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Cell membrane status and cations in essential and malignant hypertension.

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Appendix A. Abbreviations

AAS	atomic absorption spectrophotometry
ADH	antidiuretic hormone
ANF	atrial natriuretic factor
ATP	adenosine triphosphatase
ATPase	adenosine triphosphatase
Ca^{2+}	calcium
cAMP	cyclic adenosine 3'5' monophosphate
cGMP	cyclic guanosine 3'5' monophosphate
DBP	diastolic blood pressure
DG	diacyl glycerol
DLA	digitalis like activity
DLF	digitalis like factor
E	erythrocyte
EDRF	endothelial derived relaxing factor
FFA	free fatty acids
GTP	guanosine triphosphate
HR	heart rate
IP_3	inositol trisphosphate
IP_3	phosphoinositide
K^+	potassium
LPC	lysophospholipid
MAP	mean arterial pressure
Mg^{2+}	magnesium
MLC	myosin light chain
Na^+	sodium
P	platelet

P_i	inorganic phosphate
PIP_2	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PRP	platelet rich plasma
QI	Quetelet index
RBC	red blood cell
S	serum
SAIMR	South African Institute for Medical Research
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
WI	Wistar rats
WKY	Wistar-Kyoto rats

Appendix B. Anticoagulants

Serum and erythrocyte studies

Heparin was used as the anticoagulant for serum and erythrocyte studies. It is the most widely used anticoagulant for clinical chemical analyses as it causes the least interference with tests. This anticoagulant acts as an antithrombin to prevent the transformation of prothrombin to thrombin and consequently the formation of fibrin from fibrinogen. Heparin is a mucocoitin polysulfuric acid and is available as sodium, potassium, lithium and ammonium salts. In this study, since serum sodium and potassium levels were analysed, the lithium salt was used in preference to the sodium and potassium salts. Although lithium is used as an internal standard for flame photometry, the amount of lithium added as lithium heparin has an insignificant effect on sodium and potassium measurements.

Platelet studies

Anticoagulants used in platelet studies include sodium citrate, heparin, ethylenediaminetetraacetic acid (EDTA) and sodium oxalate (Newhouse & Clark, 1978). The most commonly used anticoagulants for platelet preparations are sodium citrate and heparin. Citrate appears to preserve labile procoagulants. High concentrations of citrate

inhibit aggregation by excessive binding of calcium (Newhouse & Clark, 1978). When heparin is utilised to prepare platelets, small platelet aggregates may occur (Zucker, 1974).

Anticoagulants used in this study

In this study, the anticoagulants used for platelet preparation were heparin and sodium citrate. Two anticoagulants were utilised because:-

1. The sodium citrate eliminated the microaggregates formed by the heparin.
2. For the measurement of intracellular platelet divalent cations the extracellular levels of these ions must be reduced to almost zero. Since citrate solutions chelate extracellular magnesium and calcium, this was an ideal anticoagulant to use for the platelet cation studies.

The concentration of lithium heparin (0,2 mg heparin/ml blood) and sodium citrate (3,8%) was kept constant throughout the study.

Appendix C.

Sources of chemicals and reagents used

Chemicals	Source
Adenosine triphosphate	Sigma
Ammonium molybdate	Sigma
Bovine serum albumin	Sigma
Calcium chloride	Merck
Calcium nitrate	BDH chemicals
Citric acid	BDH chemicals
Copper sulphate	BDH chemicals
Dextrose	Merck
Dithiothreitol	Merck
Dimethylsulfoxide	Merck
Ethylene diamine tetraacetic acid (EDTA)	Merck
Ethylene glycol-bis (p amino ethyl ester) N N N' N' tetraacetic acid (EGTA)	Merck
Ferrous sulphate	BDH chemicals
Folin phenol reagent	Unilab
Fura-2 AM	Boehringer Mannheim
Hepes	Boehringer Mannheim
Hydrochloric acid	Merck
Hydrogen phosphate	Sigma
L-histidine	BDH chemicals
Lanthanum oxide	Merck

Magnesium chloride	Merck
Magnesium nitrate	BDH chemicals
Ouabain	Sigma
Potassium chloride	Sigma
Potassium dihydrogen phosphate	Merck
Potassium hydrogen orthophosphate	Unilab
Potassium sodium tartrate	BDH chemicals]
Saponin	Merck
Sodium carbonate	Merck
Sodium chloride	Merck
Sodium dihydrogen orthophosphate	Unilab
Sodium hydroxide	Merck
Sucrose	Merck
Sulfuric acid	Merck
Trichloracetic acid	Unilab
Tris (hydroxymethyl) aminomethan	Merck
Trisodium citrate	Unilab
Triton X-100	BDH chemicals

Appendix D. Effect of centrifugation time on platelet count

Ideal conditions for obtaining PRP have not been well defined. Some researchers have reported low gravitational forces for a long time - 100 - 160 g for 20 minutes (Mustard et al, 1972; Resink et al, 1984; Rao et al, 1985), while others have reported high gravitational forces for a short time - 700 g for 5 minutes (Hallam and Rink, 1985). Prolonged centrifugation results in sedimentation of the large blood cells as well as platelets.

The optimal centrifugation time in this study was determined by centrifuging blood samples for varying lengths of time. Six blood samples were centrifuged for 10, 15, 20, 30 and 60 minutes at 160 x g. Platelet counts were performed after each centrifugation time. The results were compared to the platelet count from blood that had sedimented at room temperature for 120 minutes (Figure 1). Centrifugation at 160 x g for 15 minutes was selected as the ideal centrifuging time, as this yielded the highest platelet count from PRP and had the least contamination by other cells. These centrifugation conditions were kept constant throughout the study.

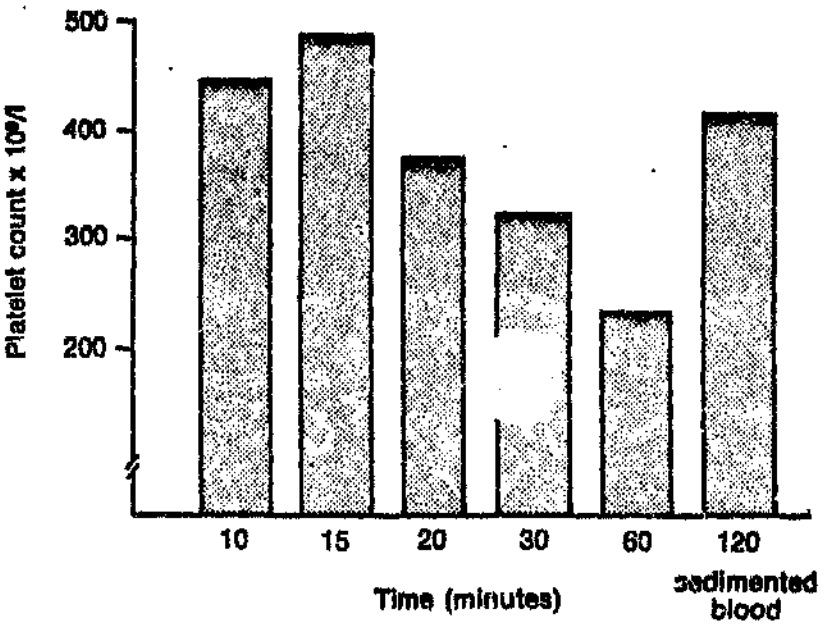

Fig. 1 Determination of optimal centrifugation time for platelet rich plasma preparation. Blood samples were centrifuged at 120 g for various time periods. The results are compared to blood that was left to stand for 120 minutes (sedimented blood).

Appendix E. Methods for separating platelets from plasma

(i) Steric exclusion chromatography (gel filtration)

Steric exclusion chromatography depends on molecular size selectivity to separate molecules (Bouers, 1986). This technique has been effectively used to isolate platelets (Tangen et al, 1971). Washed platelets are applied onto a column filled with an uncharged agarose gel (Sepharose - 2B) consisting of non-compressed beads with a diameter of 60-200 U. The gel has an exclusion limit for molecules greater than 4×10^7 MW. Due to their size, platelets are restricted to the fluid between the beads and pass rapidly through the column, eluting in the void volume i.e. the volume of fluid that passes, without restraint, through a column. Separations made on the basis of molecular size, assume that there is no interaction between the solutes and the support. This is often not the case. There are disadvantages of using gel filtration for platelet isolation. Gel filtration induces Factor VIII and prostaglandin E2 synthesis (which are associated with release of granule contents) and the platelet count is only about 50% of that of the original PRP applied to the column (Day et al, 1975).

(ii) Density gradient centrifugation

For platelet separation using this method, PRP is applied to a concentrated solution of albumin. When centrifuged, an albumin concentration gradient is formed and the centrifugal force drives the platelets into that region where their density is equal to that of the albumin solution. This method has the disadvantage of the isolated platelets containing a high concentration of albumin which could interfere with further biochemical measurements.

(iii) Platelet isolation by repeated centrifugation and resuspension in physiological solutions

Platelets are isolated by centrifuging PRP at high speeds. The platelet pellet obtained is then washed repeatedly by centrifugation and resuspension in physiological buffers. The main disadvantage of this method is that it is time consuming to separate the platelets from all plasma constituents. In this study, platelets were isolated by repeated centrifugation and resuspension in physiological buffers.

Appendix F. Validation of techniques

F. 1. Validation of technique for isolating platelets

The method described to isolate platelets was based on that described by Resink et al (1984). This technique was modified for platelets prepared for Na^+ and K^+ analysis. The washing buffer contained 100 mM $MgCl_2$ and 1 mM Mg $HOP_4.3H_2.C$ instead of the previously described 145 mM NaCl and 1 mM NaH_2PO_4.

The technique of isolating platelets by repeated washing and centrifuging has been previously described. This technique has the following advantages:

i) it is simple

ii) it produces a high yield of platelets

iii) the platelets remain intact

iv) there is minimal or no contamination of the platelets by erythrocytes or leukocytes

F. ii. Validation of technique for preparing washed erythrocytes

The method described for preparing washed erythrocytes was based on that described by Fortes Mayer and Starkey (1977). This method has the following advantages:-

i) It is relatively simple

ii) The cells remain intact

iii) There is minimal leukocyte and platelet contamination.

iv) Trapped extracellular fluid is accounted for in the final analysis.

Washing erythrocytes free of plasma by repeated centrifugation and resuspension in iso-osmolar solution has been extensively reported (Astrup, 1974; Goolden et al, 1977). The combined use of the washing procedure and PCV determination obviates the estimation of the trapped plasma cations. A variety of methods, including isotope markers, have been used to correct the erythrocyte cation concentration for trapped ions (Maizels and Remington, 1959; Vettore et al, 1974). These procedures however are complex with potential for technical errors. The use of isotonic salt solutions to wash erythrocytes free of plasma has been criticized on the basis that ions migrate across the erythrocyte during washing (Valdberg et al, 1965). However, in the method used in this study, there was no detectable Na^+ and K^+ in the final $MgCl_2$ wash solution and no Mg^{2+} and Ca^{2+} in the final NaCl wash solution.

This implies that the conditions employed in the study do not give rise to a significant leakage of the cations across the erythrocyte membrane, These findings are supported by Astrup (1966).

F. iii. Validation of techniques for preparing cells for cation determination

The method of washing blood cells free of plasma by repeated centrifugation and resuspension in iso-osmolar solutions has been previously described (Goolden et al. 1971; Freeman and Spirtes, 1963; Astrup, 1974; Ladefoged and Hagen, 1988). The absence of the relevant ions in the final supernatant wash solutions in the methods used in this study, implies that the conditions employed results in no significant leakage of the cations from the erythrocytes.

Previous reports have corrected erythrocyte ion concentration for residual plasma ions by the use of markers. These markers include haemoglobin, insulin, lactose, plasma proteins, radiolabelled ions, ^{59}Fe and indocyanine green (Maizels and Remington, 1959; Maizels, 1965; Funder and Wieth, 1967; Cividalli and Loker 1969; Vettore et al, 1974). These methods however are complex and involve increased cell handling and manipulation. The potential error of such methods is increased. The use of washed erythrocytes coupled to packed cell volume

determination obviates the estimation of the amount of trapped plasma. This technique was used in the present study Reports by Astrup (1974) and Fortes Mayer and Starkey (1977) support this method.

For platelet preparation, the trapped intercellular plasma was removed by repeated washings. All platelet samples were adjusted to counts of 1 x 10^8 cells / ml and all platelet samples had normal platelet volumes. By controlling the washing procedure, platelet count and volume, the technique for measuring platelet cations was standardised between specimens.

F. iv. Lysing procedures

In previous studies, concentrated acid was used to lyse platelets (Baker et al, 1978). Relatively large volumes of acid are needed and when added to the platelets they do not always dissolve immediately. Its effect is quicker and more efficient than concentrated acids and methods that depend on freezing and thawing or subjecting cells to osmotic shock (Funder and Wieth, 1966; Millart et al, 1985). Saponin has been used extensively to lyse erythrocytes (Astrup, 1966; Fortes Mayer and Starkey 1977). For the reasons stated above, Saponin was used as the lysing agent in this study.

Appendix G. Methods for the determination of sodium, potassium, magnesium and calcium

Appendix G.a. Sodium and potassium analysis

The most commonly used method for the quantitative measurement of sodium and potassium in body fluids is flame emission photometry (Velapoldi et al, 1978a; 1978b).

Reagents

1. Standard lithium 3000 mmol/l
2. Electrolyte standards for serum:- Na^+ - 140 mmol/l
   K^+ - 5 mmol/l
3. Electrolyte standards for erythrocytes and platelets:
   Na^+ - 5 mmol/l
   K^+ - 100 mmol/l

To prevent leaching of Na^+ and K^+, the electrolyte standards were stored in polyethylene bottles.

Procedure

The IL 543 digital flame photometer (Instrumentation Laboratory Inc. Lexington, MA 02173) was used. After 'zeroing', with distilled water, the machine was calibrated with the lithium diluent (to the lithium reference range) and then with the appropriate sodium and potassium standards. Serum samples were aspirated directly into the diluter. (For serum sample analysis, no manual dilutions

were necessary). After processing five samples, the flame photometer was reset to zero and recalibrated.

Appendix G.b. Methods for the determination of magnesium and calcium.

A variety of methods have been described for the quantitative analysis of magnesium in biological fluids (Alcock and MacIntyre, 1967). Atomic absorption spectroscopy (AAS) is sensitive, specific and accurate and is the current definitive technique for magnesium analysis. It is regarded as the reference method (Wills et al, 1986).

The definitive method for total calcium measurement is isotope dilution followed by mass spectroscopy (Moore and Machlan, 1972). This method serves as a reference for atomic absorption spectroscopy, the secondary reference method (Cali et al, 1973). The principles of AAS are described in Appendix G.d.

(i) Magnesium analysis

The basis for magnesium determination is the formation of specific soluble or insoluble complexes which are then quantitated by AAS. Specimens are diluted with an acidic lanthanum chloride and aspirated directly into the AAS flame without further treatment. Levels are calculated

from a linear calibration curve of standards made up with the same lanthanum diluent.

Reagents

i) Stock lanthanum solution.

a) 58,76 g of lanthanum oxide was placed in a 1,0 l volumetric flask and wet with water.

b) 250 ml concentrated HCl was slowly added until the lanthanum was dissolved.

c) The solution was diluted in 1,0 l of water, giving 5% lanthanum ion solution in 4N HCL.

d) The stock solution was stored at 4°C.

ii) Working lanthanum solution (0,2% w/v). 20 ml of the stock solution was diluted to 500 ml with deionised water.

iii) Stock standard magnesium.

Magnesium nitrate : 1 ml = 1 mg Mg^{2+} = 41,1 mmol/l

iv) Working standards for calibration

0 - 4,0 mmol/l calibration standards were prepared from the stock solution. The standards were stored in polyethylene bottles at 4°C and made up monthly.

Analytical procedure

1. Magnesium was measured at a wavelength of 285.2 nm.
2. The specimen was diluted 50 times with the salt-free lanthanum oxide diluent (100 ml of specimen diluted to 5 ml) and vortexed.
3. The standards were similarly prepared with the diluent.
4. The AAS was zeroed with the lanthanum.
5. The machine was calibrated with the diluted standards.
6. The sample was aspirated and the concentration read directly off the machine.
7. The concentration of the element could also be determined by measuring the absorbance and applying the formula:-

$$\frac{\text{test absorption}}{\text{standard absorption}} \times \text{standard concentration} = \text{test concentration (mmol/l)}$$

8. After measuring five samples, the instrument was reset to zero with the working lanthanum blank and recalibrated with the standard solutions.

(ii) Calcium analysis

Lanthanum is added to the diluent for samples and standards because:- 1) It eliminates the effects of anions which depress the light absorption by atomic magnesium and calcium; 2) it serves to liberate calcium from its ligands and; 3) it decreases the interferences from protein and

variations in sodium and potassium concentrations in the sample.

Reagents

i) Stock lanthanum solution

1. 58,7 g of lanthanum oxide was placed in a 1,0 l volumetric flask and wet with water.
2. 250 ml concentrated HCl was added until the lanthanum was dissolved.
3. The solution was made up to 1,0 l, giving a 5% lanthanum solution in 4N HCl.
4. The stock solution was stored at 4°C.

ii) Stock lanthanum solution (0,1% w/v).

10 ml of the stock solution was diluted to 500 ml with deionised water.

iii) Stock standard Ca^{2+}.

Calcium nitrate: 1 ml = 1 mg Ca^{2+} = 24,9 mmol/l

iv) Working standards for calibration.

0-3,0 mmol/l calibration standards were prepared from the stock solution. The standards were stored in polyethylene bottles at 4°C and prepared monthly.

Analytical procedure

The procedure for determining calcium by atomic absorption spectroscopy was similar to that for magnesium analysis, except that the wavelength was set at 422,7 nm and the

working lanthanum solution was a 0,1% concentration instead of a 0,2% concentration.

All samples were prepared in duplicate and each sample was analysed three times. The mean of the multiple recordings was taken for final analysis.

Appendix G.c. Principles of flame photometry (Frei, 1974)

Atoms of elements, such as sodium and potassium, when exposed to sufficiently high temperatures, will be forced into an excited state through thermal collision. A specific amount of thermal energy is absorbed by an orbital electron. The electrons, being unstable in this excited state, release their excess energy as photons of a particular wavelength as they change from the excited to their previous state. The energy may be dissipated in many ways, one of which is light emission. Each atom has associated with it a set of energy levels. In the atomised state, excited atoms will emit a characteristic set of wavelengths. The intensity of the emitted light is directly proportional to the number of atoms undergoing the transition. Thus, by selectively monitoring a specific wavelength of an element being volatilised and excited in a flame, the concentration of that element may be measured directly. Sodium and potassium can be analysed by flame photometry with greater precision and accuracy than by any other technique.

Components of the flame photometer

The sample is diluted in a lithium diluent and aspirated into a propane-air flame. Sodium and potassium, when excited, emit spectra at 589 and 768 nm respectively. Light emitted from the thermally excited ions is directed through separate interference filters to corresponding photodetecters. The lithium emission signal is taken as a reference (internal standardisation) against which sodium and potassium signals are compared separately. The system is standardised relative to low and high concentrations of each analyte, and the relation of signal to concentration is defined by an associated microprocessor. Upon aspiration of controls and blood samples into the standardised instrument, results are presented in mmol/l on a digital readout device.

The 'internal standard' method was employed. In this method, lithium is added to all blanks, standards and unknowns in equal concentrations. Lithium has a high-emission intensity, is normally absent from biological fluids and emits at a wavelength sufficiently removed from sodium and potassium to allow spectral isolation. The photometer compares the emission of the element with that of the reference lithium. By measuring the ratios of emissions, small variations in atomisation rates, flame stability and solution viscosity are compensated for.

Appendix G.d. Principles of AAS (Bradbury et al, 1968; Brandenburger, 1974)

AAS may be considered to be the inverse of flame photometry. In AAS, the element is not excited by the flame, but dissociated from its chemical bonds and placed in an unexcited state. The atom is at a low energy level and is capable of absorbing radiation at a narrow bandwidth corresponding to its own line spectrum. A hollow cathode lamp, made of the material to be analysed, is used to produce a wavelength of light specific for the kind of metal in the cathode. When the light from the lamp enters the flame, some is absorbed by the ground-state atoms in the flame, resulting in a net decrease in the intensity of the beam from the lamp.

Components of the AAS

The hollow cathode lamp is the light source. The sample is sprayed into the flame by a nebuliser. The aerosol is reduced to the constituent atoms in a flame. The flame lies in the light path between the lamp and the line detector (monochromator and photomultiplier tube). If any of the element is present in the sample, absorption of the characteristic line will occur; the amount of this absorption will provide an accurate measure of the concentration of the metal. The degree of absorption is concentration dependent.

There are a few disadvantages of using atomic absorption spectroscopy to measure magnesium and calcium. These include:-

i) the expense of the equipment

ii) it is technically difficult to automate

iii) the use of flammable gases.

The advantages however outweigh the disadvantages and include:-

i) the AAS is highly specific-using the 285,2 nm line of magnesium and 422,7 nm line of calcium hollow cathode lamps. One element in a sample of complex composition can be isolated and measured.

ii) there is no interference from other metals.

iii) the method is sensitive enough to ensure precision in small samples.

iv) concentrations as low as 1 /ug/l can be measured.

v) analytical precision is around 1 percent relative.

In this study, for the above mentioned reasons, atomic absorption spectroscopy was the method of choice for magnesium and calcium analysis.

The Varian Techtron (AA175) atomic absorption spectrophotometer was used. The light source was a hollow cathode lamp (Mg^{2+} or Ca^{2+}). The fuel was acetylene and the oxidant, compressed air. The manufacturers standard setting was used for the lamp current (5 mAmps). Initial

fuel and air pressure were 80 kPa and 6 kPa respectively. A slit width of 0,5 mm was used and Mg^{2+} absorption at 285,2 nm and Ca^{2+} absorption at 422,7 nm were recorded. The fuel-air mixture was adjusted to give a blue flame tinged with yellow for Mg^{2+} and a yellow reducing flame for Ca^{2+}.

Appendix H. Methods for measurements of serum GGT, creatinine and albumin

(a) Serum GGT

(i) Principles

Serum GGT was determined by an enzymatic method. GGT catalyses the transfer, or removal in some compounds of the gamma glutamyl group from gammy glutamyl peptides to another peptide or 4 amino acid. The measurement of GGT in serum is based on the principles developed by Orlowski and Meister (1963) and Szasz (1969):-

L- -glutamyl-3-carboxy-4-nitroaniliole + glycylglycine $\xrightarrow{\gamma GT}$ L- -glutamylglycylglycine + 5 amino-2-nitrobenzoate

Formation of 5 amino-2-nitrobenzoate produces colour measurable at 450 nm.

(ii) Methods

A S.A.I.M.R. Laboratory Reagent's Service GGT Kit (Sandringham, S.A.) containing buffer and substrate was used. The buffer (pH 8,25) comprised tris and glycylglycine and the substrate was L- -glutamyl-3-carboxy-4-nitroanilide. The buffer and substrate were mixed. A 2 ml sample of working reagent was placed in a water bath at 37° for 5 minutes. 200 µl of sample was added. Using an Encore instrument (Baker machines, S.A.

Scientific) set at wavelength of 405 nm, readings were taken at minute intervals for 3 minutes. The mean absorbance change per minute was calculated:- A/min

The final serum GGT was obtained according to the formula:-

U/l = A/min x 1158

(b) Serum creatinine

(i) Principles

The creatine method is based on the Jaffe reaction, first described in 1856. The reaction occurs between saturated picric acid with creatinine in an alkaline medium producing a red-orange adduct. The intensity of this colour complex is measured at 510 nm. This method was automated in 1961.

(ii) Method

The automated method is based on the manual procedure of Jaffe (1886). The serum sample was diluted with creatinine sample diluent. The diluted sample was dialysed against the creatinine recipient solution to remove the creatinine in the sample from protein and other endogenous serum interferences. Sodium hydroxide solution and creatinine colour reagent were added to the recipient stream to form the red-coloured chromogen in alkaline medium. The reaction mixture was heated to 37°C to accelerate

development of the chromogen. The absorbance of the analytical stream was measured at 505 nm in flowcell with a 10 mm light path and an inside diameter of 0,5 mm. The Technicon Smac system (Technicon Instruments, Tarrytown, New York) was used and the final creatinine concentration presented on the digital printout. Serum creatinine units are expressed as µmol/l.

(c) Serum albumin

(i) Principles

Serum albumin determination is based on the binding behaviour of the protein with the anionic dyes bromocresol green (BMG) of bromocresol purple (BCP) in a manual or automated procedure (Peters et al, 1982). In this study, an automated procedure using the bromocresol green method was employed.

Albumin and BCG are allowed to bind at pH 4,2 and absorption of the BCG-albumin complex is determined spectrophotometrically at 628 nm. At pH 4,2, albumin acts as a cation to bind the anionic dye.

(ii) Methods

The Encore Spectrophotometer (Baker Instruments, S.A. Scientific) was used. The instrument was set at a

wavelength of 628 nm and standardised with the BCG reagent (Technicon Instrument Corp., Tarrytown, New York, 10591) containing bromocresol green, succinic acid, sodium azide and Brij-35. Known albumin standards were added to the BCG reagent to calibrate the machine. 20 µl of the serum samples were added to 5,0 ml BCG reagent and the absorbance recorded at 628 nm at 30 seconds after the addition.

The calculations for the final concentrations were based on the formula:-

$$\text{albumin, g/100 ml} = A_u/A_s \times C_s \text{ (g/100 ml)}$$

where A_u = unknown absorbance

A_s = absorbance of standard

C_s = concentration of standard

The result was obtained directly from the instrument or the digital printout, and in this study, albumin concentration is expressed as g/l.

Appendix I. Accuracy of techniques for measuring cations

(a) Serum cations

Accuracy of techniques for serum cations was established by measuring the cation content of ten samples of pooled serum for 8 consecutive weeks. The samples were stored at 0°C. The mean coefficient of variation for the serum cation varied between 0,80 and 3,7% (Table 1).

Variable	Mean ± SD	Mean coefficient of variation	P
Serum Mg^{2+}	0,90 ± 0,01	1,1%	NS
Serum Ca^{2+}	2,3 ± 0,04	1,7%	NS
Serum Na^{+}	138 ± 1,2	0,80%	NS
Serum K^{+}	3,7 ± 0,10	2,7%	NS

Table 1 Mean coefficient of variation for serum cations. NS = not significant

In addition to performing weekly measurements on the same samples, alternate serum samples (from the control subjects) were analysed blind by an independent laboratory [South African Institute for Medical Research) (S.A.I.M.R.)], using an automated technique. The Student-t and the Wilcoxon tests showed no significant differences in

the results obtained by myself (RMT) and the SAIMR (Table 2).

	Serum Mg^{2+} mmol/l	Serum Ca^{2+} mmol/l	Serum Na^{+} mmol/l	Serum K^{+} mmol/l
RMT (n = 78)	0,90 ± 0,09	1,99 ± 0,13	138 ± 2,6	4,0 ± 0,34
SAIMR (n = 39)	0,92 ± 0,06	2,0 ± 0,10	138 ± 2,3	4,0 ± 0,34
p	0,0001	0,0001	0,0001	0,0001

Table 2 Cation values, and probabilities between the two laboratories.

To determine within-day precision of the methods, 100 ml of venous blood was collected from a healthy volunteer (RMT). The blood was centrifuged and serum, erythrocytes and platelets prepared for analysis. Eighteen aliquots of serum from the same sample were determined on the same day. Three readings were made per sample and the mean taken for analysis. Table 3 presents the serum cation values and the mean coefficients of variation.

Variable	Mean ± SD (mmol/l)	Coefficient of variation (%)	Significance
Serum Mg^{2+}	0,89 ± 0,008	0,9	NS
Serum Ca^{2+}	2,3 ± 0,04	1,7	NS
Serum K^{+}	3,5 ± 0,09	2,5	NS
Serum Na^{+}	139 ± 1,0	1,0	NS

Table 3 Within-day precision data for serum. Results were obtained from 18 aliquots of the same sample.

These results indicate a high degree of reproducibility and precision.

(b) Erythrocyte cations

The accuracy of the methods for determining erythrocyte cation concentrations was assessed by analysing samples of pooled erythrocytes on 8 separate occasions. The erythrocytes were prepared for cation analysis according to the method described in section (8.3.2.1.). Table 4 depicts the results.

Variable	Mean ± SD	Coefficient of Variation (%)	P
EMg^{2+}	2,3 ± 0,03	1,3	NS
ECa^{2+}	5,2 ± 0,10	1,9	NS
ENa^{+}	9,5 ± 0,30	3,0	NS
EK^{+}	86 ± 0,70	0,8	NS

Table 4 Precision data for cations in aliquots of the same erythrocyte sample analysed on different days.

Within-day repeatability of these methods was determined by analysing 18 aliquots of erythrocytes from the same person (RMT) on the same day. Three readings were obtained from each sample. Table 5 presents the within-day results.

Variable	Mean ± SD (mmol/l)	Coefficient of Variation	P
$E.Mg^{2+}$	2,2 ± 0,04	1,8	NS
ECa^{2+}	5,1 ± 0,1 (/umol/l)	1,9	NS
ENa^{+}	9,4 ± 0,1	1,0	NS
EK^{+}	80 ± 0,3	0,4	NS

Table 5 Within-day precision data for erythrocyte cations. The results were obtained from 18 aliquots of the same sample.

(c) Platelet cations

The precision and accuracy of the technique described for platelet cation determination was determined by analysing samples of pooled platelets on 8 separate occasions. The platelets were prepared as described in section (8.3.2. 1). Nine samples of lysed platelets were pooled and the cations analysed [method described in section (8.3.2.2.)]. Table 6 presents the results.

Variable	Mean ± SD (mmol/l)	Coefficient of Variation	P
PMg^{2+}	2,2 ± 0,01	0,7	NS
PCa^{2+}	1,9 ± 0,03	1,5	NS
PNa^{+}	1,5 ± 0,05	3,3	NS
PK^{+}	5,2 ± 0,1	1,9	NS

Table 6 Precision data for cations in aliquots of the same platelets. Samples of pooled lysed platelets were analysed on 8 separate occasions. P = platelet.

Within-day precision for platelet cation determination was assessed in 10 aliquots of platelets obtained from the same subject (RMT). These samples were all analysed on the same day. The results are presented in Table 7.

Variable	Mean ± SD (/umol/l)	Coefficient of Variation	P
PMg^{2+}	2,5 ± 0,02	0,9	NS
PCa^{2+}	2,0 ± 0,02	1,0	NS
PNa^{+}	1,38 ± 0,02	1,4	NS
PK^{+}	4,10 ± 0,05	1,0	NS

Table 7 Within-day precision data for platelet cations. Results were obtained from 10 aliquots obtained from the same sample, measured on the same day.

The effects of preparative procedures on the platelet cation content was also examined. The platelet cation levels did not change significantly when the platelet rich plasma was allowed to stand for 90 minutes compared to platelets that had been processed immediately. Also, no change was found when platelet pellets were prepared by increasing the centrifugation time from 10 to 30 minutes.

The results of the above 'accuracy studies' demonstrate that i) the standard deviations of the mean values are small; ii) the coefficients of variation for the variables are high.

For these reasons it can be concluded that there is a high degree of reproducibility, precision and accuracy with respect to the methods employed for cation determination in serum, platelets and erythrocytes.

Appendix J. Platelet-free and platelet-total calcium concentrations

The contractile state of vascular smooth muscle is largely dependent on the concentration of free cytosolic calcium (Kuriyama et al, 1982). An increased free calcium concentration has been described in platelets of patients with essential hypertension (Erne et al, 1984; Bruschi et al, 1985). The free cytosolic component constitutes less than 10% of the total intracellular calcium concentration. It is unknown what the total intracellular calcium status is in hypertension. For these reasons, the total cellular calcium concentration was measured in this study.

In order to determine whether the total cytoplasmic calcium concentration is a marker of the free calcium component the free and total concentrations were measured from platelets of the same subjects.

Subjects

Six healthy normotensive subjects were studied. Thirty millilitres of venous blood was obtained from the antecubital fossa according to methods described in Section 5.3.2. The blood was divided into two equal aliquots one for measuring platelet free calcium concentrations and the other for platelet total calcium concentrations. For the total calcium determinations, platelets were isolated and

prepared as described in Section 8.3.2.1. Platelet total calcium concentrations were measured according to the methods described in Section 8.3.2.2.2 (a).

Platelet free calcium determination

Preparation of platelets - fura-2 loading of platelets

The blood samples were centrifuged at 200 xg for 15 minutes at 21°C. The platelet-rich plasma was incubated in 3 µmol of fura-2-acetoxymethyl ester (fura-2) per litre at 37°C for 30 minutes and centrifuged at 650 x g for 10 minutes at 21°C. The plasma and extracellular fura-2 was removed by aspiration. The platelet pellet was then suspended in a calcium-free HEPES (10 mM) buffer (pH 7,4) and centrifuged at 650 x g. The washed platelets were suspended at a concentration of 1 x 10^8 cells per millilitre in HEPES (10 mM) containing calcium (1,5 mM). This method of preparing platelets by differential centrifugation yields results indistinguishable from those obtained in platelets purified by gel filtration (Standley et al, 1989; Zemel et al, 1990).

Fura-2 method

Intracellular free calcium was measured in platelets using a nondisruptive fluorescent dye technique based on the methods described by Tsien et al (1982) and Rink et al

(1982). Emission measurements were performed using a Perkin-Elmer LS50 spectrofluoreimeter at excitation wavelengths of 340 and 380 nm, an emission wavelength of 495 nm and entry and exit slits at 5 nm.

The intracellular calcium concentration was calculated by taking the ratio of the fluorescent intensities at 340 nm and 380 nm and comparing them to a calibration curve (obtained from known calcium concentrations in solution) by using the following equation (Grynkiewiez et al, 1985).

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{(Fo)}{(F_s)}$$

where:-

- K_d = dissociation constant for Fura-2, taken as 224 nM at 37°C or 135 nM at 25°C.

- R_{max} = 340/380 ratio of the signal obtained after cell lysis by adding 0,2% Triton x-100 to obtain a saturating calcium concentration.

- R_{min} = 340/380 ratio of the signal obtained after the addition of 10 mM EDTA to obtain a calcium concentration of zero.

- F_o and F_s = the fluorescent intensities at 380 nM in zero $[Ca^2]_o$ and saturating $[Ca^{2+}]_o$ respectively.

Three readings were made for each sample, and the mean was taken for analysis.

Statistical analysis

Values are expressed as means ± SD. Pearsons correlation coefficients were determined for correlations between the intracellular free calcium concentration and the intracellular total concentration.

Results

Table 1 presents the calcium concentrations for each subject and the mean calcium concentrations for the 6 subjects. There was a significant positive correlation between the platelet free calcium concentrations and the platelet total calcium concentrations ($r = 0,886$; $p < 0,001$).

Table 1 Platelet calcium concentrations

	Platelet free $[Ca^{2+}]$ (nM)	Platelet total $[Ca^{2+}]$ (μmol/l x 10^8)
Subject Number		
1	1,4	115,3
2	1,6	116,6
3	1,3	115,0
4	1,5	125,0
5	2,0	138,0
6	1,6	126,9
mean $\pm$ SD	1,56 $\pm$ 0,24	122,8 $\pm$ 9,03

Discussion

These results suggest that the platelet total calcium concentration may reflect the free calcium concentration.

Appendix K. The principles and methods for protein measurement (Reider, 1974).

Proteins react with Cu (II) in alkaline solution to form copper-peptide bond-protein complexes. When the Folin-Ciocalteu reagent is added, the copper-protein complexes join with tyrosine and tryptophan residues in the reduction process. Approximately 75% of the colour produced depends on the Cu (II) complex. The absorbance of the coloured complex is measured at a wavelength between 650-750 nm. This method is sensitive to protein concentrations of 10-60 /ug/ml, which is about 100 times more sensitive than previously described methods (biuret method) (Reider, 1974). The Lowry method is used to quantitate tissue proteins and enzyme protein in purified preparations and was the ideal method for cell membrane protein analysis in this study. A modified method based on the Lowry technique was employed (Hartree, 1972).

The Lowry method has two disadvantages which are accounted for in the adapted procedure. The disadvantages are:- i) that the colour yields of different proteins vary considerably and ii) the relationship between colour yield and protein concentration is not linear. By incubating the protein in a concentrated alkaline copper tartrate reagent at temperatures greater than ambient, the colour yield is significantly increased. This modification was described by Hartree (1972) whose adapted technique was used to

measure protein in this study.

Materials and methods for protein determination

(i) Materials

1. Bovine serum albumin. 1 mg/ml bovine serum albumin (BSA) was prepared in 0,85% NaCl (i.e. 1 mg in 1 ml) i.e. 25 mg BSA was added to 0,85 g NaCl in 100 ml of water.
2. Solution A. 0,02 g potassium sodium tartrate and 10 g Na_2CO_3 were dissolved in 50 ml 1 N NaOH and diluted with water to 100 ml.
3. Solution B. 2 g potassium sodium tartrate and 1 g $CuSO_4 \cdot 5H_2O$ were dissolved in 90 ml water and 10 ml 1 N NaOH was added.
4. Solution C. One volume Folin-Ciocalteau reagent was diluted with 15 volumes of water. This solution was prepared daily and the acidity was between 0,15 N and 0,18 N when titrated to pH 10 with 1 N NaOH.

(ii) Methods

Assays were performed in medium-weight test tubes of 13 mm diameter. The tubes were all matched for wall thickness and identical tubes were used for all assays. Standard albumin samples of 10, 20, 40, 60, 80, 100 µl were made up to 1 ml with water. Fifty microlitres of water made up to

1 ml with water was used as the blank. The membrane samples were similarly prepared - 50 µl made up to 1 ml with water. The samples were all treated with 0,9 ml Solution A, vortexed and then placed in a water bath at 50° for 10 minutes. The samples were allowed to cool at room temperature (21-25°C). One hundred microlitres of Solution B was added. The tubes were vortexed and left to stand at room temperature for 10 minutes. Three millilitres of solution C was added rapidly to each sample. The samples were vortexed for a few seconds and again heated at 50°C for 10 minutes. The tubes were then cooled at room temperature. Absorbances were read in 1 cm disposable cuvettes using a Gilford (Stasar III; VI Instruments, Johannesburg, South Africa) spectrophotometer. The wavelength was set at 650 nm. A standard curve of concentration versus absorbance was plotted, and the unknown membrane protein concentrations read off the curve.

Since Triton-X-100 can influence the assay, 50 µl of 0,2% Triton-X-100 was added to all the samples (blanks, standards and membrane suspensions) to correct for increased background absorbance.

Appendix L. Principles and methods for the determination of inorganic phosphate

The method for assay of inorganic phosphate is based on the formation of a complex of phosphate ion with a molybdate compound. The colourless hexavalent molybdenum phosphate complex $(NH_4)_3[PO_4\ (MoO_3)_{12}]$ is reduced to the pentavalent coloured form and then measured spectrophotometrically at wavelengths greater than 400 nm, usually about 700 nm. This colorimetric method was described in 1925 (Fiske and Subbarow, 1925). Reducing agents suitable for producing the coloured pentavalent complex include aminonaphtholsulfonic acid, stannous chloride, methyl-p-aminophenol sulfate, ascorbic acid, ferrous sulphate and N-phenyl-p-phenylene diamine. Ferrous sulphate was used in this study as it has the following advantages over the other reducing agents:-

1) it produces less breakdown of the labile phosphate esters and; 2) it produces a more stable colour.

The method is based on the principle that proteins are precipitated with liberation of P_i from ligands by trichloracetic acid. The protein-free filtrate is mixed with ammonium molybdate in acid solution to form ammonium phosphomolybdate i.e.

$$7H_3PO_4 + 12\ (NH_4)_6\ Mo_7O_{24}.4H_2O \longrightarrow$$
$$7\ (NH_4)_3\ [PO_4\ (MoO_3)_{12}] + 51\ NH_4^+ + 51\ OH^- + 33\ H_2O$$

Ferrous sulphate is added to reduce the hexavalent complex to the pentavalent blue coloured complex. The intensity of the blue colour is measured spectrophotometrically.

The Gilford spectrophotometer, set at 680 nm was used in this study.

L. Measurement of inorganic phosphate

The amount of inorganic phosphate released from the above reactions was measured according to the method described by Taussky and Shorr (1953). The technique used here is a micromethod for the determination of inorganic phosphate in small tissue samples. Previously described methods for P_i measurements have the disadvantages of instability of solutions and interference of substances like creatinine, glycocyamine, creatine, urea, uric acid, glycogen, dextrose, cysteine, cystine, and various acids. In the method employed here, the reduction of phosphomolybdic acid was carried out by ferrous sulfate in weakly acid solution. This adaptation eliminated the effects of interfering substances and improved the sensitivity of the method.

(i) Reagents

1. Ammonium molybdate - 10%. 5 g $(NH_4)_6\ MO_7\ O_{24}.4H_2O$ was added to 40 ml of 10 N sulphuric acid. This was made up to 50 ml with sulphuric acid.

2. Sulphuric acid - 10 N. On ice, 278 ml of sulphuric acid was added to 700 ml of water and then made up to 1000 ml of water.
3. Colour reagent - 10 ml of 10% ammonium molybdate was mixed with 70 ml of water. 5g $FeSO_4.7H_2O$ was added to the diluted ammonium molybdate and made up to 100 ml with water. The colour reagent was stored in a dark bottle at 4°C.
4. Standard phosphate solution. 0,05 g K_2HPO_4 was added to 50 ml of water i.e. 1 mg/ml.

(ii) Methods

Standards of 0, 5, 10, 15, 20, 25 /ug were pipetted into test tubes. The volumes were made up to 250 /ul with water. Two hundred and fifty microlitres of 10% trichloracetic acid was added to each tube. To 0,5 ml of membrane suspension prepared for ATPase assay, and 0,5 ml of standards, 0,5 ml of colour reagent was added. The tubes were vortexed and then allowed to stand at room temperature for 10 minutes. Using a Gilford spectrophotometer (Stasar III; VI Instruments, Johannesburg, South Africa), absorbance of the samples were determined. The instrument was set at a wavelength of 680 nm. A standard curve of absorbance versus concentration was plotted and the unknown sample concentrations were read off the curve.

Appendix M. Enzymatic analysis

The adenosinetriphosphatase (ATPase) enzymes

The ATPases, which are discrete enzyme proteins, are ubiquitous in nature. The membrane-bound form of the enzyme catalyses the terminal transphosphorylating reaction of oxidative phosphorylation, that is, the synthesis of ATP from ADP and inorganic phosphate (P_i) (Penefsky, 1979; Cross, 1977). Other types of ATPases also participate in ion transport. These include the plasma membrane Na^+-K^+-pump and the Ca^{2+}-ATPase of the plasma membrane and the sarcoplasmic reticulum (de Meis and Bianna, 1977; Cantley, 1981).

The formation of P_i and ADP from ATP reflects ATP 'utilisation'. Measurements of ATPase activity are based on the release of P_i from ATP. A divalent metal ion is required for most assays. Cell membrane Na^+-K^+-ATPase requires Mg^{2+} while the sarcoplasmic reticulum requires Ca^{2+} as well (Penefsky, 1974).

Methods of ATPase analysis

A number of methods have been described for enzymatic analysis. The most important are regenerating systems for ATP, measurement of the release of P_i from ATP and a pH-metric procedure.

These methods are based on the underlying premises.

i) $ATP + H_2O \xrightarrow[Mg^{2+}]{ATPase} ADP + Pi$

ii) $ADP + PEP \xrightarrow[K^+]{Pyruvate\ Kinase} ATP + pyruvate$

iii) $Pyruvate + NADH + H^+ \xrightarrow{lactate\ dehydrogenase} lactate + NAD^+$

In this study ATPase activity was measured using the first premise where the amount of P_i released from ATP was determined.

In this study, ATPase activity was measured in cell membranes of erythrocytes and platelets. ATPase analysis was expressed as the amount of P_i released per ATP molecule per mg membrane protein per minute at 37°C. Membrane protein was measured according to the method of Hartree (1972) (Appendix K).

M.a. Assay for Mg^{2+} - ATPase activity in platelet and erythrocyte membranes.

(i) Reagents

1. Incubation buffer containing 120 mM KCl, 5 mM $MgCl_2$, 20 mM N-tris (hydroxymethy-2-aminoethane-sulfonic acid TES - (NaOH) (pH 7-5 at 37°C).
2. EGTA (ethyleneglycol-bis- N,N,N,N^1-tetra acetic acid) - 1 mM.

3. ATP - 1 mM.

4. Trichloracetic acid - 5 g in 100 ml of water.

(ii) Methods

The Mg^{2+}-ATPase activity was based on the method of Niggli et al (1981). In this study, for all ATPases, the spectrophotometer coupled enzyme system was replaced by colorimetric quantitation. ATPase activities are comparable using both methods (Resink et al, 1986). All ATPase assays were performed in duplicate.

The membrane suspensions were suspended in the buffer containing KCl, $MgCl_2$, N-tris, EGTA and ATP. The reaction was initiated by adding 50 µl of membrane suspension to 350 µl of incubation buffer and 100 µl of ATP. The buffer and ATP had been prewarmed at 37°C for 5 minutes. The final incubation volume was 500 µl. The samples were incubated at 37°C for 15 minutes. Studies showed that under these conditions, ATPase activity was linear with time. Less than 5% of the ATP was hydrolysed after 15 minutes incubation at 37°C (See chapter 9.3.5). Reactions were terminated by adding 500 µl of 5% trichloracetic acid. Inorganic phosphate released from ATP was measured by the colorimetric method of Taussky and Shorr (1953).

M.b. Assay for Ca^{2+}-ATPase activity in cell membranes

(i) Reagents

1. Incubation buffer A-120 mM KCl, 5 mM $MgCl_2$, 20 mM N-tris (hydroxymethy p 2-aminoethane-sulfonic acid (TES) - NaOH) (pH 7-5 at 37°C), 1 mM EGTA (ethyleneglycol - bis - N, N, N, N^1 - tetra acetic acid).
2. Incubation buffer B - 120 mM KCl, 5 mM $MgCl_2$, 20mM N-tris, 0,1 mM $CaCl_2$.
3. ATP - 1 mM.
4. Trichloracetic acid - 5 g in 1000 ml.

(ii) Methods

Ca^{2+}-ATPase activity was based on the methods described by Niggli et al (1981) and Resink et al (1985). For each sample, ATPase activity was measured in the presence of EGTA (buffer A) and $CaCl_2$ (buffer B). Fifty microlitres of membrane suspension were added to prewarmed incubation buffer (350 µl) and ATP (100 µl). The buffer contained either EGTA or $CaCl_2$. The samples (final volume 500 µl) were incubated at 37°C for 15 minutes (see chapter 9.3.5). Reactions were terminated by adding 500 µl of trichloracetic acid. Inorganic phosphate released was measured by the method of Tausky and Shorr (1953).

The Ca^2-ATPase activity was determined by subtracting activity measured in the absence of $CaCl_2$ from the activity measured in the presence of $CaCl_2$.

i.e. [ATPase activity measured in buffer B ($+CaCl_2$)]-
[ATPase activity measured in buffer A (+EGTA)]
= Ca^{2+}-ATPase activity (nmol P_i/mg/min).

Determination of the free calcium concentration in the ATPase buffers

Calcium-magnesium buffers for calibration were made up with calcium-magnesium-EGTA formulae calculated on the basis of apparent dissociation constants for calcium-EGTA and magnesium-EGTA at pH 7,5 and 37°C. The apparent dissociation constants (K_d) were calculated from the absolute stability constants and enthalpies tabulated by Bartfai (1979).

The formula to determine free calcium concentration is based on the equation:-

$$Ax^2 + Bx + C = 0$$

where $[Ca^{2+}_{free}]^2 + [Ca^{2+}_{free}]\{[EGTA_{total}]-[Ca^{2+}_{total}]+K_d\} - K_D[Ca^{2+}_{total}] = 0$

The dissociation constants are dependent on pH and temperature. In this study, ATPase assays were performed at pH 7,5 and 37°C.

- K_D for Ca^{2+}-EGTA at pH 7,5 and temperature 37°C = 318 nm
- K_D for Mg^{2+}-EGTA at pH 7,5 and temperature 37°C = 14,4 mM

The above formula was used to determine the free calcium concentration. In the method for Ca^{2+}-ATPase and Mg^{2+}-ATPase, the assay conditions of 0,1 mM $CaCl_2$ and 1 mM EGTA gives a free calcium concentration of less than 1 nm. Since basal activity (not maximal activity) of Ca^{2+}/Mg^{2+}-ATPase was measured, this free calcium concentration was low enough to determine basal conditions.

To determine Ca^{2+}-ATPase activity, the ATPase activity in the absence of Ca^{2+} (without Ca^{2+}, with EGTA) was subtracted from the activity in the presence of Ca^{2+}, without EGTA). This corrects for the activity of the Mg^{2+}-ATPase component.

M.c. Determination of Na^+-K^+-ATPase activity in the cell membranes

(i) Reagents

1. Incubation buffer containing 50 mM N-tris (pH 7,2 at 37°C), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$.
2. ATP - 3 mM.
3. Ouabain - 2 mM

(ii) Methods

Determination of Na^+-K^+-ATPase activity was based on the method described by Ringel et al (1987) and Rahman et al 1986. The technique was similar to that for Mg^{2+}- and Ca^{2}-ATPase activity measurement, except that the assay buffers were different. The incubation buffer and ATP were prewarmed at 37°C for 5 minutes. For each sample, ATPase was measured in the presence and the absence of ouabain. Ouabain inhibits ouabain-sensitive ATPase.

Preparation of samples without ouabain contained:-

- 50 µl membrane suspension
- 350 µl buffer
- 100 µl ATP
- 500 µl total volume

Preparation of samples with ouabain contained:-

- 50 µl membrane suspension
- 100 µl ATP
- 200 µl ouabain
- 150 µl buffer
- 500 µl total volume

The samples were incubated at 37°C for 15 minutes.

Reactions were terminated by adding 500 µl of trichloracetic acid. Inorganic phosphate released from ATP was determined by the method of Taussky and Shorr (1953).

(Appendix L).

The Na^+-K^+-ATPase activity was defined as the difference between the enzymatic activity measured in the presence and the absence of ouabain.

i.e. (ATPase activity measured in buffer - oubain) -
(ATPase activity measured in buffer and oubain)
= Na^+-K^+-ATPase activity (nmol P_i/mg/min)

ATPase activities were expressed as nanomoles of inorganic phosphate released per ATP molecule per milligram membrane protein per minute at 37°C (nmol P_i/mg/min).

Effect of incubation time on ATPase activity

In order to determine optimal conditions for ATPase measurement, samples of erythrocyte and platelet membrane suspensions were incubated in the buffers with ATP for varying periods of time (5 minutes to 30 minutes). ATPase activity was linear with time when incubated for 15 minutes at 37°C. Less than 5% of the ATP was hydrolysed after 15 minutes. Results are presented in Figures i and ii.

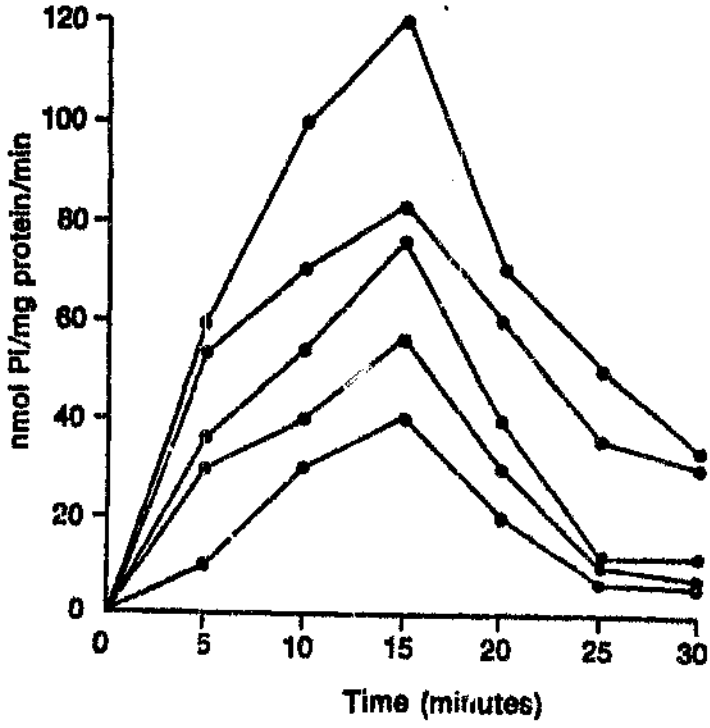

Figure 10:
Effect of incubation time on platelet Mg^{2+}-ATPase activity

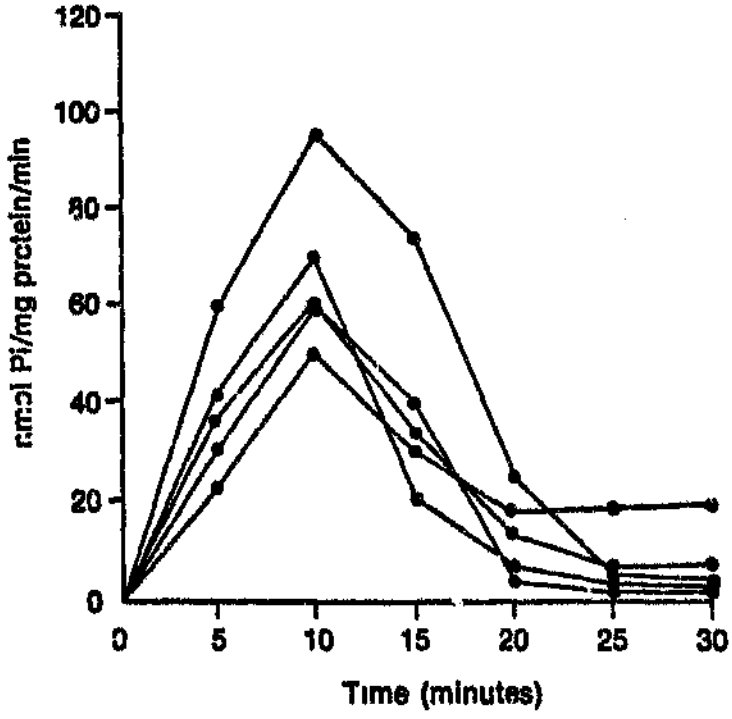

Figure 11:
Effect of incubation time on erythrocyte Mg^{2+}-ATPase activity

Appendix N. Cell membrane calcium binding

Calcium binds to various components of the cell membrane including anionic phospholipids and proteins. The major calcium-binding proteins include calmodulin, calpain, Ca^{2+}-ATPase and calcium binding protein (Carafoli et al, 1989). Calcium is bound at the inner and outer aspect of the plasma membrane. The intracellular site of the cell membrane is exposed to ionic calcium concentrations about 1,0 µmol/l or less and high affinity is involved. The outer aspect of the membrane is exposed to ionic calcium concentrations about three orders of magnitude greater than the inner aspect and both high and low affinity binding are important. A variety of techniques have been described to measure the amount of calcium binding to the outer, and inner cell membranes. These include the use of radioactive isotopes, determination of potassium efflux with varying calcium concentrations (Jones, 1974) and by measuring the amount of calcium released when exposed to various chelators (Zsoter et al, 1977; Postnov et al, 1977).

In this study calcium binding was based on the method described by Postnov et al (1977) where the amount of calcium bound to the outer cell membrane is measured.

Principles of method

This method is based on the determination of the amount of calcium that is released from the outer membrane when exposed to chelating agents and isoosmotic $MgCl_2$. The cells are washed and then incubated in a $MgCl_2$ solution. The amount of calcium removed by the $MgCl_2$ is measured by atomic absorption spectroscopy. The cells undergo repeated washings and are then exposed to a calcium chelating agent (EDTA) after which the concentration of calcium removed is again measured by atomic absorption spectroscopy.

Validation of technique

The technique is validated by the following supporting facts:-

1. Mg^{2+} ions compete with Ca^{2+} for binding sites at the cell membrane and take the place of a certain quantity of Ca^{2+} (Harrison and Long, 1968).
2. The EDTA and the Ca^{2+}-EDTA complex do not penetrate the intracellular space of cells (Reed and Bygrave, 1975).
3. Washing of cells with EDTA removes approximately 90% of membrane bound Ca^{2+} (Harrison et al, 1968).
4. Washing of cells with monovalent cation solutions does not alter the intracellular Ca^{2+} concentration (Takashi and Tatsuzo, 1974).

This method provides an almost complete removal of calcium from the outer cell membrane. A small residue of calcium probably remains bound to the membrane. This membrane bound calcium will not be detected by the described method, as the calcium-determination technique (atomic absorption spectroscopy) is not sensitive enough to determine minute amounts of calcium.

Appendix N. Methods for the determination of calcium binding to the outer cell membranes of erythrocytes and platelets

N.a. Erythrocyte membranes

Reagents

1. Choline chloride solution - 310 mosm
2. $MgCl_2$ - 107 mM
3. EDTA - 2 mM

Methods

The cells were washed with the choline chloride solution at centrifugation speed of 450 x g for 15 minutes at room temperature. The supernatant was aspirated and discarded. The washed cells were then incubated in 4,5 ml of the $MgCl_2$ solution for 15 minutes at 22°C after which the incubated preparation was centrifuged at 500 x g for 5 minutes.

Using a siliconised pipette the supernatant was aspirated and retained for calcium measurement. The packed cell volume of the erythrocyte sediment was determined using a microcentrifuge and microneedles (Hereus Christ Gmbh Microcentrifuge). The erythrocytes were washed twice in the choline chloride solution, aspirating and discarding the supernatant. The washed erythrocytes were incubated in 4,5 ml of the EDTA solution (pH 7,4) for 15 minutes at 22°C. The incubated cells were centrifuged at 500 x g for 5 minutes and the supernatant retained for calcium analysis. The packed cell volume of the erythrocyte sediment was again determined. The amount of calcium in the supernatants following washing of the erythrocytes with $MgCl_2$ and EDTA solutions was measured by atomic absorption spectroscopy (Appendix G describes the method for measuring calcium).

The final unit for expressing the amount of calcium removed from the outer erythrocyte membranes was /umol of calcium per litre of packed cells.

The packed cell volume (PCV) obtained ranged between 85% and 92%. The final calcium concentration was corrected by multiplying it by $\frac{100}{PCV}$

i.e. final calcium concentration (/umol/l packed red cells)

$$= \text{calcium value recorded} \times \frac{100}{PCV}$$

N.b. Determination of calcium binding to the platelet membranes.

Reagents

1. Choline chloride - 310 mosm
2. $MgCl_2$ - 107 mM
3. EDTA - 2 mM

Methods

The principles and methods used to measure platelet membrane calcium binding were based on the methods described by Postnov et al (1977) for erythrocytes. The platelet count was initially adjusted to 1 x 10^8 cells/ml. After incubating the platelet suspension in the $MgCl_2$ and EDTA solutions, the platelet count was again checked to ensure that the count was kept constant at 1 x 10^8 cells/ml.

The unit to express the amount of calcium released from the outer platelet membrane was:

umol Ca^{2+} released/1 x 10^8 cells

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