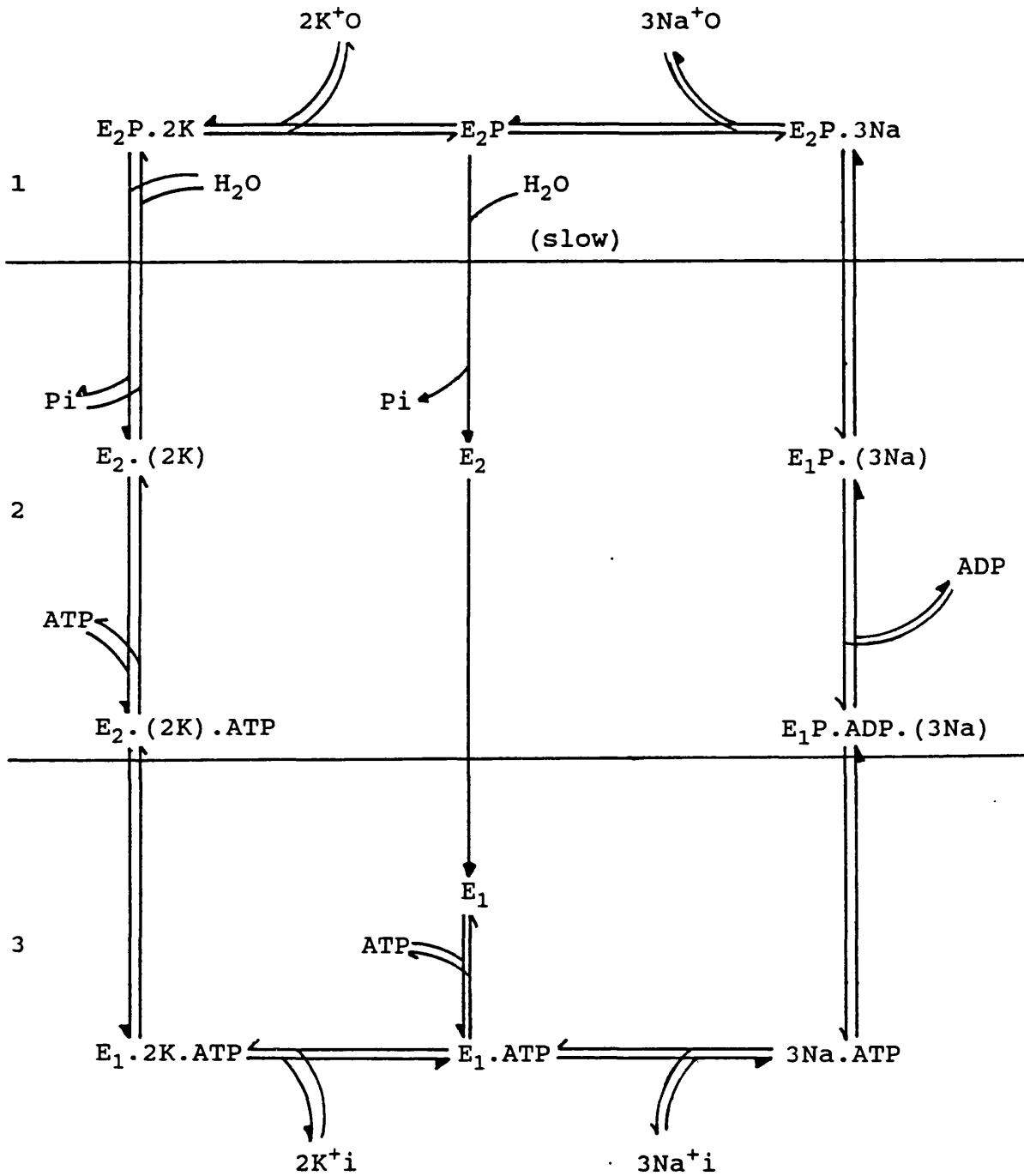
1. Cation loading sites are accessible from the exterior of the cell and prefer K^+ to Na^+.

2. Cation loading sites are occluded.

3. Cation loading sites are accessible from the interior of the cell and prefer Na^+ to K^+.

0 = outside i = inside

<u>Modes of transport in the sodium pump (Kaplan, 1985;</u>

<u>Skou,1986; Glynn, 1988; Glynn, 1989)</u>

There are six modes of transport:

a) <u>Forward flux mode</u>: The pump, under physiological conditions will run in an anti-clockwise direction as shown in the above model. Three sodium ions are expelled and two potassium ions are taken up at the expense of an ATP molecule.

b) <u>Reverse mode</u>: As the reactions of the sodium pump are reversible in certain non-physiological conditions, it should be possible to drive the pump backwards. Under these artificial conditions ATP is produced, potassium is expelled and sodium enters the cell (Garrahan & Glynn, 1967a).

c) <u>Na-Na exchange</u>: The pump in a medium containing no potassium shows a one for one exchange of internal sodium for external sodium that requires ATP and ADP to be present with no net consumption of ATP (Garrahan & Glynn, 1967b). There is a second type of Na-Na exchange which is much slower and occurs in the absence of ADP. It is accompanied by the hydrolysis of ATP and is electrogenic (Lee & Blostein, 1980).

d) <u>K-K exchange</u>: In conditions where sodium is absent but extracellular potassium is present, the pump shows a one for one exchange of internal potassium for external potassium. As expected, this transport requires the presence of ATP and Pi with no net hydrolysis of ATP (Simons, 1975).

e) <u>Uncoupled Na efflux</u>: In an extracellular medium with no Na^+ or K^+, three sodium ions are transported out of the cell for every ATP molecule hydrolysed. This is a slow reaction (Sachs, 1986) which led to the addition of the slow pathway to

the model ($E_2P \to E_2 \to E_1$).

f)   Uncoupled K efflux: Sachs (1986) demonstrated the transport of K^+ out of the cell using the slow pathway that also allows uncoupled Na^+ efflux. Karlish & Stein (1982) also demonstrated very small ouabain-sensitive fluxes in the complete absence of ATP and Pi.

Other transport mechanisms

a)   H-K exchange:

Na^+,K^+-ATPase can be incorporated into the membranes of proteoliposomes.   An   ATP-induced   ouabain-sensitive acidification of the intravesicular medium was seen when Na^+ was absent.   This   finding   demonstrates   the   transport   of hydrogen ions (H^+) by the sodium pump. Therefore, Hara and Nakao concluded that protons can be transported in the place of sodium ions (Hara & Nakao, 1986).

b)   Na-Ca exchange:

The model for this exchange is that the membrane contains a mobile carrier which combines with one calcium ion (Ca^{2+}) or with three or more Na^+ ions and the exchange can occur in either direction (Horackova & Vassort, 1979). This antiport is electrogenic and can be found in excitable tissues (Miyamoto & Racker, 1980). It has also been suggested that platelets have this exchange mechanism as vesicular plasma membrane enriched fraction obtained from human platelets exhibited Ca^{2+} uptake in exchange for intravesicular Na^+ (Rengasamy et al, 1987). The direction of the movement will depend on the

intracellular and extracellular concentrations of Na^+ and Ca^{2+} and also on the membrane potential as the exchange is electrogenic. This system has been studied in the heart and smooth muscle (Brading & Lategan, 1985). Inhibition of the sodium pump with ouabain would be expected to alter the internal:external ratio of Na^+ in smooth muscle cells. The increase in intracellular Ca^{2+} seen suggests that the increase in intracellular sodium due to the blocked sodium pump is compensated for by this exchange mechanism causing an increase in intracellular calcium instead (Pritchard & Ashley, 1986; Ahmad & Bloom, 1989).

c) Na/H exchange:

Another system that is present is the influx of Na^+ in exchange for the efflux of H^+ (Escobales & Canessa, 1986; Siffert & Akkerman, 1987). This electroneutral antiport has been seen in many mammalian cell types including erythrocytes (Escobales & Canessa, 1986) and platelets (Siffert & Akkerman, 1987). Stimulated platelets take up sodium ions and release hydrogen ions using this mechanism. The Na/H exchange is a major pathway for the uptake of Na^+ and may cause an intracellular Na^+ load which would stimulate the sodium pump (Rasmussen et al, 1989).

Inhibitors of the pump

a) Cardiac Glycosides (Schwartz et al, 1975; Schuurmans
    Stekhoven & Bonting, 1981; Skou, 1986):
    The active transport of sodium and potassium is

inhibited, with almost absolute specificity, by cardiac glycosides. The most widely used cardiac glycoside in experiments is ouabain (strophanthin-G) as it is the most water soluble. It inhibits the binding of ATP and dephosphorylation of the phosphointermediate by binding to the extracellular side of the system. Mg^{2+} is required on the cytoplasmic side for the ouabain-ATPase interaction. With Mg^{2+} alone, the rate of binding is low. When ATP or Pi is present and phosphorylation of the enzyme to form E_2P can occur, the binding rate of ouabain is drastically increased. However, the final concentration of ouabain that is bound   is   the same whether or not phosphorylation occurs. This and data on the energies of activation for the binding reactions under different conditions indicates that there 'is only one state where the ATPase coincides with the orientation of the receptor required for binding. Therefore, the various rates of binding caused by different conditions may reflect the steady-state levels of the particular reactive enzyme state compatible with glycoside binding.

The binding affinity of ouabain varies from tissue to tissue and the different glycosides do not have the same affinities. Ouabain at a concentration of 10^{-7}-10^{-3} M inhibits the sodium pump of most tissues.

b)   Endogenous digoxin-like immunoreactive substance (DLIS):

Factors that inhibit sodium pump activity have been stated to be elevated in certain human diseases. These include hypertension during pregnancy (Graves & Williams, 1984),

preeclampsia (Gudson et al, 1984), hepatic failure (Sewell et al, 1982), advanced alcoholic liver cirrhosis (Sewell et al, 1984), chronic renal failure (Kelly et al, 1986) and essential hypertension (Hamlyn et al, 1982). During the third trimester of pregnancy women have an endogenous digoxin like immunoreactive substance (DLIS) that disappears post-partum (Graves et al, 1984) just like in preeclampsia. Cord serum also has this substance (Morris et al, 1987). Kelly et al (1986) identified that the digoxin-like immunoreactive substances seen in normal plasma as lysophospholipids and non-esterified fatty acids (Kelly et al, 1986) as these are present in the plasma fractions that have Na^+,K^+-ATPase inhibitory action. LaBella et al (1985) showed that derivatives of progesterone will bind to the ouabain receptor site. More recently, evidence suggests that the digoxin-like factor is ouabain (Anon, 1991; Schoner, 1991) and that it is a steroid hormone produced by the adrenal cortex.

c)   Other inhibitors:

There are other agents that inhibit the sodium pump such as sulfhydryl reagents, oligomycin, vanadate, butanedione and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (Schwartz et al, 1975; Skou, 1986). Since these inhibitors are not used in this study detail of their properties and mechanisms of action has not been included.

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Factors affecting Na^+,K^+-ATPase activity

Skou (1957) showed that Na^+,K^+-ATPase activity is dependent on the relative concentrations of Na^+, K^+, magnesium (Mg^{2+}) and Ca^{2+}. Both Na^+ and K^+ are necessary. The requirement for intracellular Na^+, as a substrate, is absolute. K^+ can be replaced with thallium (Tl^+), rubidium (Rb^+), caesium (Cs^+), ammonium ($NH4^+$) and less efficiently, with lithium (Li^+). The ratio of Na^+/K^+ is also important and should be in the range of 5-10 for optimal Na^+,K^+-ATPase activity (Schwartz et al, 1975).

a)   External [K^+]:

Both K^+ influx and Na^+ efflux are affected by external K^+ concentration (Parker & Welt, 1972). As external K^+ increases, the pump activity increases such that a sigmoid-shaped curve is produced. Therefore, high concentrations of K^+ (>15 $mmol.l^{-1}$; normal concentration in buffer = 6 $mmol.l^{-1}$) (Baron & Kahn 1985) are inhibitory (Schuurmans Stekhoven & Bonting, 1981).

b)   Internal [Na^+]:

The sodium pump activity increases as internal Na^+ is raised to form a sigmoid-shaped curve. Small variations in intracellular Na^+ concentration will greatly influence the rate at which ions are pumped (Parker & Welt, 1972; Schuurmans Stekhoven & Bonting, 1981).

c)   Internal [K$^+$]:

The behaviour of the sodium pump is significantly influenced by the intracellular K$^+$ concentration. For a given Na$^+$ concentration, the pump activity is greater for a low intracellular K$^+$ concentration than for a high concentration (Parker & Welt, 1972). As the internal K$^+$ increases, the pump will be inhibited because K$^+$ competes with Na$^+$ for the Na$^+$ activation site (Schuurmans Stekhoven & Bonting, 1981).

d)   External [Na$^+$]:

K$^+$ influx is inhibited by the presence of external Na$^+$ ions as they compete for the inward carrier activation site. Therefore, K$^+$ influx is increased when there is no external Na$^+$ (Parker & Welt, 1972; Schuurmans Stekhoven & Bonting, 1981).

e)   Other cations:

Magnesium is required because it is the Mg-ATP complex that is the actual substrate for the enzyme. Other divalent cations (manganese and cobalt) can be used instead of Mg^{2+} but with $^1/_{10}$th the efficiency. Therefore, Mg^{2+} is a cofactor for the forward cycle of the pump. However, iron (Fe^{2+}), Ca^{2+}, zinc (Zn^{2+}), copper (Cu^{2+}), barium (Ba^{2+}), strontium (Sr^{2+}) and beryllium (Be^{2+}) inhibit the hydrolysis of ATP and consequently, the activity of the sodium pump (Schuurmans Stekhoven & Bonting, 1981).

Calcium inhibits the enzyme because at high concentrations it competes with Mg^{2+} for the cofactor site and

with Na$^+$ for its binding site (Skou, 1957; Schuurmans Stekhoven & Bonting, 1981).

f)   Adenosine triphosphatase:

The primary substrate for the enzyme is ATP (Schwartz et al, 1975). Pump activity increases linearly with ATP concentration, up to a saturation value (Parker & Welt, 1972; Negendank & Shaller, 1982).

g)   Adenosine diphosphate and inorganic phosphate:

Adenosine diphosphate (ADP) and inorganic phosphate (Pi) both inhibit the forward cycle of the pump and the hydrolysis of ATP (Apell et al, 1986).

h)   Temperature:

Na$^+$,K$^+$-ATPase activity is temperature-dependent. A rise in the incubation temperature will cause a gradual increase in enzyme activity. However, as there is little change from 37 ^{o}C to 40 ^{o}C, the optimum temperature is taken as body temperature (37 ^{o}C) (Baron & Khan, 1985). ATP production and utilisation are markedly diminished as temperature decreases. However, human leucocytes can maintain near normal intracellular levels of K$^+$ and Na$^+$ at 10 ^{o}C due to a mechanism other than the sodium pump (Negendank & Shaller, 1982).

i)   pH:

pH alters the macromolecular conformation of the enzyme. Therefore, it is important to use the optimal pH for efficient

functioning of the pump. Maximal activity of the pump has been reported at pH 7.1-7.4 (Schuurmans Stekhoven & Bonting, 1981; Baron & Khan, 1985)

j)   Hormones:

Hormones have a significant influence on Na^+,K^+-ATPase activity. The response of the sodium pump depends on the hormone and the tissue under consideration. Some of these hormones will now be considered individually.

I)   Progesterone:

Progesterone has been shown to stimulate Na^+,K^+-ATPase activity in the brain and liver (Garcia et al, 1985), but to have no effect on the sodium pump activity in the kidney (Rayson & Edelman, 1982) and the erythrocyte (Grichois et al, 1986) of the same species, the rat. However, other evidence indicates that Na^+,K^+-ATPase activity is decreased in the rat kidney (Lijnen et al, 1985) and liver (Pimplikar et al, 1980) by progesterone. Therefore, there is some confusion as to the effect of progesterone on the sodium pump. The study of the movement of ions also shows the same confusing profile. Membrane permeability is affected such that progesterone inhibits K^+ influx (Swierczynski et al, 1973), indicating a decrease in pump activity. Also sodium influx has been shown to decrease (Neumann et al, 1986) and sodium efflux to increase (Mendoza & De Mello, 1974) in the presence of progesterone, indicating an increase in pump activity. Therefore, the overall picture of the effect of progesterone on Na^+,K^+-ATPase activity is confused.

II)   Oestrogen:

Oestrogens, in various forms, have been shown to increase the Na^+,K^+-ATPase activity in lamb endometrial tissue (Zanca et al, 1983), rat brain (Garcia et al, 1985; Rodriguez del Castillo et al, 1987; Litteria, 1987) and kidney (Aperia et al, 1981); to have no effect on lamb cell (endometrial) homogenates (Zanca et al, 1983), rat erythrocytes (Grichois et al, 1986) and kidney (Lijnen et al, 1985; Rayson & Edelman, 1982); and to decrease Na^+,K^+-ATPase in the rat brain (Rodriguez del Castillo et al, 1987), kidney (Aperia et al, 1981) and liver (Garcia et al, 1985). Once again, the profile is too confused to draw any firm conclusions. Oestrogens have also been reported as causing an increase in the electrolyte and water content of the rat uterus (Talbot et al, 1940; Spaziani & Szego, 1958; Fujimoto & Morrill, 1978). The most commonly used oestrogen is 17β-oestradiol, but occasionally α-oestradiol and oestradiol benzoate are used.

Estradiol has also been shown to affect calcium homeostasis (Sommerville et al, 1989). Therefore, reports of estradiol causing changes in Na^+,K^+-ATPase are not surprising when the close links between sodium and calcium, via the Na-Ca exchange system, are considered.

III)   Testosterone:

Testosterone increases Na^+,K^+-ATPase activity in the lamb endometrium (Zanca et al, 1983), the rat brain (Guerra et al, 1987; Garcia et al, 1985) and liver (Garcia et al, 1985); but has been found to have no effect on the rat kidney (Rayson & Edelman, 1982). It is interesting to note that testosterone

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and not dihydrotestosterone (DHT) has been used in these studies. DHT is the active form of testosterone. Maybe the results would have been more clear if DHT was used, rather than testosterone.

IV) Oral contraceptives:

The oral contraceptive (O/C), of the oestrogen and progesterone combined type, decreases Na^+,K^+-ATPase activity of erythrocytes in women. There was no significant difference in subjects, whether taking the oral contraceptive or not, that are due to the stage of the cycle (Gallery et al, 1986). Smith et al (1986) found that the O/C (Demulen, Ortho novum, Ovral, Lo-ovral, Nordette) did not alter Na^+,K^+-ATPase activity in women but that men had a significantly higher activity than ovulatory women. These results contradict each other probably because the researchers assessed different indices of Na^+,K^+-ATPase activity. Intracellular erythrocyte sodium (M'Buyamba-Kabangu et al, 1985) and plasma sodium (Mira et al, 1984) concentrations have been shown to be lower in the luteal compared to the follicular phase of normal women. In the present study, leucocytes did not show a significant difference in the intracellular sodium concentration and the decrease in plasma sodium was not confirmed. Also pregnancy has been recorded to increase Na^+,K^+-ATPase activity (Grichois et al, 1986). Therefore, since Na^+,K^+-ATPase activity differs in men and women, a variation has been reported during the menstrual cycle and the use of O/C alters the sodium pump activity it may be concluded that there is some degree of control exerted by the sex hormones.

V)   Others:

Adrenocortical hormones (Guerra et al, 1987; Turaihi et al, 1987; Turaihi et al, 1988), and analogues of predominantly glucocorticoid and mineralcorticoid activity (aldosterone, cortisol, fludrocortisone, carbenoxolone) (Baron & Green, 1986) have also been shown to influence the sodium pump by increasing its activity.

k)   Ethnic origin:

The ethnic origin of humans may influence sodium pump activity. It has been shown that Na^+,K^+-ATPase activity is lower in black subjects than in whites who live in the USA. This theory is supported by the erythrocyte intracellular Na^+ concentration of blacks being higher than that in whites (Lasker et al, 1985). Also Beutler et al (1983) found that Jewish, Asian and black subjects had a significantly lower Na^+,K^+-ATPase activity than a non-Jewish white population. Those with Scandinavian ancestry had a higher Na^+,K^+-ATPase activity than the rest of the non-Jewish white group. However, Arumanayagam et al (1987) reported that there was no significant difference between Chinese males and European males. Since there is obviously some variation between ethnic groups care must be taken in the selection of subjects for patient and control groups to ensure that an ethnic bias does not influence the results.

l)   Sex:

Men have been shown to have significantly lower

Na^+,K^+-ATPase activity and higher intracellular Na^+ concentrations than women (Lasker et al, 1985; M'Buyamba-Kabangu et al, 1985). This is supported by the finding that women have a higher mean pump number than men (Hasstedt et al, 1989). Therefore, studies must take the gender of the subjects into consideration.

m)   Genetic expression:

Genetic control of the sodium pump has been indicated in a study of identical twins compared to unrelated individuals. Twins showed no intrapair differences for pump number and activity whereas unrelated individuals showed two to three fold variation in pump number (DeLuise & Flier, 1985). It has also been noted that first degree relatives show closer pump numbers than the population in general. Genetic effects account for 77% of the variance in sodium pump number (Hasstedt et al, 1989).

n)   Serotonin:

Serotonin levels in platelets are closely linked to the sodium pump because inhibition of serotonin transport and uptake will occur with inhibition of Na^+,K^+-ATPase (Chong & Kay, 1977; Wolfel et al, 1989). As the sodium pump controls Na^+ concentration, it exerts some control over platelet activity and hence serotonin levels (Bogdaski et al, 1970; Pales et al, 1989). If ouabain is added, both the sodium pump and serotonin uptake are inhibited.

o)   <u>Ethanol</u>:

Ethanol inhibits sodium pump activity in vitro and in vivo. However, to inhibit the sodium pump in leucocytes a concentration of ethanol of 80 mmol.l^{-1} was required. This concentration would be toxic in vivo (Green & Baron, 1986). It has, however, been seen that 17 mmol.l^{-1} ethanol alters the sodium content of leucocytes. This may be due to reduced sodium influx rather than an increase in sodium efflux as a result of greater pump activity (Main & Thomas, 1990). A reduction in sodium influx must eventually effect the pump activity since if the internal sodium concentration is decreased, Na^+,K^+-ATPase activity will also decrease (Parker & Welt, 1972; Schwartz et al, 1975).

<u>The sodium pump and disease</u>

There is a long list of diseases where abnormalities of the sodium pump activity have been documented. These include hypertension (Berntorp et al, 1987; Glynn, 1989), diabetes mellitus (MacGregor & Matschinsky, 1986; Berntorp et al, 1987; Llewelyn & Thomas, 1987; Bergstrom et al, 1989), hypokalaemic periodic paralysis (Oh et al, 1990; Chan et al, 1991) and hyperthyroidism (Rubython et al, 1983; Arumanayagam et al, 1990). Sodium pump activity has been claimed to be altered in encephalopathy (Ede et al, 1987), ischemic brain oedema (Betz et al, 1990), acute brain trauma (Cohadon et al, 1989), spinal injury (Faden et al, 1987), Reye's syndrome (Olson et al, 1989), convulsions induced by kaninic acid (Sztriha et al, 1987), leukaemia (Logan & Newland, 1982), hepatic failure

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(Alam et al, 1978), constipation and diarrhoea (Ewe, 1988). In addition stomatocytosis, sickle cell anaemia, sick cell syndrome, uremia, burns, metastatic cancer, severe infections, malaria (caused by plasmodium knowlesi), cystic fibrosis and schizophrenia have all been said to be associated with erythrocyte Na^+,K^+-ATPase abnormalities (Parker & Welt, 1972).

III. Considering Present Methods

Cell Model

The present method for studying the sodium pump in human subjects uses leucocytes as the model cell. We question whether this is the best choice and attempted to develop a new and improved method. We firstly considered the advantages and disadvantages of the cell types that are available to choose the most suitable model.

The sodium pump has been studied in a variety of cell types including liver, heart, brain, kidney, skeletal muscle, erythrocytes, leucocytes and platelets. Blood cells have been widely used because they can be obtained rapidly by a relatively non-invasive method. Erythrocytes were first used because they are numerous and easily available. However, erythrocytes are highly specialised, atypical cells that have anaerobic metabolism and no nucleus (Baron & Levin, 1978; Green, 1986 pp168-169). Even though leucocytes form only a small portion of the body mass, they are more representative

of the body than erythrocytes because they have a nucleus, a full complement of enzymes and the Na^+,K^+-ATPase activity of these cells reflects the pump activity of other body cells, unlike that of erythrocytes. However, using leucocytes as representative cells does have disadvantages. These include heterogeneity of the cell population (cells differ in function and composition) (Baron, 1969), the large volumes of blood required (100 ml), and the relatively time consuming (60 min) procedure to isolate them from other blood cells (Green, 1986 pp168-169). In contrast, erythrocytes can be isolated by centrifugation at 1300g for 10 min and 20 ml of blood would be required (Green, 1986 p68). Platelets are simple and rapid to isolate (15 min) (Turaihi et al, 1989) and are a homogeneous population of cells with metabolic pathways similar to those of other cells. In addition, smaller volumes of blood are required (18 ml). The disadvantage in using platelets is that, like erythrocytes, they do not have a nucleus. Platelets are however, more useful than erythrocytes because they have a shorter life-span than erythrocytes. Platelets survive only 10-12 days (Stuart et al, 1975) whereas erythrocytes remain in the blood for 120 days (Landaw, 1991). Therefore, if the nucleated "mother" cell (megakaryocyte) responds to an environmental change the altered platelets will appear in the circulating blood much faster than an altered erythrocyte. Also platelets may be useful because unlike erythrocytes and leucocytes their function is easy to evaluate by aggregation and size change (Mikhailidis et al, 1990) studies.

The study of sodium and / or rubidium transport

In leucocytes, the use of ^{86}Rb or ^{22}Na to study the sodium pump are comparable with respect to the time taken to obtain results. Both require two days before the final results can become available. In platelets, however, the study of sodium transport does not require a protein assay hence results are obtained the same day. The protein assay requires the cell pellets to be left overnight in buffer V for the protein to form a solution, therefore the results can not be obtained until the following day. Rubidium transport in platelets, as with leucocytes, requires two days to obtain the results. Therefore, it may be beneficial to study sodium transport in platelets to obtain rapid results.

Comparing the cost of using each isotope to study Na$^+$,K$^+$-ATPase activity in platelets shows that the ^{22}Na$^+$ transport assay and the ^{86}Rb$^+$ influx assay have similar costs (£2.00 and £2.50, respectively). However, the half-life of ^{86}Rb is 18.6 days whereas that of ^{22}Na is 2.6 years. Therefore, after two weeks the costing looks very different because twice as much ^{86}Rb is required. This results in the ^{86}Rb$^+$ influx assay costing twice the original value while the ^{22}Na$^+$ cost remains the same. Therefore, as sodium has a much longer half-life it is more cost effective to use on long term studies where subjects must be reviewed over several weeks.

Platelets are also less expensive to study when ^{22}Na$^+$ is the preferred system. ^{22}Na$^+$ transport in platelets cost £2.00 for each experiment whereas ^{22}Na$^+$ efflux in leucocytes cost £7.20. This is due to the methodology where the incubation

volume required for the leucocyte assay is three times greater than that required for the platelet study. Therefore, to obtain the same concentration of ^{22}Na more radioactivity must be used making the leucocyte assay much more expensive.

Using either isotope (^{22}Na & ^{86}Rb) to study the sodium pump in platelets may reflect different properties because each method relies on changes in either intracellular sodium or potassium content. This is relevant because the intraplatelet potassium content has been recorded as 122 mmol.kg^{-1}(range: 62-151) whereas the intracellular sodium is only 27 mmol.kg^{-1}(25-28) (Letner, 1984). Therefore, the potassium pool is much larger than the sodium pool. Because of the difference in pool size, the transport kinetics of each ion may also be considerably different. A small pool size should result in ^{22}Na influx reaching an equilibrium more rapidly. Sodium should, therefore, reach an equilibrium with its radio-isotope before potassium. Whether this property is a methodological advantage or a disadvantage must be considered in future studies.

There is a case for the study of both ions to obtain a clear picture of the sodium pump because under certain conditions sodium and potassium transport differ. Thus, we should consider:

(i) the argument above regarding pool size which states that due to the difference in intracellular concentrations, the two ions may show different transport kinetics.

(ii) we have shown that although ^{86}Rb influx and ^{22}Na transport in human platelets were influenced by almost identical

agonists and antagonists, they do react differently to salbutamol, a $\beta2$-agonist. Salbutamol stimulated ^{86}Rb influx but had no effect on [22]Na transport in human platelets.

(iii) it has been previously noted that at temperatures between 37 °C and 10 °C, the rate of exchange of potassium decreases markedly while the rate of exchange of sodium remains normal (Negendank & Shaller, 1982). Therefore, the rate of exchange for potassium and sodium are dissociated, at least at low temperatures.

(iv) N-ethylmaleimide will inhibit the sodium pump and hence will induce a rise in sodium content of platelets but without significantly changing the potassium content (Wolfel et al, 1989).

These findings suggest that in platelets, the sodium and potassium transport are not as closely linked as may have been first thought (Wolfel et al, 1989).

IV.   Aims of the Present Project

The aim of the present project was to study the sodium pump in women with PMS to try and substantiate our hypothesis that although there is no fluid retention there is a redistribution of fluid within the body compartments. This redistribution would result in an increase in intracellular fluid and a decrease in sodium pump activity occurring in the luteal phase of the menstrual cycle.

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As most women complain of bloatedness and water retention, researchers for many years have tried to establish scientific proof for this phenomenon, but without much success. In cases where water retention occurs in disease such as brain oedema (Betz et al, 1990), spinal (Faden et al, 1987) and brain injury (Cohadon et al, 1989) there are documented changes in sodium pump activity. Also there is evidence that hormones influence the sodium pump (see p41-44) and there is obviously a cyclical change in hormones in these patients. Finally, women with PMS suffer psychological symptoms which can be linked to serotonin levels which are in turn related to water balance.

Therefore, there are several indirect links between PMS and the sodium pump and for this reason we studied the sodium pump and sodium status in leucocytes obtained from these patients.

There is little doubt that ovarian hormones influence the physical and emotional wellbeing of women. As they have a cyclical pattern and the symptoms of PMS occur in a cyclical manner, it seemed likely that the ovarian hormones had a causal role in PMS. Hence, an attempt was made to study the effect of these hormones on the sodium pump in vitro. Technical problems eventually put a halt to this part of the project. Originally, it was thought that although ethanol affects the sodium pump in leucocytes at high concentrations, at lower concentrations (< 40 mmol.1^{-1}) the pump would be unaffected (Green & Baron, 1986). Ethanol was required for these experiments because the steroid hormones are insoluble

in aqueous solution. After investigation, the sodium pump was found to be affected by ethanol concentrations as low as 2 mmol.l^{-1} and at this concentration the hormone did not remain in solution. Also the leucocytes must be resuspended in an aqueous buffer which results in the hormone coming out of solution as can be seen by the poor recovery of 17β-oestradiol.

Ouabain, which is used to block the sodium pump, seems to interact with 17β-oestradiol and alters its availability to the radio-immuno-assay used to measure the concentration of this hormone. Therefore, as ethanol affects the pump and ouabain, an essential part of the assay, reacts with the hormone the technical difficulties were too great to overcome in order to carry out this work.

The reaction of ouabain with 17β-oestradiol was an interesting discovery and gave rise to considering the effect of ouabain on other hormones. Hence the effect of ouabain on testosterone was investigated.

The ex vivo study was carried out on leucocytes, which are difficult to use in patient-based studies (see p47-48). Hence, human platelets were chosen as an alternative model cell for future work and a method devised to study sodium transport in this model. The new method was validated by comparing our findings with those previously obtained when studying the sodium pump in platelets, using rubidium influx. This method could then be used for further clinical investigations or to study the effect of chemicals, in vitro.

Milrinone is a cardiotonic drug, administered to patients in heart failure. This drug was under investigation within the department at the same time as this project was in progress. Previous work indicated that milrinone had no effect on the sodium pump but will stimulate Na^+ influx through the fast Na^+ channels. Since the current platelet project assessed the whole picture of sodium transport, it was thought that it would be useful to study the effects of milrinone using the newly developed methodology. A further reason for using this methodology was to enable the evaluation of the effect of milrinone in the presence of adrenaline and to estimate the effect that this drug may have on the heart, in vivo.

CHAPTER 2: SUBJECTS, MATERIALS AND METHODS

SUBJECTS

Twenty women with PMS were recruited from the Premenstrual Syndrome clinic of the Royal Free Hospital by Dr S Y Hussain (Registrar of Dept Obstetrics & Gynaecology). Ten asymptomatic women were selected from the hospital staff as a control group. There was no significant difference between the women with PMS and the asymptomatic controls for their age, height or weight (see Table 2.1). None of the women had used medication for 2 months prior to recruitment and all were on an unrestricted diet. A general health questionnaire was completed in the mid-follicular and mid-luteal days of the cycle to exclude women with underlying psychological problems. The Moos Modified Menstrual Distress Questionnaire was used to diagnose PMS. A linear visual analogue scale (O'Brien, 1987) was completed daily for assessment of symptoms (Hussain et al, 1990). Three of the women with PMS were removed from the study because their symptoms did not comply with the diagnosis of PMS.

Blood samples were taken in the follicular (4-9th day) and in the luteal (18-24th day) phase of the menstrual cycle. The women were tested in either the follicular or the luteal phase on their first visit and this phase of the cycle was selected in a random manner. Each blood sample was taken at the same time of day (09:30) when the women had fasted from the previous night.

All patients and healthy controls gave informed written consent and the project was approved by the local ethics

committee.

Table 2.1: Characteristics of women with PMS and asymptomatic

controls

Median and (range) shown below.

	PMS Group (n=17)	Control Group (n=10)
Age (years)	31 (23 - 40)	30 (22 - 41)
Weight (kg) Follicular	62.0 (52.4 - 83.1)	62.9 (53.0 - 87.4)
Luteal	62.8 (52.1 - 85.8)	62.8 (53.0 - 88.6)
Height (m)	1.63 (1.52 - 1.72)	1.66 (1.62 - 1.75)

n= number of PMS or control subjects

II. **MATERIALS**

1. Chemicals

<u>From Amersham International, Aylesbury, Bucks, UK</u>

[51]Chromium-EDTA

[86]Rubidium chloride in aqueous solution

[22]Sodium chloride in aqueous solution

<u>From BDH Ltd., Poole, Dorset, UK</u>

$CaCl_2.2H_2O$

Choline chloride ($[CH_2(OH).CH_2.N(CH_3)_3]Cl$)

$CuSO_4.5H_2O$

D-Glucose

Folin Ciocalteu's Phenol Reagent

HNO_3

HCl

KCl

$MgCl_2.6H_2O$

$MgSO_4.7H_2O$

Na_2CO_3 anhydrous

$Na_2CO_3.10H_2O$

$NaHCO_3$

$NaH_2PO_4.2H_2O$

NaOH

Ouabain (Strophanthin-G)

Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$)

From C D Pharmaceuticals Ltd., Wrexham, UK

Heparin sodium (1000 units per ml, mucous)

From Dupont De Nemours (Deutschland), Dreieich, GERMANY

^{22}Sodium chloride in water

From James Burrough Public Ltd. Company, London, UK

Ethanol (absolute alcohol, analytical grade)

From Sigma Chemicals Company Ltd., Poole, Dorset, UK

Acetylsalicylic Acid (aspirin)

Adrenaline (bitartrate salt)

Atenolol

β-estradiol

Dextran (MW 266 000)

Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid)

Protein standard (human albumin 50g.l^{-1} & mixed globulin 30g.l^{-1})

Sodium potassium tartrate ($C_4H_4KNaO_6.4H_2O$)

Salbutamol

Timolol (maleate salt)

From Smith Kline Beecham Pharmaceuticals, Epsom, Surrey, UK

BRL 37344

From Sterling Winthrop, Guildford, Surrey, UK

Milrinone

2. Instruments

Automated Stat/Routine Analyzer System: Synchron Clinical
Systems AS4, Beckman
Ltd., High Wycombe,
Bucks, UK.

Balance: Sartorius research balance R160P,
Sartorius-Instruments Ltd.,GB-Belmont, Surrey, UK.

Centrifuge: ICE CENTRA-7R, International Equipment Company,
USA.

Coulter T-890 Counter: Coulter Electronics Ltd, Luton, Beds,
UK.

Coulter ZM Counter: Coulter Electronics Ltd, Luton, Beds, UK.

Eppendorf centrifuge: Anderman Eppendorf centrifuge 5414,
Eppendorf, Hamburg, GERMANY.

Flame photometer: IL543, Instrumentation Laboratories,
Cheshire, UK.

γ-counter: LKB Wallac 1282 compugamma, universal gamma
counter, LKB Wallac, Turku, FINLAND.

Magnetic stirrer: B and T Flat spin, Baird and Tatlock,
Chadwell Heath, Essex, UK.

Microscope: Microsystem 70, Watson, Barnet, London, UK.

Oven: Hotbox oven size 1, Gallenkamp, London, UK.

pH meter: Corning pH meter 140, Corning Science Products,
Halstead, Essex, UK.

Pipettes: Gilson pipetman, Anachem, Luton, UK.
BCL 8000 Repetitive pipette, BCL, Sussex, UK.

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<u>Spectrophotometer:</u> Unicam SP1800 ultraviolet

spectrophotometer, Pye Unicam Ltd.,

Cambridge, UK.

<u>Spinmix:</u> Gallenkamp, London, UK.

<u>Waterbath:</u> Shaking waterbath, Grant Instruments (Cambridge)

Ltd., UK.

<u>Whole-blood Aggro-meter model 540:</u> Chrono-log Corporation,

Havertown, Philadelphia,

USA.

3. <u>Buffers and Solutions</u>

I <u>Leucocyte Buffer</u>

NaCl	115.0 mmol.l^{-1}
KCl	6.0
CaCl$_2$.2H$_2$O	1.8
MgSO$_4$.7H$_2$O	0.8
NaH$_2$PO$_4$.2H$_2$O	1.0
Glucose	5.5
Hepes	3.0
NaHCO$_3$	24.0

pH 7.35 at 37 °C

II X2 Concentration of Leucocyte Buffer (shown above), i.e.

NaCl	230.0 mmol.l^{-1}
KCl	12.0
$CaCl_2.2H_2O$	3.6
$MgSO_4.7H_2O$	1.6
$NaH_2PO_4.2H_2O$	2.0
Glucose	11.0
Hepes	6.0
$NaHCO_3$	48.0

pH 7.35 at 37 °C

III Rubidium Assay Buffer

NaCl	115.0 mmol.l^{-1}
$[CH_2(OH).CH_2.N(CH_3)_3]Cl$	6.0
$CaCl_2.2H_2O$	1.8
$MgSO_4.7H_2O$	0.8
$NaH_2PO_4.2H_2O$	1.0
Glucose	5.5
Hepes	3.0
$NaHCO_3$	24.0

pH 7.35 at 37 °C

IV Dextran Buffer

Dextran	2.0 g
Buffer I	100.0 ml

V Folin Buffer

$NaCO_3$	200 mmol.l^{-1}
NaOH	200 mmol.l^{-1}

VI Alkaline Sodium Carbonate Solution

Na_2CO_3	200 mmol.l^{-1}
NaOH	100 mmol.l^{-1}

VII Copper Sulphate Solution

$CuSO_4$	40 mmol.l^{-1}

VIII Sodium Potassium Tartrate Solution

Na K Tartrate	80 mmol.l^{-1}

IX Lowry A Solution

Alkaline sodium carbonate solution	100 ml
Copper sulphate solution	1 ml
Sodium potassium tartrate solution	1 ml

X Folin Ciocalteu's Phenol Reagent Solution

1:1 dilution of BDH commercial reagent with distilled water.

XI Saline

NaCl	154.0 mmol.l^{-1}

XII Ouabain Solutions

a. Ouabain 2.0 mmol.l^{-1} in buffer I.

b. Ouabain 2.0 mmol.l^{-1} in buffer III.

c. Ouabain 4.0 mmol.l^{-1} in saline solution.

XIII Magnesium Chloride Solution

$MgCl_2$ 22.91 g.l^{-1} (113 mmol.l^{-1})

XIV Lithium Nitrate and Nitric Acid Solution

$LiNO_3$ 15.0 mmol.l^{-1}

HNO_3 100.0 mmol.l^{-1}

XV Acetylsalicylic Acid Solution (ASA Solution)

Acetylsalicylic Acid 4.0 g.l^{-1} (22 mmol.l^{-1})

1.   Leucocytes

a)   Collection of blood

Venous blood (60-100 ml) was collected from an antecubital vein in two 50 ml tubes containing sodium heparin at a final concentration of 3 units.ml^{-1}. The blood was gently inverted to ensure adequate mixing of the heparin and to prevent coagulation.

b)   Isolation of leucocytes (Baron & Ahmed, 1969)

After collection, the blood was equally shared into 50 ml tubes so that for each 20 ml of whole blood; 30 ml of dextran buffer (buffer IV), at 37 °C, was added and the tubes gently inverted. Any bubbles on the surface of the blood were aspirated with a plastic pastette. The tubes were placed at 37 °C for the erythrocytes to sediment under gravity for 20 min or until there was a clear demarcation line between the erythrocytes and the supernatant, if this happens to be longer. The supernatant was removed and centrifuged for 3 min, at 200 g. This creates a cell pellet of leucocytes with a few contaminating erythrocytes. These erythrocytes were removed by hypotonic lysis which involves adding distilled water for 10 s while mixing with the Spinmix and then, immediately, adding an equal volume of X2 concentration leucocyte buffer (buffer II) to restore isotonicity. The cell suspension was centrifuged again for 3 min, at 200 g, to form a cell pellet

where the leucocytes are below a layer of erythrocyte ghosts. The ghosts were resuspended in the supernatant by carefully blowing at the suface of the pellet with a plastic Pasteur pipette (pastette) and the supernatant discarded. The remaining leucocytes were resuspended in leucocyte buffer and placed in a shaking waterbath for 15 min to allow the cells to return to an equilibrium state.

c)   <u>Measurement of protein concentration</u> (Lowry et al, 1951)

<u>Standards:</u> made from Sigma Chemicals Company protein standard (80 g.l^{-1}) diluted in folin buffer (Buffer V) to give working standards with final concentrations of 0.05, 0.10, 0.15 and 0.20 g.l^{-1}.

Each sample to be analysed for protein content had been previously digested by leaving the leucocyte pellets in 1 ml of buffer V overnight. Two aliquots of 0.2 ml of the digested pellet sample or protein standard were each placed into a sample tube. The blank was 0.2 ml of buffer V. Lowry A Solution (1 ml) (Solution IX) was added to each and left for a minimum of 10 min but never longer than 15 min. After this 0.1 ml of Folin Ciocalteu's Phenol Reagent Solution (Solution X) was added to each sample tube and immediately mixed using the Spinmix. The reaction solutions were transferred to microcuvettes after 30 min and read against distilled water using the Unicam SP1800 ultraviolet spectrophotometer. The zero was set on the spectrophotometer by reading the blank against distilled water. The other settings used were λ at 700 nm, band width at 2.8 nm and slit width at 0.9 mm.

Fig 2.1: Typical Standard Curve for Lowry Protein Estimation

Absorbance at 700nm

Protein Concentration (g/l)
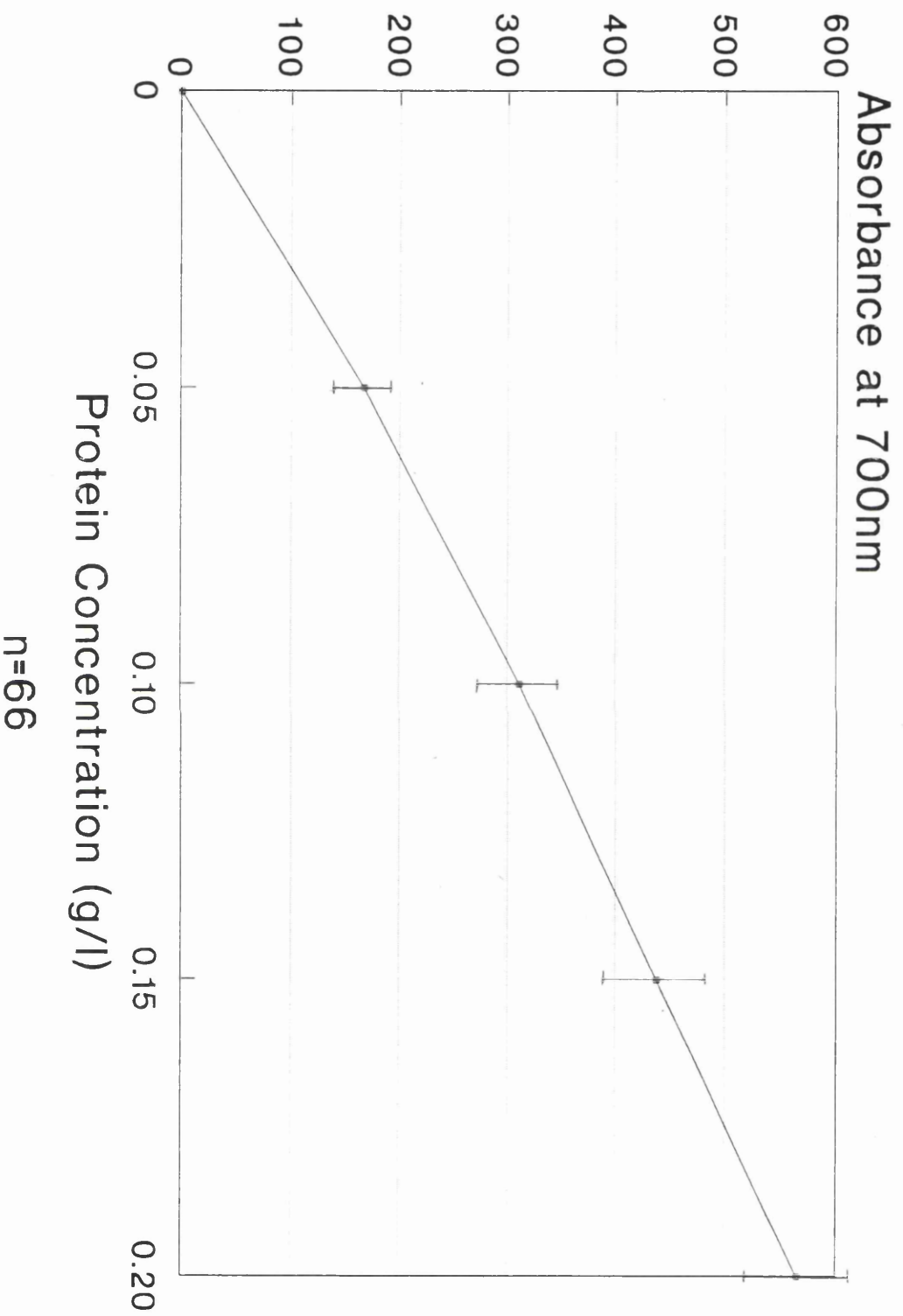
n=66

Bars represent (SD) Standard deviation

The protein content was then calculated from the standard curve obtained in each assay from the prepared protein standards. A typical curve is shown in Fig.2.1.

d)   Sodium Influx

To the leucocyte suspension, at 37 °C, ^{22}NaCl (final concentration [FC]: 74kBq.ml^{-1}) was added and a clock immediately started. At precisely 12 min, two 1 ml aliquots were centrifuged in the Eppendorf centrifuge for 4 s to form a pellet. Supernatant, 20 μl from each tube, was then transferred to preweighed Eppendorf tubes and the rest discarded. The leucocytes were washed twice in buffer I, at 4 °C. The supernatant tubes were weighed again and the leucocytes and supernatants counted in a γ-counter. The cells were then digested by adding 1 ml buffer V and the protein concentration estimated (Lowry et al, 1951) as previously described (p.66). The supernatant counts were calculated using the volume estimated by difference in weight of the Eppendorf tube and the counts from the γ-counter. The sodium influx rate calculation was derived from Sacks and Welt (1967) and described by Hilton & Patrick (1973). The calculation is shown below:

$$\dfrac{\left[\dfrac{\text{Cell count in 1 h (count/h)}}{\text{Protein (mg)}}\right]}{\text{Supernatant count in 1 ml (count/ml)}} \cdot \quad X \; \begin{array}{l}\text{Concentration of} \\ \text{Sodium in} \\ \text{buffer (mmol.l}^{-1}\text{)}\end{array}$$

Units: $\text{mmol.kg}^{-1}\text{Protein.h}^{-1}$

Where:

Cell counts in 1 h is the radioactive count that would be produced by a   cell   pellet if influx continued for 1 h.

Protein is the total protein content of the   cell pellet.

Supernatant counts in 1 ml is the radioactive count that would be present in 1 ml of supernatant collected from the incubation mixture.

The coefficient of variation (CV) for this method was calculated to be 1.3 %; n=39. CV was previously measured at 6.8 %; n=10 (Khan, 1985). The difference in the values may be due to slight changes in the method and an improvement of the technique with the benefit of more experience.

e)   Sodium Efflux Rate Constant

Leucocytes suspended in buffer I were incubated for 30 min with $^{22}\text{NaCl}$ (FC: 74 kBq.ml^{-1}) in a shaking waterbath, at 37 °C. The leucocytes will take in labelled sodium during this time. The cells were removed and washed twice in warm buffer I to remove excess external labelled sodium by centrifuging at 200 g for 3 min and resuspension. The leucocytes were resuspended in 6 ml of buffer I. Half this suspension was

added to a reaction tube containing ouabain at a final concentration of 0.1 mmol.l^{-1} (Solution ^{XII}Xa) and at the same time the other half to a control tube containing an equal volume of buffer I, with no ouabain. A clock was immediately started and the tubes replaced in the waterbath. Samples of 1 ml were taken at 3, 8, 12 min and immediately centrifuged in the Eppendorf centrifuge for 4 s. Each sample was washed twice in cold buffer I and counted in a γ-counter. The cell pellets were digested using buffer V and a protein estimation (Lowry et al, 1951) carried out on each. The sodium efflux rate constant was calculated (Hilton & Patrick, 1973) as follows.

The slope of the graph of:

$$-\ln \left[\frac{\text{Counts in cells (count)}}{\text{Protein (mg)}} \right] \text{plotted against time (min)}$$

gives the rate at which the amount of radioactivity in the cells falls, which is directly proportional to the rate at which radioactivity is being pumped out. The slope is multiplied by 60 to give the efflux rate constant.

Units: h^{-1}

Where:

counts in cells is the radioactive count of each cell pellet

protein is the total protein content of the cell pellet

The control reaction tube gives the total efflux rate constant (TERC) and the reaction tube containing ouabain gives the ouabain insensitive efflux rate constant (OIERC). The pump activity is measured by the ouabain sensitive efflux rate constant (OSERC) which was calculated as below

OSERC = TERC - OIERC

The CV of this method has previously been calculated to be CV of TERC = 7.9 %; n=10, CV of OSERC = 6.3 %; n=10, CV of OIERC = 9.0 %; n=10 (Khan, 1985).

f)   Intracellular electrolyte concentrations and percentage intracellular water (Baron & Ahmed, 1969)

The day before the test, Eppendorf tubes were washed and dried overnight in an oven at 100 °C. These tubes were weighed before use. The equilibrated leucocytes were washed twice in cold $MgCl_2$ solution (solution XIII). After the final wash, the leucocyte pellet was resuspended in 1 ml of $MgCl_2$ solution. ^{51}Cr-EDTA (FC: 185 kBq.ml^{-1}) was added and after thorough mixing the cells were transferred to a pre-dried, pre-weighed Eppendorf tube and centrifuged in the Eppendorf centrifuge, for 4 s, to form a pellet. The supernatant (100 μl) was transferred to a pre-weighed tube and the remainder discarded. The pellet and supernatant tubes were weighed and counted in the γ-counter. The cell pellet was dried overnight in the oven and re-weighed. Finally, the cell pellet was digested with solution XIV.

The digested pellet was run through a flame photometer and read against standards of 0, 0.1, 0.2, 0.5 and 1.0

mmol.l^{-1} potassium and sodium to obtain the total sodium and potassium from the leucocyte pellet.

The intracellular electrolytes and intracellular water were calculated as below:

Extracellular fluid = <u>Cell count</u> X Supernatant mass
(ECF)   Supernatant count

where:

cell counts is the radioactive count produced by the cell pellet

supernatant counts is the radioactive count produced by 100 μl of supernatant

supernatant mass is the weight of supernatant that is counted.

Total fluid = Wet cell mass - Dry cell mass

where:

wet cell mass is the weight of the wet cell pellet

dry cell mass is the weight of the dry cell pellet.

Intracelluar fluid (ICF) = Total fluid - ECF

We assume that the mass of intracellular fluid in mg is equal to the volume in ml.

Intracellular electrolyte concentration

$$= \underline{\text{Electrolyte concentration in pellet}} \times 1000$$
$$\text{ICF}$$

where:

electrolyte concentration is the concentration of the electrolyte in the solution produced by dissolving the cell pellet in 1 ml of solution XIV.

Units: $mmol.l^{-1}$cell water

$$\text{\% Cell water} = \underline{\text{ICF}} \times 100$$
$$\text{Dry cell mass} + \text{ICF}$$

The CV of intracellular sodium and potassium concentrations have been calculated to be 6.3 %; n=5 and 7.0 %; n=5 respectively. They have also been previously recorded as 6.8 %; n=12 and 4.6 %; n=12 respectively (Khan 1985). The percentage cell water method has a CV of 1.4 %; n=5 and previously was 8.8 %; n=12 (Khan, 1985).

Subjects with intracellular K:Na ratio < 3.0 were removed from the study as a low value suggests cell damage.

g) <u>Plasma sodium concentration & plasma potassium concentration</u>

Plasma sodium and plasma potassium were measured using a Synchron Clinical Systems AS4 Automated Stat/Routine Analyser System. The method used by this equipment is that of a low

dilution selective pH electrode.

h)   Plasma urea concentration

The plasma urea was measured using a SMAC-1 Technicon, Basingstoke, UK. The method has a CV of 1.5 %.

i)   Plasma osmolality

The osmolality was calculated using the formula (Whitby et al, 1988):

$$Osmolality = 2[Na^+] + 2[K^+] + [Urea] + [Glucose]$$

Units: $mmol.l^{-1}$

The plasma glucose was estimated to be between 4.0 and 5.0 $mmol.l^{-1}$ as the women in the study were all non-diabetic and had fasted from midnight. The overall contribution of fasting plasma glucose levels in non-diabetics is in the order of 1-2 % of the total plasma osmolality. Since the plasma glucose was not measured, the osmolality was calculated using a presumed plasma glucose value of 4.5 $mmol.l^{-1}$.

j)   Cell Viability

The viability of the leucocytes after isolation and equilibration was tested by the trypan blue exclusion method (Green 1985, p87). A 0.4 % solution of trypan blue in buffer I was prepared and an equal volume of cell suspension, with bovine serum albumin added to a concentration of 0.1 %, was

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mixed with trypan blue solution. The solution was applied to a haemocytometer and the number of unstained and stained cells counted using a microscope. Non-viable cells stain blue. The mean of the number of stained and unstained cells in five squares is obtained and the result expressed as a percentage of viable cells.

2.   Platelets

a)   Isolation of Platelets

Venous blood (20-30 ml) was collected from an antecubital vein. Aliquots (9 ml) of whole blood were placed in tubes containing 1 ml of 3.8% tri-sodium citrate and gently mixed. The blood was then centrifuged at 150 g for 15 min to produce platelet rich plasma (PRP). The PRP, which is essentially free of erythrocyte and leucocytes, was decanted off and placed in a waterbath, at 37 °C, for 15 min to equilibrate. (PRP was analysed with a T-890 Coulter counter to ensure its purity, see p.101).

b)   Rubidium Influx in Platelets (Turaihi et al, 1989)

After equilibration of the PRP, the incubation tubes were set up. The chemical to be tested (dissolved in buffer III) was placed into 4 incubation tubes and to 2 of these ouabain (50 μl solution XIIb) was added. Four control incubation tubes were used for comparison, 2 of these had ouabain added. [86]RbCl (FC: 55 kBq.ml^{-1}) was added to each incubation tube and the volume of each made up to 1 ml with buffer III. PRP (500 μl)

was added to each tube and gently mixed with a Whirlimixer. The tubes where then placed in a shaking waterbath, at 37 °C. After precisely 12 min (timing commenced at the beginning of mixing), 1 ml aliquots from each tube were placed in Eppendorf tubes containing 4 μl acetylsalicylic acid solution (2 g.l^{-1}) and centrifuged in an Eppendorf 5414 centrifuge for 1 min at 10 000 g to form a cell pellet. A portion (100 μl) of each supernatant was transferred to Eppendorf tubes and the remainder discarded. The pellets were washed twice in cold buffer. The cell pellets and supernatants were counted in a γ-counter. Protein concentration was estimated in each cell pellet (Lowry et al, 1951). The rubidium influx was calculated according to the method of Hilton & Patrick (1973) and derived from Sacks & Welt (1967).

$$\frac{\left[\dfrac{\text{Cell count in 1 h (count/h)}}{\text{Protein (mg)}}\right]}{\text{Supernatant count in 1 ml (count/ml)}} \times \begin{array}{l}\text{Concentration of}\\\text{Potassium in}\\\text{buffer (mmol.l}^{-1}\text{)}\end{array}$$

Where:

Cell count in 1 h is the radioactive count that would be produced by a platelet pellet if influx continued for 1 h.

Protein is the total protein content of the platelet pellet.

Supernatant count in 1 ml is the radioactive count that would be present in 1 ml of supernatant collected from the incubation mixture.

The total ^{86}Rb influx is derived from the incubation tubes without ouabain. The passive ^{86}Rb influx is the ^{86}Rb

influx that occurs in the presence of ouabain, an inhibitor of the sodium pump. The active influx is calculated from subtracting the passive from the total ^{86}Rb influx and represents the influx due to the sodium pump.

c)   Sodium transport in platelets (Ozin et al, 1992b)

After equilibration, the PRP was placed into cuvettes used for the Whole blood Aggro-meter. The aggro-meter and cuvettes had been prewarmed to 37 °C. Magnetic stirrers were switched on, and 1 min later the inhibitor (ouabain, atenolol, timolol dissolved in saline) was added. The platelets were stirred for a further 2 min and then allowed to rest for 3 min. In control experiments, the same volume of saline replaced the inhibitor and the stimulator. At this time (6 min from the start of the experiment) the stimulant (adrenaline, salbutamol, BRL 37344 dissolved in saline) was added and the platelets stirred for 2 min and rested for 3 min. After this ^{22}NaCl (FC: 37 kBq.ml^{-1}) was added, the platelets stirred and a sample taken 2, 6 and 10 min after the addition of ^{22}NaCl. The samples taken were washed twice in cold saline and the pellets counted on an LKB Wallac 1282 compugamma universal gamma counter. The raw counts, of radioactivity, were corrected for the number of platelets in the PRP, to a value of 350 x 10^9 platelets/l of PRP, to standardise the method.

Where no inhibitor was required in the procedure, while investigating the effect of an agonist, platelets were stirred for 1 min prior to addition of the stimulant and then the procedure continued as above.

Single measurements of sodium transport were carried out. In early experiments, the variation between duplicates was so small that it was judged unecessary to perform measurements in duplicate. It is difficult to assess the intra-assay variation because only four wells were available to stir platelets. Thus, performing several sequential measurements would mean that the time lapse between the first and last set of measurements would be considerable. This delay, could in turn, alter platelet responses. However, we considered the CV of nine measurements and their duplicates. The CV was found to be 0.8 % for this method.

d)   <u>Extracellular Fluid Trapping in Platelets</u>

Fluid trapped in the extracellular space of the pellet could result in errors. Hence, ^{51}Cr-EDTA was used to assess the amount of radioactivity likely to be trapped in the platelet pellet. However, the presence of ^{51}Cr-EDTA may be attributed to extracellular fluid trapped in the pellet or due to adherence of the radio-chemical to the plastic of the Eppendorf tubes. PRP (1 ml) was placed in each of 2 cuvettes with magnetic stirrers. The PRP was mixed for 3 min and then allowed to rest for 3 min as in the experiments. Then, ^{51}Cr-EDTA (FC: 30 kBq.ml^{-1}) was added to one cuvette and saline to the other cuvette. The samples were stirred and 2 min after the addition of the ^{51}Cr-EDTA a sample was taken from each and transferred to an Eppendorf tube. At this time, the same amount of ^{51}Cr-EDTA was added to 1 ml saline in a third Eppendorf tube to estimate if the radioactivity adheres to the

plastic. All 3 Eppendorf tubes were washed twice in cold saline and the samples were then counted on a LKB Wallac 1282 Compugamma universal gamma counter, as before.

3. In vitro assessment of the effect of milrinone using platelets

(Ozin et al, 1992c)

After equilibration, the PRP in the cuvettes was mixed using magnetic stirrers. Milrinone (FC: 1 or 5 μmol.l^{-1}) was added to one cuvette and saline to another after 1 min of mixing. Final concentrations of 20, 200 and 500 nmol.l^{-1} milrinone were also investigated in preliminary experiments in order to establish an effective concentration. The platelets were mixed for a further 2 min and then allowed to rest for 3 min. At this time, ^{22}NaCl (FC: 37 kBq.ml^{-1}) was added to each cuvette and the stirrers switched on. Samples were taken at 2, 6 and 10 min after the addition of ^{22}NaCl. Each sample was immediately centrifuged at 10,000 g for 1 min in the Eppendorf centrifuge and washed twice in cold saline. The cell pellets were counted using the LKB compugamma counter.

The effect of pre-incubation with ouabain (FC: 0.2 mmol.l^{-1}) on the milrinone response was investigated as was the effect of milrinone on adrenaline (FC: 50 nmol.l^{-1}) stimulation. When ouabain was used, the stirrers were switched on and 1 min later the ouabain was added. The platelets were stirred for a further 2 min and then allowed to rest for 3 min. In control samples an equal volume of saline replaced

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the ouabain. After this, ^{22}NaCl (FC: 37kBq.ml^{-1}) was added and the experiment continued as above. When adrenaline was used, it was added simultaneously with the milrinone to one cuvette and saline replaced the adrenaline in its control. The method was carried out as for the milrinone vs saline experiment above.

All results were corrected for the number of platelets in the PRP samples to a value of 350 x 10^9 platelets.l^{-1}. The platelet count was established using the Coulter ZM Counter. The reason for this is explained later.

4.   In vitro work with ethanol and the sodium pump

The effect of ethanol concentration and length of exposure on sodium efflux rate constant was investigated.

a)   Prolonged exposure (120 min)

Blood (100 ml) was collected from an antecubital vein and divided into two equal volumes. The first portion was sedimented as previously described (p65) with buffer IV and the second with ethanol added to buffer IV. The leucocytes were isolated as before using the appropriate buffer with and without ethanol added. The leucocytes were placed in buffer I and buffer I with added ethanol and left in a shaking waterbath, at 37 °C, for 30 min. After this time ^{22}NaCl (FC: 74 kBq.ml^{-1}) was added to both portions and the cells loaded with radioactivity for 30 min. The TERC and OIERC were measured, as previously described (p64) and the OSERC calculated for each portion.

b)   Short exposure (30 min)

Leucocytes from 100 ml of blood were isolated as previously described (p65). The leucocytes suspended in buffer I were divided into two portions. Ethanol was added to one portion and then ^{22}NaCl (FC: 74 kBq.ml^{-1}) was added to both. The cells were placed in a shaking waterbath, at 37 °C, for 30 min to load the cells with radioactivity. After this both cell suspensions were removed and washed twice in buffer I. Each cell pellet obtained was resuspended in 6 ml buffer I and the TERC and OIERC were measured, as previously described (p69) and the OSERC was calculated for each sample.

5.   17β-oestradiol recovery when diluted with Buffer I

Samples of 17β-oestradiol were prepared by dissolving the hormone in ethanol and diluting with buffer I to get a FC of 30, 50 and 300 pmol.l^{-1}. These samples contained a very small amount of ethanol (FC: 1.8 mmol.l^{-1})

6a.   Ouabain / 17β-oestradiol Reaction (Ozin et al, 1992d)

Platelet poor plasma (PPP) was prepared by centrifugation of whole blood for 15 min, at 1000 g. A pooled PPP from several normal healthy men was prepared to use as a diluent. PPP from 8 heathly men was prepared and stored at -70 °C; these are the sample PPP. 17β-oestradiol was dissolved in 1 ml ethanol and diluted with the pooled PPP to give a final 17β-oestradiol concentration of 500 pmol.l^{-1} in the stock solution. The ethanol had an FC of 1.8 mmol.l^{-1}. 17β-oestradiol stock solution (100 μl) was placed into three

sample tubes. Ouabain solution XIIa (50 μl) was added to one, 50 μl of buffer I was added to another and nothing was added to the third. The volume was made up to 1 ml with each of the eight sample PPP and the samples well mixed. The concentration of 17β-oestradiol was measured by a radio-immuno-assay (RIA) by the Endocrine Laboratory, Royal Free Hospital. The oestradiol RIA kit was obtained from Diagnostics Products Ltd, UK and uses [125]I.

6b.   <u>Ouabain / Testosterone Reaction</u> (Ozin et al, 1992d)

PPP was prepared from the whole blood of normal healthy males. Ouabain solution XIIa (50 μl) was added to one sample tube, 50 μl of buffer I was added to another and nothing was added to the third. The volume was made up to 1 ml with the sample PPP and the sample well mixed. The concentration of testosterone was measured by a RIA by the Endocrine Laboratory, Royal Free Hospital. The testosterone kit was obtained from Medgenix Diagnostics Ltd, High Wycombe, Bucks, UK and uses [125]I.

<u>Statistical Analysis</u>

The Wilcoxon matched pairs signed ranks test was used to compare the follicular with the luteal phase within the group of control subjects or patients. It was also used to compare samples in the platelet method. The Mann Whitney U test was used to compare control subjects with patients in the same stage of the menstrual cycle. Variables are considered to be statistically different where $p < 0.05$. All tests were two-

tailed.

The coefficient of variation (CV) was measured using the formula below:

$$s = \sqrt{\frac{\Sigma d^2}{2n}}$$

where $\underline{n}$ = number of duplicate measurements

d = difference between duplicate samples.

$$CV = \frac{s \times 100}{x}$$

where x = mean of measurements.

Units for the CV = %.

CHAPTER 3: RESULTS

The results chapter may be considered as consisting of three separate sections. Section one includes the results of the patient-based study where the sodium pump activity and the electrolyte status of leucocytes of women with PMS was compared to a control group. These investigations were carried out in the follicular and the luteal phase to make a comparison between the asymptomatic and symptomatic phases.

Section two has three parts. Part one covers the development of a novel method for the assessment of Na^+,K^+-ATPase activity in human platelets using ^{22}Na. Following this, in part two, is the validation of this method by comparing the responses with certain agents to those obtained with the established ^{86}Rb influx assay in platelets, that also measures Na^+,K^+-ATPase activity. Finally, in part three, the method is used to characterise the effect of milrinone on this system.

Section three includes the results of the attempt to investigate the effect of hormones, in vitro, on the sodium pump in leucocytes.

I. **Ex vivo study of leucocyte Na^+,K^+-ATPase activity in women with PMS and asymptomatic controls in the follicular and the luteal phase**

There were no significant difference in weight when the follicular and luteal phase were compared (see Table 2.1) in controls or in women with PMS.

a)   Comparisons within the control group

There were no significant differences between the follicular and the luteal phase for any of the indices measured (see Tables 3.1B & 3.2B).

b)   Comparisons within the patient group

There was no significant difference between the follicular phase and the luteal phase for sodium influx, total efflux rate constant (TERC), ouabain sensitive efflux rate constant (OSERC), ouabain insensitive efflux rate constant (OIERC), intracellular sodium and potassium concentration, plasma sodium and potassium concentration, intracellular K:Na ratio, plasma Na:K ratio, intracellular Na + K concentration and body weight. The percentage intracellular water was significantly increased (p<0.04) in the luteal phase, 72.8% (63.1 - 75.6), compared to the follicular phase, 64.7% (41.1 - 75.0) (see Tables 3.1A & 3.2A).

c)   Comparisons between the patient and the control groups

The patient group and control group were not statistically different for any of the indices measured during the follicular phase.

The TERC of the patients, 2.38 h^{-1} (1.95 - 3.38), was significantly (p<0.04) lower than that of the controls, 2.86 h^{-1} (2.06 - 3.50), in the luteal phase. The OSERC of the patients,

1.63 h^{-1} (1.14 - 2.53), was significantly (p<0.03) lower than the controls, 2.34 h^{-1} (1.37 - 3.38), in the luteal phase (see

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Tables 3.1A and 3.1B and Fig 3.1 - 3.3).

The percentage cell water for the patient group 72.8 (63.1 - 75.6) was significantly (p<0.015) higher than the control group 69.1% (60.1 - 72.1) in the luteal phase (see Tables 3.2A and 3.2B and Fig 3.4).

Table 3.1: Sodium transport studies

A) PMS PATIENTS (n=16) Median value and (range) stated below.

	FOLLICULAR	LUTEAL
Corrected Influx $(mmol.kg^{-1}Pr.h^{-1})$	337 (264 - 726)	336 (198 - 551)
Total Efflux Rate Constant (TERC) (h^{-1})	2.60 (1.88 - 3.90)	2.38 @ (1.95 - 3.38)
Ouabain Sensitive Efflux Rate Constant (OSERC) (h^{-1})	2.00 (1.08 - 3.18)	1.63 @@ (1.14 - 2.53)
Ouabain insensitive Efflux Rate Constant (OIERC) (h^{-1})	0.68 (0.10 - 1.25)	0.70 (0.37 - 1.13)

B) CONTROLS SUBJECTS (n=8) Median value and (range) stated below.

	FOLLICULAR	LUTEAL
Corrected Influx ($mmol.kg^{-1}Pr.h^{-1}$)	322 (230 - 764)	391 (199 - 480)
Total Efflux Rate Constant (TERC) (h^{-1})	2.90 (2.22 - 3.56)	2.86 @ (2.06 - 3.50)
Ouabain Sensitive Efflux Rate Constant (OSERC) (h^{-1})	2.28 (1.53 - 3.11)	2.34 @@ (1.37 - 3.38)
Ouabain insensitive Efflux Rate Constant (OIERC) (h^{-1})	0.56 (0.22 - 0.91)	0.44 (0.12 - 1.07)

PMS = premenstrual syndrome

Pr = protein

@@ = $p < 0.03$ comparing the PMS patients with the control subjects in the luteal phase

@ = $p < 0.04$ comparing the PMS patients with the control subjects in the luteal phase

One PMS patient and two controls were not included in the analysis of results due to probable cell damage during separation suggested by a low potassium:sodium ratio.

Table 3.2: Electrolyte concentrations and percentage cell water

A) PMS PATIENTS

	FOLLICULAR	LUTEAL
Intracellular sodium Concentration (n=12) (mmol.l^{-1} cell water)	19.6 (7.8 - 42.5)	18.6 (12.4 - 29.0)
Intracellular potassium Concentration (n=12) (mmol.l^{-1} cell water)	130 (93 - 227)	140 (98 - 183)
Percentage Cell Water (n=12)	64.7 (41.1 - 75.0)	72.8 #@@ (63.1 - 75.6)
Plasma sodium Concentration (n=14) (mmol.l^{-1})	139 (137 - 142)	138 (136 - 142)
Plasma potassium Concentration (n=14) (mmol.l^{-1})	4.0 (3.5 - 4.3)	3.8 (3.5 - 4.3)
Intracellular K:Na ratio (n=14)	6.7 (4.4 - 13.9)	7.0 (5.0 - 14.2)
Plasma Na:K ratio (n=14)	35 (32 - 40)	36 (32 - 46)
Intracellular Na + K concentration (n=12) (mmol.l^{-1})	156 (114 - 261)	157 (112 - 212)
Plasma urea concentration (n=14) (mmol.l^{-1})	4.5 (2.6 - 5.8)	4.7 (2.5 - 7.4)
Plasma osmolality (n=14) (mmol.l^{-1})	296 (290 - 299)	293 (287 - 305)

B) CONTROLS SUBJECTS

	FOLLICULAR	LUTEAL
Intracellular sodium Concentration (n=12) (mmol.1^{-1} cell water)	17.5 (11.7 - 25.7)	16.5 (7.9 - 34.2)
Intracellular potassium Concentration (n=12) (mmol.1^{-1} cell water)	153 (114 - 166)	147 (110 - 198)
Percentage Cell Water (n=12)	68.0 (62.9 - 69.5)	69.1 # (60.1 - 72.1)
Plasma sodium Concentration (n=14) (mmol.1^{-1})	139 (137 - 143)	139 (137 - 140)
Plasma potassium Concentration (n=14) (mmol.1^{-1})	3.9 (3.8 - 4.1)	4.1 (3.7 - 4.4)
Intracellular K:Na ratio (n=14)	7.9 (6.3 - 12.9)	7.9 (5.8 - 15.2)
Plasma Na:K ratio (n=14)	37 (33 - 39)	35 (31 - 38)
Intracellular Na + K concentration (n=12) (mmol.1^{-1})	168 (130 - 189)	160 (126 - 232)
Plasma urea concentration (n=14) (mmol.1^{-1})	4.2 (2.2 - 5.3)	4.1 (3.4 - 5.2)
Plasma osmolality (n=14) (mmol.1^{-1})	296 (294 - 303)	294 (292 - 297)

PMS = premenstrual syndrome

\#   = p< 0.015 comparing the PMS patients with the control

      subjects in the luteal phase

@@   = p< 0.04 comparing the follicular with the luteal phase

      in the PMS patients

One PMS patient and two controls were not included in the analysis of the results due to probable cell damage during separation as suggested by a low potassium:sodium ratio. Insufficient sample was obtained to carry out all the electrolyte analysis in four patients and two controls.

Fig 3.1: Total Efflux Rate Constant

TOTAL EFFLUX RATE CONSTANT (/h)

PATIENT FOLLICULAR □

PATIENT LUTEAL ■

CONTROL FOLLICULAR ○

CONTROL LUTEAL ●
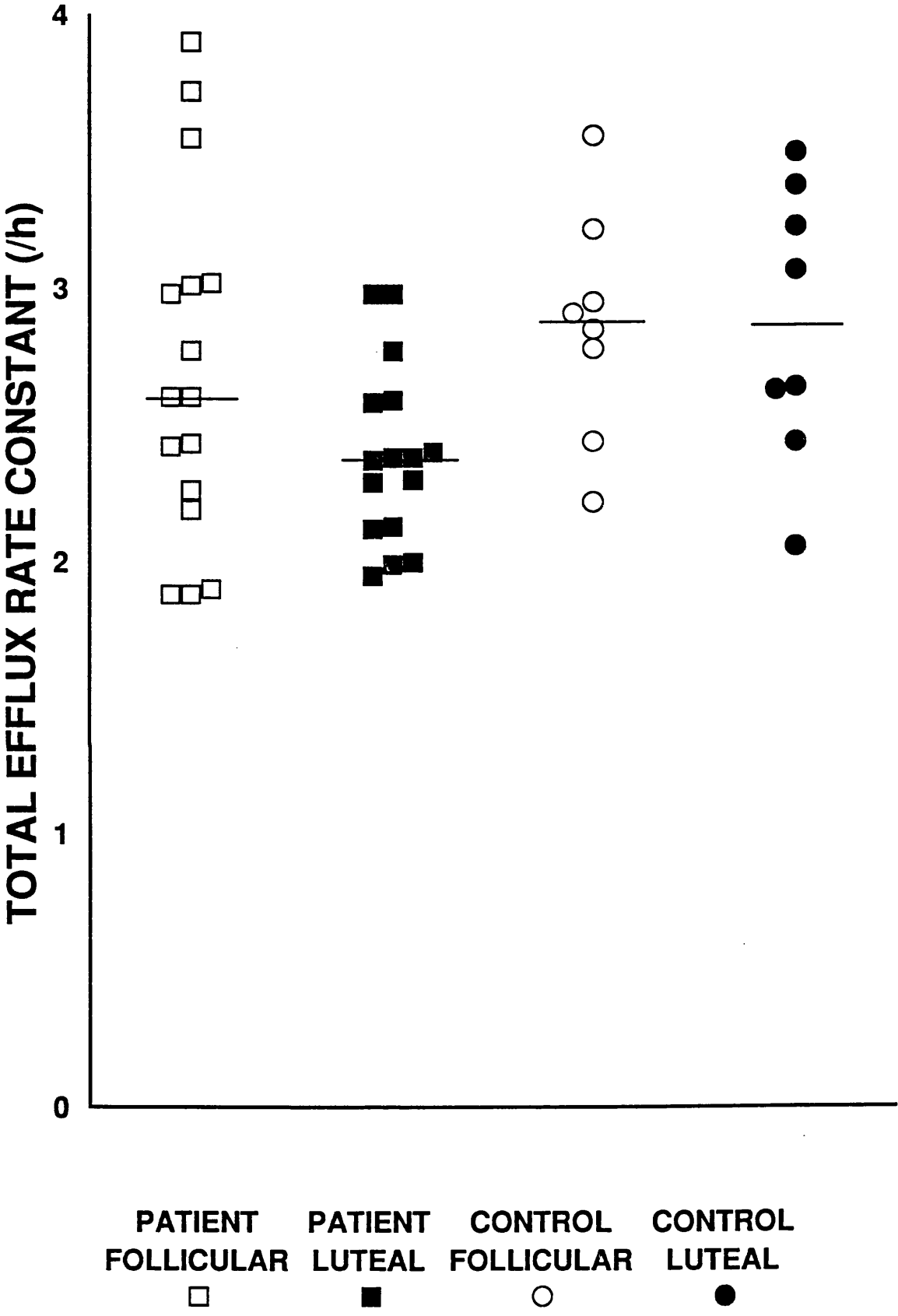
Fig 3.2: Ouabain Sensitive Efflux Rate Constant
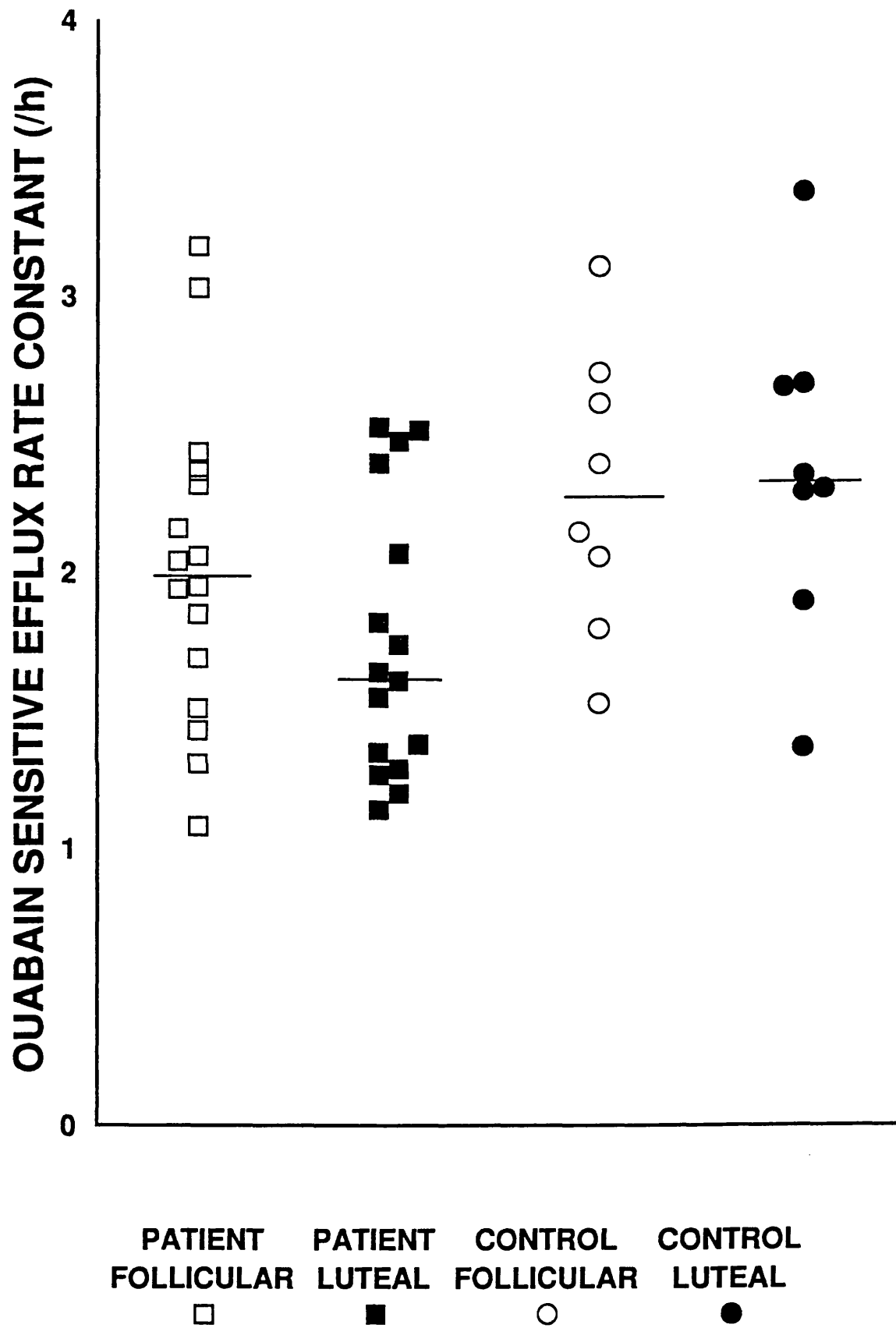
Fig 3.3:Ouabain Insensitive Efflux Rate Constant
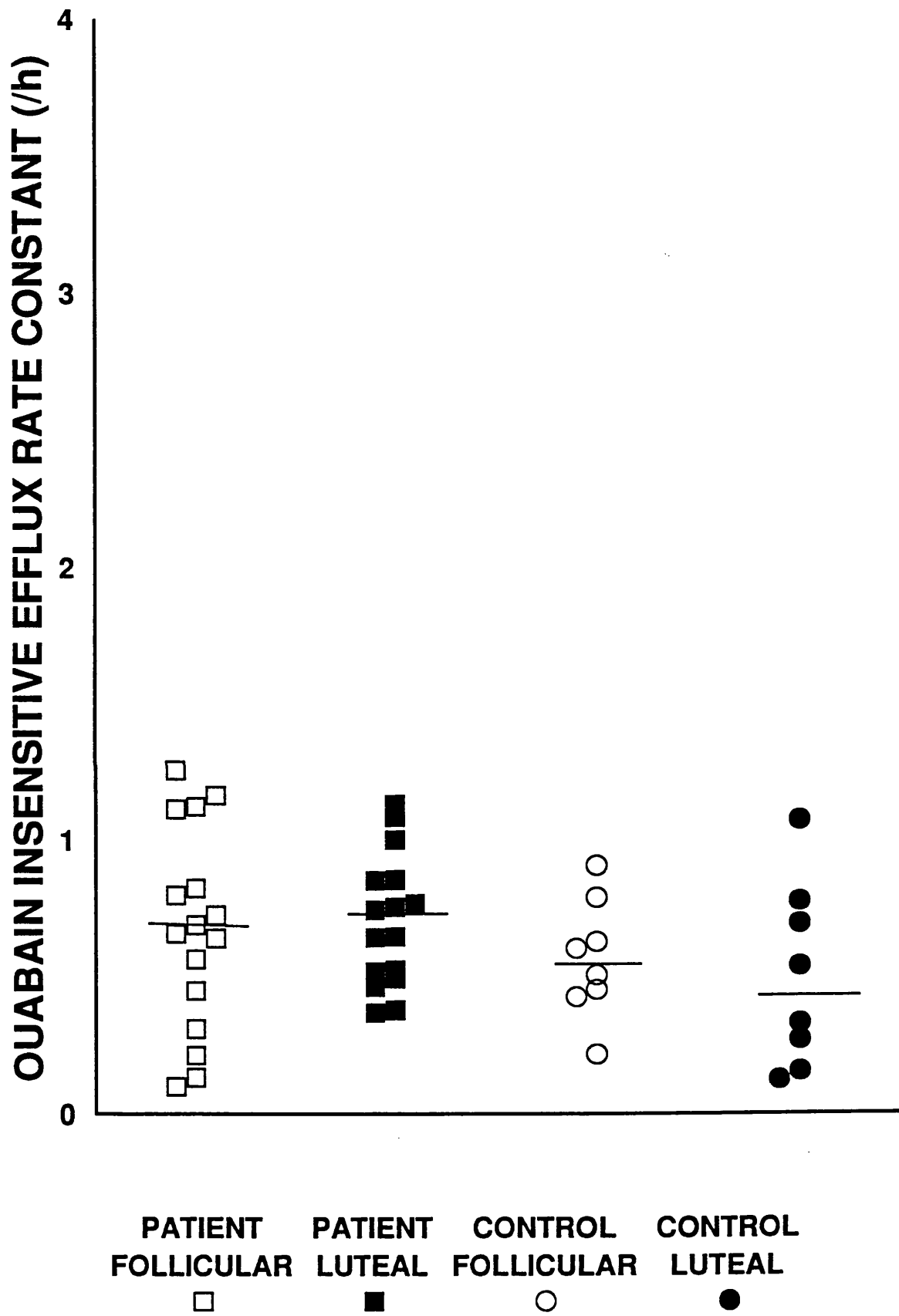
Fig 3.4: Percentage Cell Water
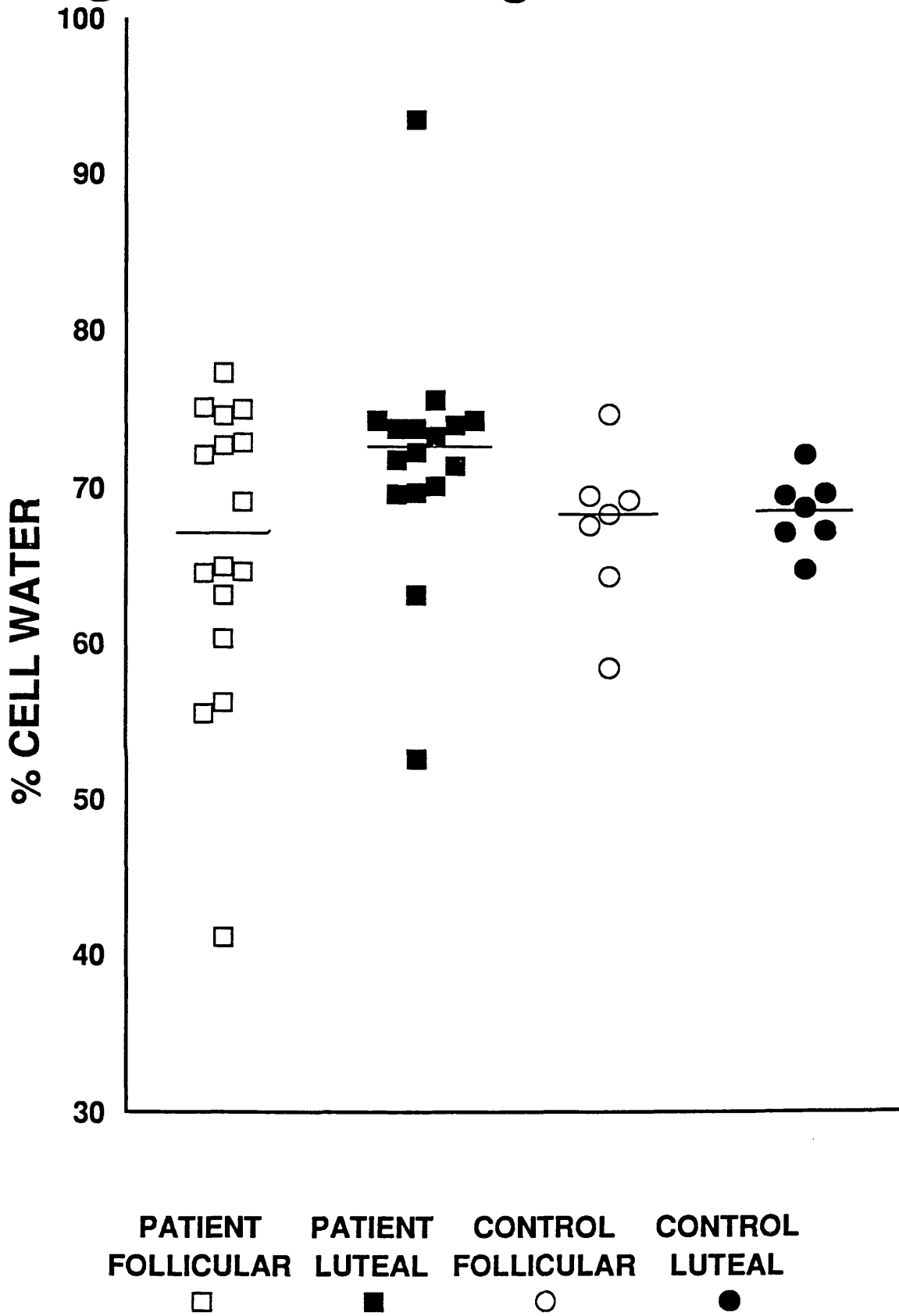
II.   Ex vivo study of human platelets from healthy controls

A.   Development of the Na^+,K^+-ATPase assay in platelets

The concentration of radioactive sodium (^{22}Na) in the platelet increases with time (see Table 3.3). The ^{22}Na content at t=6 min is significantly (p<0.0001) higher than the value at t=2 min. Also the value observed at t=10 min is significantly (p<0.0005) higher than the value at t=6 min.

Table 3.3: The uptake of sodium in platelets

Median value and (range) stated below. (n=50)

Units: count per minute (cpm).

Time (min)	Median count of radioactivity in pellet		
2	251 (116 - 559)	⎤ p<0.0001	
6	335 (148 - 801)	⎦	
10	363 (149 - 631)	⎤ p<0.0005	

To prove that variation in platelet number or size does not occur during the incubation and cause the increase in the pellet sodium content, the platelet count was measured. The results can be seen in Table 3.4. and show that the increase in platelet pellet ^{22}Na content was not due to an increase in the number of platelets.

Table 3.4: Platelet Count

Median value and (range) stated below. (n=7)

Units: $x10^9$ platelets.l^{-1}.

Time (min)	Platelet Count
2	349 (276 - 548)
6	348 (248 - 572)
10	348 (248 - 560)

Since the number of platelets in each PRP sample varies, the platelet count was corrected to a value of 350×10^9 platelets.l^{-1} to standardise the method. This procedure makes each set of results more comparable to others in the same group of experiments as can be seen by comparing Fig 3.5 and 3.6.

The count per minute (cpm) in the supernatant for a set of experimental conditions at a particular time will vary. Hence, whether to correct for the cpm value was also considered. By comparing Fig 3.5 and 3.7 it was decided that no additional benefit was gained from this correction and this calculation was not considered necessary.

Fig 3.5: Sodium Uptake by Platelets
Uncorrected Values
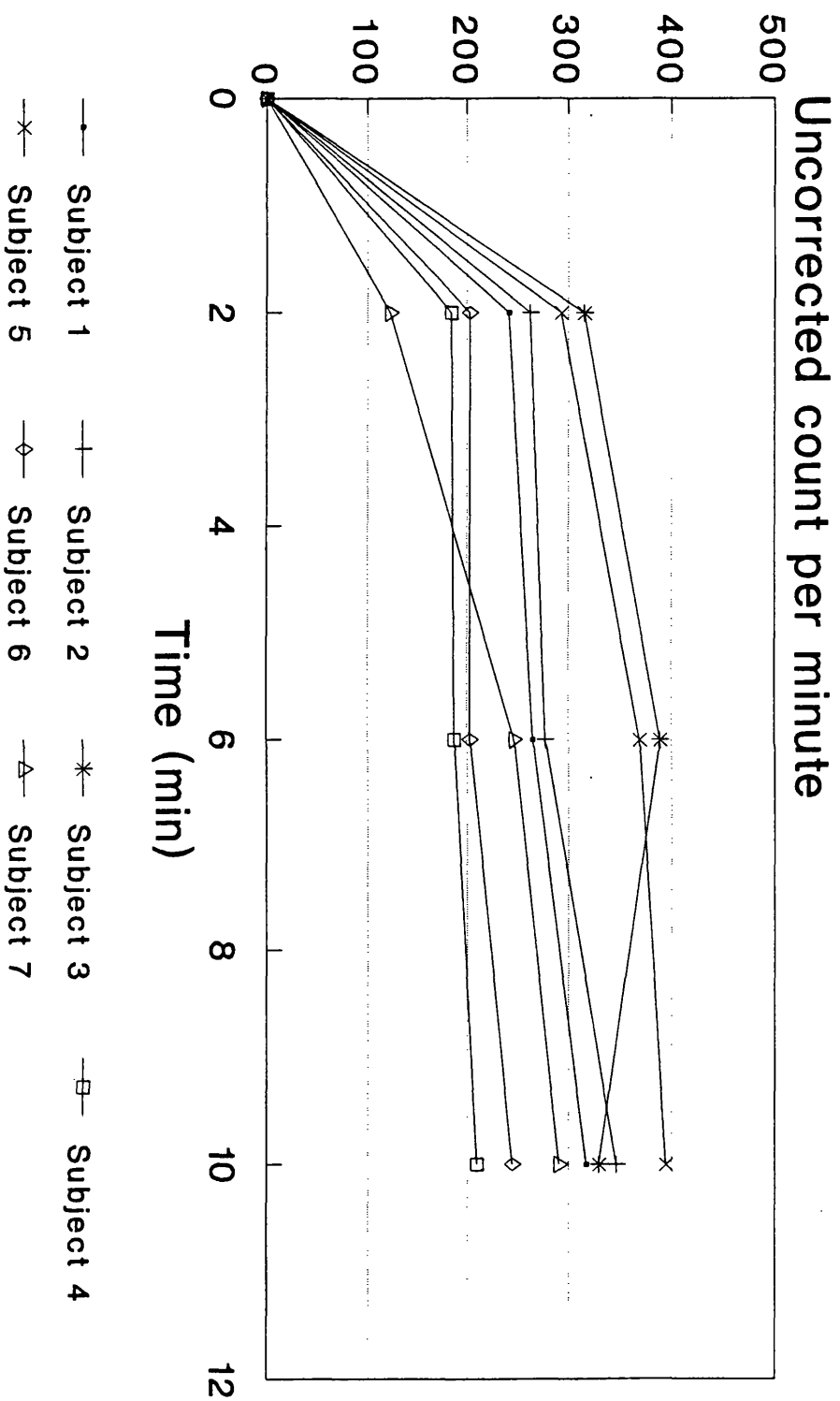
Fig 3.6: Sodium Uptake by Platelets
Corrected for platelet number
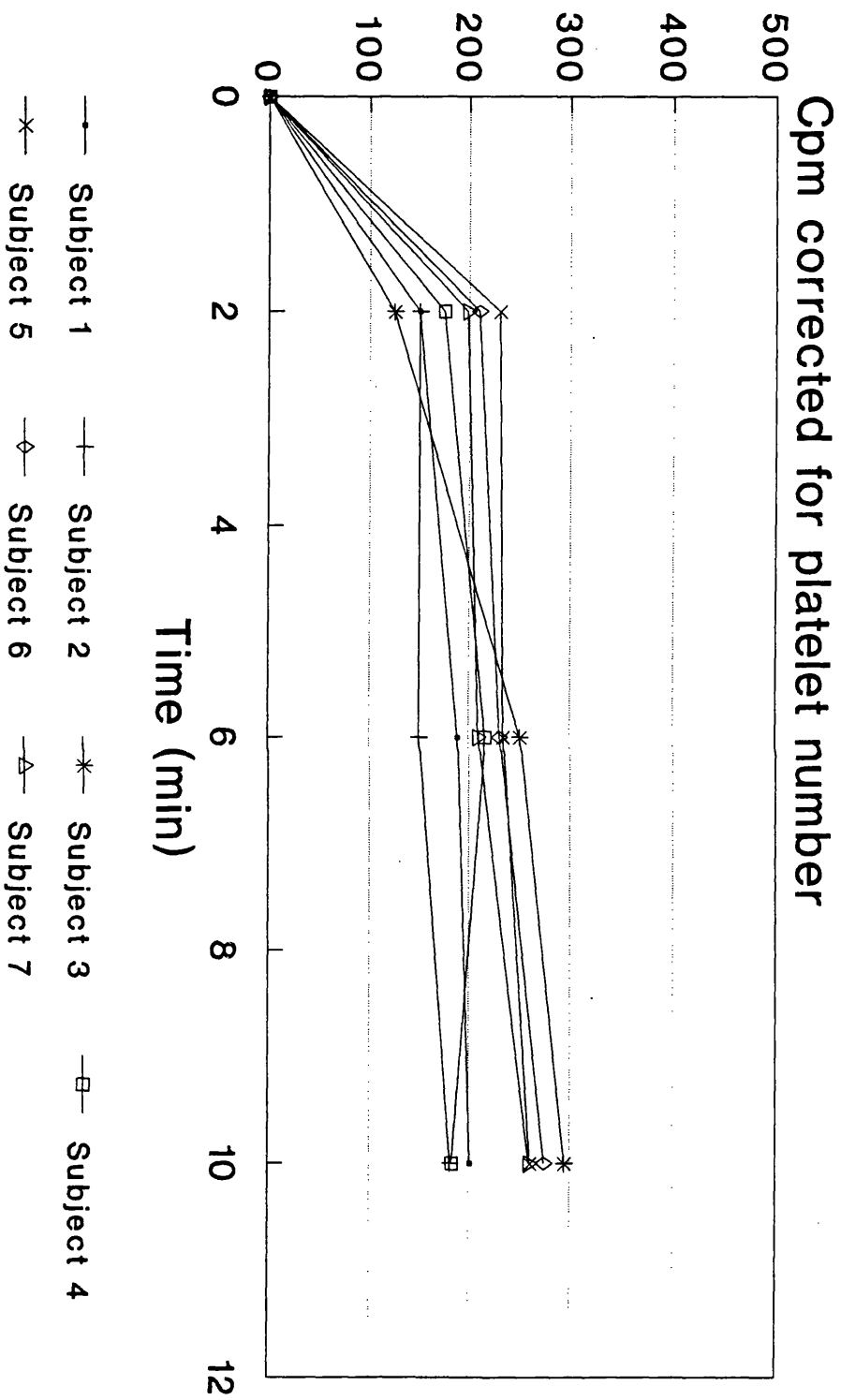
Fig 3.7: Sodium Uptake by Platelets
Corrected for cpm in supernatant
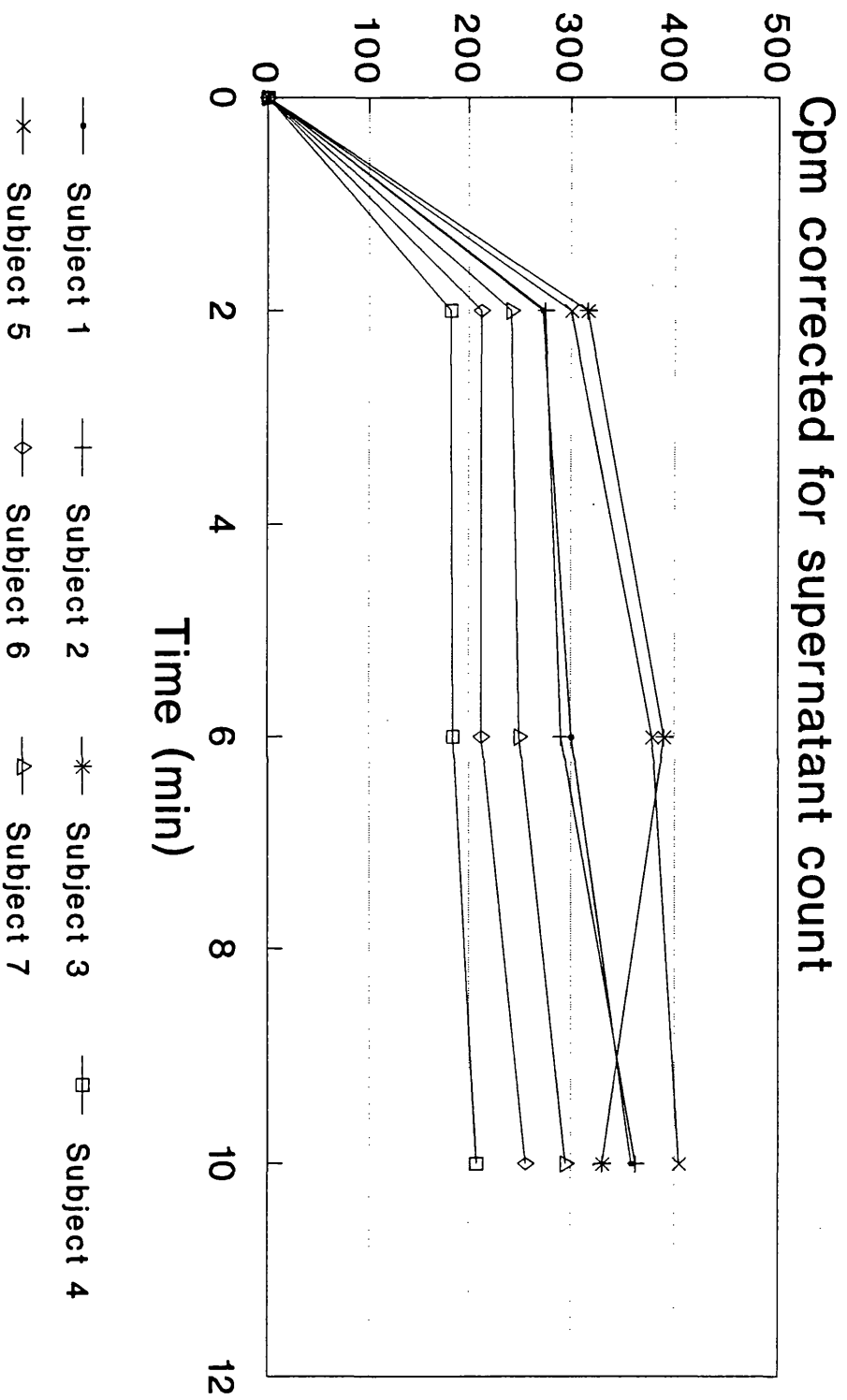
Finally, the supernatants of the platelet pellets were collected and counted to ascertain whether it was better to study the rate of increase of radioactivity in the pellet or the rate of decrease of radioactivity in the supernatant. The results in Table 3.5 show that a fall in the cpm of the supernatant could not be consistently detected.

Table 3.5: Supernatant count

Median value and (range) stated below. (n=3)

Units: $x10^4$ cpm.1^{-1} supernatant.

Time (min)	Supernatant Count
2	227 (212 - 228)
6	216 (205 - 220)
10	221 (205 - 234)

Purity of Platelet Rich Plasma

Table 3.6: Verification of the contamination of PRP

Median value and (range) stated below, (n=14).

Platelets x 10^9/l	Leucocytes x 10^9/l	Erythrocytes x 10^{12}/l
369 (180 - 480)	0.1 (0.1 - 0.4)	0.00 (0.00 - 0.02)

These results show that the erythrocyte count is undetectable in most cases and the leucocyte count represents 0.066 % of the platelet count.

Extracellular Fluid Trapping in Platelets

Table 3.7: Verification of ^{51}Cr-EDTA adherence to plastic

and fluid trapped in the platelet pellet

Median value and (range) of the cpm of washed platelet pellets in Eppendorf tubes stated below (except saline where because there is no PRP there cannot be a pellet), (n=7).

^{51}Cr-EDTA in saline	^{51}Cr-EDTA in PRP	PRP alone
10 (0 - 43)	19 (0 - 34)	4 @@@ (0 - 8)

@@@ = $p < 0.03$ when comparing PRP to ^{51}Cr-EDTA in PRP.

Comparing ^{51}Cr-EDTA in saline to that in PRP show no significant difference. However, PRP with and without ^{51}Cr-EDTA are significantly different.

B. <u>Validation of the Na$^+$,K$^+$-ATPase assay using ^{22}Na in</u>
   <u>platelets</u>

Table 3.8: The accumulation of sodium in the presence of
         ouabain

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	Ouabain (0.2 mmol/l)	Increment
2	175 (140 - 245)	258 (160 - 359)	+ 58.3 % @@
6	220 (187 - 297)	360 (232 - 423)	+ 65.1 % @@
10	253 (188 - 342)	444 (387 - 571)	+ 88.1 % @@

@@ = $p < 0.03$ comparing platelet ^{22}Na uptake in the
presence and absence of ouabain.

The median values, seen in Table 3.8, indicate that
ouabain causes a significant ($p < 0.03$) increase in the ^{22}Na
uptake of the platelet pellet compared to a saline control.
The ouabain-induced increment increases with incubation time.
Thus, at t=2 min, the increment is +58.3%; at t=6 min, +65.1%
and at t=10 min, +88.1%.

Table 3.9: The adrenaline-induced increase in platelet

sodium uptake

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	Adrenaline (50 nmol/l)	Increment
2	175 (140 - 245)	254 (192 - 270)	+ 45.1 % @@@
6	220 (187 - 297)	239 (173 - 333)	+ 8.6 %
10	253 (188 - 342)	280. (201 - 547)	+ 10.7 %

@@@ = p< 0.02 comparing platelet ^{22}Na uptake in the presence and absence of adrenaline.

The presence of adrenaline (50 nmol.l^{-1}) caused a temporary significant (p< 0.02) increase in platelet ^{22}Na uptake at t=2 min but there was no significant increase in the 6 and 10 min samples (see Table 3.9).

Table 3.10: The effect of adrenaline (2.5 nmol.l^{-1}) on

platelet sodium uptake

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Adrenaline (2.5 nmol/l)	Increment
2	290 (233 - 386)	308 (238 - 371)	+ 6.2 %
6	407 (316 - 726)	399 (313 - 433)	- 2.0 %
10	409 (334 - 592)	447 (364 - 1082)	+ 9.3 %

Table 3.11: The effect of adrenaline (25 nmol.l^{-1}) on

platelet sodium uptake

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Adrenaline (25 nmol/l)	Increment
2	344 (264 - 559)	343 (271 - 437)	0.0 %
6	469 (344 - 801)	443 (311 - 476)	- 5.5 %
10	531 (402 - 631)	522 (389 - 711)	- 1.7 %

Preliminary experiments showed that adrenaline at a final concentration of 2.5 and 25 nmol.l^{-1} had no significant effect on the ^{22}Na uptake of platelets (see Tables 3.10 & 3.11).

Table 3.12: The effect of ouabain on the adrenaline

-induced increase in platelet sodium uptake

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline + Ouabain	Adrenaline + Ouabain	Increment
2	258 (160 - 359)	252 (204 - 355)	- 2.3 %
6	360 (232 - 423)	375 (249 - 411)	+ 4.2 %
10	444 (387 - 571)	414 (333 - 512)	- 6.5 %

In the presence of ouabain, adrenaline did not induce a further increase in the platelet ^{22}Na uptake (see Table 3.12).

Fig 3.8 is a summary of Tables 3.8, 3.9 and 3.12. It shows that both adrenaline and ouabain increase ^{22}Na uptake at t=2 min. However, the two agents act in different ways because the ouabain is a permanent effect that continues to increase the ^{22}Na content at t=6 and 10 min whereas the platelets seem to recover from the adrenaline effect at these times. Thus, the ^{22}Na uptake moves towards the saline control value at t=6 and 10 min in the presence of adrenaline.

Fig 3.8: Sodium Uptake in Platelets

Count per minute

Time (min)

— Saline   + Ouabain   * Adrenaline   ☐ Adrenaline & Ouabain
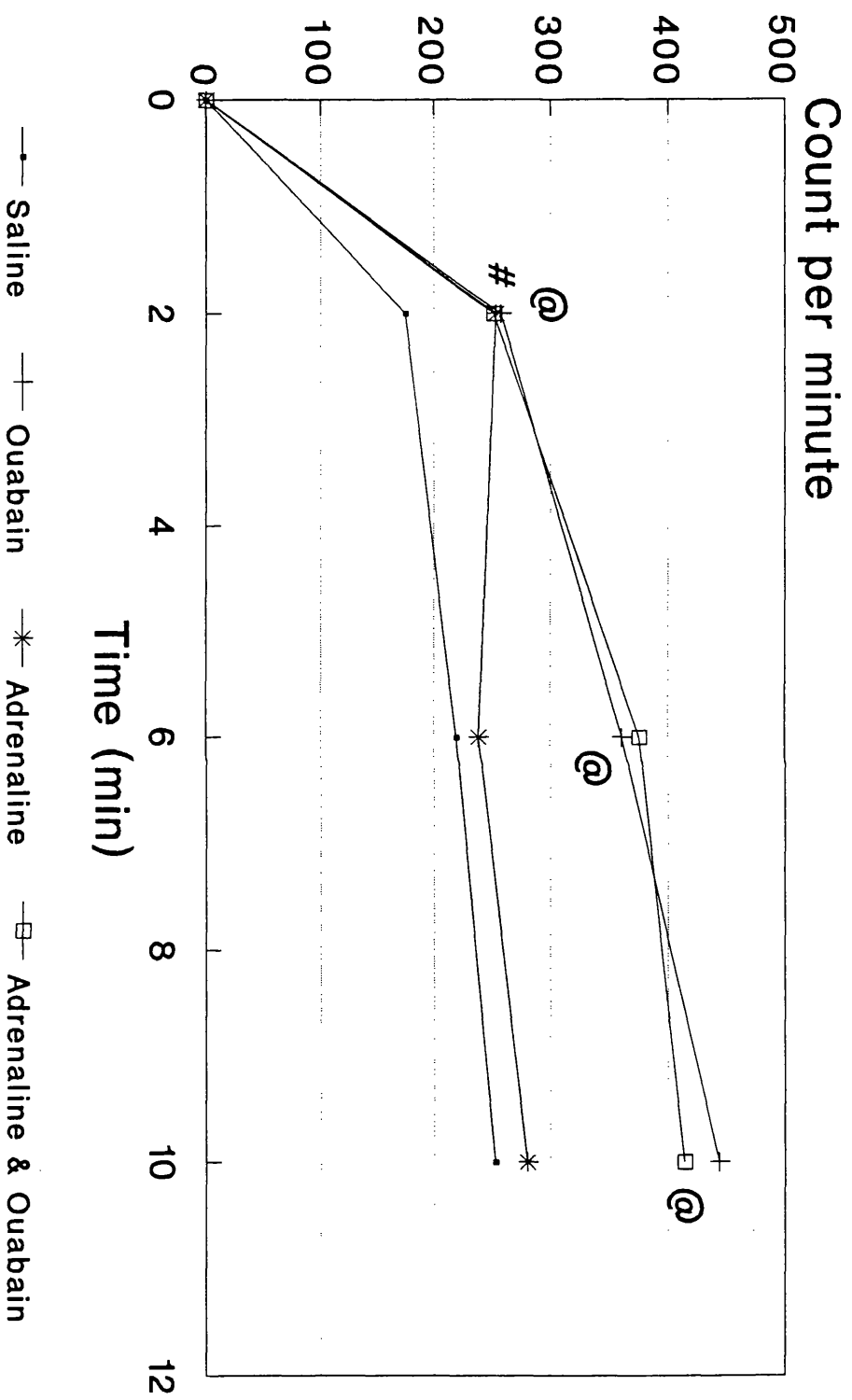
p<0.02 Saline vs Adrenaline #
p<0.03 Saline vs Ouabain   @

Timolol (a β_1, β_2 adrenoceptor antagonist) and atenolol (a β_1 adrenoceptor antagonist) were used to identify which subtype of adrenoceptor causes the adrenaline-induced increase seen. Investigations showed that at t=2 min atenolol (30 nmol.l^{-1}) did not inhibit the adrenaline-induced increase in platelet ^{22}Na uptake (see Table 3.13). However, timolol significantly (p<0.02) inhibits the adrenaline-induced increase in platelet ^{22}Na uptake (see Table 3.14).

Table 3.13: The lack of inhibition of the adrenaline -induced increase in platelet sodium uptake with atenolol

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Adrenaline	Adrenaline + Atenolol (30 nmol/l)	Increment
2	324 (204 - 532)	320 (215 - 498)	- 1.3 %

Table 3.14: The inhibition of the adrenaline-induced
increase in platelet sodium uptake with
timolol

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Adrenaline	Adrenaline + Timolol (30 nmol/l)	Increment
2	594 (243 - 666)	451 (215 - 556)	- 31.7 % @@@

@@@ = p< 0.02 comparing platelet ^{22}Na uptake in the presence and absence of timolol.

The results so far, indicate that the adrenaline-induced increase in platelet ^{22}Na uptake is mediated by the β_2 adrenoceptor site. Xamoterol, a β_1 agonist, and salbutamol, a β_2 agonist, were used in an attempt to confirm this. However, the results show that both xamoterol (50 nmol.l^{-1}) and salbutamol (50 nmol.l^{-1}) do not cause an adrenaline-like increase in platelet ^{22}Na uptake (see Tables 3.15 & 3.16). Both xamoterol and salbutamol were also studied at a concentration of 2.5 nmol.l-1 and similarly they did not cause an adrenaline-like increase (see Tables 3.17 & 3.18).

Table 3.15: Platelet sodium uptake in the presence of

xamoterol (50nmol.l^{-1})

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Xamoterol (50 nmol/l)	Increment
2	327 (199 - 502)	385 (163 - 604)	+ 17.7 %
6	408 (228 - 651)	375 (285 - 558)	- 8.1 %
10	473 (296 - 692)	479 (280 - 659)	+ 1.3 %

Table 3.16: Platelet sodium uptake in the presence of

salbutamol (50 nmol.l^{-1})

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Salbutamol (50 nmol/l)	Increment
2	184 (158 - 276)	181 (147 - 243)	- 1.6 %
6	268 (193 - 445)	253 (186 - 307)	- 5.6 %
10	258 (192 - 788)	259 (196 - 582)	+ 0.4 %

Table 3.17: Platelet sodium uptake in the presence of

xamoterol (2.5 nmol.l^{-1})

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Xamoterol (2.5 nmol/l)	Increment
2	218 (196 - 289)	221 (172 - 369)	+ 1.4 %
6	264 (200 - 365)	253 (173 - 430)	- 4.2 %
10	325 (209 - 971)	366 (270 - 523)	+ 12.6 %

Table 3.18: Platelet sodium uptake in the presence of

salbutamol (2.5 nmol.l^{-1})

Median value and (range) stated below. (n=6)

Units: cpm.

Time (min)	Saline	Salbutamol (2.5 nmol/l)	Increment
2	255 (172 - 317)	196 (184 - 260)	- 23.1 %
6	267 (227 - 349)	259 (199 - 333)	- 3.0 %
10	305 (192 - 788)	287 (196 - 582)	- 5.9 %

The results for salbutamol are not in agreement with the findings using ^{86}Rb influx which indicate that the Na,K-ATPase activity is linked to the β_2 receptor (Turaihi et al, 1989).

To clarify the results noradrenaline, a $\alpha_1,\alpha_2,\beta_1$ adrenoceptor agonist, and isoprenaline, a β_1,β_2 adrenoceptor agonist, were used. As expected isoprenaline caused a significant (p< 0.02) increase in platelet ^{22}Na uptake and noradrenaline did not (see Tables 3.19 & 3.20). The lack of stimulation by noradrenaline is evidence that there is no significant α-component to the adrenaline-stimulation that has been observed.

Table 3.19: Platelet sodium uptake in the presence of isoprenaline

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	Isoprenaline (50 nmol.1)	Increment
2	199 (153 - 758)	264 (165 - 785)	+ 32.7 % @@@

@@@ = p< 0.02 comparing platelet ^{22}Na uptake in the presence and absence of isoprenaline.

Table 3.20: Platelet sodium uptake in the presence of noradrenaline

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	Noradrenaline (50 nmol/1)	Increment
2	241 (163 - 1204)	273 (165 - 1073)	+ 13.3 %

The stimulatory effect of adrenaline on ^{86}Rb influx was confirmed in preliminary experiments (see Table 3.21). The results for ^{86}Rb and ^{22}Na methods showed a similar increase due to the presence of adrenaline, at the same concentration.

Table 3.21: ^{86}Rb influx stimulation of human platelets in the presence adrenaline

Median value and (range) stated below. (n=6)

Units: mmol.kg^{-1} protein.h^{-1}.

	Saline	Adrenaline (50 nmol/l)	Increment
Total	1240 (817 - 1675)	1361 (973 - 2117)	+ 15.1 % @@
Passive	520 (309 - 914)	538 (327 - 1120)	+ 9.8 %
Active	631 (508 - 1076)	726 (566 - 1512)	+ 15.1 % @@

@@= $p < 0.03$ comparing ^{86}Rb influx in platelets in the presence and absence of adrenaline.

^{86}Rb influx also shows a dose-dependent response with adrenaline, where the percentage increase over the saline control increases with the concentration of adrenaline used as can be seen in Fig 3.9.

Fig 3.9: Stimulation of rubidium influx due to the presence of adrenaline

% increase over saline control

Concentration of Adrenaline (nmol/l)

Total   Active   Passive
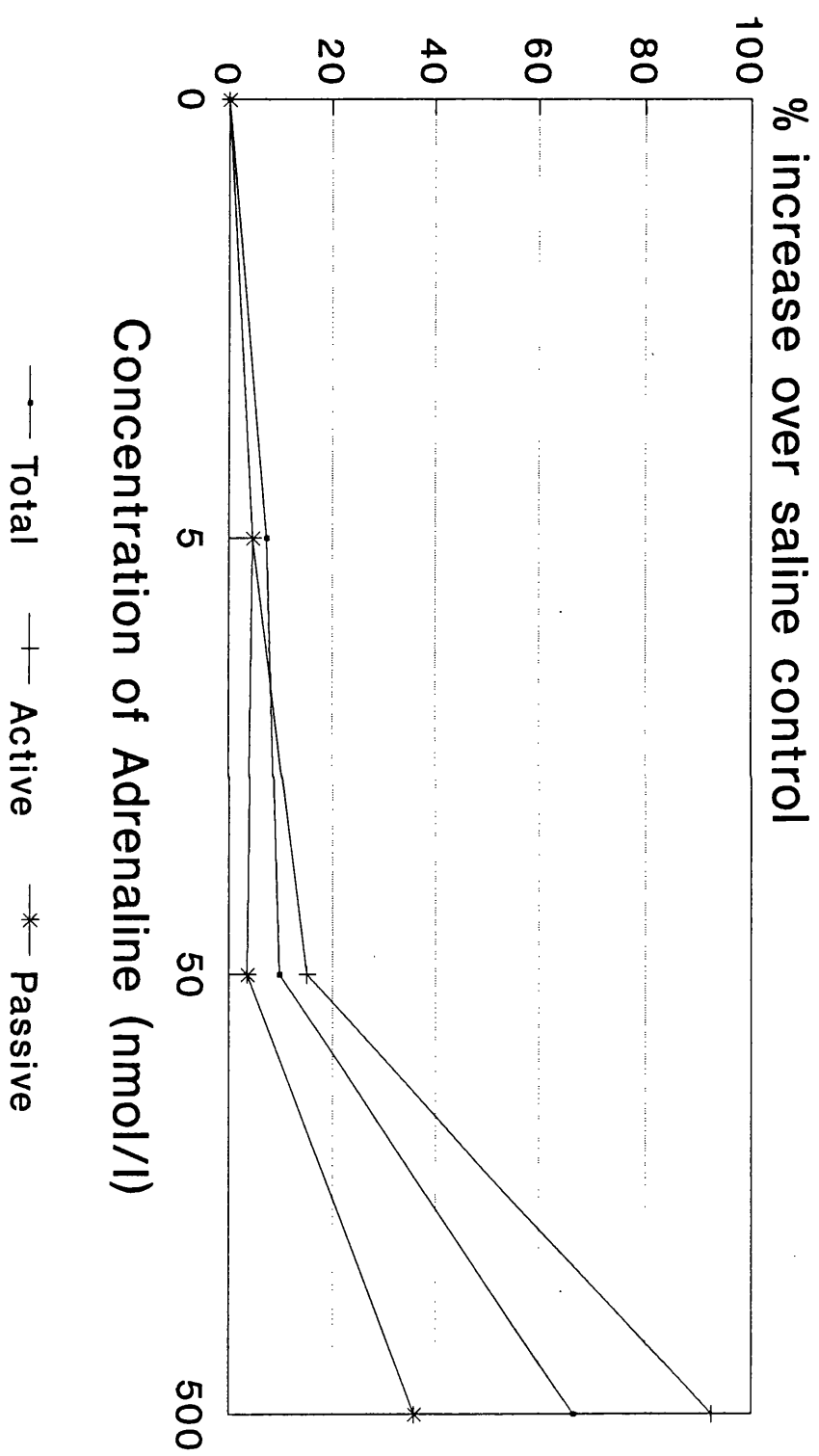
5 nmol/l n=4; 50 nmol/l n=6; 500 nmol/l n=2

^{86}Rb influx was significantly (p< 0.02) stimulated by salbutamol (see Table 3.22).

Table 3.22: ^{86}Rb influx stimulation in the presence salbutamol

Median value and (range) stated below. (n=7)

Units: mmol.kg^{-1} protein.h^{-1}.

	Saline	Salbutamol (50 nmol/l)	Increment
Total	1412 (748 - 2096)	1636 (786 - 1904)	+ 15.9 %
Passive	736 (386 - 1176)	747 (354 - 957)	+ 1.5 %
Active	676 (362 - 920)	889 (432 - 1069)	+ 31.5 % @@@

@@@ = p< 0.02 comparing platelet ^{86}Rb uptake in the presence and absence of salbutamol.

Since adrenaline and salbutamol both have β_2 agonist activity the stimulation of ^{86}Rb was considered to be mediated through the β_2 adrenoceptor. However, the findings with ^{22}Na, suggested that the adrenaline-induced increase in platelet ^{22}Na uptake must be due to an "atypical" β-adrenoceptor (adrenaline stimulated, salbutamol did not). BRL 37344, an "atypical" β_3-agonist, was therefore used to further clarify the mechanism of adrenaline action. BRL 37344, final concentration 2.5 nmol.l^{-1}, caused an adrenaline-like increase in platelet ^{22}Na uptake (see Table 3.23). As with adrenaline, the increase is significant (p< 0.002) only at t=2

min (see Table 3.23). However, BRL 37344 at a final concentration of 50 $nmol.l^{-1}$ did not cause a significant increase in platelet ^{22}Na uptake (see Table 3.24).

Table 3.23: Platelet sodium uptake in the presence of
BRL 37344 (2.5 $nmol.l^{-1}$)

Median value and (range) stated below. (n=8)

Units: cpm.

Time (min)	Saline	BRL 37344 (2.5 nmol/l)	Increment
2	227 (167 - 392)	335 (161 - 588)	+ 47.6 % @@
6	313 (200 - 453)	315 (218 - 462)	+ 0.6 %
10	346 (258 - 511)	340 (211 - 525)	- 1.7 %

@@ = $p < 0.03$ comparing platelet ^{22}Na uptake in the presence and absence of BRL 37344.

Table 3.24: Platelet sodium uptake in the presence of

BRL 37344 (50 $nmol.l^{-1}$)

Median value and (range) stated below. (n=3)

Units: cpm.

Time (min)	Saline	BRL 37344 (50 nmol/l)	Increment
2	255 (253 - 323)	245 (232 - 336)	+ 3.9 %
6	369 (339 - 633)	331 (290 - 423)	- 10.3 %
10	383 (371 - 551)	389 (356 - 515)	+ 1.6 %

Table 3.25: ^{86}Rb influx stimulation in the presence

BRL 37344

Median value and (range) stated below. (n=7)

Units: $mmol.kg^{-1}$ $protein.h^{-1}$.

	Saline	BRL 37344 (2.5 nmol/l)	Increment
Total	1228 (857 - 2096)	1408 (1059 - 2273)	+ 14.7 % @
Passive	606 (351 - 1176)	579 (348 - 869)	- 4.5 %
Active	653 (362 - 1143)	901 (475 - 1404)	+ 38.0 % @@@@

@ = $p < 0.04$ comparing active ^{86}Rb influx in the presence and absence of BRL 37344.

@@@@ = $p = 0.005$ comparing active ^{86}Rb influx in the presence and absence of BRL 37344.

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BRL 37344 (2.5 nmol.l^{-1}) also significantly stimulates both the total ^{86}Rb influx (p<0.04) and the active ^{86}Rb influx (p< 0.005) (see Table 3.25).

The adrenaline-like significant (p< 0.02) increase caused by BRL 37344 in platelet ^{22}Na uptake is inhibited by timolol (see Table 3.26) and not by atenolol (see Table 3.27).

Table 3.26: The inhibition of the BRL 37344-induced
increase in platelet sodium uptake with
timolol

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	BRL 37344	BRL 37344 + Timolol (60 nmol/l)
2	167 (109 - 398)	223 (141 - 410)	173 @@@ (143 - 442)
6	187 (142 - 478)	230 (124 - 717)	168 (136 - 462)
10	238 (189 - 579)	252 (189 - 560)	245 (168 - 535)

@@@ = p< 0.02 comparing platelet ^{22}Na uptake of the saline control to that in the presence of BRL 37344.

Table 3.27: The lack of inhibition of the BRL 37344
-induced increase in platelet sodium uptake
with atenolol

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	BRL 37344	BRL 37344 + Atenolol (60 nmol/l)
2	286 (185 - 415)	260 (218 - 409)

BRL 37344-induced a significant (p< 0.02) increase in total and active ^{86}Rb influx. Timolol significantly (p< 0.02) decreased the BRL 37344-stimulated active ^{86}Rb influx as can be seen in Table 3.28.

Table 3.28: ^{86}Rb influx stimulation in the presence

BRL 37344 and its inhibition with timolol

Median value and (range) stated below. (n=7)

Units: mmol.kg-1 protein.h-1.

	Saline	BRL 37344	BRL 37344 + Timolol
Total	994 (536 - 2440)	1129 @@@ (585 - 2596)	1098 (534 - 2246)
Passive	426 (258 - 1019)	439 (248 - 869)	492 (258 - 948)
Active	556 (278 - 1421)	687 @@@ (337 - 1794)	579 ### (276 - 1298)

@@@ = p< 0.02 comparing ^{86}Rb influx in the presence and absence of BRL 37344.

= p< 0.02 comparing BRL 37344-stimulated ^{86}Rb influx in the presence and absence of timolol.

C.   Using the Na+,K+-ATPase assay to investigate the effect of milrinone on sodium transport in platelets

Milrinone appears to act in the same way as adrenaline, causing an increase in sodium platelet uptake initially and reaching an equilibrium so that there is no significant difference between the saline control and milrinone in later samples.

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Table 3.29: Platelet sodium uptake in the presence of

milrinone (1 μmol.l^{-1})

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Saline	Milrinone (1 μmol/l)	Increment
2	218 (160 - 265)	254 (191 - 327)	+ 16.5 % @@@
6	302 (232 - 359)	304 (230 - 328)	+ 0.7 %
10	327 (284 - 417)	345 (294 - 442)	+ 5.5 %

@@@ = p< 0.02 when comparing platelet ^{22}Na uptake in the presence and absence of milrinone.

Table 3.30: Platelet sodium uptake in the presence of

milrinone (5 μmol.l^{-1})

Median value and (range) stated below. (n=3)

Units: cpm.

Time (min)	Saline	Milrinone (5 μmol/l)	Increment
2	253 (186 - 310)	310 (222 - 349)	+ 22.5 %
6	272 (199 - 325)	319 (248 - 362)	+ 17.3 %
10	280 (235 - 334)	347 (295 - 390)	+ 23.9 %

The results in Table 3.30 show that responses at 5 μmol.l^{-1} milrinone are in a similar direction to those with 1 μmol.l^{-1}. However, the higher dose appears to produce

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qualitatively more definitive results. However, the 1 μmol.l^{-1}

milrinone dose is more relevant because therapeutic levels

rarely exceed

3 μmol.l^{-1}.

Table 3.31: Platelet sodium uptake of ouabain-treated

cells in the presence of milrinone

(1 μmol.l^{-1})

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Ouabain + saline	Ouabain + milrinone	Increment
2	259 (181 - 353)	259 (207 - 397)	0 %
6	342 (252 - 493)	321 (247 - 546)	- 6.1 %
10	421 (309 - 552)	402 (310 - 515)	- 4.5 %

The pretreatment of platelets with ouabain masks the

milrinone-induced increase as ouabain itself causes an

increase in platelet sodium uptake (see Table 3.31). However,

this result does indicate that the milrinone-induced increase

is linked to the sodium pump because ouabain is an inhibitor

of this pump. Adrenaline and milrinone were added

simultaneously to see if they were acting through the same or

different mechanisms. As can be seen in Table 3.32 synergism

does not occur suggesting that adrenaline and milrinone do not

act through independent mechanisms.

Table 3.32: The effect of milrinone (1 μmol.l^{-1}) on
sodium uptake of platelets stimulated with
adrenaline (50 nmol.l^{-1})

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Adrenaline + saline	Adrenaline + milrinone	Increment
2	210 (145 - 292)	195 (163 - 330)	- 7.1 %
6	299 (199 - 360)	258 (202 - 387)	- 13.7 %
10	307 (234 - 401)	297 (244 - 430)	- 3.3 %

Table 3.33: The effect of milrinone (5 μmol.l^{-1}) on
sodium uptake of platelets stimulated with
adrenaline (50 nmol.l^{-1})

Median value and (range) stated below. (n=7)

Units: cpm.

Time (min)	Adrenaline + saline	Adrenaline + milrinone	Increment
2	207 (154 - 245)	184 (138 - 240)	- 11.1 %
6	218 (157 - 261)	195 (166 - 290)	- 10.6 %
10	259 (183 - 311)	200 (171 - 288)	- 22.8 % @@@

@@@ = $p < 0.02$ when comparing adrenaline stimulation of
platelet ^{22}Na uptake in the presence and absence of milrinone.

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Table 3.33 shows that adrenaline and milrinone do not enhance each other. They seem to interfere with each other as the sodium uptake is reduced in the presence of milrinone.

Table 3.34: Platelet sodium uptake stimulation with adrenaline (50 nmol.1-^{1}) and the effect of milrinone (5 μmol.1^{-1})

Median value and (range) stated below. (n=3)

Units: cpm.

Time (min)	Saline	Adrenaline	Adrenaline + milrinone
2	264 (149 - 279)	284 (172 - 335)	261 (168 - 268)
6	279 (179 - 344)	339 (287 - 415)	293 (241 - 315)
10	273 (210 - 365)	392 (249 - 405)	381 (208 - 415)

The results above indicate that milrinone inhibits the adrenaline-induced increase so that the results with adrenaline and milrinone resemble those of the control.

IIIA. In vitro work with ethanol and the sodium pump in

leucocytes

a) Prolonged Exposure

Table 3.35: The effect of prolonged exposure of ethanol

on efflux rate constants in human leucocytes

Units: h^{-1}.

	Control	Ethanol	Increment
Subject 1		(18 mmol/l)	
TERC	2.06	2.33	+ 14.0 %
OSERC	1.28	1.96	+ 54.9 %
OIERC	0.79	0.37	- 53.3 %
Subject 2			
TERC	2.59	2.94	+ 13.4 %
OSERC	1.66	2.45	+ 48.0 %
OIERC	0.93	0.49	- 47.8 %
Subject 1		(9 mmol/l)	
TERC	2.90	3.11	+ 7.4 %
OSERC	2.44	2.23	- 8.7 %
OIERC	0.45	0.88	+ 48.5 %
Subject 1		(1.8 mmol/l)	
TERC	3.50	3.58	+ 2.5 %
OSERC	1.85	2.18	+ 18.2 %
OIERC	1.65	1.41	- 15.0 %
Subject 2			
TERC	2.16	3.02	+ 40.0 %
OSERC	1.55	2.53	+ 63.6 %
OIERC	0.62	0.50	+ 19.5 %

The results were obtained from different subjects on
different days.

b) <u>Short Exposure</u>

Table 3.36: The effect of short exposure of ethanol on

efflux rate constants

Units: h^{-1}.

	Control	Ethanol	Increment

Subject 1
  TERC
  OSERC
  OIERC
	
	1.78
	1.25
	0.54
		(9 mmol/l)
		1.51
		1.00
		0.51
			
			− 15.5 %
			− 20.0 %
			− 5.1 %

Suject 2
  TERC
  OSERC
  OIERC
	
	3.12
	1.99
	1.13
		
		2.32
		1.53
		0.79
			
			− 25.7 %
			− 30.5 %
			− 23.0 %

Subject 3
  TERC
  OSERC
  OIERC
	
	3.93
	3.38
	0.55
		
		3.10
		2.71
		0.39
			
			− 21.2 %
			− 28.8 %
			− 19.9 %

Suject 1
  TERC
  OSERC
  OIERC
	
	3.53
	3.16
	0.37
		(1.8 mmol/l)
		3.25
		2.48
		0.77
			
			− 7.9 %
			− 21.6 %
			+ 110.0 %

Subject 2
  TERC
  OSERC
  OIERC
	
	3.83
	3.22
	0.62
		
		3.27
		1.39
		1.88
			
			− 14.7 %
			− 56.9 %
			+ 20.6 %

With an incubation time of 2 h, it appears that the addition of ethanol will affect the sodium pump at much lower concentrations than originally thought. From this preliminary work it can be seen that ethanol increases sodium pump activity (OSERC) even at concentrations of 1.8 $mmol.l^{-1}$ when the incubation time is extended to 2 h.

Short incubation times show a confused set of results. Ethanol at a concentration of <40 $mmol.l^{-1}$ should have no effect on the sodium pump activity as was previously shown by Green (1985). However, at a concentration of 9 $mmol.l^{-1}$ a

decrease in sodium pump activity, OSERC, was seen. This is consistent with the effect of higher concentrations of alcohol on short exposure. At 1.8 mmol.l^{-1} the sodium pump activity appears to be increased. Even though this is not consistent with previous work, the ethanol is still influencing the assay.

IIIB.The recovery of 17β-oestradiol when diluted with the
Buffer I

As can be seen from Fig 3.10 the recovery of 17β-oestradiol from the buffer I is very poor. The maximum recovery was approximately 50 % which we do not consider sufficient to warrant further investigation using buffer I as the diluent.

Fig 3.10: % Recovery of 17β-oestradiol

% recovery

Concentration 17β-oestradiol (pmol/l)
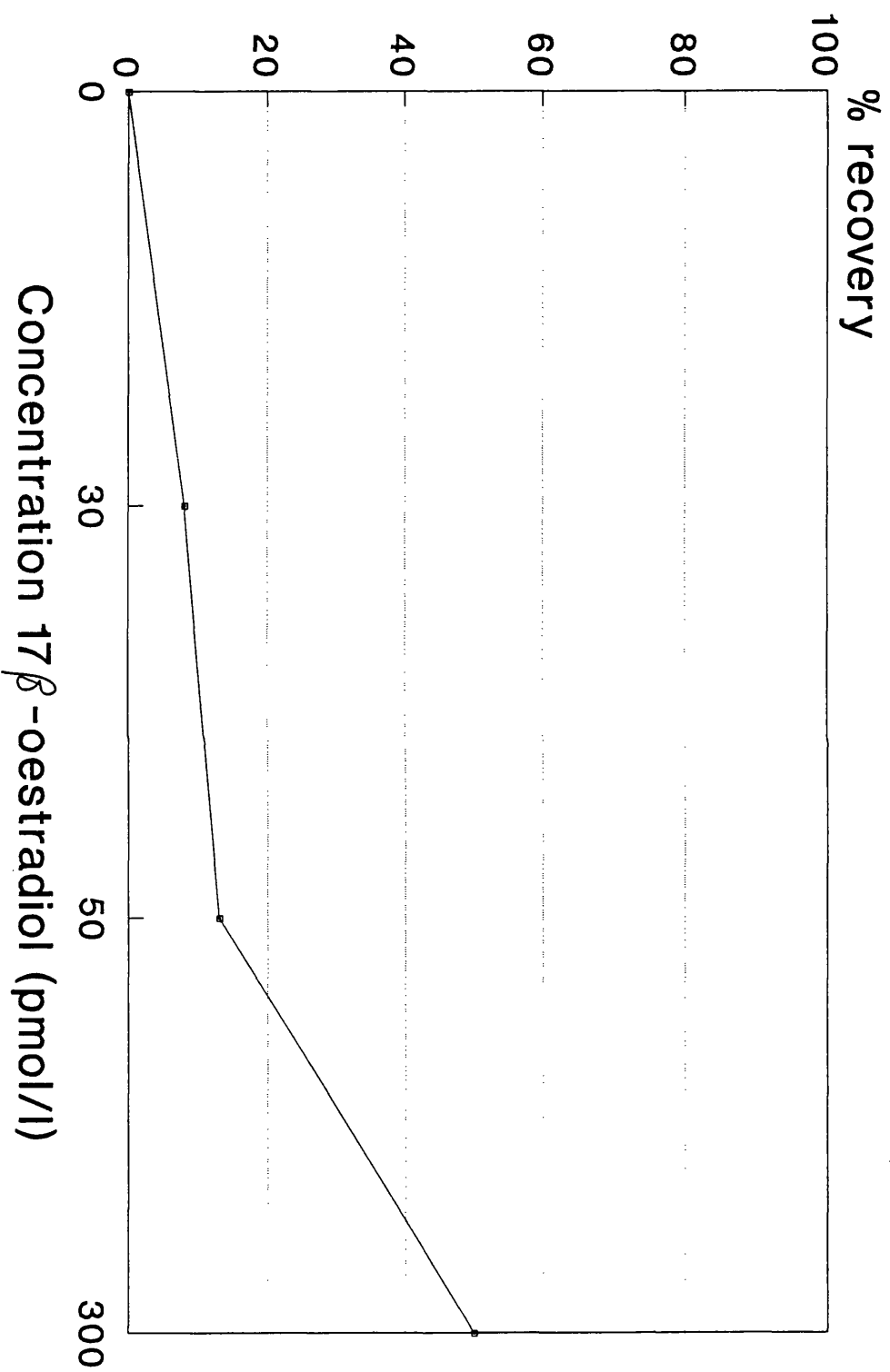
30 pmol/l n=3; 50 pmol/l n=2;
300 pmol/l n=2

IIIC. <u>The interaction of ouabain with 17β-oestradiol or</u>

   <u>testosterone</u>

i)   <u>Ouabain and 17β-oestradiol</u>

   Table 3.37: The effect of the addition of buffer I or

           ouabain solution XIIa to pooled PPP on

           17β-oestradiol concentration in PPP

   Median value and (range) stated below, (n=8).

   Units: $pmol.l^{-1}$.

PPP + Buffer I	PPP + Buffer and Ouabain	PPP Alone
385 @ (347 - 495)	421 (301 - 499)	443 (348 - 503)

@ = $p < 0.04$ when comparing the addition of buffer I to

PPP to a solution of 17β-oestradiol in pooled plasma.

There is significantly less 17β-oestradiol when buffer I

(p< 0.04) is added to PPP than when PPP alone is present.

This shows that the addition of aqueous solutions

significantly lowers the concentration of 17β-oestradiol in

PPP samples. However, on addition of ouabain, the

17β-oestradiol concentration was no longer significantly lower

than when the ouabain vehicle alone was added. In fact, the

concentration of 17β-oestradiol appears to be increased,

although not significantly.

ii)   <u>Ouabain and testosterone</u>

Table 3.38: The effect of the addition of buffer I or

ouabain solution XIIa to PPP

Median value and (range) stated below, (n=7).

Units: nmol.l^{-1}.

PPP + Buffer I	PPP + Ouabain	PPP
8.4 (6.8 - 11.4)	8.3 (7.4 - 11.7)	8.7 (7.1 - 11.7)

The concentration of testosterone detected by the radio-immuno-assay was not significantly altered by either the addition of the aqueous buffer or of ouabain to the buffer.

CHAPTER 4: DISCUSSION

This chapter is divided into three sections to correspond with the results chapter.

I. Ex vivo study of leucocytes in women with PMS and asymptomatic controls in the follicular and the luteal phase

Electrolyte and water movements are regulated by Na^+,K^+-ATPase (Ewe, 1988). Consequently the sodium pump plays a major role in the regulation of intracellular volume and intracellular electrolytes (Lopes et al, 1988). The rate of the sodium pump is dependent on the intracellular (Weissberg, 1983) and extracellular (Baron & Khan, 1985) sodium and potassium concentrations. A decrease in Na^+,K^+-ATPase activity will cause a decrease in intracellular potassium and an increase in intracellular sodium (Dux et al, 1990). The sodium pump transports $3Na^+$ out of the cell and $2K^+$ into the cell for each unit of ATP that is used. Therefore, an equimolar exchange does not occur and hence a decrease in pump activity may result in an increase in the concentration of intracellular ions. This increase may lead to an increase in cell water, by osmosis, leading to cellular oedema (Betz et al, 1990; Shigeno et al, 1989; Cohadon et al, 1989; Arrigoni et al, 1987; Sztriha et al, 1987).

Suggestions have been made that in many diseases where cellular oedema occurs, the sodium pump activity is altered. For example, in diabetic animals where endoneurial oedema occurs there is also decreased perineurial Na^+,K^+-ATPase activity (Llewelyn & Thomas, 1987). Decreased sodium pump

activity has also been recorded in cases where spinal cord oedema (Faden et al, 1987), encephalopathy (Ede et al, 1987), epilepsy (Arrigoni et al, 1987) and Reyes syndrome (Olson et al, 1989) occur. Hence, increased cell volume and decreased sodium pump activity appear to be linked in many disease states.

In the present study, there was a decrease in pump activity with a corresponding increase in percentage cell water when comparing patients to controls in the luteal phase. However, there was no significant change in the intracellular or plasma electrolyte concentrations. This is not surprising because although the decreased pump activity will lead to an increased intracellular sodium, the cell will increase its water content (and volume) to maintain the correct intracellular concentration of sodium. Therefore, our findings agree with the previous work that a decrease in pump activity leads to cellular oedema.

M'Buyamba-Kabangu et al (1985) recorded a significant decrease in red cell intracellular sodium concentration in the luteal phase of normal women. Although significant, this decrease was very small in magnitude (1.29 mmol.l^{-1}). Consequently, any change in leucocyte electrolyte concentration may also be expected to be equally small. The change in intracellular potassium of erythrocytes was even smaller (1.1 mmol.l^{-1}) and not surprisingly insignificant (M'Buyamba-Kabangu et al, 1985). In the present study, controls show a slight decrease (1.0 mmol.l^{-1}) in intracellular sodium and in intracellular potassium

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concentrations (6.0 mmol.l^{-1}) in leucocytes in the luteal phase. Both these changes were not significant. Interestingly, the PMS patients show a similar slight decrease (1.0 mmol.l^{-1}) in intracellular sodium and an increase (10.0 mmol.l^{-1}) in intracellular potassium. Again these changes were not significant. Our results do not agree with those of M'Buyamba-Kabangu et al (1985), perhaps because we used leucocytes and not erythrocytes. It has been previously noted that leucocytes and erythrocytes behave in an opposing manner when studied in the same disease state (Green, 1985 p170).

The women with PMS do however, show a different trend to the controls. An increase in intracellular sodium concentration and a decrease in intracellular potassium concentration would have been expected to result from the decrease in sodium pump activity in the PMS patients. The increase in intracellular sodium concentration may have been masked by the documented increase in intracellular water. As both the results for the PMS group are insignificant they fit with the assumption that the increase in intracellular sodium concentration in the patients cannot be demonstrated because water enters the cell in response to the increase in intracellular sodium. However, a concomitant decrease in intracellular potassium concentration would have been expected but was not seen. This apparent discrepancy may be explained if these changes are not large enough to be measured accurately and hence show no significance. Furthermore as the sodium pump transports 3Na$^+$ for every 2K$^+$, the increase in

sodium may be more marked than the decrease in potassium. Therefore, the decrease in potassium concentration may be too small to be measured with the methodology used. Also the transport of potassium ions is not exclusively controlled by the sodium pump. There are at least 20 potassium channels (Triggle, 1990). Therefore, changes in the sodium pump may be counteracted by other channels such that the results observed do not concur with the decrease in sodium pump activity.

Another possibility is that due to the pool size in the intracelular environment a much larger change in potassium may be needed to obtain a significant result. The intracellular pool of sodium is much smaller than for potassium [intracellular sodium concentration = 27 mmol.kg-1(range: 25-28); intracellular potassium concentration = 122 mmol.kg-1(62-151)] (Letner, 1984). Therefore, if there is a quantitatively similar increase in sodium and decrease in potassium then the change in sodium is more likely to be significant.

There were no significant differences in plasma osmolality either between the women with PMS and the controls or between the follicular and the luteal phases. Therefore, changes in cell water are unlikely to be due to the free movement of water into cells as a consequence of cells originating from plasma of different osmolality. These findings are therefore compatible with the hypothesis that there is an increase in cell water due to the decrease in sodium pump activity.

Dr S Y Hussain (Department of Obstetrics & Gynaecology,

Royal Free Hospital) measured plasma volume (PV) and total body water (TBW) during the menstrual cycle in the same controls and PMS subjects as those considered in the present thesis. Her preliminary findings (personal communication of unpublished data) suggest that there is no significant increase in PV or TBW. Total body water consists of the intracellular and the extracellular comparments. The latter includes a small transcellular compartment. The extracellular fluid consists of the plasma and the interstitial fluid. As the plasma volume, a marker for the extracellular fluid, and the total body water do not change this suggests that there can only be a redistribution of fluid between the intracellular compartment and the interstitial compartment.

In this study, the evidence points to an increase in intracellular fluid with no concomitant gain in body weight. This combination of findings suggests that a fluid redistribution occured between the compartments rather than a net gain in any one compartment.

Serotonin is involved in the regulation of the body's water balance (Batmanghelidj, 1987) because a decrease in serotonin transport may result from a decrease in sodium pump activity (Wolfel et al, 1989; Chong & Kay, 1977). As the sodium pump regulates electrolyte and water movements (Ewe, 1988) serotonin influences the body water. Also, tryptophan is a precursor of serotonin and changes in cellular water may alter tryptophan metabolism because "free" water is required to produce the energy to conformationally change the tryptophan (Batmanghelidj, 1987). Serotonin, and therefore

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tryptophan, have been associated with alterations in mood (Wurtman et al, 1989; Wurtman, 1988; Rapkin et al, 1987). In PMS, depression is common in the luteal phase. We have already observed that cell water is increased in association with altered pump activity in this stage of the cycle. The decreased pump activity will result in a diminished serotonin uptake (Wolfel et al, 1989). It is therefore, of interest that a decrease in serotonin in whole blood, platelet rich and platelet poor plasma has been observed in the luteal phase of PMS patients (Rapkin et al, 1987; Taylor et al, 1984). It has previously been suggested that changes in serotonin status may account for the mood disturbances of PMS (Taylor et al, 1984).

In this study, it has been documented, for the first time, that in PMS there is a decrease in Na^+,K^+-pump activity. This may result in an increase in intracellular sodium and, due to osmosis, an increase in intracellular water. Cellular oedema could be a result of increased cell water leading to a compartmental shift in fluid. These changes may make the patient feel that they are "bloated" or have "water retention". This could certainly upset the water balance of the body affecting the serotonin levels and explain the mood change that occurs in PMS. If this mechanism is confirmed, it may provide ideas of how to treat PMS in the future.

The exact sequence of events remains unclear, however it can be said that these patients have abnormal intracellular water and sodium pump activity in the luteal phase of the cycle, when symptoms occur.

II. <u>Ex vivo study of human platelets from healthy controls</u>

A. <u>Development of the Na$^+$,K$^+$-ATPase assay using ^{22}Na uptake in platelets</u>

The results show that ^{22}Na is taken up by human platelets in a time-dependent manner so that the cpm at t=2 min is significantly different (p<0.0001) from that at t=6 min and the t=6 min results are significantly different (p<0.0005) from those at t=10 min. This finding is in agreement with previous work (Feinberg et al, 1977) which showed that human platelets uptake ^{22}Na.

The increase in ^{22}Na content may be due to an artefact. Hence the possibility that it is due to an increase in platelet number, trapped extracellular fluid or contaminating leucocytes or erythrocytes containing the ^{22}Na were investigated.

The platelet number was measured and the results showed that there was no significant increase in this variable. This proves that the increase in ^{22}Na was not due to an increase in platelet number in the later samples. This could have happened if the sample was not stirred sufficiently and the platelets had settled to the bottom of the cuvette.

The trapped extracellular fluid (ECF) was investigated to ensure that the increase in ^{22}Na counts were due to changes in ^{22}Na content of the platelet pellet and not due to trapped ECF, which also contains ^{22}Na. The results showed that ECF trapped between platelets is negligible as there is no significant difference in counts between the saline control or

PRP, both with ^{51}Cr-EDTA. The significant difference between PRP with ^{51}Cr-EDTA and PRP alone can be explained by considering that ^{51}Cr-EDTA adheres to the plastic Eppendorf tube. This also explains the residual activity seen in the saline control where ^{51}Cr-EDTA is present but, as there are no cells, this finding can not be attributed to ECF in the pellet. Therefore, the results show the there is no significant trapped ECF and that the changes in ^{22}Na content can be attributed to intraplatelet concentrations. The error caused by radioactivity adhering to the plastic of the sample tubes is present in all samples and therefore does not account for the increase in ^{22}Na over the time period investigated.

PRP samples were investigated for contamination and it was found that there were no erythrocytes and the few leucocytes present represented only 0.066% of the platelet count. Therefore, the PRP samples are considered to be uncontaminated.

The method was also standardised by accounting for the variation in the number of platelets in each PRP sample. The number of platelets was corrected to a value of 350 x 10^9 platelets.1^{-1}. This was considered beneficial because it makes PRP samples with very different platelet numbers have cpm values that are more comparable. It also shows that the responses seen were not due to platelet number because the response remains after the correction was carried out.

When a sample of supernatant was collected from the 2 min sample it was be seen that the cpm in each supernatant varies a little. When the method was standardised by correcting for

this variation it had virtually no effect on the results, (see Fig 3.5 and 3.7), and hence, as it was not beneficial this correction was not carried out.

When an efflux study with radioactivity is carried out the efflux may estimated in two ways. Either by recording the decrease of radioactivity in the cell pellet or by recording the increase of radioactivity in the supernatant. Therefore, the supernatants were collected and counted to ascertain whether it was better to study these rather than the cell pellets. As there was no obvious change in the supernatant cpm the cell pellets were studied.

B.   Validation of the sodium method in platelets

As previously stated, the results show that ^{22}Na is taken up by human platelets in a time-dependent manner. Wolfel et al (1989) also showed a significant increase in intracellular sodium (non-radiolabelled) in the presence of ouabain. Using ouabain, to block the sodium pump, indicates that Na$^+$,K$^+$-ATPase plays a significant role in ^{22}Na uptake (Andersson & Vinge, 1988). These findings suggest that ^{22}Na accumulates intracellularly because it cannot leave the platelet in exchange for potassium. Sodium influx, on the other hand, will not be directly affected by ouabain since this is a passive pathway. This interpretation is compatible with the observed increase in intraplatelet ^{22}Na content and concurrent decrease in intracellular potassium following the addition of ouabain to rabbit platelets (Clausen & Flatman, 1980). Feinberg et al (1977) also arrived at the same

conclusion; however, we have shown the same result in 10 min instead of 3.5 h experiments. This is an advantage as it allows a more rapid tunover of experiments.

Adrenaline is known to stimulate the sodium pump (Turaihi et al, 1989; Baron et al, 1985). In the present study, adrenaline caused a significant (p< 0.02), transient, increase in the ^{22}Na content of platelets. This is likely to be due to a stimulation of sodium efflux (a pump-mediated effect) which in turn increases the influx of extracellular ^{22}Na. It is therefore not surprising that in the presence of ouabain, (an inhibitor of the sodium pump) adrenaline did not cause a further increase in platelet ^{22}Na content. These findings confirm previous observations with adrenaline (Baron et al, 1985; Turaihi et al, 1989).

The adrenaline-mediated increase in ^{86}Rb influx in platelets has been attributed to the β_2-adrenoceptor (Turaihi et al, 1989). Hence, we examined β-adrenoceptor specificity with agonists and antagonists using the sodium transport method. Atenolol (a β_1 adrenoceptor antagonist) did not inhibit adrenaline-induced increases in platelet ^{22}Na content whereas timolol (a β_1,β_2 adrenoceptor antagonist) did. These findings are compatible with previous work which showed that the sodium pump is linked to the β_2-adrenoceptor in leucocytes (Baron et al, 1985), platelets (Turaihi et al, 1989; Kerry & Scrutton, 1983) and muscle (Wood et al, 1990). However, as adrenaline has both α and β adrenoceptor agonist activity we cannot exclude an α-adrenoceptor-linked component without including experiments using adrenaline in the presence of

α-adrenceptor antagonists. However, noradrenaline did not stimulate sodium uptake. Therefore the α-receptor is unlikely to play a major role in the adrenaline stimulation. From the results, it seems likely that adrenaline is acting at the β_2-adrenoceptor site and if this is the case, then using xamoterol, a β_1 agonist, and salbutamol, β_2 agonist, should clarify this further. Xamoterol, as expected, did not significantly alter platelet ^{22}Na content. Salbutamol did not significantly increase platelet ^{22}Na content whereas it significantly ($p< 0.02$) stimulated active ^{86}Rb influx. The latter observation is compatible with earlier findings (Turaihi et al, 1989; Wood et al, 1990). This apparent discrepancy between ^{22}Na and ^{86}Rb methods suggests that sodium transport is linked to an "atypical" β_3 adrenoceptor or that technical differences do not always produce the same responses. ^{86}Rb and ^{22}Na transport may also have different properties, even in the same cell type. An additional explanation is that salbutamol may act as a partial β-agonist (Kerry & Scrutton, 1983) and BRL 37344 as a full β-agonist. Wood et al (1990) suggest that salbutamol stimulates cation transport via Na^+,K^+-ATPase in vivo in some tissues but not in all which also indicates that salbutamol may be a partial β-agonist.

The possibility of an "atypical" β-adrenoceptor was investigated by adding BRL 37344, a β_3 adrenoceptor agonist (Gill et al, 1991). This agonist caused a significant ($p< 0.03$) increase in platelet ^{22}Na content in a manner similar to that observed with adrenaline. This observation supports the

view that an "atypical" β_3 adrenoceptor is involved. Others, using similar agonists and antagonists to influence calcium transport in human platelets, have also provided evidence favouring the presence of a β_3 adrenoceptor on human platelets (Gill et al, 1991). Active ^{86}Rb influx was also significantly (p< 0.02) stimulated to a similar extent with BRL 37344. This agonist was more potent than adrenaline, in both systems studied, since the concentration required to give the same magnitude of increase was 20 times less. In other systems, BRL 37344 has also been found to be more potent than other β agonists (salbutamol, isoprenaline and fenoterol) (Gill et al, 1991; Arch, 1989; Arch et al, 1984).

As with adrenaline, the increase in platelet [22]Na content due to BRL 37344 was inhibited by timolol. This suggests that BRL 37344 is acting in a similar way to adrenaline. The BRL 37344-mediated stimulation of ^{86}Rb influx was also inhibited by timolol. This suggests that BRL 37344 is acting at the same site to cause stimulation of both [22]Na and ^{86}Rb influx. Therefore, we suggest that, like [[45]Ca^{2+}] uptake by human platelets (Gill et al, 1991), sodium transport is linked to an "atypical" β adrenoceptor possibly of the "β_3" subtype.

It is of interest that sodium and calcium transport appear to be linked (Pritchard et al 1989; Pales et al, 1989). It has been suggested that inhibition of the pump leads to an increase in Na-Ca exchange (Ahmad & Bloom, 1989), where sodium is transported out of the cell and calcium into the cell. Therefore, if the pump is no longer able to keep the intracellular sodium concentration at the correct value, it

may be that Na-Ca exchange is altered. As potassium is not involved in this latter form of exchange, this phenomenon could explain a discrepancy between ^{86}Rb and ^{22}Na transport. This phenomenon would not be apparent in leucocytes because there is no evidence that the Na-Ca exchange system occurs in this cell type (Shore et al, 1984).

Another system that does not involve potassium is the influx of Na$^+$ in exchange for the efflux of H$^+$ (Escobales & Canessa, 1986). The Na/H exchange is a major pathway for the uptake of Na$^+$ and may cause an intracellular Na$^+$ load which would stimulate the sodium pump (Rasmussen et al, 1989). If adrenaline stimulates this transport, as well as the sodium pump, this could explain the discrepancies between the ^{86}Rb and ^{22}Na methods.

The Na,K-pump, Na/H exchange and Na-Ca exchange appear to be associated with each other. They all influence intracellular [Na$^+$] which has a direct effect on membrane potential and conductance. Ouabain and adrenaline increase membrane conductance and inhibitors of conductance cause compensatory changes in the sodium pump (Burke & Sanders, 1990; Ehara & Ishihara, 1990; Kone et al, 1989). Therefore, a possible explanation for the observed increased ^{22}Na$^+$ content due to ouabain and adrenaline is a change in membrane conductance. However, adrenaline-induced increases in membrane conductance cannot be inhibited by some β-adrenoceptor antagonists (Earm et al, 1983) in contrast to our observations. Furthermore, the studies on membrane conductance use adrenaline at concentrations, 10-100 fold higher (Ehara &

Ishihara, 1990; Matsuoka et al, 1990; Earm et al, 1983) than the present study. Therefore, membrane conductance may not be altered at the low concentrations of adrenaline we used.

If membrane conductance does, however, influence the results it may account for some of the discrepancy between the ^{86}Rb and ^{22}Na methods because sodium is more efficient than potassium (or rubidium) at facilitating adrenaline-mediated effects on conductance (Matsuoka et al, 1990).

^{86}Rb influx and ^{22}Na transport in platelets were influenced by almost identical agonists and antagonists. The only difference being salbutamol, a β_2 agonist, which stimulated ^{86}Rb influx but had no effect on ^{22}Na transport. This discrepancy may be related to methodological sensitivities or to differences in factors controlling the intracellular status of potassium and sodium. The latter is likely since there are numerous pathways that have been identified which influence the transport of these two ions (Triggle, 1990; Shore et al, 1984; Sweadner & Goldin, 1980). Also the study of the sodium pump using either isotope may be measuring different properties because each method relies on changes in intracellular sodium or potassium content. This is relevant because the intraplatelet potassium content is much greater than the intracellular sodium (Lentner, 1984). Because of the difference in pool size, the transport kinetics of each ion may be considerably different (see above).

The case for the study of both ions to obtain a clear picture of the sodium pump is supported because under certain conditions sodium and potassium transport differ. As we have

shown that although ^{86}Rb influx and ^{22}Na transport in human

platelets were influenced by almost identical agonists and

antagonist, they do react differently to salbutamol, a β_2-

agonist. Also it has been previously noted that at

temperatures between 37 $^{\circ}$C and 10 $^{\circ}$C, the rate of exchange of

potassium decreases markedly while the rate of exchange of

sodium remains normal (Negendank & Shaller, 1982). Therefore,

the rate of exchange for potassium and sodium are dissociated,

at least at low temperatures. Finally, N-ethylmaleimide will

inhibit the sodium pump and hence will induce a rise in sodium

content of platelets but without significantly changing the

potassium content (Wolfel et al, 1989). This suggests that in

platelets, the sodium and potassium transport are not as

closely linked as may have been first thought (Wolfel et al,

1989).

Therefore, it would appear that to study the sodium pump

thoroughly, both rubidium and sodium transport should be

investigated. This would be impossible using leucocytes

because too large a blood volume would be required. For

example, for ^{22}Na estimations, the leucocyte methodology would

require 100 ml compared to 18 ml for platelets. As the study

of the sodium pump in platelets requires relatively small

volumes of blood it may now be possible to carry out

sequential measurements in any one study. Therefore, the

future of Na$^+$,K$^+$-ATPase investigation in clinical studies may

lie with platelets.

C. Using the Na$^+$,K$^+$-ATPase assay to investigate the effect of milrinone on sodium transport in platelets

Na$^+$,K$^+$-ATPase activity and therefore the sodium pump, is essential for excitability and contractility of muscle tissue (Norgaard et al, 1990a; Norgaard et al, 1990b). The propagation of heart muscle contraction occurs following a sudden influx of sodium causing a potential difference across the membrane which is reversed by the sodium pump (Sokolow & McIlroy, 1981). If the sodium transport of cardiac muscle cells is altered then the muscles may not contract properly causing the heart to function inefficiently.

Cardiac Na$^+$,K$^+$-ATPase activity has been shown to be altered in animals with heart disease (Ganguly et al, 1990; Kuroda et al 1990). In the leucocytes and erythrocytes of patients with heart failure the intracellular potassium concentration is decreased and the intracellular sodium concentration is increased (Lui et al, 1990). This indicates that the sodium pump is inhibited. The leucocytes and erythrocytes of these patients support this hypothesis as the Na$^+$,K$^+$-ATPase activity is reduced. This inhibition appears to be linked to an increase in the circulating level of plasma digoxin-like immunoreactive substance (Lui et al, 1990). This factor has recently been identified as endogenous ouabain produced from the adrenal cortex (Anonymous, 1991; Schoner, 1991).

Both digoxin and adrenaline are considered to improve heart function however, their effects on the sodium pump are quite different. Cardiac glycosides inhibit Na$^+$,K$^+$-ATPase

whereas adrenaline stimulates sodium pump activity. The sodium pump regulates the efficiency of muscle contraction by modulating the concentration of cytosolic calcium in the cells (Rossier et al, 1987). When the sodium pump is blocked the intracellular concentration of sodium will increase (Dux et al, 1990) and consequently so will Ca-Na exchange. This may cause an increase of intracellular calcium which would result in more Ca^{2+} available for release. The more Ca^{2+} released the stronger the muscle contraction (Sokolow & McIlroy, 1981).

Adrenaline causes an increase in pump activity and so it increase sodium efflux. However, from this study (Ozin et al, 1992b) in platelets, adrenaline has been seen to cause a transient increase in the uptake of sodium. If this effect was associated with an increase in intracellular sodium then it would also increase the Ca-Na exchange as described above. However, the adrenaline effect would be shortlived and could only be of benefit to overcome a crisis whereas the cardiac glycoside effect would be longer lasting and useful in long term therapy. This can be seen in the clinical situation where adrenaline is thought to play a role in maintaining cardiac function (Poole-Wilson & Lindsay, 1992). Therefore, the increase in endogenous ouabain may be the natural way to combat heart failure.

Milrinone appears to act like adrenaline since it increases the ^{22}Na content of the platelets when compared to saline controls. However, milrinone does not mediate its effect through the sodium pump as it has previously been seen to cause no inhibition of Na^+, K^+-ATPase activity (Goyal &

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McNeill, 1986). Milrinone also does not effect the sodium pump activity like adrenaline (Bagli et al, 1988). Instead, it causes an increase in sodium influx through the sodium fast channel (Grima, et al 1988; Goyal & McNeill, 1986). This would cause stronger contractions of the muscle by increasing the intracellular calcium through the Ca-Na exchange mechanism. The observations that milrinone increases the force of contractions in isolated cardiac cells (Brown et al, 1986) and stimulates calcium influx (Alousi & Johnson, 1986) support this hypothesis.

The pretreatment of cells with ouabain shows that the increase caused by milrinone cannot occur in the presence of ouabain. This is clinically important as it suggests that the administration of milrinone to patients receiving digoxin is unlikely to be of benefit as the digoxin may, like ouabain, negate the actions of milrinone (Harris et al, 1988). This has been demonstrated in the clinical setting since the treatment of patients with heart failure with digoxin and milrinone was of no extra benefit when compared to the treatment with digoxin alone (Dibianco et al, 1989). These findings are also compatible with the observation that ouabain attenuates the vasodilator action of milrinone (Harris et al, 1988).

Adrenaline and milrinone are not acting through independent pathways because their simultaneous addition did not show synergism when compared to adrenaline alone. Our hypothesis is that adrenaline increases the efflux rate of intracellular sodium and hence increases the influx of ^{22}Na. Milrinone is also supposed to increase the influx of sodium

(Grima et al, 1988). Therefore we conclude that these two modes of influx are closely related or mutually exclusive. Hence, these two agents increase sodium influx and this will stimulate the Na-Ca exchange, increasing the influx of Ca^{2+} (Alousi & Johnson, 1986). Prolonged exposure to both agents appears to have a negative influence compared to adrenaline alone. This can be explained because as the sodium pump, in the presence of adrenaline, reaches an equilibrium the efflux will be equivalent to the influx of sodium. If milrinone is also present, sodium influx is stimulated through the fast channel (Grima et al, 1988; Goyal & McNeill, 1986) as well as due to an increase in sodium efflux rate. This may, on prolonged exposure, cause the sodium pump not to respond to adrenaline if milrinone interferes with the sodium pump stimulation by controlling the influx of sodium. Our results suggest that milrinone, on prolonged use, will negate some of the beneficial effects of endogenous adrenaline on the cardiac muscle of patients with severe heart failure, where adrenaline may play a vital role (Poole-Wilson & Lindsay, 1992). Also these patients have raised levels of DLIS which may, like digoxin, negate the actions of milrinone. Therefore, milrinone may not be suitable for the longterm therapy of heart failure.

IIIA. <u>In vitro studies with ethanol and the sodium pump in</u>

     <u>leucocytes</u>

The aim of the in vitro work was to study the effect of 17β-oestradiol and progesterone on the sodium pump of leucocytes. The steroid hormones must be dissolved in nonpolar solvents, such as ethanol, as they are insoluble in aqueous solutions. Hence, the effect of ethanol on the sodium pump must be investigated to ensure that ethanol does not effect the sodium pump activity.

Leucocytes were exposed to ethanol for 30 min and 2 h at various concentration. An exposure time of as long as possible was chosen to maximise the chance of seeing an effect because the hormone is most likely to cause protein synthesis to mediate their actions. This requires an exposure time of at least 2 h, which is within the capabilty of this assay. However, the exposure of leucocytes to ethanol for 2 h, even at very low concentrations, influences the assay. An increase of 40 % in the TERC was observed at an ethanol concentration of 1.8 mmol.l^{-1}. Hence, an exposure time of 30 min was chosen to minimise the alcohol effect. Even at this shorter exposure time and very low concentrations, ethanol still influences the efflux rate constants of the sodium pump. A decrease of up to 15 % in TERC and up to 57 % in OSERC was observed using 1.8 mmol.l^{-1} for 30 min.

Previously, ethanol at concentration of <40 mmol.l^{-1} did not effect the sodium pump (Green 1986). However, these experiments used the very short exposure time of 15 min. The difference in exposure time may account for the different

sensitivity to ethanol when using the same assay for assessing the sodium pump. Ethanol has also been seen to lower the intracellular sodium content of leucocytes at concentrations of 17-170 mmol.l^{-1} (Main & Thomas, 1990). Therefore, it is possible that the sodium pump is affected by exposure to ethanol at 17 mmol.l^{-1} for 20 min (Main & Thomas 1990). Our results and the findings of others make the use of ethanol inadvisable in assessments of the sodium pump. As the hormones are insoluble in aqueous solution this work could not be carried out using the methodology available at the present time.

IIIB. The recovery of 17β-oestradiol when diluted with

   Buffer I

The results showed that when buffer I is used to dilute the hormone the concentration of hormone available to the RIA is not as high as it should be. The best recovery achieved was 50 %. As half of the hormone is lost before the experiment has begun this would limit the effects seen. Also the loss of hormone is not linear and hence the amount of active hormone in each sample may vary considerably. Therefore, this method of preparing the hormone for use in leucocyte studies can not be used.

IIIC. The reaction of ouabain with 17β-oestradiol or

testosterone

i)   Ouabain and 17β-oestradiol

Developing a method to study the sodium pump in plasma was considered as the steroid hormones would be more likely to be soluble in plasma than in an artificial buffer. The platelet method was an ideal way to avoid the use of an aqueous buffer.

Ouabain, a cardiac glycoside, is an essential part of the sodium efflux assay. Digitonin (another cardiac glycoside) will combine with oestradiol to form a precipitate (Estradiol, Merck Index). Therefore, the stability of oestradiol in the presence of ouabain was investigated. Since buffer I is usually used as the vehicle for ouabain, the effect of adding this buffer to the oestradiol preparation was also considered.

The results showed that the addition of buffer to 17β-oestradiol in plasma reduced the 17β-oestradiol that is available to the RIA compared to the addition of PPP. This indicates that oestradiol preparations are unstable even in partially aqueous solution. Therefore, the aqueous buffer is an unsuitable vehicle for ouabain or any other agents when oestradiol is being used. The presence of ouabain in aqueous buffer appears to have less effect on the 17β-oestradiol concentration than buffer alone. This suggests that ouabain stabilises the oestradiol by helping the oestradiol to remain soluble in a partially aqueous solution. This is not as strange as it may seem because ouabain is more water soluble than digitonin (Digitonin, Merck Index). Therefore, ouabain

combining with oestradiol may cause the oestradiol to be held in solution.

In the clinical situation, it may be that occasional reports of gynecomastia in patients taking digoxin (LeWinn, 1953) are due to the ouabain-oestrogen interaction in the plasma or at the receptor level. Such an interaction may influence oestradiol bioactivity. This possibility is supported by the results we obtained since the ouabain-oestradiol interaction leaves most of the oestradiol available to the radioimmunoassay (Ozin et al, 1992d).

ii)   Ouabain and testosterone

The ouabain does not have the same effect on samples with testosterone as it does with 17β-oestradiol. This indicates that the responses found with 17β-oestradiol may be relatively specific to oestrogens.

IV   SUGGESTIONS FOR FUTURE WORK

1)   In future studies, it may be beneficial to select PMS patients after a placebo period so that those who report an improvement or a worsening of symptoms can be excluded from further study. This procedure would remove those women where suggestion plays a significant role in the severity of the symptoms recorded. If this is the case then the results should become more significant as the patient sample would only include women with a more exact definition of PMS.

2)   It would be interesting to investigate whether the abnormalities documented in leucocytes of PMS patients are mirrored in their platelets. This study can now be carried out using the Na^+, K^+-ATPase assay that has been developed in this project. Since less blood is required using this method there is the option to collect more frequent samples over the investigation period and to study the sodium pump using both ^{22}Na and ^{86}Rb. These modifications would be of benefit because they would give a more complete profile of the changes that occur.

3)   Recent work has indicated that digoxin-like immuno-reactive substance (DLIS) is ouabain. Since we recorded a decrease in the sodium pump activity in the luteal phase of women with PMS, compared to controls, it may be possible that this phenomenon can be attributed to an excess of DLIS. Therefore, it would be interesting to investigate the levels of DLIS in women with PMS and controls during the menstrual cycle. DLIS is known to be increased in patients with heart failure, therefore, it may be interesting to extend this idea to study the platelet Na^+, K^+-ATPase activity in these patients to see if an increase in DLIS does in fact cause a significant change in platelet Na^+, K^+-ATPase activity.

4)   Our results indicate that the platelet β-adrenoceptor is associated with Na^+, K^+-ATPase activity. Given that adrenaline is both an α- and a β-agonist, there may also be an α component to the responses seen. This possibility could

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investigated with specific α-adrenoceptor agonists (and antagonists). The results of these experiments would establish whether the effect of adrenaline was mediated through the α-adrenoceptors as well as via β-adrenoceptors.

5)   It may be speculated that part of the adrenaline-mediated platelet response is due to changes in membrane conductance. Since the membrane conductance can be measured directly it would be useful to carry out such a study to clarify this matter.

6)   Leucocyte Na^+,K^+-ATPase activity correlates well with changes in muscle Na^+,K^+ATPase activity in several conditions. It would therefore be interesting to investigate platelet Na^+,K^+-ATPase activity in conditions where leucocytes have been shown to have altered pump activity to see if the findings in these two cells types correlate well. This investigation would also serve to further validate the platelet method.

7)   Platelets are easy to prepare and the measurement of sodium pump activity and cell function (by aggregation and size change studies) can be evaluated. It may be interesting to evaluate both the platelet function indices and the pump activity simultaneously, in the same blood samples. This investigation would indicate if the two processes are interrelated. The functional assessment of leucocyte function would be technically much more complex.

V   <u>CONCLUSION</u>

The aims of this project were (see p51):

i) to substantiate the hypothesis that there is a redistribution of fluid between the body compartments in PMS,

ii) to test whether ovarian hormones may influence this redistribution and,

iii) to improve the established methodology for the assessment of Na^+,K^+-ATPase activity, used in patient-based studies.

We have shown that there is a decrease in the sodium pump activity and an increase in percentage cell water in the luteal phase of the menstrual cycle in PMS. The decrease in sodium pump activity suggests that there is increased intracellular sodium content. Since there is also an increase in cell water the findings of this project support the hypothesis of fluid redistribution. PMS symptoms may be caused by this fluid redistribution. Therefore, an assay for the sodium pump may become an important research tool to investigate the pathogenesis and treatment of PMS.

Ovarian hormones proved to be technically impossible to use with the established methodology. Therefore, we could not investigate whether these hormones can mimic the changes in the luteal phase of women with PMS, that we have demonstrated.

A new method, using platelets, was devised and validated. It is more rapid and less expensive to carry out than the leucocyte technique. Also, a much smaller volume of blood is

required. Therefore, more frequent sampling, or alternatively, both sodium and potassium (rubidium) transport may be studied on the same subject. The leucocyte method would require an unreasonably large volume of blood to carry out such a study.

In conclusion, it has for the first time, been shown that PMS is associated with a change that can be objectively measured. The study of the sodium pump, using the platelet method developed in this project, is easier to carry out. This may facilitate the study of PMS in the future.

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EFFECT OF MILRINONE ON SODIUM TRANSPORT IN HUMAN PLATELETS

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Abstract

Milrinone significantly increases sodium uptake in isolated human platelets at t=2 minutes suggesting an increase in Na^+, K^+-ATPase activity. In platelets pre-treated with ouabain, milrinone does not cause a further increase in sodium uptake. This indicates that the milrinone action is directly or indirectly associated with the sodium pump. There is no synergism between adrenaline and milrinone suggesting that they do not act through independent mechanisms. Milrinone, rather than causing a synergistic effect, appears to reverse the adrenaline action. These effects may explain why clinical trials do not show an additional benefit when milrinone is administered to patients, with heart failure, receiving digoxin.

Key words: Milrinone, sodium pump, adrenaline

Introduction

The sodium pump is driven by Na^+, K^+-ATPase which transports sodium out of and potassium into cells to maintain the low sodium and high potassium concentrations which occur intracellularly [1]. Na^+, K^+-ATPase activity, and therefore the sodium pump, is essential for excitability and contractility of muscle tissue [2,3]. The propagation of the heart muscle contraction occurs following a sudden influx of sodium causing a potential difference across the membrane which is reversed by the sodium pump [4]. Therefore, if the sodium transport of cardiac muscle cells is altered then the heart may not function efficiently.

The sodium pump also modulates the concentration of cytosolic calcium [5]. When the sodium pump activity is diminished, the intracellular concentration of sodium will increase [6] as will Ca-Na exchange. This may result in an increase in intracellular calcium and consequently stronger muscle contractions [4].

Cardiac Na^+, K^+-ATPase activity has been shown to be altered in animal models of heart disease [7–9]. Low Na^+, K^+-ATPase activity has also been found in the erythrocytes of patients with heart failure [10]. It appears that this latter inhibition is related to an increase in the circulating plasma digoxin-like immunoreactive substance (DLIS) level [10]. This factor has recently been identified as endogenous ouabain produced from the adrenal cortex [11,12].

In this study we investigate the effect of milrinone, a drug designed to improve cardiac function, on platelet Na^+, K^+-ATPase. Milrinone is a bipyridine derivative that increases the force of cardiac muscle contraction probably by inhibiting the cAMP-specific phosphodiesterase III activity thus increasing the intracellular concentration of cAMP [13].

The human platelet has been developed, in our laboratory [14], as a cell model for Na^+, K^+-ATPase activity. This allows both the *in vitro* assessment of Na^+, K^+-ATPase activity and serial sampling of healthy volunteers or patients. The assay measures the uptake of ^{22}Na which is associated with the rate of Na^+ efflux. Since Na^+, K^+-ATPase controls the rate of sodium efflux, this assay is an index of the activity of this enzyme. We have used this method to estimate the effect of ouabain or adrenaline on milrinone action with respect to Na^+, K^+-ATPase.

Materials and methods

Reagents

Reagent	Obtained from
$^{22}NaCl$	Amersham International, Aylesbury, Bucks, UK.
Ouabain (Strophanthin-G)	BDH, Poole, Dorset, UK.
Adrenaline	Sigma Chemicals Company, Poole, Dorset, UK.
Milrinone	Sterling Winthrop, Guildford, Surrey, UK.
All other chemicals	BDH, Poole, Dorset, UK.

Methods

Collection of blood

Blood (18ml) was collected from an antecubital vein of young healthy volunteers and anticoagulated using one volume of 3.8% tri-sodium citrate for every nine volumes of whole blood.

Isolation of platelets

After collection, the whole blood was centrifuged at 160g for 15 minutes to prepare platelet rich plasma (PRP). The PRP was decanted off and placed in a waterbath, at 37°C, for 15 minutes to equilibrate.

After equilibration, the PRP in the cuvettes was mixed using magnetic stirrers in a Whole-blood Aggro-meter model 540, (Chrono-log Corporation, Havertown, Philadelphia, USA). Milrinone (dissolved in saline) was added to one cuvette (to a final concentration of 1 or 5μmol/L) and saline alone to another after one minute of mixing. The concentration of milrinone was selected to be comparable to the therapeutic levels reported *in vivo* [15]. The platelets were mixed for a further two minutes and then allowed to rest for three minutes. At this time $^{22}NaCl$ (final concentration: 37kBq/ml) was added to each cuvette and the stirrers switched on. Samples were withdrawn at two, six and 10 minutes after the addition of $^{22}NaCl$. Each sample was immediately centrifuged at 1,000g for one minute in an Eppendorf centrifuge 5414 (Eppendorf, Hamburg, Germany) and washed twice in cold saline. The cell pellets were counted using a LKB Wallac 1282 compugamma, universal gamma counter, (LKB Wallac, Turku, Finland).

The effect of pre-incubation with ouabain (final concentration: 0.2mmol/L) on the milrinone response, and the effect of milrinone on adrenaline stimulation was investigated. When ouabain was used the stirrers were switched on and one minute later the ouabain (dissolved in saline) was added. The platelets were stirred for a further two minutes and then allowed to rest for three minutes. In control samples, the same volume of saline replaced the ouabain. After this, $^{22}NaCl$ (final concentration: 37kBq/ml) was added and the experiment continued as above. When adrenaline (final concentration: 50nmol/L) was used, it was added simultaneously to milrinone to one cuvette and saline replaced the adrenaline in its control. The method was carried out as for the milrinone *vs.* saline experiment above.

All results were corrected for the number of platelets to a value of 350×10^9 platelets/L. The platelet count was established using a Coulter ZM counter (Coulter Electronics Ltd, Luton, Beds, UK).

The coefficient of variation between duplicate measurements using the saline

controls for the method without ouabain was calculated to be 0.8%.

Statistical analysis

Results are expressed as the median and (range). The Wilcoxon matched pairs signed ranks test was used to compare results.

Results

Effect of milrinone on sodium uptake in platelets

Milrinone (1μmol/L) caused a significant (p<0.02) increase in sodium uptake by platelets initially (two minutes) and reached an equilibrium so that there was no significant difference between the saline and milrinone at later samples (six and 10 minutes, Table 1). Responses (n=3) at 5μmol/L milrinone, were similar in direction but qualitatively more definitive than those at 1μmol/L. Milrinone appears to act in a similar way to adrenaline in the present assay system.

Effect of ouabain on the milrinone action

The pretreatment of platelets with ouabain masks the milrinone-induced increase, that was observed above, because ouabain itself causes an increase in platelet sodium uptake [11] (Table 2). Our observations, however, suggest that the milrinone-induced increase is associated (directly or indirectly) with the sodium pump since milrinone does not further

Table 2. Median (range) platelet sodium uptake (CPM) in ouabain-treated cells in the presence of milrinone (n=7).

Time (min)	Ouabain + saline	Ouabain + milrinone	Increment
2	259 (181–353)	259 (207–397)	0%
6	342 (252–493)	321 (247–546)	−6.1%
10	421 (309–552)	402 (310–515)	−4.5%

elevate sodium uptake in the presence of ouabain, an inhibitor of the sodium pump.

Effect of adrenaline on the milrinone action

Adrenaline and milrinone were added simultaneously to investigate if they were both acting through the same mechanism. As can be seen in Table 3, synergism does not occur suggesting that adrenaline and milrinone do not act through independent mechanisms.

Table 4 shows that adrenaline and milrinone do not enhance each other; instead they seem to interfere with each other since the sodium uptake is significantly (p<0.02) reduced in the presence of milrinone. This was only seen at the higher concentration of milrinone (5μmol/L and not 1μmol/L).

Table 1. Median (range) platelet sodium uptake (CPM) in the presence of milrinone (n=7).

Time (min)	Saline	Milrinone (1μmol/L)	Increment
2	218 (160–265)	254 (191–327)	+16.5%*
6	302 (232–359)	304 (230–328)	+0.7%
10	327 (284–417)	345 (294–442)	+5.5%

*p<0.02 comparing platelet sodium uptake in the presence and absence of milrinone.

Table 3. The effect of milrinone (1μmol/L) on median (range) sodium uptake (CPM) by platelets stimulated with adrenaline (50nmol/L) (n=7).

Time (min)	Adrenaline + saline	Adrenaline + milrinone	Increment
2	210 (145–292)	195 (163–330)	−7.1%
6	299 (199–360)	258 (202–387)	−13.7%
10	307 (234–401)	297 (244–430)	−3.3%

Table 4. The effect of milrinone (5μmol/L) on median (range) sodium uptake (CPM) by platelets stimulated with adrenaline (50nmol/L) (n=7).

Time (min)	Adrenaline + saline	Adrenaline + milrinone	Increment
2	207 (154–245)	184 (138–240)	–11.1%
6	218 (157–261)	195 (166–290)	–10.6%
10	259 (183–311)	200 (171–288)	–22.8%*

*p<0.02 comparing adrenaline stimulation of platelet sodium uptake in the presence and absence of milrinone.

Discussion

The present findings indicate that milrinone does not mediate its effect through inhibition of the sodium pump. This conclusion is compatible with previous reports where milrinone was found to have no inhibitory effect on Na^+, K^+-ATPase activity in rat cardiac preparations[16]. Instead, milrinone elevates intracellular sodium concentration by increasing influx through the fast sodium channel[16,17]. This suggests that an increase in Ca-Na exchange occurs causing a stronger muscle contraction. This mechanism is in agreement with the observation that milrinone increases the force of contractions in isolated cardiac cells [13] and stimulates calcium influx [18]. Our results show that an additional increase in sodium uptake caused by milrinone does not occur in the presence of ouabain. This may be of clinical importance since it suggests that the administration of milrinone to patients receiving digoxin (another cardiac glycoside) is unlikely to result in additional benefit.

This hypothesis is supported by the observation that the treatment of chronic heart failure with digoxin and milrinone shows no benefit over treatment with digoxin alone [19]. These findings are also compatible with the observation that ouabain attenuates the vasodilator action of milrinone [20].

Adrenaline and milrinone do not appear to act through independent pathways because their simultaneous addition did not show synergism. In fact, at t=2 minutes, adrenaline significantly enhances[14] sodium uptake and this phenomenon appears to be reversed, at least in part, by high doses of milrinone (5μmol/L; Table 4). A similar pattern was seen at t=10 minutes where high doses of milrinone (5μmol/L) significantly decrease adrenaline-induced ^{22}Na uptake (Table 4). However, these latter observations should be considered with caution since therapeutic plasma levels of milrinone [14] rarely exceed 3μmol/L.

Adrenaline causes an increase in pump activity and, therefore, sodium efflux. However, we have previously shown [14], in platelets, that adrenaline also causes a transient increase in sodium uptake. This effect may, transiently, increase Ca-Na exchange, as described in the introduction. These changes are likely to be short-lived. In contrast, cardiac glycoside action would be more useful in long-term therapy. This hypothesis is mirrored in the clinical situation where adrenaline is thought to play a role in maintaining cardiac function in heart failure [21,22].

It is of interest that the plasma levels of endogenous digoxin-like substances are higher in patients with congestive cardiac failure [10]. Therefore, it is tempting to speculate that an increase in circulating endogenous ouabain concentrations may be a natural response to compensate for heart failure.

Our results suggest that milrinone, in prolonged use, may negate some of the beneficial effects of endogenous adrenaline on the cardiac muscle of patients with severe heart failure. This effect may be limited to severe heart failure where adrenaline may play an important role [21,22]. Our findings also provide a possible explanation of why treatment with milrinone does not appear to be of additional benefit in combination with digoxin [19].

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LETTER TO THE EDITOR

Endogenous Ouabain: a new steroid hormone?

Dear Sir

Ouabain is becoming accepted both as a new steroid hormone and as the digitalis-like factor found in plasma [1]. For example, La Bella et al. [2] reported that derivatives of progesterone bind to ouabain receptors.

In order to investigate potential relationships between ouabain and oestrogens we prepared a solution of 17β-oestradiol in pooled platelet poor plasma (PPP). The PPP was prepared from normal men (n=8); 17β-oestradiol was dissolved in a small amount of ethanol, diluted in pooled PPP and then added to each PPP sample. The 17β-oestradiol concentration was measured with a specific radioimmunoassay (Diagnostics Products Ltd), and approximately 90% recovery was obtained in PPP. In the presence of the ouabain vehicle (buffer) [3,4], the concentration of 17β-oestradiol in the PPP samples was significantly lower (p<0.04; 385: 347–495 vs. 443: 348–503pmol/L) than without the ouabain vehicle. However, on addition of ouabain (0.1mmol/L) the 17β-oestradiol concentration was no longer significantly lower (421: 301–499) than when the ouabain vehicle alone was added. This increase in 17β-oestradiol concentration, although not significant, implies an interaction between ouabain and oestrogens.

Thus, both in vitro and in vivo, ouabain and oestrogens may interact and modify their bioactivity (e.g. binding to sex hormone binding globulin, SHBG).

These observations may be relatively specific to oestrogens since we could not show a similar effect of ouabain on physiological testosterone concentration in PPP (obtained from healthy males; n=7).

In the clinical situation, one can speculate that occasional reports of gynaecomastia in patients taking digoxin [5] may be due to ouabain-oestrogen interactions in the plasma or at the receptor level. Interestingly, in premenstrual syndrome we have documented a decrease in sodium pump activity [3] a finding which might be expected if endogenous ouabain were present. Future studies should consider that endogenous ouabain may interact with ovarian hormones to cause pathology.

Yours faithfully

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104 CHANGES IN SODIUM AND WATER TRANSPORT IN
PREMENSTRUAL SYNDROME

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The aim of this project was to study changes in
intracellular electrolyte concentrations and their
transport in premenstrual syndrome (PMS). Na^+, K^+-ATPase
was studied in leucocytes during the follicular (4-9th
day) and luteal (18-24th day) phase. The indices
measured were sodium influx, sodium efflux,
intracellular sodium and water concentration, plasma
sodium and potassium concentration and body weight. PMS
patients (n=17) and female controls (n=10) were
studied.

The percentage cell water for the patient group
72.8% (52.6 - 93.5) was significantly higher ($p<0.015$)
than in the control group 68.7% (64.7 - 72.1) in the
luteal phase. Also the percentage intracellular water
of PMS patients was significantly increased ($p<0.05$) in
the luteal phase , 72.8% (52.6 - 93.5), compared to the
follicular phase, 67.0% (41.1 - 77.3). The total efflux
rate constant of the patients 2.37 h^{-1} (1.95 - 3.38)
was significantly lower ($p<0.02$) than in the controls
3.01 h^{-1} (2.06 - 3.50) in the luteal phase. The ouabain
sensitive efflux rate constant of the patients,
1.64 h^{-1} (1.14 - 2.53), was significantly lower
($p<0.03$) than in the controls, 2.33 h^{-1} (1.37 - 3.38),
in the luteal phase. Therefore, the efflux rate
constant, which is controlled by the sodium pump, is
decreased during the luteal phase in PMS.

This study has documented, for the first time,
that in PMS there is a decrease in Na^+, K^+-pump
activity. Although the exact sequence of events remains
unclear, it can be said that these patients have
increased intracellular water and decreased sodium pump
activity in the luteal phase of the cycle. This is the
symptomatic phase of the cycle when patients complain
of bloatedness and "water retention".

PLATELETS

Sodium Transport in Human Platelets as an Index of Na,K-ATPase Activity

R. L. Ozin, D. P. Mikhailidis, D. N. Baron

SUMMARY. Both sodium (^{22}Na) and rubidium (^{86}Rb) influx, an index of potassium transport, were studied in human platelets to establish a simple and rapid method for the study of the sodium pump in platelets.

^{22}Na was taken up by platelets in a time dependent manner. Ouabain, an inhibitor of the sodium pump, significantly ($p < 0.03$) increased intracellular ^{22}Na content. Adrenaline significantly ($p < 0.02$) increased ^{22}Na content initially. This adrenaline-induced increase did not occur in the presence of ouabain. Timolol (a β_1,β_2-adrenoceptor antagonist) but not atenolol (a β_1-adrenoceptor antagonist) inhibited these adrenaline-induced responses. Xamoterol (a β_1-adrenoceptor agonist) did not cause an increase in platelet ^{22}Na content and unexpectedly, nor did salbutamol (a β_2-adrenoceptor agonist). BRL 37344 (an 'atypical' β_3-adrenoceptor agonist) caused a significant ($p < 0.002$) increase in platelet ^{22}Na content that was significantly ($p < 0.02$) inhibited by timolol. Active ^{86}Rb influx was significantly ($p < 0.02$) stimulated by salbutamol. BRL 37344 also significantly ($p < 0.005$) stimulated active ^{86}Rb influx; this process was inhibited by timolol.

There are considerable similarities between ^{22}Na and ^{86}Rb transport in the human platelet. The only discrepancy, with salbutamol, may be due to methodological sensitivity or to different factors controlling the transport of these two ions. The findings of the present study indicate that it may be necessary to assess both ^{22}Na and ^{86}Rb transport in order to make conclusions concerning the sodium pump, in human platelets.

The sodium pump has for many years been studied in erythrocytes,[1-4] leucocytes[5-10] and more recently in platelets.[11-15] Leucocytes replaced erythrocytes because they have a nucleus and a full complement of enzymes whereas erythrocytes are highly specialised, atypical cells which have anaerobic metabolism and no nucleus.[16] Furthermore, leucocyte ATPase activity appears to reflect the pump activity in other body cells whereas erythrocyte ATPase activity does not.[17] However, there are disadvantages in using leucocytes. Large volumes (100 ml) of blood are required and the cell population, which is heterogeneous (cells differing in function and composition),[18] requires relatively time consuming (60 min) procedures for separation from erythrocytes and platelets. The platelet is a blood cell that is simple and rapid (15 min) to isolate by centrifugation of citrated whole blood.[11] Platelets will give a homogeneous population and considerably smaller volumes (18 ml) of blood are required. These cells also have metabolic pathways similar to those of other cells whereas erythrocytes rely on glycolysis. In addition, they may well be more useful because unlike erythrocytes and leucocytes, their function is easily evaluated with aggregation[19] and size change[20] studies. The disadvantage with platelets, as with erythrocytes, is that they are anucleate. However they both originate

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from a nucleated 'mother' cell. This disadvantage may be somewhat compensated for because the platelet population is replenished every 10–12 days[21] as opposed to 120 days with the erythrocyte. Thus, the platelet population may respond much faster to 'environmental' changes.

Rubidium (^{86}Rb) influx studies,[11] an index of potassium transport, and sodium efflux studies[14,15] have demonstrated that the sodium pump is present in platelets. To study the sodium pump by either method may be measuring different properties because each method relies on changes in intracellular sodium or potassium content. This is relevant because the intraplatelet potassium content has been recorded as 122 mmol.kg^{-1} (range: 62–151) whereas the intracellular sodium is only 27 mmol.kg^{-1} (25–28).[22] Therefore, the potassium pool is much larger than the sodium pool. Because of the difference in pool size the transport kinetics may be considerably different. A small pool size should result in [22]Na influx reaching an equilibrium more rapidly. Sodium should, therefore, reach an equilibrium with its radio-isotope before potassium. Whether this property is a methodological advantage or a disadvantage must be considered when studying these two ions.

Therefore, we evaluated sodium transport directly in human platelets. The assay was designed by adapting the influx and efflux methods that have been previously used in human leucocytes.[23] In order to validate the method we compared our findings with those of the previously reported ^{86}Rb influx method in platelets.

Materials and Methods

Reagents

[22]NaCl and [86]RbCl were obtained from Amersham International, Aylesbury, Bucks, UK. Ouabain (Strophanthin-G) was obtained from BDH, Poole, UK. Adrenaline, atenolol, salbutamol and timolol were obtained from Sigma Chemicals Company, Poole, Dorset, UK. BRL 37344 was a gift from SmithKline Beecham Pharmaceuticals, Epsom, Surrey, UK. All other chemicals were obtained from BDH, Poole, UK.

Buffer

NaCl 115.0 mmol.l^{-1}, KCl 6.0 mmol.l^{-1}, CaCl$_2$.2H$_2$O 1.8 mmol.l^{-1}, MgSO$_4$.7H$_2$O 0.8 mmol.l^{-1}, NaH$_2$PO$_4$.2H$_2$O 1.0 mmol.l^{-1}, Glucose 5.5 mmol.l^{-1}, HEPES 3.0 mmol.l^{-1}, NaHCO$_3$ 24.0 mmol.l^{-1}, pH 7.35, at 37°C.

Methods

Collection of Blood. Venous blood (18 ml) was collected from an antecubital vein of young healthy volunteers and anticoagulated using one volume of 3.8% tri-sodium citrate for every 9 volumes of whole blood.

Isolation of Platelets. After collection the whole blood was centrifuged at 160 g for 15 min to prepare platelet-rich plasma (PRP). The PRP was decanted off and placed in a waterbath, at 37°C, for 15 min to equilibrate. All flux measurements were carried out on platelets in plasma (PRP).

PRP contains median values of 302 × 10^9 platelets/l (180–480), 0.2 × 10^9 leucocytes/l (0.1–0.4) and 0.00 × 10^{12} erythrocytes/l (0.00–0.02) when n = 14. The leucocyte count represents 0.066% of the platelet count and the median erythrocyte count is zero, hence the PRP is essentially free of erythrocytes and leucocytes.

Rubidium Influx in Platelets. After equilibration of the PRP, the incubation tubes were set up. The chemical to be tested (dissolved in buffer) was placed into 4 incubation tubes and to 2 of these ouabain (final concentration 0.1 mmol.l^{-1}) was added. Four control incubation tubes were used for comparison, 2 of these had ouabain added. [86]RbCl (55 kBq.ml^{-1}) was added to each incubation tube. An appropriate volume of buffer and 500 μl of PRP was added to each tube to give a final incubation volume of 1 ml. The tubes were then gently mixed with a whirlimixer and placed in a shaking waterbath, at 37°C. After precisely 12 min (timing commenced at the beginning of mixing), the entire contents of each tube were placed in Eppendorf tubes containing 4 μl acetylsalicylic acid solution (2 g.l^{-1}) and centrifuged in an Eppendorf 5414 centrifuge (Eppendorf, Hamburg, Germany) for 1 min at 10 000 g to form a cell pellet. A portion (100 μl) of each supernatant was transferred to Eppendorf tubes and the remainder discarded. The pellets were washed twice in cold buffer. The cell pellets and supernatants were counted in a γ-counter. Protein concentration was estimated in each cell pellet.[24] The ^{86}Rb influx was calculated using the formula below according to the method of Hilton and Patrick[23] and derived from Sacks and Welt.[25]

$$\frac{\dfrac{\text{Cell count in 1 h (counts/h)}}{\text{Protein (mg)}}}{\text{Supernatant count in 1 ml (counts/ml)}} \times \begin{array}{l}\text{Concentration of} \\ \text{Potassium in} \\ \text{buffer (mmol.l}^{-1}\text{)}\end{array}$$

Where: cell counts in 1 h is the counts that would be produced by a platelet pellet if influx continued for 1 h, obtained by multiplying the result for 12 min by 5. As Turaihi et al[11] showed there is no difference between samples taken at 12 and 30 min we assume that influx is linear.

Protein is the total protein content of the platelet pellet.

Supernatant counts in 1 ml is the counts that would be present in 1 ml of supernatant collected from the incubation mixture.

The total ^{86}Rb influx is derived from the incubation tubes without ouabain. The passive ^{86}Rb influx is the ^{86}Rb influx that occurs in the presence of ouabain, an inhibitor of the sodium pump. The active influx is calculated from subtracting the passive from the total ^{86}Rb influx and represents the influx due to the sodium pump.

Sodium Transport in Platelets. After equilibration, the PRP was placed into cuvettes in a Whole blood Aggro-meter (Chrono-log Corporation, Havertown, Philadelphia, USA) that had been prewarmed to 37°C. Magnetic stirrers were switched on and 1 min later the inhibitor (ouabain, atenolol, timolol dissolved in saline) was added. The platelets were stirred for a further 2 min and then allowed to rest for 3 min. In controls the same volume of saline replaced the inhibitor and the stimulator used in the experiment. At this time the stimulant (adrenaline, salbutamol, BRL 37344 each dissolved in saline) was added and the platelets stirred for 2 min and rested for 3 min. After this ^{22}NaCl (37 kBq.ml^{-1}) was added, the platelets stirred and a sample taken 2, 6 and 10 min after the addition of ^{22}NaCl. The samples taken were washed twice in cold saline and the pellets counted on an LKB Wallace 1282 compugamma universal gamma counter (Turku, Finland). The raw counts, of radioactivity, were corrected for the number of platelets in the PRP, to a value of 350×10^9 platelets/ l of PRP, to standardise the method.

Single measurements of sodium transport were carried out. In early experiments, the variation between duplicates was so small that it was judged unecessary to perform measurements in duplicate. It is difficult to assess the intra-assay variation because only four wells were available to stir platelets. Thus, performing several sequential measurements would mean that the time lapse between the first and last set of measurements would be considerable. This delay, could in turn, alter platelet responses. However, we considered the median of nine measurements and their duplicates. Each set of duplicates was performed on a different subject on different days. The values obtained were:

	Median	Range
Original	337	(231–485)
Duplicate	334	(235–474)

Thus, it can be seen that the duplicate measurements show very good reproducibility.

Where no inhibitor was required in the procedure, while investigating the effect of an agonist, platelets were stirred for 1 min prior to addition of the stimulant and then the procedure continued as above.

Fluid trapped in the extracellular space of the pellet could result in errors. Hence ^{51}Cr-EDTA was used to assess the amount of radioactivity likely to be trapped in the platelet pellet. PRP (1 ml) was placed in each of 2 cuvettes with magnetic stirrers. The PRP was mixed for 3 min and then allowed to rest for

3 min as in the experiments. Then, ^{51}Cr-EDTA (30 kBq.ml^{-1}) was added to one cuvette and saline to the other cuvette. The samples were stirred and 2 min after the addition of the ^{51}Cr-EDTA a sample was taken from each and transferred to an Eppendorf tube. At this time, the same amount of ^{51}Cr-EDTA was added to 1 ml saline in an Eppendorf tube and all 3 Eppendorf tubes were washed twice in cold saline. The samples were then counted on a LKB Wallace 1282 Compugamma universal gamma counter, as before. Seven experiments were conducted. There was no evidence of any significant trapping of radioactivity in the cell pellet.

Statistical Analysis

Results are expressed as the median and (range) of the data. The Wilcoxon matched pairs signed ranks test was used to compare results.

Results

The concentration of radioactive sodium (^{22}Na) in the platelet increases with time (see Table 1). The ^{22}Na content at t = 6 min is significantly (p < 0.0001) higher than the value at t = 2 min. Also the value observed at t = 10 min is significantly (p < 0.0005) higher than the value at t = 6 min.

The median values, seen in Table 2, indicate that ouabain causes a significant (p < 0.03) increase in the ^{22}Na content of the pellet compared to a saline control. The ouabain-induced increment increases with incubation time. Thus, at t = 2 min, the increment is + 58.3%; at t = 6 min, + 65.1% and at t = 10 min, + 88.1%. The presence of adrenaline (50 nmol.l^{-1}) caused a temporary significant (p < 0.02) increase in platelet ^{22}Na content at t = 2 min but there was no significant increase in the 6 and 10 min samples. Preliminary experiments showed that adrenaline at a final concentration of 2.5 and 25 nmol.l^{-1} had no significant effect on the ^{22}Na content of platelets (results not shown). In the presence of ouabain, adrenaline did not induce a further increase in the platelet ^{22}Na content (see Table 2).

Timolol (a β_1, β_2 adrenoceptor antagonist) and atenolol (a β_1 adrenoceptor antagonist) were used to

Table 1 The uptake of ^{22}Na in platelets. Median value and (range) shown below, (n = 50). Time is after addition of ^{22}NaCl. Units: counts per minute (cpm)

Time (min)	Median counts of radioactivity in pellet	
2	251 (116–559)	p < 0.0001
6	335 (148–801)	
		p < 0.0005
10	363 (149–631)	

Table 2 The effect of ouabain (0.2 mmol.l^{-1}) and adrenaline (50 nmol.l^{-1}) on ^{22}Na platelet content. Median values and (range) shown below, (n = 7). Units: cpm

| | Time (min) after addition of ^{22}NaCl | | |
	2	6	10
Saline	175	220	253
	(140–245)	(187–297)	(188–342)
Ouabain	258*	360*	444*
(0.2 mmol.l^{-1})	(160–359)	(232–423)	(387–571)
Adrenaline	254†	239	280
(50 nmol.l^{-1})	(192–270)	(173–333)	(201–547)
Adrenaline	252	375	414
+ Ouabain	(204–355)	(249–411)	(333–512)

* = $p < 0.03$ when comparing platelet ^{22}Na content with saline alone to that with ouabain present.
† = $p < 0.02$ when comparing platelet ^{22}Na content with saline alone to that with adrenaline present.

identify which subtype of adrenoceptor causes the adrenaline-induced increase seen. Investigations showed that at t = 2 min atenolol (30 nmol.l^{-1}) did not inhibit the adrenaline-induced increase in platelet ^{22}Na content. Adrenaline alone resulted in an intracellular ^{22}Na content of 324 counts per minute [cpm] (204–532) and in the presence of atenolol the result was 320 cpm (215–498) (n = 7). However when timolol was used the results were; adrenaline alone, 594 cpm (243–666) and adrenaline with timolol, 451 cpm (215–556) (n = 7). Thus, timolol significantly ($p < 0.02$) inhibits the adrenaline-induced increase in platelet ^{22}Na content.

The median values show that both xamoterol (50 nmol.l^{-1}), a β_1 agonist, and salbutamol (50 nmol.l^{-1}), a β_2 agonist, do not cause an adrenaline-like increase in platelet ^{22}Na content (see Table 3). Both xamoterol and salbutamol were also studied at a concentration of 2.5 nmol.l^{-1} and similarly they did not cause an adrenaline-like increase (results not shown). The results for salbutamol contradict the findings with ^{86}Rb influx which indicate that the Na,K-ATPase activity is linked to the β_2 receptor. The stimulatory effect of adrenaline on ^{86}Rb influx was confirmed in preliminary experiments (results not shown). The results for ^{86}Rb and ^{22}Na methods showed a similar increase due to the presence of adrenaline at the same concentration. ^{86}Rb influx was also stimulated by salbutamol ($p < 0.02$, n = 7) as can be seen in Table 4. As adrenaline and salbutamol both have β_2 agonist activity the stimulation was

Table 3 The effect of xamoterol (50 nmol.l^{-1}) and salbutamol (50 nmol.l^{-1}) on ^{22}Na platelet content. Median values and (range) shown below, (n = 7). Units: cpm

| | Time (min) after addition of ^{22}NaCl | | |
	2	6	10
Control for	327	408	473
xamoterol	(199–502)	(228–651)	(296–692)
Xamoterol	385	375	479
(50 nmol.l^{-1})	(163–604)	(285–558)	(280–659)
Control for	184	268	258
salbutamol	(158–276)	(193–445)	(192–788)
Salbutamol	181	253	259
(50 nmol.l^{-1})	(147–243)	(186–307)	(196–582)

Table 4 ^{86}Rb influx stimulation in the presence salbutamol. Median value and (range) stated below, (n = 7). Units: mmol.kg^{-1} protein.h^{-1}

	Saline	Salbutamol (50 nmol.l^{-1})	Increment
Total	1412	1636	+15.9%
	(748–2096)	(786–1904)	
Passive	736	747	+ 1.5%
	(386–1176)	354–957)	
Active	676	889*	+31.5%
	(362–920)	(432–1069)	

* = $p < 0.02$ comparing platelets ^{86}Rb influx in the presence and absence of salbutamol.

considered to be mediated through the β_2 adrenoceptor.

These findings suggested that the adrenaline-induced increase in platelet ^{22}Na content must be due to an 'atypical' β-adrenoceptor. BRL 37344, an 'atypical' β_3-agonist, was therefore used to further clarify the mechanism of adrenaline action.

BRL 37344, final concentration 2.5 nmol.l^{-1}, caused an adrenaline-like increase in platelet ^{22}Na content (see Table 5). However, BRL 37344 at a concentration of 50 nmol.l^{-1} did not cause a significant increase in platelet ^{22}Na content (results not shown). As with adrenaline, the increase is significant ($p < 0.002$) only at t = 2 min (see Table 5). BRL 37344 (2.5 nmol.l^{-1}) also significantly stimulates both the total ^{86}Rb influx ($p < 0.04$) and the active ^{86}Rb influx ($p < 0.005$) (see Table 6).

The adrenaline-like significant ($p < 0.02$) increase caused by BRL 37344 in platelet ^{22}Na content is inhibited by timolol (see Figure).

BRL 37344-induced a significant ($p < 0.02$) increase

Table 5 The effect of BRL 37344 (2.5 nmol.l^{-1}) on ^{22}Na platelet content. Median values and (range) shown below, (n = 15). Units: cpm

| | Time (min) after addition of ^{22}NaCl | | |
	2	6	10
Control for	175	234	346
BRL 37344	(109–398)	(142–478)	(124–602)
BRL 37344	236*	286	281
(2.5 nmol.l^{-1})	(141–558)	(124–602)	(189–560)

* = $p < 0.002$ comparing platelets ^{22}Na content in the presence and absence of BRL 37344.

Table 6 ^{86}Rb influx stimulation in the presence BRL 37344. Median value and (range) stated below, (n = 7). Units: mmol.kg^{-1} protein.h^{-1}

	Saline	BRL 37344 (2.5 nmol.l^{-1})	Increment
Total	1228	1408*	+14.7%
	(857–2096)	(1059–2273)	
Passive	606	579	− 4.5%
	(351–1176)	(348–869)	
Active	653	901**	+38.0%
	(362–1143)	(475–1404)	

* = $p < 0.04$ comparing total ^{86}Rb influx in the presence and absence of BRL 37344.
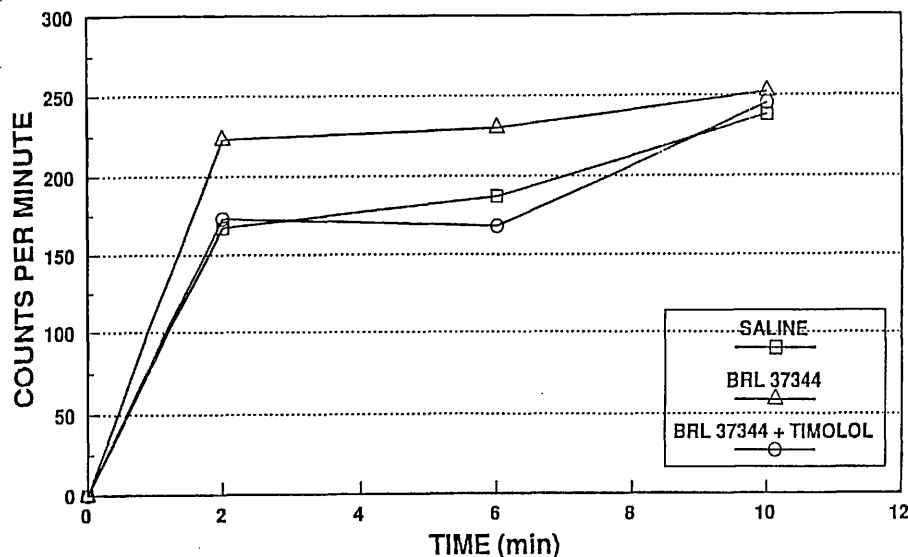** = $p = 0.005$ comparing active ^{86}Rb influx in the presence and absence of BRL 37344.

Figure The BRL 37344 (2.5 nmol.l^{-1}) stimulated ^{22}Na transport and its inhibition with timolol (60 nmol.l^{-1}). Each point represents the median of n = 7 subjects. Units: cpm.

in total and active ^{86}Rb influx. Timolol significantly (p < 0.02) decreased the BRL 37344-stimulated active ^{86}Rb influx as can be seen in Table 7.

Discussion

The results show that ^{22}Na is taken up by human platelets in a time-dependent manner. This finding is in agreement with previous work[12] which showed human platelets uptake ^{22}Na. Wolfel et al[26] also showed a significant increase in intracellular sodium (non-radiolabelled) in the presence of ouabain. Using ouabain, to block the sodium pump,[13] indicates that Na$^+$,K$^+$-ATPase plays a significant role in ^{22}Na uptake. These findings suggest that ^{22}Na accumulates intracellularly because it can not leave the platelet in exchange for potassium. Sodium influx, on the other hand, will not be directly affected by ouabain since this is a passive pathway. This interpretation is compatible with the observed increase in intraplatelet ^{22}Na content and concurrent decrease in intracellular potassium following the addition of ouabain to rabbit platelets.[27] Feinberg et al[12] also arrived at the same

Table 7 ^{86}Rb influx stimulation in the presence BRL 37344 and its inhibition with timolol. Median value and (range) stated below, (n = 7). Units mmol.kg^{-1} protein.h^{-1}

	Saline	BRL 37344	BRL 37344 + Timolol
Total	994 (536–2440)	1129* (585–2596)	1098 (534–2246)
Passive	426 (258–1019)	439 (248–869)	492 (258–948)
Active	556 (278–1421)	687* (337–1794)	579** (276–1298)

* = p < 0.02 comparing ^{86}Rb influx in the presence and absence of BRL 37344.
** = p < 0.02 comparing BRL 37344-stimulated ^{86}Rb influx in the presence and absence of Timolol.

conclusion; however, we have shown the same result in 10 min instead of 3.5 h experiments.

Adrenaline is known to stimulate the sodium pump.[8,11] In the present study, adrenaline caused a significant (p < 0.02), transient, increase in the ^{22}Na content of platelets. This is likely to be due to a stimulation of sodium efflux (a pump-mediated effect) which in turn increases the influx of extracellular ^{22}Na. It is therefore not surprising that in the presence of ouabain, (an inhibitor of the sodium pump) adrenaline did not cause a further increase in platelet ^{22}Na content. These findings confirm previous observations with adrenaline.[8,11]

^{86}Rb influx in platelets has been attributed to the β_2-adrenoceptor.[11] Hence, we examined the receptor specificity with agonists and antagonists using the sodium transport method. Atenolol (a β_1 adrenoceptor antagonist) did not inhibit adrenaline-induced increases in platelet ^{22}Na content whereas timolol (a β_1,β_2 adrenoceptor antagonist) did. These findings are compatible with previous work which showed that the sodium pump is linked to the β_2 adrenoceptor in leucocytes,[8] platelets[11] and muscle.[28] However, as adrenaline has both α and β adrenoceptor agonist activity we can not exclude an α-adrenoceptor-linked component without including experiments using adrenaline in the presence of α-adrenoceptor antagonists. From the results, it seems likely that adrenaline is acting at the β_2-adrenoceptor site and if this is the case, then using xamoterol, a β_1 agonist, and salbutamol, a β_2 agonist, should clarify this further. Xamoterol, as expected, did not significantly alter platelet ^{22}Na content. Salbutamol did not significantly increase platelet ^{22}Na content whereas it significantly (p < 0.02) stimulated active ^{86}Rb influx. The latter observation is compatible with earlier findings.[28] This apparent discrepancy between ^{22}Na and ^{86}Rb methods suggests that sodium transport is linked to

an 'atypical' β_3 adrenoceptor or that technical differences do not always produce the same responses. [86]Rb and [22]Na transport may also have different properties, even in the same cell type. An additonal explanation is that salbutamol may act as a partial β-agonist[29] and BRL 37344 as a full β-agonist.

The possibility of an 'atypical' β-adrenoceptor was investigated by adding BRL 37344, a β_3 adrenoceptor agonist.[30] This agonist caused a significant ($p < 0.03$) increase in platelet [22]Na content in a manner similar to that observed with adrenaline. This observation supports the view that an 'atypical' β_3 adrenoceptor is involved. Others, using similar agonists and antagonists to influence calcium transport in human platelets, have also provided evidence favouring the presence of a β_3 adrenoceptor on human platelets.[30] Active ^{86}Rb influx was also significantly ($p < 0.02$) stimulated to a similar extent with BRL 37344. This agonist was more potent than adrenaline, in both systems studied, since the concentration required to give the same magnitude of increase was 20 times less. In other systems, BRL 37344 has also been found to be more potent than other β agonists[30-32] (salbutamol, isoprenaline and fenoterol).

As with adrenaline, the increase in platelet [22]Na content due to BRL 37344 was inhibited by timolol. This suggests that BRL 37344 is acting in a similar way to adrenaline. The BRL 37344-mediated stimulation of ^{86}Rb influx was also inhibited by timolol. This suggests that BRL 37344 is acting at the same site to cause stimulation of both [22]Na and ^{86}Rb influx. Therefore, we suggest that like [[45]Ca^{2+}] uptake by human platelets,[30] sodium transport is linked to an 'atypical' adrenoceptor possibly of the 'β_3' subtype.

It is of interest that sodium and calcium transport appear to be linked.[33,34] It has been suggested that inhibition of the pump leads to an increase in Na-Ca exchange,[35] where sodium is transported out of the cell and calcium into the cell. Therefore, if the pump is no longer able to keep the intracellular sodium concentration at the correct value, it may be that Na-Ca exchange is altered. As potassium is not involved in this latter form of exchange, this phenomenon could explain a discrepancy between ^{86}Rb and [22]Na transport. This phenomenon would not be apparent in leucocytes because there is no evidence that the Na-Ca exchange system occurs in this cell type.[36]

Another system that does not involve potassium is the influx of Na$^+$ in exchange for the efflux of H$^+$.[37] The Na/H exchange is a major pathway for the uptake of Na$^+$ and may cause an intracellular Na$^+$ load which would stimulate the sodium pump.[38] If adrenaline stimulates this transport, as well as the sodium pump, this could explain the discrepancies between the ^{86}Rb and [22]Na methods.

The Na,K-pump, Na/H exchange and Na-Ca exchange appear to be associated with each other. They all influence intracellular [Na$^+$] which has a direct effect on membrane potential and conductance. Ouabain and adrenaline increase membrane conductance and inhibitors of conductance cause compensatory changes in the sodium pump.[39-41] Therefore, a possible explanation for the observed increased [22]Na$^+$ content due to ouabain and adrenaline is a change in membrane conductance. However, adrenaline-induced increases in membrane conductance can not be inhibited by some β-adrenoceptor antagonists[42] in contrast to our observations. Furthermore, the studies on membrane conductance use adrenaline at concentrations, 10 to 100-fold higher[41-43] than the present study. Therefore, membrane conductance may not be altered at the low concentrations of adrenaline we used.

If membrane conductance does, however, influence the results it may account for some of the discrepancy between the ^{86}Rb and [22]Na methods because sodium is more efficient than potassium (or rubidium) at facilitating adrenaline-mediated effects on conductance.[43]

[86]Rb influx and [22]Na transport in platelets were influenced by almost identical agonists and antagonists. The only difference being salbutamol, a β_2 agonist, which stimulated ^{86}Rb influx but had no effect on [22]Na transport. This discrepancy may be related to methodological sensitivities or to differences in factors controlling the intracellular status of potassium and sodium. The latter is likely since there are numerous pathways that have been identified which influence the transport of these two ions.[36,44,45]

Therefore, it would appear that to study the sodium pump thoroughly, both rubidium and sodium transport should be investigated. This would be impossible using leucocytes because too large a blood volume would be required. For example, for [22]Na estimations, the leucocyte methodology would require 100 ml compared to 18 ml for platelets. As the study of the sodium pump in platelets requires relatively small volumes of blood it may now be possible to carry out sequential measurements in any one study. Therefore, the future of Na$^+$, K$^+$-ATPase investigation in clinical studies may lie with platelets.

Acknowledgements

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