Chapter 2

Theoretical background and experimental evaluation of the techniques for the determination of intracellular free calcium, transmembrane calcium fluxes and intracellular distribution of calcium

1) Introduction

In the present study aspects of intracellular calcium regulation and signalling were investigated in the neutrophil, both in healthy subjects and in chronic renal failure patients on maintenance haemodialysis treatment (MHT). The various aspects of intracellular calcium regulation and signalling investigated included: basal intracellular free calcium, transmembrane calcium fluxes upon agonist stimulation, and the intracellular distribution of calcium between the various subcellular compartments. The techniques available to determine any one of these aspects are cumbersome and requires experimental evaluation as their success rate vary from cell type to cell type and small variations in the techniques would appear to have a major impact on the results. The following 4 paragraphs will refer to the techniques originally considered. Where after the techniques eventually employed will be discussed in more detail.

1.1) Basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation

The determination of basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation by using the fluorescent calcium indicators were evaluated. The determination of intracellular free calcium using the fluorescent calcium indicators,

introduced into the cytosol of the cell as the membrane permeant acetoxy methyl esters, is said to surpass all the other available techniques regarding avoidance of disruption of cell integrity and sensitivity. It is of the utmost importance that the technique employed should involve minimum disruption of cell integrity, since experimental manoeuvres that cause cell damage can subsequently result in disturbances in basal intracellular free calcium followed by the perturbation of transmembrane calcium fluxes. In this study initial values obtained with purely laboratory experimental work the results appeared to be quite satisfactory. However, results obtained from patients versus age and sex matched individuals forced rethinking and reevaluation of the technique. A large part of this chapter is therefore committed to the theoretical background and evaluation of this technique.

1.2) Total calcium content of the neutrophil

The neutrophil is endowed with powerful mechanisms to regulate basal intracellular free calcium in the cytosol. These mechanisms buffer abnormal elevations in basal intracellular free calcium and can include: sequestration of calcium into intracellular organelles and an increase in the pool of calcium bound to intracellular molecules. A short discussion of these mechanisms is provided in chapter 1. It is therefore possible that basal intracellular free calcium can be maintained within physiological limits despite elevations in the total calcium content of the neutrophil. Eager to include a method for the total calcium content, three techniques available for determining the total intracellular calcium content in a sample were tried and tested. The techniques included conventional flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry. (1 - 3) The techniques were tested on a neutrophil sample size similar to that available from MHT samples. None of these methods

were successful in the determination of the total calcium content in the neutrophil. The major reason was most probably the small cell number available after removal of neutrophils from the isolated neutrophil pool for fluorescence and transmission electron microscopy (TEM) studies. This in combination with background calcium levels rendered the methods not suitable. Two alternatives remained, i.e., to increase the volume of blood collected at one sampling or to do the determination on separate occasions. In view of the anaemia of MHT patients, as well as the daily variability of intracellular calcium, neither alternative was feasible.

1.3) Localisation of intracellular calcium

Transmission electron microscopy was employed to investigate the distribution of intracellular calcium between the various subcellular compartments in the neutrophil. During the preparation of the neutrophils for transmission electron microscopy, in situ precipitation of calcium was performed, resulting in the formation of electron-dense particles containing calcium. The distribution of these electron-dense particles was investigated by means of the transmission electron microscope. The main concern about this technique was the specificity of the precipitation reaction for calcium, since the employed pyroantimonate anion can also precipitate other available cations. Adaptations and evaluations of the technique are presented in the second part of the chapter.

1.4) Spatial and temporal measurements of intracellular free calcium changes using video-rate confocal microscopy

It has become known that cellular calcium regulation and signalling are more complex than envisioned from calcium measurements made in cell populations. Intracellular calcium regulation and signalling involve more dynamic processes than these previous rigid concepts of a set

intracellular free calcium level at rest, and a transient rise and fall in intracellular free calcium upon agonist stimulation. These processes include temporally and spatially organised calcium oscillations and waves. These spatial and temporal patterns become obscured when measured in cell populations since cells, even from the same cell type, display heterogeneity in these intracellular calcium patterns. It is therefore necessary to investigate these spatially and temporally organised calcium oscillations and waves in a single cell. This can be achieved with the use of a video-rate confocal microscope. (4, 5) The use of the confocal microscope at the University of the Witwatersrand was tentatively envisaged and the possibility of using their facilities arranged. However, due to impracticalities such as sample size and cellular change with travel time as well as the fact that the standardisation of such a technique would fall outside the scope of the Masters degree's specifications, investigation of the temporal and spatial calcium patterns were not performed. After the evaluation and consideration of these various techniques the following techniques were considered practical although further standardisations and evaluations were necessary, i.e., determination of intracellular free Ca^{2+} by fluorescence spectrophotometry, determination of the transmembrane Ca^{2+} flux by fluorescence spectrophotometry and the localisation of intracellular calcium by transmission electron microscopy.

2) The measurement of basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation by the employment of the fluorescent calcium indicators

2.1) Theoretical background

2.1.1) Biological fluorescence measurements

Fluorescence is an electromagnetic radiation phenomenon. A molecule, capable of fluorescing is excited by exposure to electromagnetic radiation of the appropriate wavelength - which usually coincides with absorption maxima - resulting in the excitation of electrons to a higher energy level. These excited electrons, with a half-life in the order of 10^{-8} seconds, revert to ground state energy levels giving rise to detectable emitted fluorescence. (6) Spatial and temporal fluorescence measurements have widely been used in studies to investigate cellular structure and function. Since fluorescence markers fluoresce more brightly than cellular constituents, they can be used to 1) indicate the presence of a specific molecule, 2) report the change in the structure of a specific molecule or 3) measure the fluctuations in the concentrations of ions. (4)

1) Spatial studies involve the use of probes, furnished with a fluorophore, which specifically bind the molecule of interest. For example:

- immunofluorescence labeling
- fluorescent analog chemistry
- vital staining of organelles and
- assessment of cell morphology (4)

2) To indicate conformational changes in a protein, probes are directed to specific protein sites. These probes report the change in conformation taking place at that specific protein site. (6)

3) Fluorescing molecules that can directly, specifically and reversibly bind an ion, and undergo a measurable change in fluorescence spectral properties upon this binding, allow the continuous monitoring of the fluctuation in the ion's concentration with various manipulations. (4, 6)

2.1.2) The fluorescent calcium indicators

In order for a molecule to resolve and report intracellular free calcium changes it should be able to directly, specifically and reversibly bind the free calcium ions. Upon this binding the molecule should undergo a measurable change, and this measurable parameter should change at a faster rate than the changes in the free calcium ion concentrations take place. A change in emitted fluorescence upon excitation takes place at an appropriate rate to be able to resolve intracellular free calcium changes. Such fluorescent indicators need to work at suitable wavelengths, fluoresce brightly, resist bleaching, sense their intended stimulus, reject interfering influences, localise in the correct cellular or tissue compartment, and perturb cell function as little as possible. It was with this in mind that R Tsien designed and synthesised the well-known fluorescent calcium indicators. (7)

i) The characteristics of the fluorescent calcium indicators

- **The structure of the fluorescent calcium indicators**

The structure of the fluorescent calcium indicators has been modelled on the widely used calcium-chelator EGTA (ethylene glycol bis (beta-aminoethyl ether)-N, N, N', N',-tetraacetic acid). (4, 6, 7) EGTA encapsulates calcium in a ring-like cage via the 8 oxygen atoms donated from the 4 carboxylate groups. This calcium chelation ring is retained in the structure of the fluorescent calcium indicators, but the two ethylene groups of EGTA are substituted for a moiety containing a stilbene-type backbone with two aromatic rings connected via a two carbon alkyl chain. This combination affords a maximised quantum efficiency and photochemical stability for the fluorophore. A fifth carboxylate group at the end of the chromophore tail is not

involved in chelation but contributes to the indicator's hydrophilic characteristics. (8)

- **The specificity of the fluorescent calcium indicators for calcium**

The fluorescent calcium indicators show a selectivity for calcium over magnesium of 5 to 6 orders of magnitude, therefore the fluorescent calcium indicators are capable of buffering calcium in the physiological range of 10^{-8} – 10^{-6} M in the presence of physiological magnesium concentrations of 10^{-4} – 10^{-3} M. (6) This aversion for magnesium in the presence of calcium ions is the result of the smaller size of the magnesium ion, such that no more than half of the liganding groups can contact the ion simultaneously to form a stable complex. Also monovalent cations do not form detectable specific complexes, probably because their charge is inadequate to organise the binding pocket in the face of the electrostatic repulsion of the negative carboxylates.

- **Negligible interference of hydrogen ion binding with calcium binding at physiological pH**

Calcium binding to EGTA is very sensitive at physiological pH, this is the result of the binding of hydrogen ions to the lone pair electrons of nitrogen at pH 7. Hydrogen binding to these electrons, results in a decrease in the availability of the negative charges at the carboxylate anion site for calcium chelation. The fluorescent calcium indicators are insensitive to hydrogen ion binding at a physiological pH, due to the incorporation of the aromatic rings in the structure of the fluorescent calcium indicators. The incorporation of the aromatic rings results in diversion of the nitrogen lone pair electrons due to the

overlapping p-orbitals and a resulting decrease in the pKa of the amine nitrogens to 6.5 or below. (4, 6, 9)

- **The fluorescence properties of the fluorescent calcium indicators**

The fluorescence properties of the fluorescent calcium indicators are determined by the electronic configuration of the aromatic rings. The electronic configuration of these aromatic rings can be manipulated by the binding of calcium to the four carboxylate groups. The amino substituents connected to the aromatic rings contain pi-electrons that can delocalise across the aromatic rings via overlapping p-orbitals. (10) Calcium binding, involving the 4 carboxylate anions in an octa-coordinate ligand complex, results in the constraint on the conformation of the molecule and the subsequent diversion of the nitrogen lone pair electrons away from the aromatic rings. (4, 9) Thus the nitrogen lone pair electrons no longer delocalises across the aromatic rings and as a result of this change in the electronic configuration of the indicator large fluorescence spectral shifts take place. (4, 6, 9)

ii) The introduction of the fluorescent calcium indicators into the cytosol of the cell

- **Problems associated with the introduction of the fluorescent calcium indicator into the cytosol of the cell**

The fluorescent calcium indicator is a highly charged, hydrophilic molecule that cannot cross the cell membrane, therefore procedures such as various membrane permeabilisation techniques or liposome fusion must be employed to introduce the fluorescent calcium indicator into the cytosol of the cell. These procedures are all problematic. Any disruption of membrane

integrity would lead to an uncontrollable rise in basal intracellular free calcium levels and the loss of soluble cytoplasmic constituents. Introduction of the fluorescent calcium indicators into the cytosol via liposome fusion results in the incorporation of foreign lipid into the cell membrane and the releasing of only trace quantities of the trapped indicator into the cytoplasm. (5, 8, 11)

- **Synthesis of a membrane-permeable form of the fluorescent calcium indicator**

To reduce these problems an uncharged, lipophilic derivative of the fluorescent calcium indicator was synthesised that could easily be introduced into the cytosol of cells by direct crossing of the membrane. This lipophilic derivative is synthesised by the formation of ester bonds to the 5 carboxylate ion groups of the calcium indicator, resulting in the concealment of the groups responsible for the molecule's hydrophilic characteristics. (6, 8, 12) Once inside the cell, esterases hydrolyse these ester bonds randomly, resulting in the formation of membrane impermeable calcium indicator molecules. Theoretically the calcium indicator is now trapped inside the cytosol of the cell. (5, 6, 8, 12) A variety of ester derivatives of the fluorescent calcium indicators have been found to cross the cell membrane, but the acetoxy methyl ester form is the most readily activated by cellular esterases. The simplest esters, methyl or ethyl are not hydrolysed. Anhydrides, imidazolides or activated aryl esters are rejected because the end products would probably include indicator molecules bound to intracellular nucleophiles. Trimethylsilyl esters are too rapidly hydrolysed extracellularly and t-butyldimethylsilyl esters are potently haemolytic. (11)

- **Loading efficiency of different cell types**

The acetoxy methyl (AM) ester form of the fluorescent calcium indicator is the most readily activated in most cell types but loading is never more than 50% efficient. More importantly, the loading efficiency varies between different cell types. This results from the variability in esterase activity in different cell types. In some cell types it is impossible to introduce the fluorescent calcium indicator into the cytosol as the acetoxy methyl ester derivative since these cells lack the esterases necessary to cleave the ester bonds. (6) A trial and error approach is thus necessary to determine a specific cell type's ability to accumulate the acetoxy methyl ester derivative of the indicator.

iii) **The fluorescent calcium indicators as intracellular calcium binding molecules**

When introduced into the cytosol of a cell, the fluorescent calcium indicators bind the available free calcium ions. This binding of the free calcium ions results in the buffering and subsequent lowering of cytosolic free calcium. In response to the lowering of the intracellular free calcium concentration, the pump/leak balance of the cell membrane for calcium is altered and steady state intracellular free calcium concentrations are restored. (5, 13) The restoration of intracellular free calcium concentrations to basal levels, will only result if the amount of fluorescent calcium indicator introduced into the cytosol is not extreme. In any case calcium buffering by the indicators tends not to have much of an effect on basal intracellular free calcium levels or elevated basal intracellular free calcium levels. If restoration to basal levels do not occur, this buffering of the free calcium ions can result in a reduction in the magnitude and

extent of calcium fluxes upon agonist stimulation, and the prolonged reversal of calcium levels to a new steady state. (5)

iv) **The fluorescent calcium indicator-calcium chelation complex**

Upon cleavage of all the ester bonds the highly charged penta-anion form of the indicator are generated. It is the penta-anion form of the indicator that is capable of chelating calcium via the octa-coordinate binding site of the molecule. These formed chelation complexes are dynamic, reversible molecules that follow the rules of chemical equilibria. In reversible equilibrium systems the amount of bound chelate-calcium complex will depend on the total concentration of target ion – Ca^{2+}, and the affinity of the chelator for the ion. The following formula is derived from the chemical equilibrium equation:

$$[\text{Chelate-}Ca^{2+}\text{ complex}] = [Ca^{2+}] \,.\, Kd \,.\, [\text{Chelator}]$$

v) **A change in the fluorescence spectral properties of the indicator caused by the binding of calcium**

In order for a fluorescent indicator to report temporal changes in the concentration of calcium ions, the indicator must undergo significant changes in its fluorescence spectra caused by any change in the concentration of calcium ions. (6)

These spectral changes can be either one of the following:

- a change in fluorescence intensity
- a shift in the excitation wavelength maximum
- or a shift in the emission wavelength maximum

Thus two types of indicators are available, those that do not change wavelength maxima – wavelength stable calcium indicators, and those that change their wavelength maxima upon calcium binding – wavelength shifting calcium indicators.

Wavelength shifting calcium indicators allow for the treatment of fluorescence data as the ratio of fluorescence intensities at two wavelengths, rather than the absolute intensity at a single wavelength. It is this property that makes the application of wavelength shifting calcium indicators less problematic, since fluorescence intensities are dependent on the illumination intensity, emission collection efficiency and indicator concentration. To account for these factors absolute fluorescence intensity values should be corrected, (7) but treating fluorescence data as the ratio of fluorescence intensities at two wavelengths, result in the cancelling of these interfering factors, since these interfering factors have a similar effect on fluorescence intensities at both wavelengths. (4 - 8)

vi) The ratio methodology for measuring intracellular free calcium concentrations using the fluorescent calcium indicators

The ratio methodology for intracellular free calcium measurements using the fluorescent calcium indicators is best explained on the basis of the following equilibrium equation. Fura-PE3 was the fluorescent calcium indicator of choice for the present study for reasons as will be explained in subsequent sections of this chapter. Therefore fura-PE3 is specified in the following equations. Binding of calcium to the indicator molecule can be related to the following equilibrium equation:

$$[Ca^{2+}] + [FURA\text{-}PE3] \Leftrightarrow [CaFURA\text{-}PE3]$$

Following from the above equilibrium equation -

- an increase in $[Ca^{2+}]$ will result in an increase in the [CaFURA-PE3] complex and at the same time a decrease in the free [FURA-PE3] indicator

- a decrease in [Ca^{2+}] will result in a decrease in the [CaFURA-PE3] complex and at the same time an increase in the free [FURA-PE3] indicator

It is clear that an increase in the [CaFURA-PE3] complex is accompanied by a decrease in the free [FURA-PE3] indicator molecules and visa versa. The CaFURA-PE3 complex and the free FURA-PE3 indicator differ in their fluorescence properties. Firstly, both the CaFURA-PE3 complex and the free FURA-PE3 indicator molecules are highly fluorescent, but in all cases the CaFURA-PE3 complex has a quantum efficiency between 1.3 and 2.1 fold higher than the free FURA-PE3 indicator, therefore the CaFURA-PE3 complex is more fluorescent than the free FURA-PE3 indicator. (7) Secondly, the CaFURA-PE3 complex and the free FURA-PE3 indicator differ in their excitation maxima wavelengths. For optimal separation of fluorescence due to the two forms of the indicator, the excitation maxima for the CaFURA-PE3 complex are set at 340nm and the excitation maxima for the free FURA-PE3 indicator are set at 380nm. At every measured time point the fluorescence intensities are obtained at both the wavelengths. At a wavelength of 340nm the fluorescence obtained is proportional to the [CaFURA-PE3] complex and at a wavelength of 380nm the fluorescence obtained is proportional to the free [FURA-PE3] indicator molecules. Following from the explanation above, these fluorescence intensity values obtained at the two different excitation wavelengths change in opposite directions. An increase in the [CaFURA-PE3] complex results in an increase of the fluorescence emission intensity obtained at the 340nm wavelength. At the same time a decrease in the free [FURA-PE3] molecules occurs and thus results in a decrease of the fluorescence emission

intensity obtained at the 380nm wavelength. These two values are represented as a ratio. The fluorescence emission value obtained at the 340nm wavelength was arbitrarily chosen as the numerator and the fluorescence emission value obtained at the 380nm wavelength as the denominator: 340nm/380nm = ratio. Such that the ratio value changes in the same direction as the intracellular free calcium concentration. (7) Thus an increase in $[Ca^{2+}]$ resulting in an increase in the [CaFURA-PE3] complex and a decrease in the free [FURA-PE3] indicator leads to an increase in the ratio value and visa versa. (6)

vii) Calculation of intracellular free calcium concentrations

Under conditions of constant illumination and in a fixed chemical and physical environment, the intensity of fluorescence from a molecular species is proportional to its concentration. Thus, fluorescence intensity can be used as a quantitative tool. (6) As explained above, at any ratio value a mixture of the CaFURA-PE3 complex and the free FURA-PE3 indicator exist. The ratio value is obtained by measuring the emitted fluorescence at the two excitation wavelengths: 340nm and 380nm. The total fluorescence intensities at these two wavelengths will be given by the following:

$$F_{340nm} = S_{FURA\text{-}PE3(340nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(340nm)} \cdot C_{CaFURA\text{-}PE3}$$

$$F_{380nm} = S_{FURA\text{-}PE3(380nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(380nm)} \cdot C_{CaFURA\text{-}PE3}$$

Where

F = fluorescence intensities

S = proportionality coefficients (the product of excitation intensity, ln10, extinction coefficient, path length, quantum efficiency, and the instrumental efficiency of collecting emitted photons)

C = concentration

However, $C_{FURA\text{-}PE3}$ and $C_{CaFURA\text{-}PE3}$ are related to $[Ca^{2+}]$ by the above mentioned equation for 1:1 complexation:

$C_{CaFURA\text{-}PE3} = C_{FURA\text{-}PE3} [Ca^{2+}] / Kd$

Where

Kd = dissociation constant

The fluorescence ratio R is obtained by calculating:

$$F_{340nm} / F_{380nm} = S_{FURA\text{-}PE3(340nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(340nm)} \cdot C_{CaFURA\text{-}PE3} / S_{FURA\text{-}PE3(380nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(380nm)} \cdot C_{CaFURA\text{-}PE3}$$

Thus: $$Ratio = (S_{FURA\text{-}PE3(340nm)} + S_{CaFURA\text{-}PE3(340nm)} \cdot [Ca^{2+}] / Kd) / (S_{FURA\text{-}PE3(380nm)} + S_{CaFURA\text{-}PE3(380nm)} \cdot [Ca^{2+}] / Kd)$$

Solving for $[Ca^{2+}]$ yields the following calibration equation:

$$[Ca^{2+}] = Kd\ [R - (S_{FURA\text{-}PE3(340nm)} / S_{FURA\text{-}PE3(380nm)})] / [(S_{CaFURA\text{-}PE3(340nm)} / S_{CaFURA\text{-}PE3(380nm)})\text{-}R]\ [S_{FURA\text{-}PE3(380nm)} / S_{CaFURA\text{-}PE3(380nm)}]$$

In practice the S factors are obtained from the fluorescence intensities of calibration solutions containing known concentrations of the free and the calcium-saturated indicator.

Note that $(S_{FURA\text{-}PE3(340nm)} / S_{FURA\text{-}PE3(380nm)})$ is simply the limiting value that R can have at zero $[Ca^{2+}]$ and so may be considered Rmin, while $(S_{CaFURA\text{-}PE3(340nm)} / S_{CaFURA\text{-}PE3(380nm)})$ is the analogous limiting value that R can have at saturating $[Ca^{2+}]$ giving Rmax.

Thus the following Grynkiewicz equation is obtained:

$$[Ca^{2+}] = Kd\ .\ (R - Rmin / Rmax - R)\ (S_{FURA\text{-}PE3(380nm)} / S_{CaFURA\text{-}PE3(380nm)})$$

Assumptions:

1) The indicator forms a simple 1:1 complex with calcium
2) The indicator behaves in cells as it does in calibration media
3) The indicator is sufficiently dilute for fluorescence intensity to be linearly proportional to the concentrations of the fluorescent species (6, 7, 12)

- **Calibration of the obtained fluorescence values**

Ultimately, the obtained fluorescence values should be converted to intracellular free calcium concentrations. As explained above, this can be achieved by solving the Grynkiewicz equation. To be able to solve for intracellular free calcium concentrations, an end of the experiment calibration should be performed. Performing the calibration procedure, results in determination of fluorescence ratio values of the indicator in solutions with known calcium concentrations. In this case: at maximum calcium concentration – 1 mM, and at minimum calcium concentration – after EGTA addition. Both these values are accounted for in the Grynkiewicz equation.

- **Autofluorescence**

Autofluorescence is not accounted for when using the ratio method, therefore autofluorescence should be subtracted from the fluorescence intensities obtained at both the wavelengths before calculating the ratios. The generated cellular autofluorescence can mostly be attributed to the pyridine nucleotides. (6 - 8)

viii) Processes interfering with the accurate measurement of intracellular free calcium concentrations using the fluorescent calcium indicators

a) Incomplete hydrolysis of the acetoxy methyl ester bonds

Determination of intracellular free calcium concentrations by the employment of the fluorescent calcium indicators assumes the complete conversion of the AM ester precursor of the indicator to the calcium sensitive fluorescent indicator. According to the Grynkiewicz equation it is from the obtained fluorescence intensities that intracellular free calcium concentrations are determined, therefore any contribution to these fluorescence intensity values from molecular species not directly involved in calcium binding would lead to the inaccurate determination of intracellular free calcium concentrations. Such calcium-insensitive, fluorescent molecular species include unhydrolysed AM ester indicator molecules and/or incompletely hydrolysed indicator molecules. In different cell types the contribution of the different fluorescent molecular species to the total fluorescence has been described. The completely hydrolysed calcium indicator molecule is said to contribute only 11.8% to the total fluorescence in neuroblastoma cells, 7.6% in human pulmonary artery endothelial (HPAE) cells and 66.5% in hepatocytes. Of the total fluorescence the more than 30 potential incompletely hydrolysed molecules resulting from the random cleavage of the five ester bonds appear to contribute 5.1% in neuroblastoma cells, 5.5% in HPAE cells, and 23.7% in hepatocytes. (8) In a separate study involving neutrophils, it was shown that intermediates of the de-esterification process also contribute to the total fluorescence. Nevertheless, washing of the cells in a medium containing BSA, after the completion of the loading process, results in the removal of unhydrolysed and incompletely hydrolysed acetoxy methyl indicator molecules. (6, 14)

b) Unwanted binding of the fluorescent calcium indicators to cellular constituents

The acetoxy methyl ester derivatives of the calcium indicator are highly lipophilic and therefore can easily bind to cell membranes and escape enzymatic hydrolysis. Even the fully de-esterified indicator or injected free acid form can interact with cellular constituents, be it cell membranes or cellular proteins, resulting in the alteration of their fluorescent properties. As a deterrent of hydrophobic binding to membranes of the indicators via the long chromophore tail, the terminal carboxylate was incorporated into the structure of the fluorescent calcium indicator. (7)

c) Differences in the uptake and cellular processing of the indicators in various cell types

In the following table a comparison is presented of possible differences in the uptake and processing in three different cell types. These factors which include differences in the rate of hydrolysis of the acetoxy methyl ester bonds and differences in the rate of extrusion of the indicator should be taken into consideration when the fluorescent calcium indicators are employed in the measurement of intracellular free calcium concentrations. (8)

	Murine NIE-115 neuroblastoma cells	Human pulmonary artery endothelial cells	Rat hepatocytes
Indicator precursor concentration	10 micromolar	10 micromolar	10 micromolar
Rate of indicator formation	9.7 pmol/min/10^6 cells	2.6 nmol/min/10^6 cells	77.9 pmol/min/ 10^6 cells
Concentration of the indicator after 60 minutes	137 micromolar	141 micromolar	Peak concentration after 15 min 82 microM
[Indicator] after washing with medium containing 2% BSA	40 micromolar	Almost completely lost	
[Indicator precursor] after wasing with medium containg 2% BSA	90 micromolar	58 micromolar	
Half-time loss after washing	34 minutes	74 minutes	

d) Sequestration of the indicator into intracellular organelles

Fluorescent calcium indicator molecules sequestered in intracellular organelles are insensitive to the changes in the free calcium concentration in the cytosol. (12) More importantly these sequestered indicator molecules report the higher calcium concentration present in intracellular organelles. Therefore a prerequisite for employing the fluorescent calcium indicators as probes of cytosolic free calcium concentrations, is the homogenous distribution of the indicator throughout the cytosol without any sequestration of the indicator into intracellular

organelles. (15) Independent studies involving different cell types indicated three possible mechanisms resulting in the sequestration of the fluorescent calcium indicators into intracellular organelles:

- **Crossing of intracellular organelle membranes by the lipophilic acetoxy methyl ester derivative**

Not all cell types contain sufficient cytosolic esterase activity to rapidly cleave the acetoxy methyl ester bonds and produce the membrane impermeable penta-anion form of the indicator. This can result in the movement of the acetoxy methyl ester derivative across the membranes of intracellular organelles. Once inside the intracellular organelle the acetoxy methyl ester bonds can be hydrolised, since many intracellular organelles such as the secretory granules contain esterase activity. The hydrolysis of the ester bonds results in the subsequent entrapment of the membrane impermeable indicator in the organelle. (12, 16)

- **Endocytosis as the means of sequestering the fluorescent calcium indicator into intracellular organelles**

Human skin fibroblasts (HSF) and 3T3 fibroblasts loaded with the membrane permeable acetoxy methyl ester (AME) derivative displayed fluorescent spots concentrated in the perinuclear region occupied by the membrane bound acidic vesicles, lysosomes. It is suggested that the means of indicator accumulation is by endocytosis followed by the subsequent hydrolysis of the acetoxy methyl ester derivative and entrapment of the indicator, since these cells – HSF and 3T3 fibroblasts – with high endocytic activity show greater accumulation of fluorescent spots in the perinuclear region than cells with less endocytic activity. (15)

- **Sequestration of the fluorescent calcium indicator into intracellular organelles by an anion transporter**

There are indications that fluorescent calcium indicator sequestration in mouse peritoneal macrophages, J774 cells, PC12 cells, and N2A cells are mediated by an organic anion transport system, since this sequestration of the fluorescent calcium indicators can be blocked by the anion transport inhibitors, probenecid and sulfinpyrazone. In a study involving the J774 macrophage cell line, the indicator was introduced into the cells either as the acetoxy methyl ester or penta-anion forms of the indicator. In both cases, sequestration of the indicator into membrane-bound cytoplasmic vacuoles were detected 90 minutes after the onset of the loading procedures, and in both these cases sequestration could be blocked by the organic anion transport inhibitor, probenecid. (17) The concentrations of these agents necessary to effect near-maximal inhibition of the unwanted transport of the indicator in J774 cells were 5mM probenecid and 2.5 mM sulfinpyrazone. Incubation of macrophages in medium containing probenecid for as long as 3h apparently has no untoward effects on cell viability, although resting intracellular free calcium could be about 20% higher than in macrophages incubated without probenecid. Importantly, the transient increase in calcium induced by agonists such as platelet activating factor or by phagocytosis of IgG-coated erythrocytes is not affected. Other cells, i.e., PC12 rat pheochromocytoma cells and N2A mouse neuroblastoma cells incubated in probenecid, indicated a reduced rise in intracellular free calcium of 50% triggered by depolarisation of the plasma membrane and by the neuropeptide bradykinin. (17, 18)

e) Leakage of the penta-anion form of the fluorescent calcium indicator into the extracellular medium

The penta-anion form of the calcium indicators can actively be extruded from cells by an anion transporter. In the spectrofluorometer cuvette, leakage of the penta-anion form of the calcium indicator out of the cell into the extracellular medium would result in binding of the calcium indicator with extracellular calcium ions and the measurement of inaccurate cytosolic calcium concentrations. This process can be inhibited by the anion transport inhibitors, probenecid or sulfinpyrazone or by the lowering of the temperature from 37°C to 30-34°C. Extrusion of the calcium indicator can also be circumvented by the attachment of the indicator to a macromolecule such as dextran. (4, 12, 15, 17)

f) Photobleaching of the fluorescent calcium indicators

A decline in fluorescence intensities due to photobleaching is typical of fluorescent molecules and the fluorescent calcium indicators are not immune to this photobleaching effect. It was shown that exposure of the fluorescent calcium indicators to the excitation light, whether in cells or in calibration solutions, resulted in photobleaching. It was initially assumed that bleaching was functionally equivalent to decreasing the concentration of indicator, and that the ratio value would not be affected since the fluorescence intensities comprising the ratio are influenced to the same extent. (19) This assumption is incorrect, since in certain circumstances, photobleaching results in the formation of a calcium indicator intermediate that is still fluorescent but not sensitive to calcium over the same range as the completely hydrolysed fluorescent calcium indicator. The formation of such fluorescent calcium indicator intermediates

with altered calcium binding affinities violates the assumption on which the ratio method of calibration is based, that is, that the only fluorescent species present are the calcium-bound and the free anion forms.

g) Different fluorescence properties of the calcium indicator in the cytosolic milieu as compared to standard calibration solutions

There are indications of spectral differences between the indicator present in the cytosol and the indicator present in standard calcium concentration solutions. This may be the result of the actual binding of the indicator to cellular constituents or effects of the alterations in ionic strength, hydrogen ion concentration, viscosity and lipid and protein buffering of calcium intracellularly, therefore the in situ Kd may vary greatly from the in vitro Kd. If this is the case, the Kd of the fluorescent calcium indicator should be determined in situ. To accomplish this the intracellular free calcium concentration should be clamped to known values and the fluorescence ratio values measured subsequently. In short, the procedure involves the attainment of extracellular medium calcium, set to a known calcium concentration via the right combination of EGTA/calcium solutions, which subsequently must be equilibrated with the intracellular calcium concentration. The strategy for equilibration involves the utilisation of a divalent cation ionophore to elevate inherent cellular calcium leakage to such an extent that calcium pump activity is overcome. Two ionophores, ionomycin and 4-bromo-A23187, display high selectivity for calcium over other divalent cations. Ideally, after equilibration, extracellular and intracellular calcium concentrations are identical. (6, 7, 20 - 23)

ix) Available fluorescent calcium indicators / advantages and disadvantages

Quin-2

Advantages: 1) Hydrolysis of quin-2/AM seems easier, reaches higher cytosolic concentrations of the indicator and is less often complicated by compartmentalisation into membranous compartments than is observed with acetoxy methyl esters of higher molecular weight and lesser H_2O solubility

2) Low Kd for calcium – well suited for resolving resting calcium concentrations

Disadvantages: 1) Does not show a usable wavelength shift, therefore calibration of fluorescence intensities calls for the standardisation of absolute fluorescence intensities which is directly dependent on indicator concentration, specimen thickness, and instrumental factors (24)

2) Poor discrimination of calcium over magnesium, Kd for magnesium: 1mM

3) Low Kd for calcium, limiting the maximum measurable calcium concentration to about 1 μmolar, since the indicator approaches saturation and looses resolution

4) Severe photobleaching

5) Interference by heavy metal ions and pH

6) Responds to other divalent ions besides calcium

7) Relatively low quantum yield for the calcium-quin-2 complex, therefore require cellular concentrations of the indicator in the millimolar range and thus intracellular calcium buffering can be

substantial, resulting in the buffering of intracellular calcium transients

8) Unsuitable for single cell studies (13)

Fluo-3

Advantages: 1) Excitation at visible wavelengths rather than near UV wavelengths, this permits the use of these indicators in cell types that routinely exhibit significant levels of background fluorescence

2) Very large enhancement in fluorescence intensity about 40-fold upon binding calcium

3) Significantly weaker calcium affinity, Kd 400nM, permitting measurement of intracellular free calcium concentrations of up to 5-10 μmolar

Disadvantages: 1) Negligible wavelength shifts in either excitation or emission spectra, so that fluo-3 like quin-2 is limited to intensity changes without wavelength pairs to ratio

2) Difficult calibration: treatment of the cells with ionophore, heavy metals, and/or detergent at the end of every experiment (4)

Fura-2

The most widely used of the fluorescent calcium indicators and is the indicator of choice for imaging studies

Advantages: 1) 30-fold brighter fluorescence, thus smaller concentrations of the indicator is necessary to be introduced into the cells, which results in a smaller calcium-buffering effect (4, 6 - 8, 17), fura-2 – micromolar, quin-2 – millimolar

2) Lower concentration of indicator, as low as 1μM – less acidity and toxicity due to either indicator itself or acetic acid and formaldehyde by-products
3) Large spectral wavelength shifts upon calcium binding (7)
4) Lower affinities for calcium, can measure intracellular free calcium levels up to several micromolar (6)
5) Improved selectivity for calcium over magnesium, manganese, zinc and iron (6, 7, 17)
6) One to one stoichiometry upon formation of the complex with calcium (5)
7) Negligible binding to membranes (13)
8) Very much more resistant to photodestruction than quin-2
9) Extra heterocyclic bridge to reinforce the ethylenic bond of the stilbene and to reduce hydrophobicity
Disadvantages: 1) Propensity to enter subcellular organelles such as the sarcoplasmic reticulum, nucleus and secretory vesicles
2) Possibility that some of the fluorescent product or starting material remains in the cellular membranes (25)

Indo-1

Advantages: 1) Has a rigidised stilbene fluorophore with reduced hydrophobic characteristics, like fura-2, due to the extra heterocyclic bridge to reinforce the ethylenic bond of the stilbene

2) Has the unique property that its emission, not just its excitation spectrum shifts to shorter wavelengths when the molecule binds calcium

3) Less compartmentalisation than with fura-2

Disadvantages: 1) The blue and violet wavelengths of indo-1 emission overlap cellular autofluorescence from pyridine nucleotides more severely than the green of fura-2

2) Indo-1 bleaches several fold faster than fura-2 (4)

2.2) Experimental evaluation of the technique used in the present study

It was deemed necessary to evaluate various aspects of the technique employed because:

1. initial studies indicated unreliable values for both basal intracellular free calcium and the magnitude of the transmembrane calcium flux in both the healthy subjects group and the patients group
2. there are many processes interfering with the accurate measurement of intracellular free calcium as discussed in the previous section
3. there are studies involving MHT patients, which indicate inconsistencies in the values published results for basal intracellular free calcium in the neutrophil.

2.2.1) Materials and methods

a) Isolation of the neutrophils

The intracellular free calcium determinations were performed in the neutrophil. The neutrophils were isolated from ACD-anticoagulated (acid citrate dextrose) whole blood according to a modification of the method of Böyum. (26, 27) In short, ACD-anticoagulated whole blood was loaded onto a layer of hypaque-ficoll. The various blood cells were

separated into different layers according to densities. The polymorphonuclear leucocytes formed a layer on top of the red cell mass in the bottom of the tube. The contaminating red cells were lysed with an ammonium chloride solution. The polymorphonuclear leucocyte layer contained the neutrophils, eosinophils and basophils. Since eosinophils contribute only 0.5-6% and basophils only 0-1% of the total amount of circulating white blood cells (28) no further separation of the neutrophils were performed from the other polymorphonuclear leucocytes. Finally the neutrophils were washed and suspended in Hanks balanced salt solution.

b) Loading of the fluorescent calcium indicators

The suspension of neutrophils was loaded with the calcium indicators. In short the neutrophil suspension was incubated with the acetoxy methyl ester form of the indicators. During this incubation period the acetoxy methyl ester form of the indicator traverses the cell membrane where after the acetoxy methyl ester bonds are hydrolysed. Upon the hydrolysis of these bonds trapped penta-anion indicator molecules are generated. The neutrophil suspension was washed and suspended in Hanks balanced salt solution.

c) Fluorescence intensity determinations

The fluorescence intensity determinations were performed on the Perkin Elmer LS-50B Luminescence spectrometer containing a Xenon lamp excitation source.

2.2.2) Initial results according to the method

In the initial study intracellular free calcium and transmembrane calcium fluxes were performed in the neutrophils of the MHT patient versus race, sex and age matched healthy controls. Results: intracellular free calcium and transmembrane calcium fluxes in nM.

Combination	Patient		Race, age and sex matched control	
	$[Ca^{2+}]_i$	Ca^{2+} flux	$[Ca^{2+}]_i$	Ca^{2+} flux
1	89 nmolar	406 nmolar	49 nmolar	461 nmolar
2	62 nmolar	408 nmolar	87 nmolar	246 nmolar
3	60 nmolar	322 nmolar	86 nmolar	310 nmolar
4	90 nmolar	437 nmolar	91 nmolar	490 nmolar
5	110 nmolar	578 nmolar	89 nmolar	379 nmolar
6	86 nmolar	435 nmolar	86 nmolar	272 nmolar
7	154 nmolar	403 nmolar	169 nmolar	466 nmolar
8	56 nmolar	300 nmolar	89 nmolar	379 nmolar

According to these results, no consistencies were indicated for intracellular free calcium values in the control group. In combination 7 both the patient's and control subject's intracellular free calcium were unexpectedly high. Since these two determinations were performed on the same day this indicated a possible experimental flaw.

2.2.3) In search of the most suitable fluorescent calcium indicator for intracellular free calcium measurements in the neutrophil

In the present study intracellular free calcium and transmembrane calcium fluxes were determined in the neutrophil in suspension. There are many fluorescent calcium indicators available for the determination of intracellular free calcium of cells in suspension. The most common of these fluorescent calcium indicators for cuvette-based measurements is fura-2, although the use of fura-2 can be problematic in some cell types. The major problem associated with the employment of fura-2 for the measurement of intracellular free calcium is fura-2's propensity to leak out of the cytosol into the extracellular medium. In this study fura-2 was initially used according to many published intracellular free calcium studies where fura-2 was employed. A new fluorescent calcium indicator became available, namely fura-PE3 which is claimed to be less

problematic than fura-2 because of fura-PE3's resistance to leakage. Both these indicators were evaluated for their ability to measure intracellular free calcium in the neutrophil.

2.2.4) The following evaluations were performed

i) Evaluation of loading procedures

With BSA

Without BSA

ii) Evaluation of loading procedures

With agitation

Without agitation

iii) Determination of acetoxy methyl ester derivative concentrations in the loading medium in order to attain sufficient quantities of the indicator in the cytosol for calcium measurements

Fura-2

Fura-PE3

iv) Comparison of determined intracellular free calcium concentrations in neutrophil populations with different quantities of the fluorescent calcium indicator in the cytosol

v) Determination of the extent of leakage via the nickel quench technique for the indicators fura-2 and fura-PE3

vi) Determination of the extent of the inaccurately determined intracellular free calcium concentration as a result of leakage of the indicator into the extracellular medium

vii) Characterisation of the leakage process

viii) Investigation of the possible interference from heavy metals in the cytosol with the determination of intracellular free calcium

ix) Evaluation of the calibration procedures

x) Evaluation of the reproducibility of the technique for the determination of intracellular free calcium

xi) Evaluation of the sensitivity of the technique for the determination of a change in intracellular free calcium

2.2.5) Experimental evaluations

i) Evaluation of the loading procedures – with and without BSA

Bovine serum albumin (BSA) acts as a dispersing agent and assures the availability of the indicators for crossing of the membrane. An example of fura-2 loading with and without BSA is presented.

- Loading medium without BSA - Refer to figure 1, red and blue graphs
- Loading medium containing 0.25% BSA – Refer to figure 1, purple and green graphs

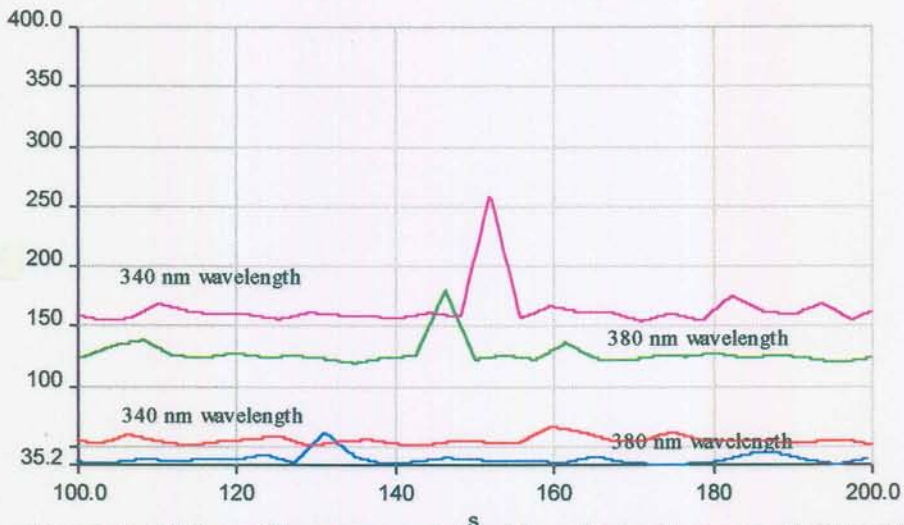

Figure 1. Different fluorescence intensities obtained as a result of loading with and without BSA

The fluorescence intensities obtained after loading in a medium without BSA were found to be equal to background fluorescence intensities resulting from cellular constituents which indicated no accumulation of the indicator in the cytosol, refer to figure 1; red and blue graphs. An increase in the fluorescence intensities well above background fluorescence was obtained when loaded in a medium containing 0.25% BSA, refer to figure 1; purple and green graphs.

ii) Evaluation of the loading procedures - with and without agitation

To facilitate the ester derivative of the indicators in crossing of the cell membrane the neutrophil suspension was continuously agitated, a shaking water bath was employed. An example of fura-2 loading with and without agitation is presented.

- Neutrophil suspension continuously agitated during the loading period – Refer to figure 2, purple and green graphs
- Neutrophil suspension kept still for the duration of the loading period - Refer to figure 2, red and blue graphs

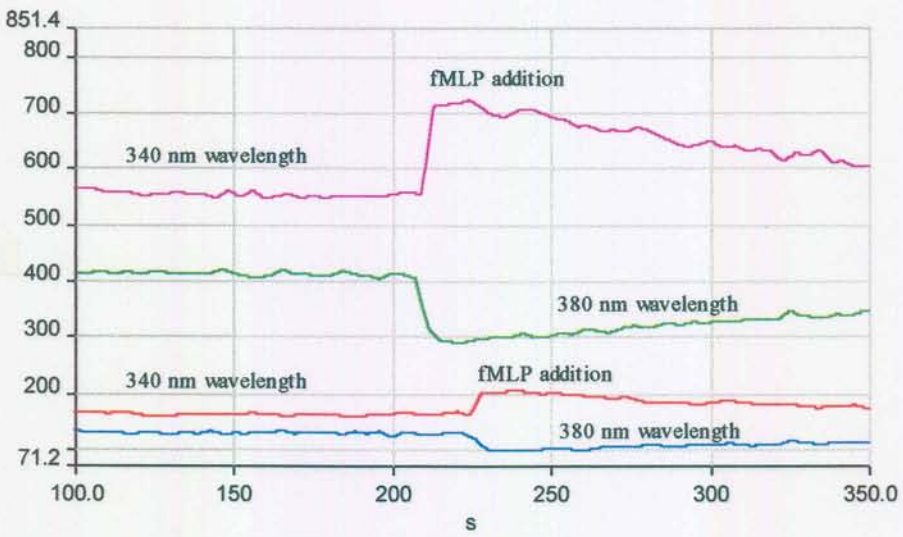

Figure 2. Different fluorescence intensities obtained as a result of continuous agitation or no agitation during the loading period

The fluorescence intensities obtained were higher when the neutrophil suspension was continuously agitated during the loading period, refer to figure 2; green and purple graphs. The red and blue graphs represent the fluorescence intensities obtained when the indicator was loaded without agitation.

iii) The concentration of the acetoxy methyl ester form of the indicator in the loading medium and the incubation time and temperature for loading of fura-2/AM and fura-PE3/AM

The fluorescent calcium indicators were introduced into the cytosol of the neutrophil as the membrane permeant acetoxy methyl ester derivatives of the indicators. Different cell types vary in their ability to accumulate the fluorescent calcium indicators due to different cytosolic esterase activities, therefore the loading protocols were determined on a trial and error basis. The following protocols were sufficient for loading of the calcium indicators fura-2 and fura-PE3.

Fura-2

- Concentration of fura-2/AM in the loading medium 5μM and % DMSO 0.05 %
- Incubation: 37°C for 10 minutes in a shaking water bath and for a further 20 minutes at room temperature
- Refer to figure 3

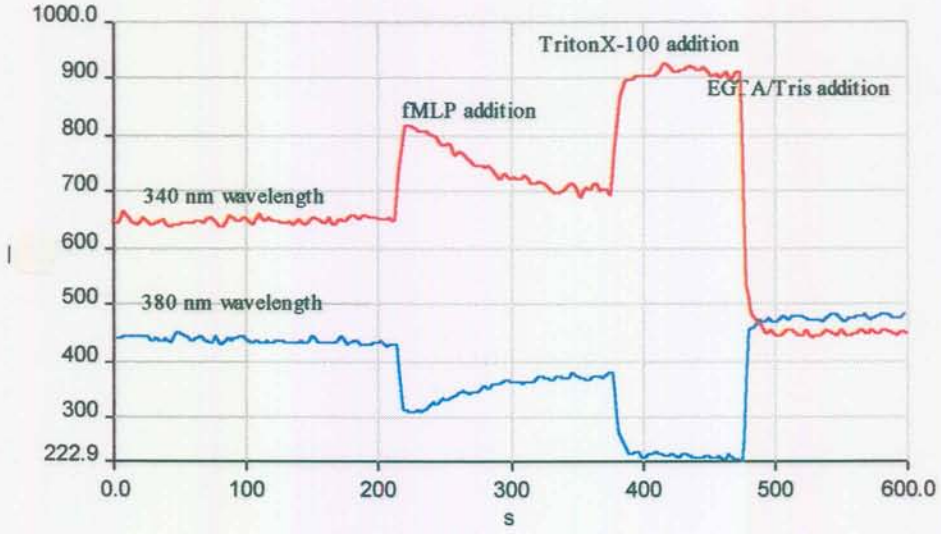

Figure 3. Determination of intracellular free calcium by employing fura-2

The lowest fluorescence intensity obtained from either excitation at the 340 nm or the 380 nm wavelength was well above 100 to be able to subtract background fluorescence. Refer to figure 3.

The concentration of the loaded neutrophils in the cuvette was chosen such that the fluorescence intensity increase upon Triton X-100 addition did not fall outside the limits for instrument detection. Refer to figure 4. Upon Triton X-100 addition the fluorescence intensities obtained for the 340 nm wavelength was outside the range for instrument detection.

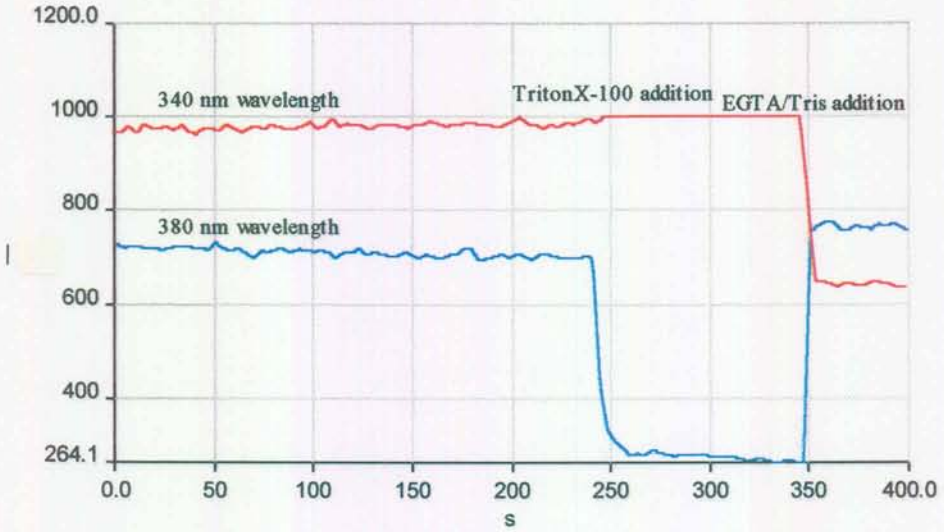

Figure 4. Too high fluorescence intensities obtained for instrument detection

According to the above results the working concentration of neutrophils in the cuvette was determined: 2 to 9 x 10^5 neutrophils/ml for fura-2.

Fura-PE3

- Concentration of fura-PE3/AM in the loading medium 2μM and the % DMSO 0.025%
- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Refer to figure 5

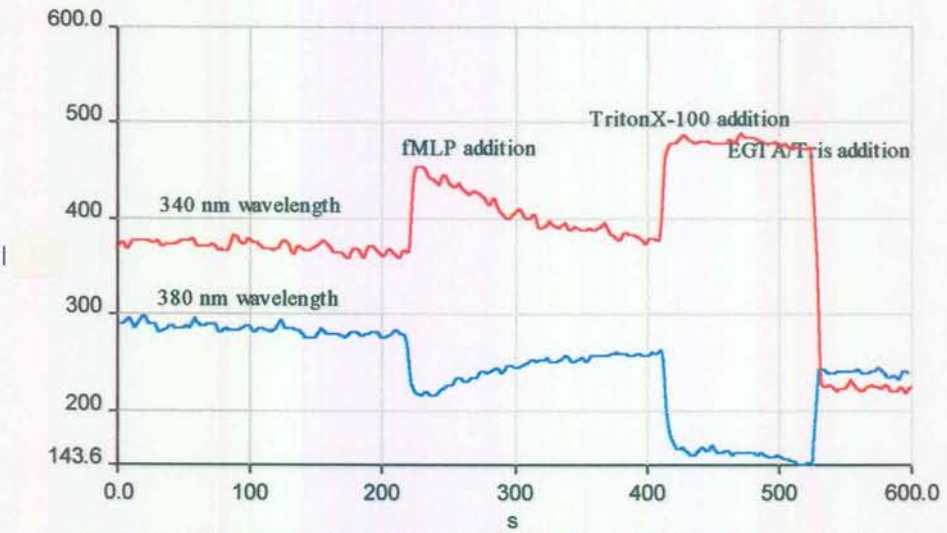

Figure 5. Determination of intracellular free calcium by employing fura-PE3

As for fura-2 evaluations were performed (not shown) in order to determine the working concentration of neutrophils in the cuvette: $9x10^5$ to $1x10^6$ neutrophils/ml for fura-PE3. This concentration range of neutrophils ensured that the maximum obtained fluorescence intensities did not fall outside instrument detection limits and that the minimum fluorescence intensities obtained were high enough to be able to subtract background fluorescence.

iv) Comparison of determined intracellular free calcium concentrations in neutrophil populations with different quantities of the fluorescent calcium indicator in the cytosol

- **The obtainment of different cytosolic indicator quantities**

1) Concentration of fura-PE3/AM in the loading medium 2μM
- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Concentration of neutrophils in the cuvette $9x10^5$/ml
- Refer to figure 6, red and blue graphs

2) Concentration of fura-PE3/AM in the loading medium 6μM
- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Concentration of neutrophils in the cuvette $9x10^5$/ml
- Refer to figure 6, purple and green graphs

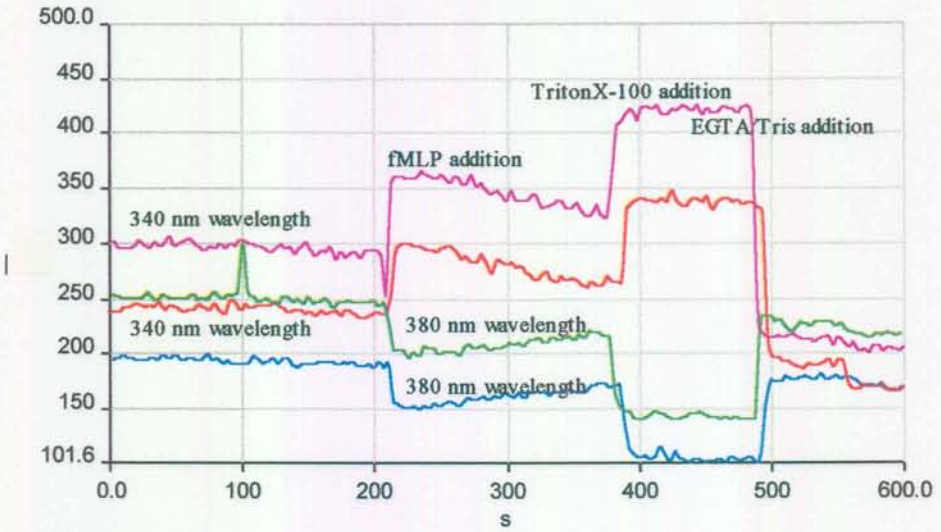

Figure 6. Different fluorescence calcium indicator quantities obtained as a result of different acetoxy methyl ester derivative concentrations in the loading medium

Different fluorescence intensities were obtained for these two protocols indicating different quantities of the indicator in the cytosol. Loading with the higher concentration of fura-PE3/AM resulted in the obtainment of higher quantities of the indicator in the cytosol (refer to figure 6; purple and green graphs) and loading with the lower concentration of fura-PE3/AM in lower quantities of the indicator in the cytosol (refer to figure 6; red and blue graphs).

- **The obtainment of similar intracellular free calcium concentrations**

Calibration of the previously obtained fluorescence intensities followed by the calculation of intracellular free calcium concentrations gave similar results. Refer to figure 7.

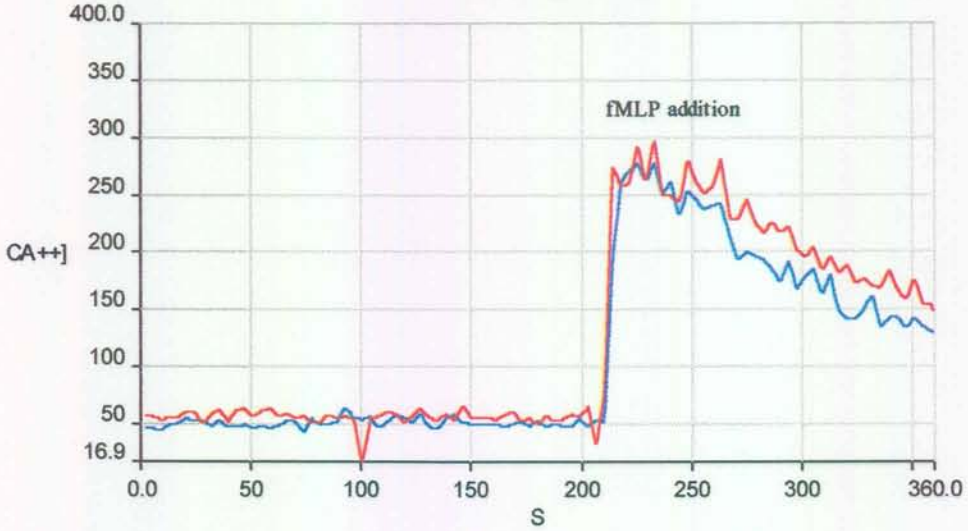

Figure 7. Similar intracellular free calcium concentrations obtained

The red graph (figure 7) represents the intracellular free Ca^{2+} concentration obtained from the purple and green graphs (figure 6). The blue graph (figure 7) represents the intracellular free Ca^{2+} concentration obtained from the red and blue graphs (figure 6). The red and blue graphs (figure 7) are identical. Therefore, different indicator quantities result in the determination of identical intracellular free Ca^{2+} concentrations.

v) Determination of the extent of leakage via the nickel quench technique for the indicators fura-2 and fura-PE3

Due to the initial results and the many published studies indicating leakage of the indicators in different cell types, it was deemed necessary to determine the leakage rate for both the indicators in the neutrophil. Determinations of the rate of leakage across the cell membrane into the extracellular medium were performed via the nickel quench technique. Nickel quenched the fluorescence output of the available indicator molecules in the extracellular medium, therefore a decrease in the measured ratio values upon nickel chloride addition were indicative of

the quantity of fluorescent calcium indicators present in the extracellular medium due to leakage from the cytosol. The extent of leakage of the two indicators were determined and subsequently compared. The protocol for loading of fura-2 was changed to the protocol for loading of fura-PE3 in order that all the conditions were the same for the two indicators. Loading of fura-2 according to the protocol for fura-PE3 still resulted in sufficient quantities of the indicator in the cytosol.

Fura-2

- Incubation: 37°C for 30 minutes in a shaking water bath and for a further 30 minutes at room temperature
- Washing and resuspension - 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes
- Working medium: the same as the washing medium, incubate on ice for a further 2 minutes and for another 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Refer to figure 8, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.26 to 1.21 was obtained

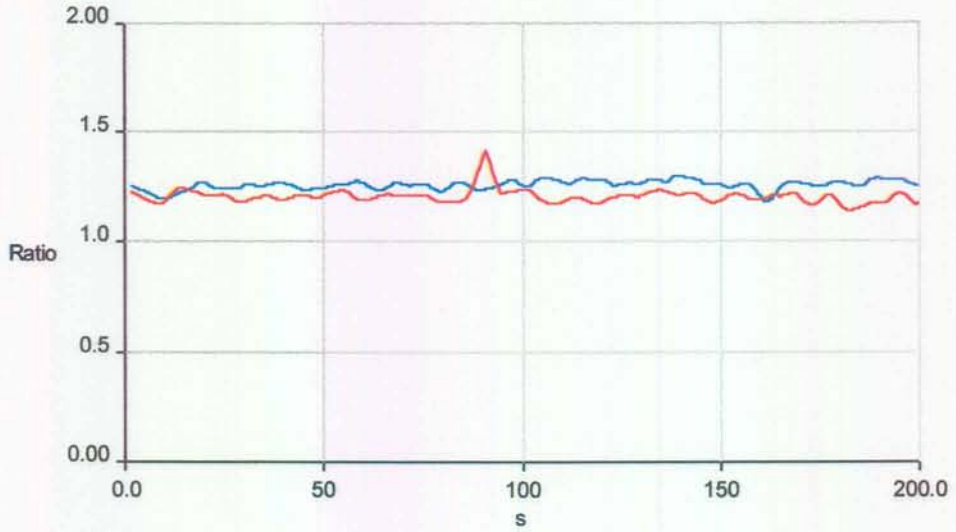

Figure 8. Decrease in the ratio value upon nickel quenching of fura-2 molecules in the extracellular medium

- A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Refer to figure 9, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.36 to 1.26 was obtained

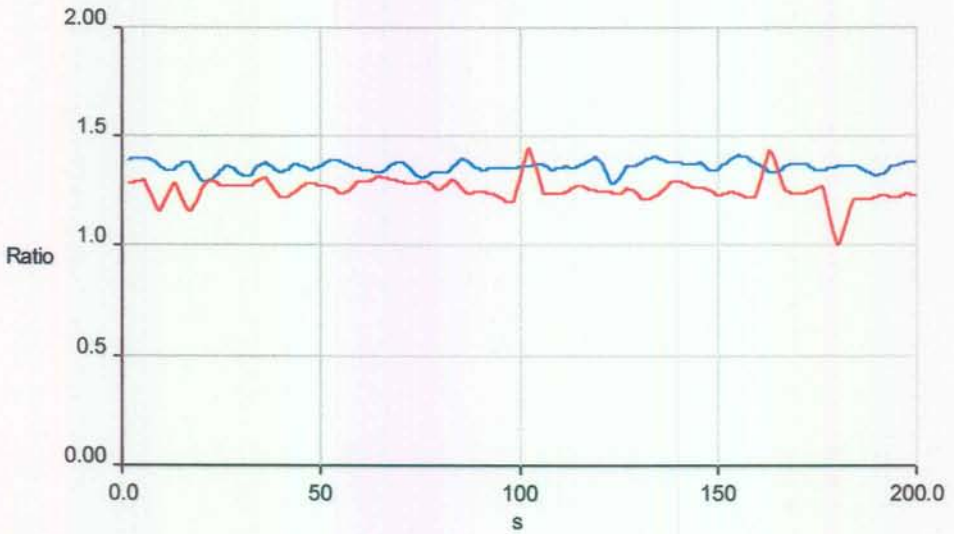

Figure 9. Decrease in the ratio value upon nickel quenching of fura-2 molecules in the extracellular medium

Fura-PE3

- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Washing and resuspension – 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes
- Working medium: the same as the washing medium, incubate on ice for 2 minutes and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Refer to figure 10, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.19 to 1.17 was obtained

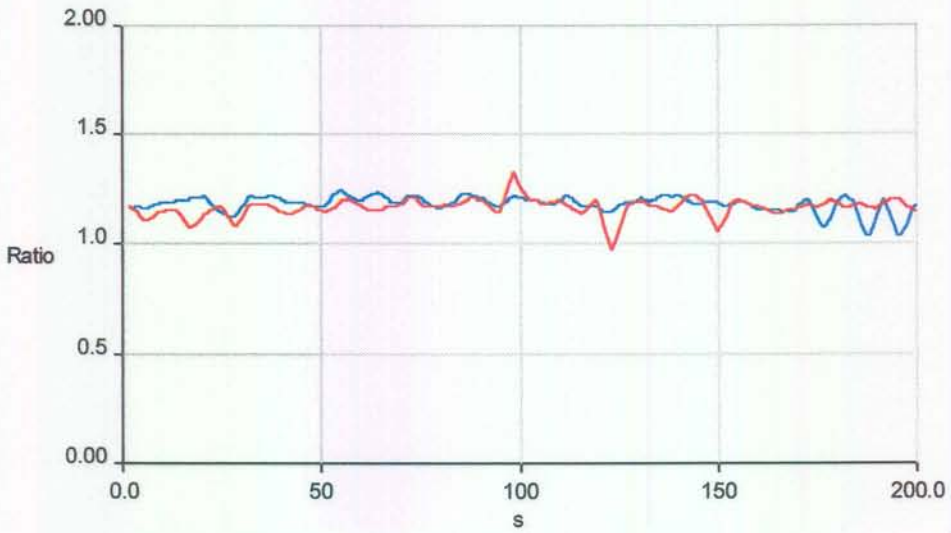

Figure 10. Decrease in the ratio value upon nickel quenching of fura-PE3 molecules in the extracellular medium

- A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

- Refer to figure 11, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.26 to 1.17 was obtained

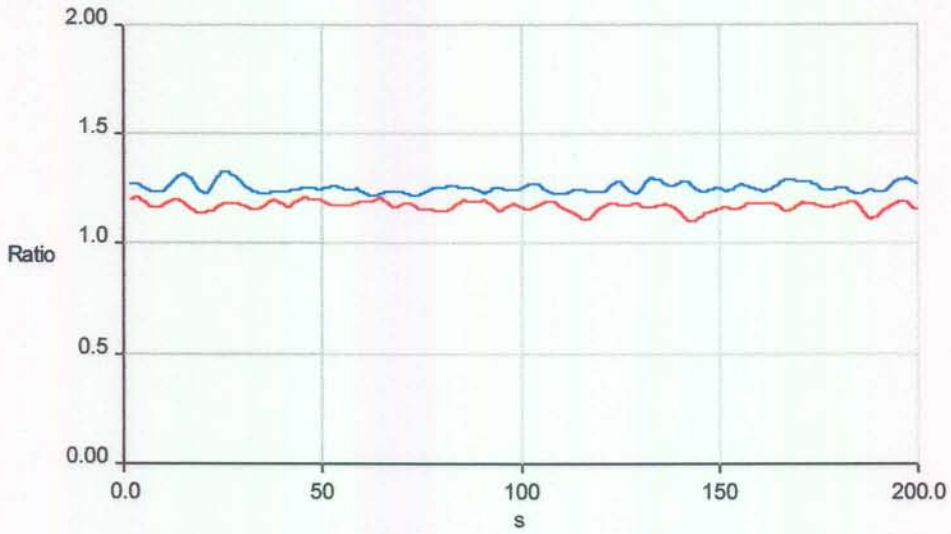

Figure 11. Decrease in the ratio value upon nickel quenching of fura-PE3 molecules in the extracellular medium

In summary: Ratio values before and after nickel chloride addition

	Before	After	Difference
Fura-2: 2 min on ice	1.26	1.21	0.05
Fura-PE3: 2min on ice	1.19	1.17	0.02
Fura-2: 50 min on ice	1.36	1.26	0.1
Fura-PE3: 50 min on ice	1.26	1.17	0.09

Both the indicators leak from the cytosol into the extracellular medium. Nevertheless, fura-PE3 to a lesser extent than fura-2 and more so during the 2 minute incubation period than during the 50 minute incubation period.

vi) Determination of the extent of the inaccurately measured intracellular free calcium concentration as a result of the leakage of the indicator into the extracellular medium

Calibration of the measured fluorescence intensities cannot be performed in the presence of nickel chloride, therefore a separate set of experiments was performed to determine the extent of the inaccurately determined intracellular free calcium concentration represented by the indicator extruded into the extracellular medium. Fura-PE3 was the indicator of choice according to the previously obtained results due to less leakage from the cytosol compared to fura-2, and was therefore used in the subsequent investigations.

- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Washing and resuspension – 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes
- Working medium: the same as the washing medium, incubate on ice for 2 minutes and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Determine the intracellular free calcium concentration at the exact time point coinciding with the nickel chloride addition as for the previous set of experiments
- Refer to figure 12, blue graph
- A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Determine the intracellular free calcium concentration at the exact time point coinciding with nickel chloride addition as for the previous set of experiments

- Refer to figure 12, red graph

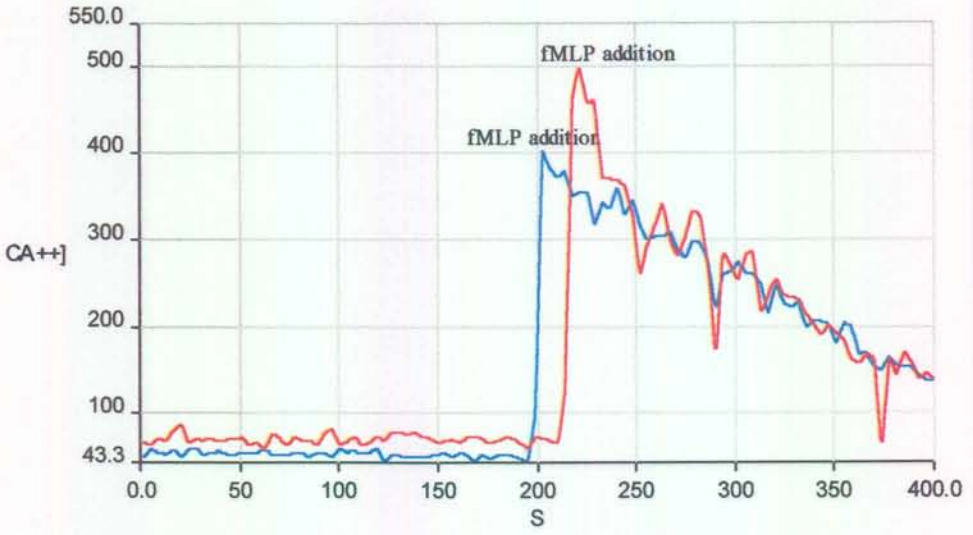

Figure 12. The extent of the inaccurately determined intracellular free calcium concentrations as a result of fluorescent calcium indicator leakage

In figure 12 the red and blue graphs represent the intracellular free Ca^{2+} concentrations determined for either after the 2 minute incubation period (blue graph) or after the 50 minute incubation period (red graph). The difference in the intracellular free Ca^{2+} concentration between these two graphs can be seen, this represent the inaccurately determined intracellular free Ca^{2+} as a result of leakage of the indicator during the 50 minute incubation period.

vii) Characterisation of the leakage process

- **The leakage process is temperature dependent**

A series of fluorescence intensity measurements was obtained at different temperature settings for a single cell suspension aliquot. The ratio values were calculated and the results plotted. Refer to figure 13 – the six ratio value graphs were obtained with a time interval of 20 minutes between measurements. The bottom two graphs – the red and blue graphs were obtained at 25°C. The following green graph was

obtained at 30°C. The remaining three graphs were obtained at 37°C with the time interval still being 20 minutes. An increase in the leakage rate occurred with an increase in temperature.

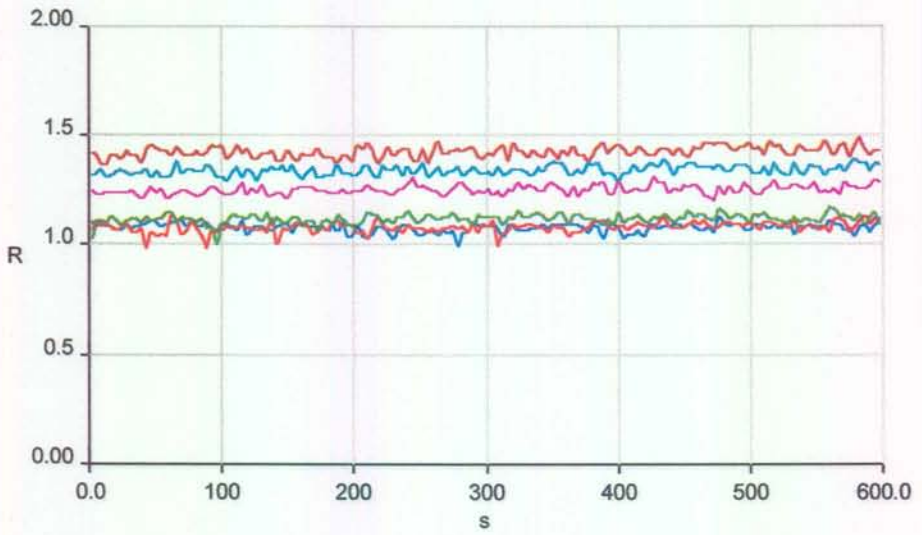

Figure 13. An increase in the rate of indicator leakage due to a rise in temperature

- **The leakage process is independent of magnetic bar stirring in the cuvette**

A series of fluorescence intensity measurements was obtained at different temperature settings and the ratio values calculated. Refer to figure 14 – nine ratio value graphs were obtained with a time interval of 20 minutes between measurements. The bottom four graphs were obtained from the first cell suspension aliquot – the red and blue graphs at 30°C and the green and pink graphs at 37°C. The following three graphs were obtained from a second cell suspension aliquot kept on the water bath and not continuously stirred. The top two graphs were obtained from a third cell suspension aliquot kept on the water bath and not continuously stirred. An increase in the leakage rate not different from the first cell suspension aliquot in the cuvette occurred for both the

cell suspension aliquots kept on the water bath. Therefore, the leakage process is not accelerated as a result of agitation of the neutrophils.

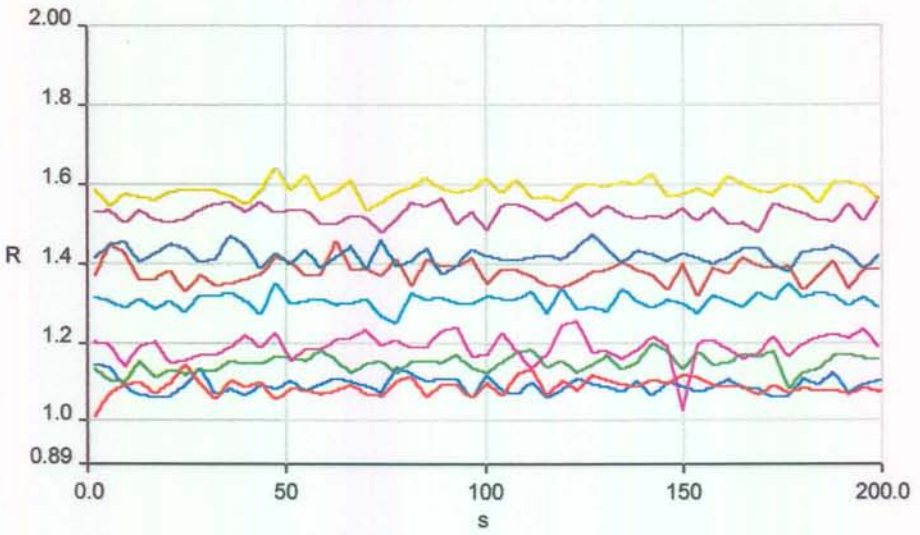

Figure 14. Comparison of the rates of the leakage process for the fluorescent calcium indicator as a result of magnetic bar stirring

- **Determination of the possible indicator leakage occurring during a 1000s period**

Fluorescence intensity measurements were performed for 1000 seconds. During this period no significant amount of leakage occurred. Refer to figure 15. The duration of the subsequent fluorescence determinations in the neutrophils for both patients and healthy subjects were less – 650 seconds in total. Therefore leakage of the indicator could not have interfered with the fluorescence intensity measurements for the duration of the experiment.

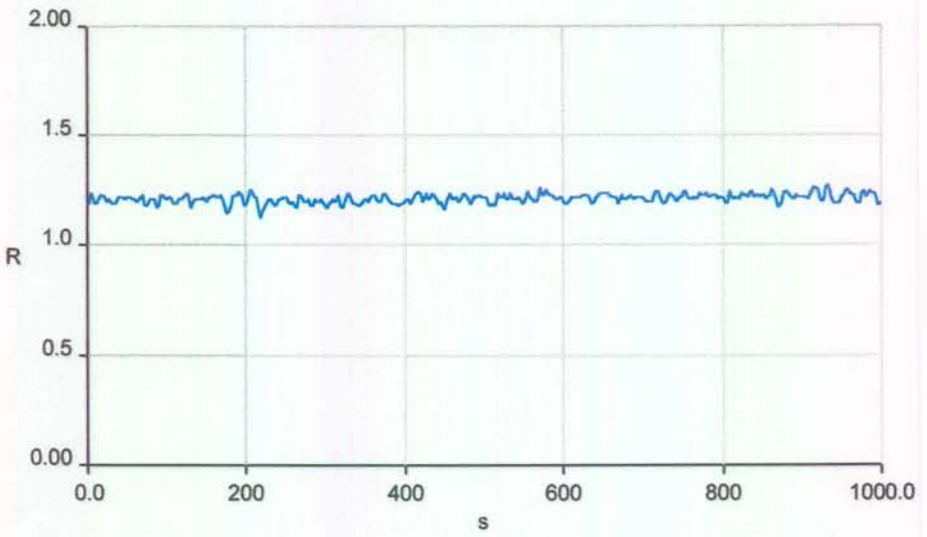

Figure 15. Constant ratio values during a similar time period as for intracellular free calcium determinations in the patients and control subjects

viii) Investigation of the possible interference from heavy metals present in the cytosol with the determination of intracellular free calcium

Possible heavy metal ion quenching of the calcium indicator was investigated in the neutrophils of both patients and healthy subjects. This was achieved by the addition of TPEN (N, N, N', N', - tetrakis(2-PYRIDYLMETHYL)ETHYLENEDIAMINE) at a concentration of 20μM during the measurement of the fluorescence intensities. A decrease in the ratio value would indicate heavy metal ion quenching of the indicator. No heavy metal ion quenching was detected.

ix) Evaluation of the calibration procedure

Intracellular free calcium concentrations were determined by solving the Grynkiewicz equation – refer to section on the theoretical background of the technique. This equation contains two calibration points – the ratio value obtained at maximum calcium and the ratio value obtained at minimum calcium. At the end of the experiment the calibration procedure is performed. The fluorescence ratio value for maximum

calcium is determined by the addition of TritonX-100 to the cell suspension. The addition of TritonX-100 results in the breakdown of the cell membranes and the subsequent releasing of the indicator into the medium containing a calcium concentration of 1mM. Fluorescence intensity determinations are continuously performed. After the addition of TritonX-100 the fluorescence ratio values at maximum calcium concentration are determined for a period of 100 seconds. At the completion of this period EGTA/Tris is added. EGTA chelates the available calcium ions resulting in attainment of fluorescence ratio values at minimum calcium concentration. Refer to figure 16.

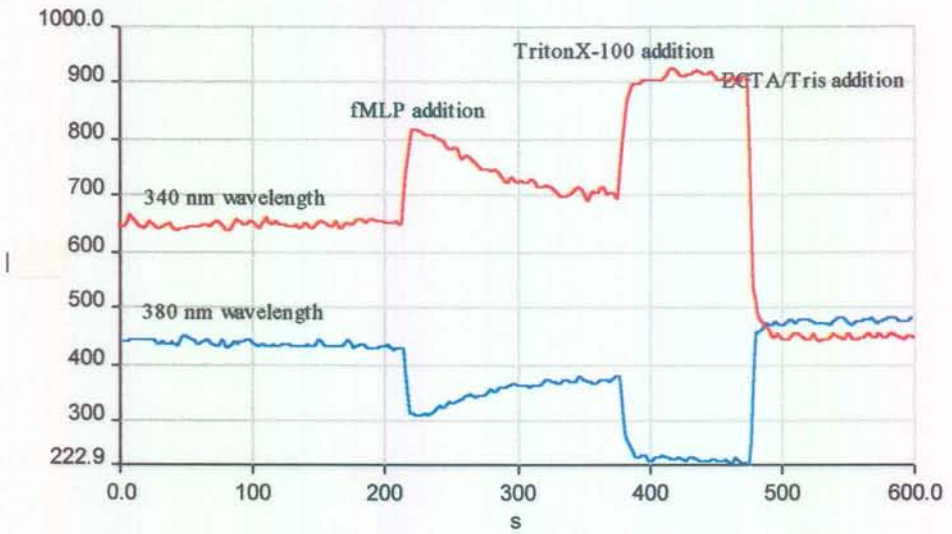

Figure 16. An end of the experiment calibration procedure, the calcium indicator is released into the extracellular medium where the calcium concentration is subsequently manipulated

For this end of the experiment calibration procedure the indicator is released into the extracellular medium where the calcium concentration is manipulated. There are indications that the fluorescence properties of the indicators are influenced by the characteristics of the medium, i.e., viscosity and ion concentrations. The cytosol differs from the extracellular medium in this regard therefore the release of the indicator into the extracellular medium might result in a change in fluorescence

ratio values independent of a change in the calcium concentration. If this is the case the calibration of the fluorescence ratio values should be performed whilst the indicator is still in the cytosol. This possibility was investigated. Intracellular free calcium concentrations should be manipulated in order to calibrate the fluorescence ratio values in situ. This was achieved by the addition of calcium ionophores to the neutrophil suspension.

- **Manipulation of intracellular free calcium concentrations in order to calibrate the fluorescence ratio values in situ – a suitable calcium ionophore**

The calcium ionophore A23187

Addition of the ionophore A23187 at a concentration of 40 μM resulted in the obtainment of fluorescence intensity values above the limits for instrument detection and obscuring of the calcium indicator's fluorescence signals, since the calcium ionophore A23187 is highly fluorescent at an excitation wavelength of 340nm and 380nm. Refer to figure 17.

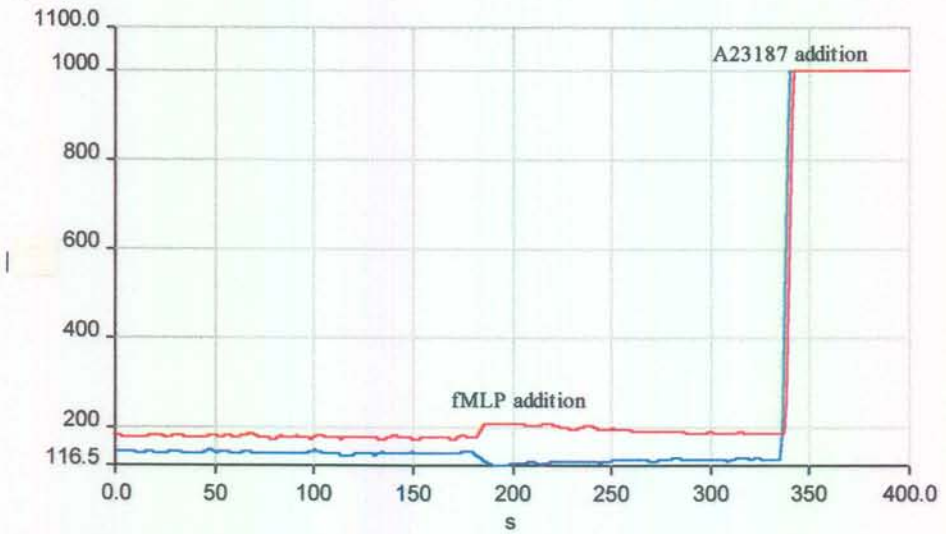

Figure 17. The addition of the calcium ionophore A23187 in order to equilibrate the intracellular free calcium with extracellular medium calcium

At a concentration of 2 μM the ionophore's fluorescence intensities were significantly less but still large enough to obscure the indicator's fluorescence signals. Refer to figure 18.

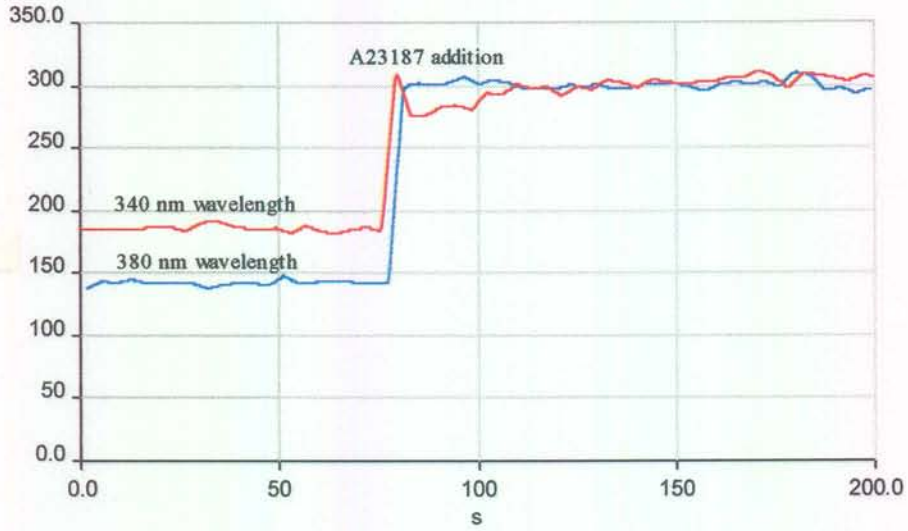

Figure 18. The addition of the calcium ionophore A23187 in order to equilibrate intracellular free calcium with extracellular medium calcium

At a lower ionophore concentration the ability to rapidly equilibrate extracellular calcium with cytosol calcium was abolished.

The calcium ionophore: ionomycin

The addition of ionomycin to a final concentration of 12.5 μM resulted in the immediate elevation of the fluorescence ratio value and thus an increase in the cytosolic free calcium concentration. Refer to figure 19.

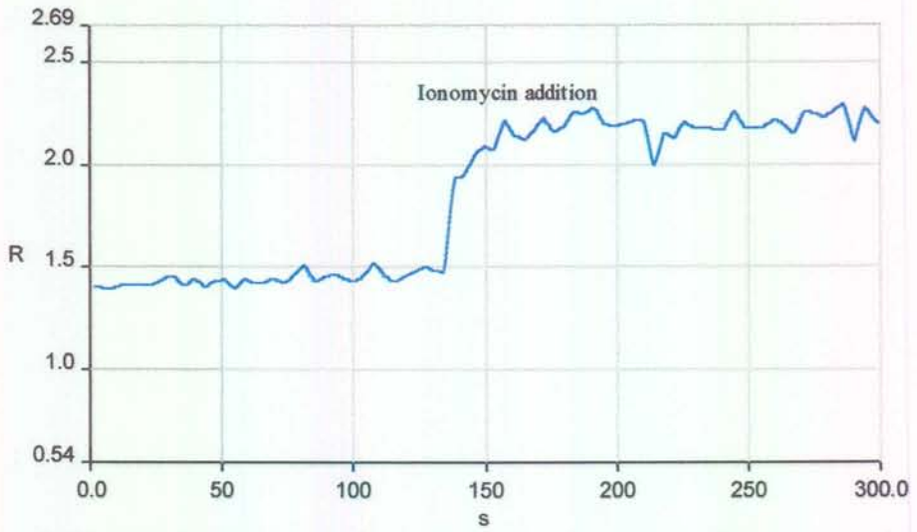

Figure 19. The addition of ionomycin in order to equilibrate intracellular calcium with extracellular medium calcium

Most importantly was the attainment of true end-point values for maximum intracellular free calcium. The evidence that ionophore-induced alterations reflect the true end points rests upon comparison to results obtained after cell lysis. To validate if maximum cytosolic free calcium is reached, it is suggested that subsequent to the addition of ionomycin TritonX-100 should be added. Any increase in the ratio value upon this TritonX-100 addition would indicate that the complete equilibration of medium calcium with cytosolic calcium did not take place. Upon the subsequent addition of TritonX-100 a small increase in the fluorescence ratio values were obtained, possibly indicating that the equilibration of cytosolic free calcium with medium calcium is incomplete. Refer to figure 20.

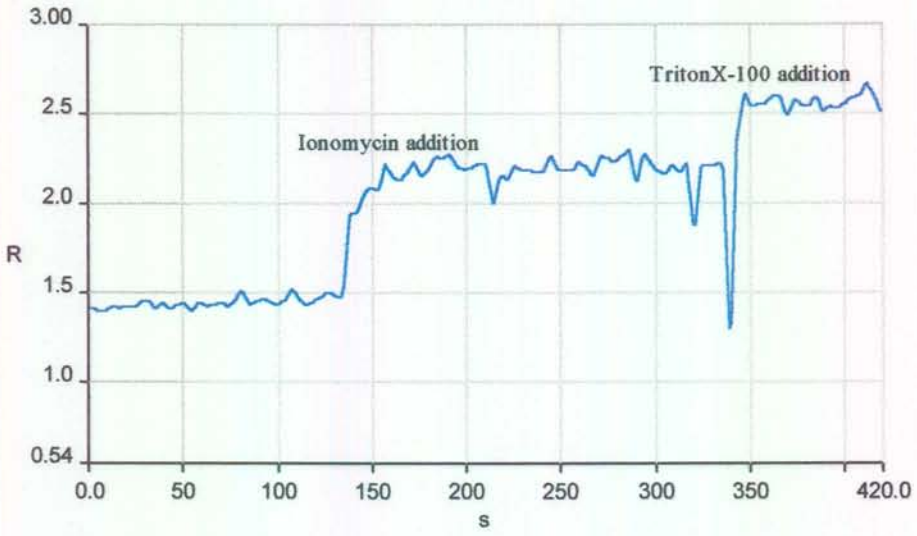

Figure 20. An increase in the ratio value upon the addition of TritonX-100 after the addition of ionomycin

However, investigation of the fluorescence intensities comprising the ratio values indicated that upon TritonX-100 addition a small, insignificant increase occurred in the 340nm wavelength graph compared to a larger decrease in the 380nm wavelength graph. Refer to figure 21.

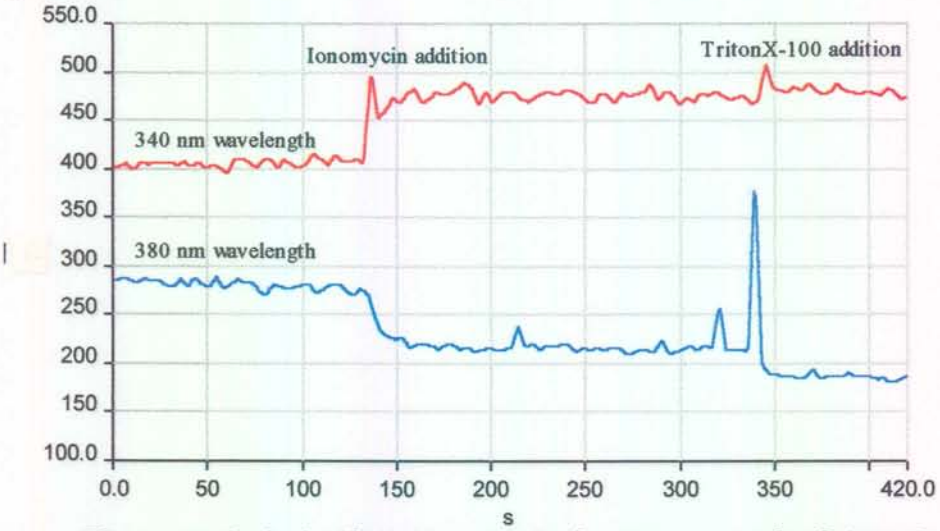

Figure 21. An insignificant increase in the 340 nm wavelenth intensity and a larger decrease in the 380 nm wavelength intensity incompatible with a rise in intracellular free calcium

This is incompatible with an increase in intracellular free Ca^{2+}, since with a true increase in intracellular free Ca^{2+} the increase in fluorescence intensity values for the 340 nm wavelength should always be greater than the magnitude of the decrease in the fluorescence intensity for the 380 nm wavelength. This subsequent increase in the ratio value upon TritonX-100 addition therefore was not due to the incomplete equilibration of extracellular medium calcium with cytosolic calcium. This increase in the ratio value could rather be the result of the change in the fluorescence characteristics of the fluorescent calcium indicator due to the release of the indicator into a medium different in constitution than the cytosol.

In order to clamp the cytosolic free calcium concentration to a minimum EGTA/Tris was added. Refer to figure 22.

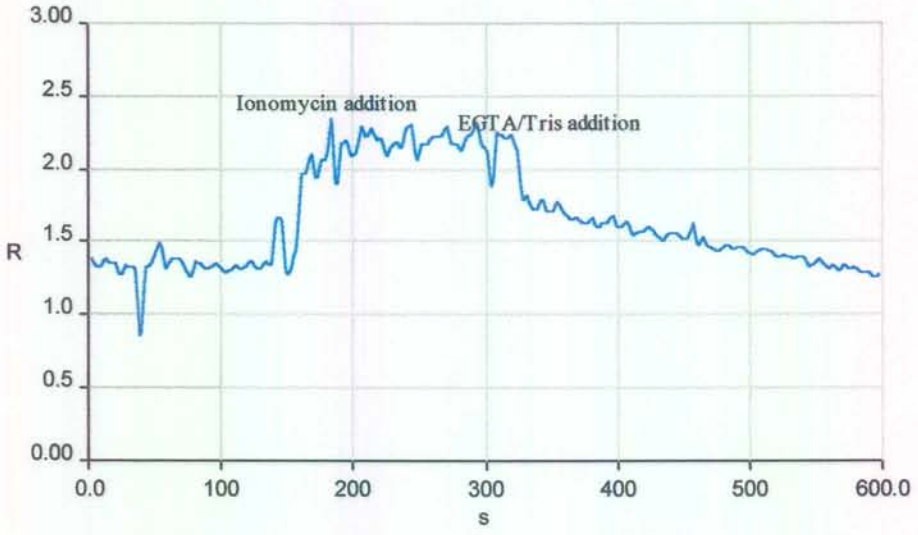

Figure 22. Addition of a EGTA/Tris solution in order to attain minimum calcium concentrations

Equilibration of the cytosolic calcium with the medium calcium containing minimum calcium was a slow process, and the attainment of minimum calcium concentrations in the cytosol uncertain.

It seems that the fluorescence characteristics of the fluorescent calcium indicator do change as a result of the releasing of the indicator into the extracellular medium but the result of this change on the ratio values are negligible. Furthermore, according to these results, the attainment of the minimum fluorescence intensity values was uncertain. Therefore the end of the experiment calibration of the obtained fluorescence intensities were performed following cell lysis.

x) Evaluation of the reproducibility of the technique for the determination of intracellular free calcium and transmembrane calcium fluxes when performed over a time period

At this stage it became necessary to determine whether the technique gave reproducible results when the intracellular free calcium and transmembrane calcium fluxes are determined on several occasions for the same subject. To evaluate the reproducibility and accuracy of the technique, basal intracellular free calcium and transmembrane calcium fluxes were determined for the same subject on different days. The attained intracellular free calcium and transmembrane calcium fluxes indicate the reproducibility of the technique and also the tight regulation of intracellular free calcium. Refer to figure 23.

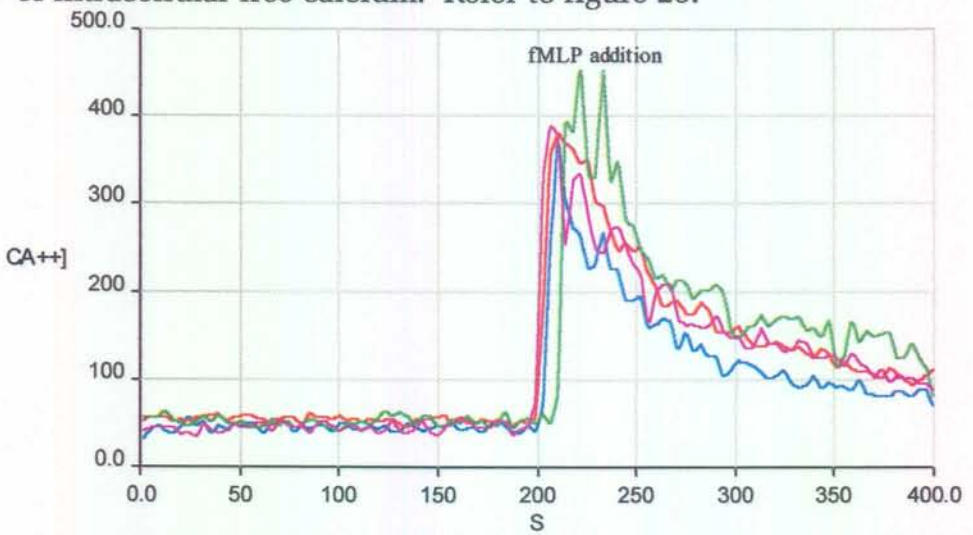

Figure 23. The attainment of reproducible results in the same subject on different occasions

The basal intracellular free Ca^{2+} concentrations were: 45 nM, 54 nM, 52 nM, 45 nM and the magnitude of the transmembrane Ca^{2+} flux upon fMLP stimulation were: 379 nM, 381 nM, 464 nM, 390 nM.

In order to determine the range of basal intracellular free calcium and transmembrane calcium fluxes in different subjects, measurements were performed for 6 subjects on 6 different days:
Refer to figure 24, white female 25y, white female 24y, black female 27y, black female 40y, white male 61y and white female 65y. The attained basal intracellular free calcium and transmembrane fluxes indicate the reproducibility of the technique and also the range of basal intracellular free calcium and transmembrane calcium fluxes in different subjects.

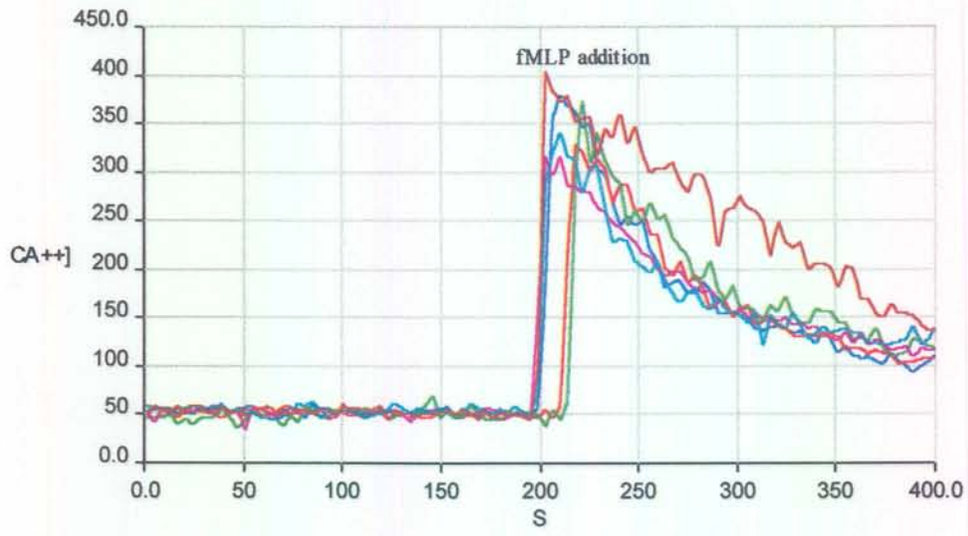

Figure 24. The attainment of reproducible results for different subjects on different occasions

The basal intracellular free Ca^{2+} concentrations were: 50 nM, 52 nM, 50 nM, 52 nM, 57 nM, 53 nM and the magnitude of transmembrane Ca^{2+} fluxes were: 328 nM, 464 nM, 380 nM, 318 nM, 341 nM, 405 nM.

xi) Evaluation of the sensitivity of the technique for the determination of a change in intracellular free calcium

The following measurements were performed to test whether the technique would indeed be sensitive enough to determine variations and whether this would follow changes in the degree of sickness such as in sepsis. Dyshomeostasis of the immune system in septic patients is the result of the dysfunction of many of the cells involved in host immunity, including the neutrophils. There are indications that one of the contributing factors to neutrophil dysfunction is intracellular calcium dysregulation. (29, 30) Intracellular free calcium at rest and upon fMLP stimulation were determined in this group of patients as an indication of the potential of the optimised experimental procedure for detecting a change in intracellular free calcium, both at rest and upon fMLP stimulation.

- **Determination of neutrophil intracellular free calcium in patients**

- Patient 1: Refer to figure 25, blue graph and upon partial recovery, red graph. Basal intracellular free calcium and the magnitude of the transmembrane calcium flux are decreased with an improvement in the patient's condition.

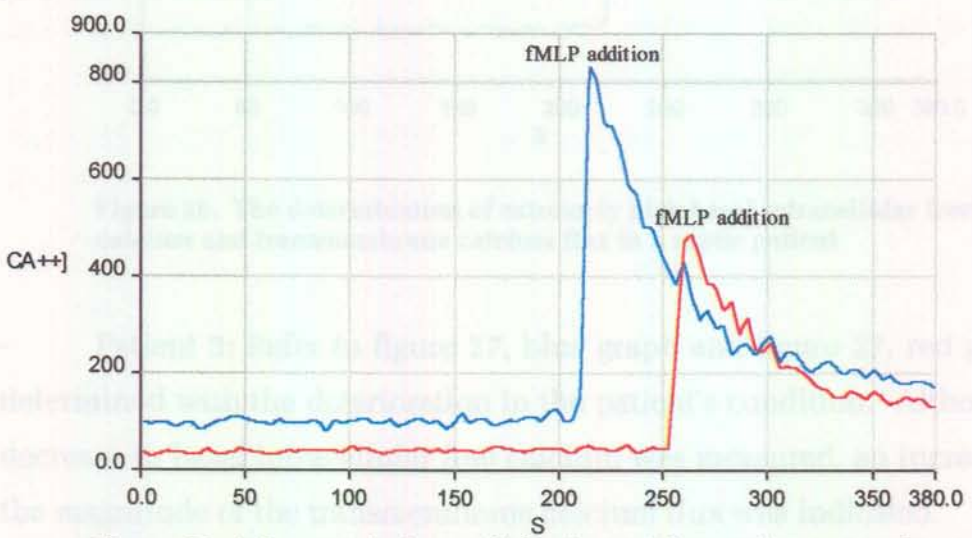

Figure 25. A decrease in intracellular free calcium and transmembrane calcium flux upon partial recovery of a septic patient

- Patient 2: Refer to figure 26, an elevation in basal intracellular free calcium and the transmembrane calcium flux was determined. The patient's condition deteriorated and the patient died, preventing a follow-up determination. The magnitude of the patient's neutrophil intracellular free calcium and transmembrane calcium flux were extremely high. Refer to figure 24 for the normal range of basal intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes.

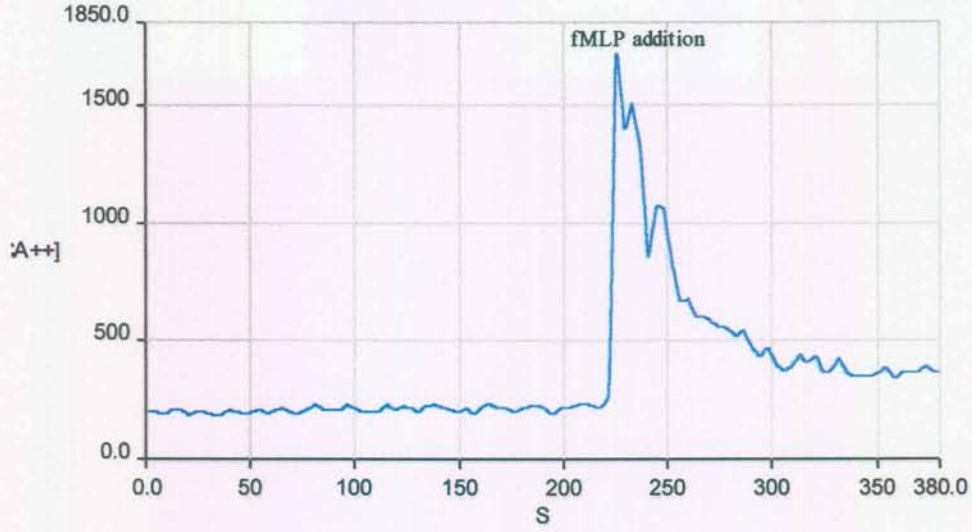

Figure 26. The determination of extremely high basal intracellular free calcium and transmembrane calcium flux in a septic patient

- Patient 3: Refer to figure 27, blue graph and figure 27, red graph, determined with the deterioration in the patient's condition. Although a decrease in basal intracellular free calcium was measured, an increase in the magnitude of the transmembrane calcium flux was indicated.

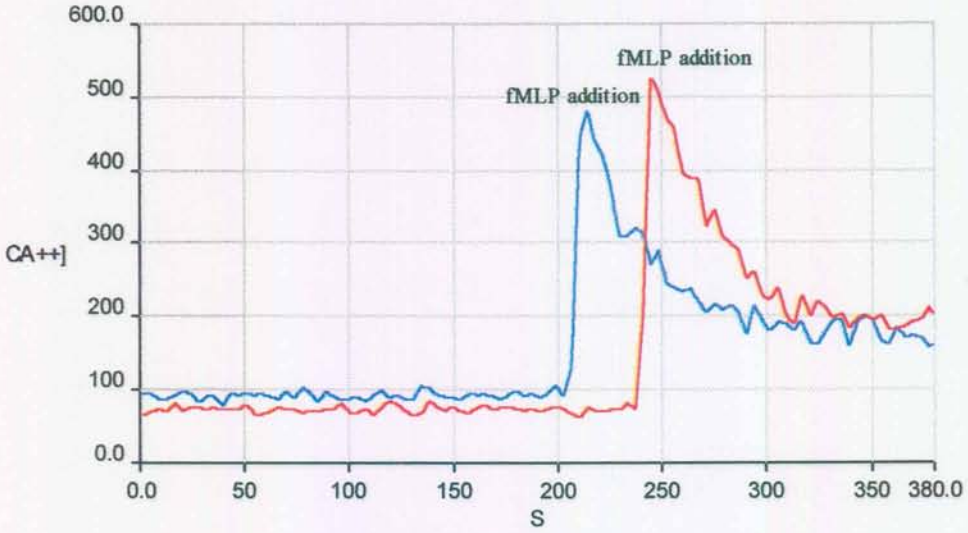

Figure 27. A decrease in basal intracellular free calcium and an increase in the transmembrane calcium flux of a septic patient with the deterioration of the patient's condition

- Patient 4: Refer to figure 28. The blue graph represents the first determination of basal intracellular free calcium and transmembrane calcium flux, whereas the red graph the second determination. A lowering in basal intracellular free calcium and the transmembrane calcium flux accompanied the improvement in the patient's condition.

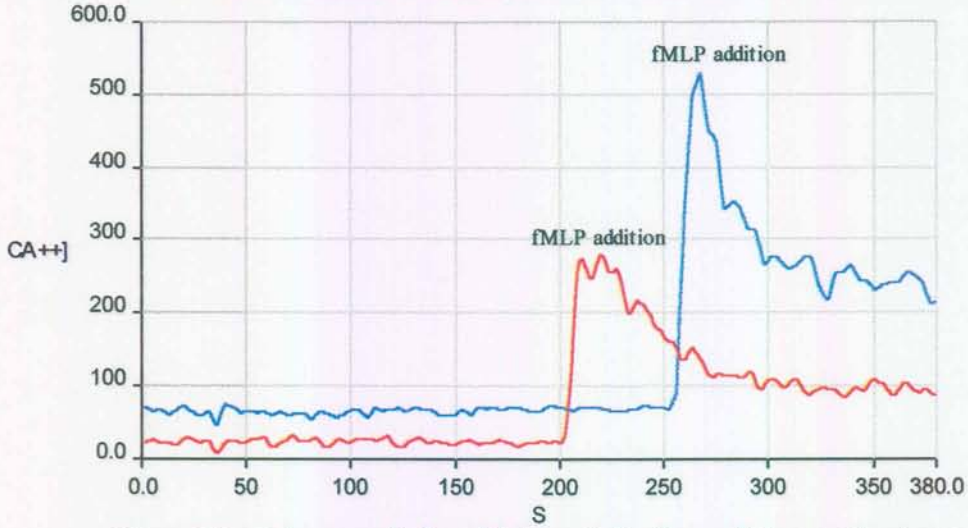

Figure 28. A decrease in basal intracellular free calcium and a decrease in the transmembrane calcium flux in a septic patient, the patient's condition improved

2.2.6) Discussion

There are many available fluorescent calcium indicators for cuvette-based measurements of intracellular free calcium. The most commonly used of these fluorescent calcium indicators is fura-2. Fura-2 is also the fluorescent calcium indicator of choice in many studies investigating intracellular free calcium in the neutrophil. The employment of this calcium indicator gave good results in many of these studies, but there are indications that leakage and compartmentalisation of the indicator into intracellular organelles might interfere with intracellular free calcium determinations in some cell types. In some cell types indicator extrusion can be so severe, that in these cell types it become difficult to load enough indicator to be sufficient for fluorescence determinations for the duration of the experiment. More commonly, cells initially load evenly, but soon the indicator leaks out of the cytosol while the remaining indicator molecules are associated with the intracellular organelles. This leakage and compartmentalisation of the indicator complicate many aspects of calcium studies, including the determination of true resting intracellular free calcium levels in cuvette-based measurements, since in the presence of external calcium a constant leakage of the indicator could be misinterpreted as a slow rise in intracellular free calcium. Indicator extrusion and compartmentalisation are not limited to cells loaded with the acetoxy methyl ester form of the indicator, since cells microinjected with the penta-anion form of the indicator display similar problems. These problems can be partly remedied with organic anion transport inhibitors such as probenecid or with an indicator linked to dextran, however the use of probenecid adds unknown variables to the experiment, and indicator-dextran conjugates must be micro-injected. (17, 18) It would therefore be desirable to develop indicators that do not leak out of cells or are compartmentalised in intracellular organelles, yet retain the ability to load as an acetoxy methyl ester. It was with this in mind that

the experimentalists developed a fluorescent calcium indicator that resisted leakage from the cytosol and compartmentalisation into intracellular organelles. The new indicator fura-PE3, an analog of fura-2 is designed to resist leakage and compartmentalisation by virtue of an added positive charge. The incorporation of an additional positive charge produced a molecule with zwitterionic characteristics, ensuring a much slower extrusion from the cytosol and compartmentalisation into intracellular organelles. It was shown by the patent holders that fura-PE3 can be loaded in much the same way as fura-2, as an acetoxy methyl ester derivative, but gives more uniform loading. In addition, cells loaded with fura-PE3 is said to remain uniformly loaded for hours, whereas fura-2 quickly accumulates in organelles and leaks out of the cell. Despite its benefits, fura-PE3 is still not the ideal cytosolic calcium indicator. Leakage and compartmentalisation are dramatically reduced but not eliminated. (31, 32) In search of the most suitable fluorescent calcium indicator for intracellular free calcium studies in the neutrophil both fura-2 and fura-PE3 were tried and tested. Both these indicators are well suited to resolve basal intracellular free calcium levels with a Kd for fura-2 of 224nM and a Kd for fura-PE3 of 250nM. Kd values closest to the concentration level to be measured, assures the largest change in fluorescence intensities as a result of a change in intracellular free calcium and thus good resolving capabilities.

Various aspects of the protocol for the determination of intracellular free calcium in the neutrophil were evaluated, since as discussed in the first section of this chapter the determination of intracellular free calcium by employing the florescent calcium indicators is not problem-free. Firstly, sufficient quantities of the fluorescent calcium indicator are to be obtained in the cytosol of the neutrophil to determine intracellular free calcium. The following precautions assisted in the attainment of

sufficient quantities of the fluorescent calcium indicators, both fura-2 and fura-PE3 in the cytosol.

- The stock solutions which consisted of the acetoxy methyl ester derivative of the indicator dissolved in DMSO was premixed in the appropriate buffer containing bovine serum albumin (BSA). Bovine serum albumin acted as a dispersing agent and assured the availability of the indicator for crossing of the membrane. Omission of BSA resulted in inadequate accumulation of the indicator in the cytosol of the neutrophil.
- The agitation of the neutrophil suspension during the loading period facilitated the acetoxy methyl ester derivative in crossing of the membrane and ensured the obtainment of sufficient quantities of the indicator in the cytosol.

Other factors also determined the efficiency of the loading procedure. These factors included the temperature at which loading of the indicator was performed, the concentration of the ester derivative of the indicator in the incubation medium and the duration of the loading procedure. These three factors were altered on a trial and error basis and the following protocols for loading of the indicator established.

- The protocol for loading of fura-2/AM is formulated according to a combination from published methods and suggestions. Attempts to optimise the loading procedure for the fura-2 indicator indicated sufficient quantities of the indicator in the cytosol with a concentration of the acetoxy methyl ester derivative of the indicator in the loading medium of 2 – 5 μM. For the loading of the fura-2 indicator a concentration of the AM ester of 5 μM was employed.
- There are no published studies on intracellular free calcium investigations in the neutrophil employing fura-PE3, but it is suggested that the concentration of the fura-PE3/AM ester in the incubation medium should be lower (1 - 2 μM) and the incubation

time longer than that for the fura-2/AM ester. This, due to the fact that the fura-PE3/AM ester has a greater tendency to crystallise or precipitate than seen with the fura-2/AM ester. For the loading of the fura-PE3 indicator a concentration of the AM ester of 2 μM was employed and found to be sufficient. The working concentration of neutrophils in the cuvette was 2 to 9 x 10^5 / ml for fura-2 and 9 x 10^5 to 1 x 10^6 for fura-PE3. This difference was the result of the obtainment of different quantities of the indicators in the cytosol due to the different loading protocols and as a result of differences in the rate of cleavage of the ester bonds for these two indicators. These working concentrations of neutrophils assured fluorescence intensities above background fluorescence values and below the limits for instrument detection.

The wavelength shifting fluorescent calcium indicators like fura-2 and fura-PE3 can be employed in ratio mode, that is at every measured time-point two fluorescence intensity values at two different wavelengths are obtained and subsequently the ratio consisting of these values is calculated for every time-point. The advantage of this feature is that factors influencing fluorescence intensity values such as indicator concentration cancel when the ratio is calculated, since indicator concentration influence the fluorescence intensity values to the same extent. To evaluate this feature two aliquots of the same neutrophil suspension were loaded with different concentrations of the fura-PE3/AM ester, resulting in the subsequent attainment of unequal quantities of fura-PE3 in the cytosol. Calibration of the obtained fluorescence intensities followed by the calculation of intracellular free calcium concentrations gave similar results. Refer to figure 7.

Indicator extrusion complicated intracellular free calcium determinations during the initial study, since leakage of the indicator into the extracellular medium resulted in an incorrectly measured higher

basal intracellular free calcium concentration. Therefore the extent of leakage for both the fluorescent calcium indicators, fura-2 and fura-PE3 were determined in the neutrophil. A negligible decrease in the ratio values occurred for both fura-2 and fura-PE3 upon nickel chloride addition after the cell suspension was kept on ice for a very short period of only two minutes – 0.05 for fura-2 and 0.02 for fura-PE3. Nevertheless, the decrease in the ratio value for fura-2 is larger than that for fura-PE3. This according to other published results in different cell types indicating that fura-PE3 is better retained in the cytosol of the specific cell types. It must be mentioned that a small amount of the indicator is not removed with repeated washing of the cell suspension after incubation in the loading medium. (6) This fraction of unremovable indicator molecules might represent indicator molecules bound to the cell membrane, therefore quenching of the fluorescence of these indicator molecules upon nickel chloride addition might have been the cause of the measured decrease in the ratio values and not as a result of the extrusion of the indicators from the cytosol. For both types of indicators a much larger decrease in the ratio values occurred upon nickel chloride addition after the 50 minute incubation period, although again a small difference existed between the two indicators – 0.1 for fura-2 and 0.09 for fura-PE3. This in contrast to other published results in different cell types indicating that fura-2 is extruded from the different cell types to a much larger extent than fura-PE3. According to these results leakage of both the fluorescent calcium indicators are mentionable during a 50 minute incubation period. But for both the indicators the leakage of the indicator during a 2 minute incubation period are negligible, more so for fura-PE3. Since leakage of the fluorescent calcium indicator into the extracellular medium results in the obtainment of higher basal intracellular free calcium, the incorrectly measured higher basal intracellular free calcium concentration as a result of the extrusion of the indicator to the extracellular medium

containing a millimolar calcium concentration was determined. An example of one of 20 determinations is presented - refer to figure 12. The blue graph was obtained after the first incubation period – 2 minutes on ice and a further 2 minutes at 37°C in the cuvette. The red graph was obtained after the second incubation period – 50 minutes on ice and a further 2 minutes at 37°C in the cuvette. The mean intracellular free calcium concentration calculated for the blue graph equals 53 nM and for the red graph 70 nM. Thus the calculated difference in the ratio values obtained before and after nickel chloride addition after an incubation period of 50 minutes which is 0.09 represents an inaccurately determined elevation of intracellular free calcium equal to: 70 – 53 nM = 17 nM. A decrease in the ratio value after the first short incubation of 0.02 would more or less represent a negligible elevation in the intracellular free calcium concentration of approximately 2 nM.

There are indications that heavy metals present in the cytosol in various cell types may interfere with intracellular free calcium determinations performed by using the fluorescent calcium indicators. Heavy metals for instance Fe^{2+} and Zn^{2+} quench the fluorescence quantum yield and would therefore result in the lowering of the determined intracellular free calcium levels. (6) It is well known that chronic renal failure patients on maintenance haemodialysis, display an increase in heavy metal ion levels in the cytosol of the red cell. (33) In both the patient's group and the healthy subject's group the possible interference of heavy metal quenching was therefore investigated. This was achieved by the addition of TPEN (N, N, N', N', - tetrakis(2-PYRIDYLMETHYL)ETHYLENEDIAMINE). TPEN is a lipid-soluble heavy metal chelator that can permeate cellular membranes and bind heavy metals in the cytosol with an extraordinary high affinity while rejecting calcium and magnesium. (34) No heavy metal ion quenching of the

calcium indicators could be detected at a final concentration of TPEN of 20μM. This could be due to fura-PE3's low affinity for heavy metals, since there is no ring nitrogen that would promote heavy metal binding.

Intracellular free calcium concentrations were determined by solving the Grynkiewicz equation – refer to section on the theoretical background of the technique. This equation contains two calibration points – the ratio value obtained at maximum calcium and the ratio value obtained at minimum calcium. At the end of the experiment the calibration procedure was performed. For this end of the experiment calibration procedure the indicator was released into the extracellular medium where the calcium concentration was manipulated. There are indications that the fluorescence properties of the indicators are influenced by the characteristics of the medium, if this is the situation calibration of the fluorescence values should be performed in situ. This was investigated. Intracellular free calcium concentrations should be manipulated in order to calibrate the fluorescence ratio values in situ. This was achieved by the addition of calcium ionophores to the neutrophil suspension. The addition of an ionophore specific for calcium would result in the equilibration of medium calcium with the intracellular free calcium. It is therefore possible to clamp intracellular free calcium levels to known concentrations and subsequently determine the fluorescence ratio values in situ. The ionophorous antibiotics are lipid-soluble molecules, capable of forming complexes with alkali metal cations followed by the transporting of the cations across a variety of membranes. (35) Physical studies indicate that the complexation-decomplexation kinetics and diffusion rates of ionophores and their complexes across lipid barriers are so favourable that their transport turnover numbers across biological membranes attain values of thousands per second, exceeding the turnover numbers of most macromolecular enzymes. (36) Initially the calcium ionophore A23187

- a carboxylic acid antibiotic with a high specificity for the divalent cations calcium and magnesium, (35) was employed for the in situ calibration procedure, but this ionophore is highly fluorescent at both the wavelengths 340 nm and 380 nm. Therefore, the non-fluorescent calcium ionophore ionomycin was subsequently employed. Ionomycin is a diacidic polyether antibiotic having high affinity for calcium ions. (37) Similar to A23187, ionomycin forms lipid-soluble complexes with calcium and traverses cellular membranes. The addition of ionomycin resulted in rapid equilibration of medium calcium with intracellular free calcium resulting in maximum cytosolic calcium. Most importantly was the attainment of true end-point values for maximum intracellular free calcium. To validate if maximum cytosolic calcium is reached, it is suggested that subsequent to the addition of ionomycin TritonX-100 should be added and any increase in the ratio value upon this TritonX-100 addition would indicate that the complete equilibration of medium calcium with cytosolic calcium did not take place. This evaluation procedure was employed, after the addition of ionomycin, TritonX-100 was added. A small increase in the ratio value did take place and according to the above-mentioned suggestion indicated incomplete equilibration of medium calcium with cytosolic calcium. However, investigation of the fluorescence intensities comprising the ratio values indicated that upon TritonX-100 addition a small, insignificant increase occurred in the 340nm wavelength graph compared to a larger decrease in the 380nm wavelength graph. Refer to figure 21. This is incompatible with an increase in intracellular free calcium, since with a true increase in intracellular free calcium the increase in fluorescence intensity values for the 340nm wavelength should always be greater than the decrease in fluorescence intensities for the 380nm wavelength. This subsequent increase in the ratio value upon TritonX-100 addition therefore was not due to the incomplete equilibration of extracellular medium calcium with cytosolic calcium, but seems to rather be the result of the change in

the fluorescence characteristics of the calcium indicator due to the releasing of the indicator into a medium different in constitution than the cytosol. In order to clamp cytosolic free calcium to a minimum EGTA/Tris was added, the attainment of minimum calcium in the cytosol is a slow process and when and if minimum calcium could be attained in the cytosol uncertain. According to these results, the releasing of the fluorescent calcium indicator into the extracellular medium resulted in a small change in the indicator's fluorescence characteristics, but since the attainment of minimum calcium concentrations in the cytosol was uncertain the calibration of fluorescence intensities were performed following cell lysis. (7)

Finally the reproducibility of the optimised protocol was evaluated, the range of basal intracellular free calcium levels determined, and the potential for the determination of a difference in basal intracellular free calcium levels and transmembrane calcium fluxes investigated. Reproducible results were obtained, refer to figures 24 and 25 and indications are that the optimised protocol is adequately sensitive to determine a change in intracellular free calcium and transmembrane calcium fluxes, refer to figures 25 to 28.

2.2.7) Conclusions

The optimised protocol is presented.

Isolation of the neutrophils

1) Pipette 3 ml of the Histopaque-1077 (8°C) in a blue topped conical tube (volume 15 ml).
2) Layer 6 ml of ACD-anticoagulated blood (room temperature) on top of the Histopaque-1077. Take care not to disturb the surface tension of the histopaque layer.
3) Centrifuge at 1800 r.p.m. (12°C) for 25 minutes.
4) Blood cells and plasma are separated in layers.
5) Discard the supernatant, consisting of plasma, platelets and MNL's.
6) Fill the tube with the remaining red blood cells and polymorphonuclear leucocytes with ammonium chloride solution (8°C) and mix well by aspiration.
7) Incubate on ice for 10 minutes.
8) Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.
9) A white pellet is formed consisting of neutrophils, eosinophils and basophils. No further separation of the neutrophils, eosinophils and basophils are necessary since eosinophils contribute only 0.5-6% and basophils only 0-1% to the total amount of circulating white blood cells.
10) If the pellet is still contaminated with red blood cells, the red blood cell lysis step can be repeated.
11) Discard the supernate and gently dissolve the pellet in 0.25% BSA supplemented Hanks (8°C).
12) Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.
13) Discard the supernate and dissolve the pellet in 2 ml 0.25% BSA supplemented Hanks (neutrophil cell suspension).
14) Incubate on ice for 45 minutes.

Counting of the neutrophils

1) Mix 50 µl of the neutrophil cell suspension with 450 µl of the white cell staining fluid (Turk's blood).
2) Fill the hemositometer.
3) Count the neutrophils in the 5 blocks indicated with a W.
4) Calculate the concentration of the neutrophil cell suspension as follows:

   Total number of neutrophils in all 5 cell chambers = N

   Number of neutrophils in one cell chamber = N/5

   Volume of one cell chamber = 0.1 mm^3

   Concentration of neutrophils in 1 mm^3 after dilution with Turk's blood solution = N/5 . 10

   Concentration of neutrophils in 1 mm^3 of the neutrophil cell suspension = N/5 . 10 . 10

   Thus the concentration of neutrophils in the neutrophil cell suspension per milliliter = N/5 . 10 . 10 . 10 . 10 . 10

   = N/5 . 10^5
5) Adjust the neutrophil cell suspension to a final concentration of 2 . 10^6 cells/ml.

Loading of the neutrophils with the fura-PE3/acetoxy methyl ester

1) Incubate the neutrophil suspension at 37°C for 10 minutes – stabilisation and equilibration of ion gradients.
2) Dissolve 2 µl of the fura-PE3/AM stock solution in 2 ml 0.25% BSA/Hanks.
3) Add 0.5 ml of this mixture to 1.5 ml of the neutrophil suspension. The final fura-PE3/AM concentration is 2 µM and the final % DMSO is 0.025%.

4) Incubate for 30 minutes at 37°C in a gently shaking water bath 50 r.p.m. - protect from light – and for a further 30 minutes at room temperature.
5) Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.
6) Wash the pellet twice with 0.25% BSA/Hanks (8°C), centrifuge at 1200 r.p.m. (12°C) for 10 minutes.
7) Discard the supernate and dissolve the pellet in 2 ml 0.25% BSA/Hanks.
8) Incubate on ice for 2 minutes prior to the commencement of the fluorescence measurements.

Measurement of the fluorescence intensities

1) Add 900 µl of the neutrophil suspension and 1100 µl 0.25% BSA/Hanks to the cuvette.
2) Place the cuvette in the spectrofluorometer cuvette chamber and incubate for 2 minutes with the magnetic bar stirrer switched on.
3) OK to start.

fMLP stimulation of the neutrophils

1) Mix 10 µl of the fMLP stock with 190 µl 0.25% BSA/Hanks.
2) Add 20 µl of the previously prepared solution to the 2 ml neutrophil suspension in the cuvette. Final fMLP concentration 1 µM and the further addition of 0.05% DMSO.

Calibration of the fluorescence ratio data

1) To obtain the fluorescence ratio value at maximum calcium – Rmax. Add 100 µl of a 1% TritonX-100 solution to the 2ml neutrophil suspension in the cuvette. TritonX-100 lyses the cells, resulting in the releasing of the fura-PE3 molecules into the extracellular medium. Since the extracellular medium contains a

calcium concentration of 1 mM, attainment of the Rmax value follows.

2) To obtain the fluorescence value at minimum calcium – Rmin. Add 100 µl of a 0.5 M EGTA/3 M Tris solution pH 8.7 to the 2 ml neutrophil suspension in the cuvette. EGTA chelates the available calcium ions and the attainment of Rmin follows.

Calculation of intracellular free calcium concentrations

The Grynkiewicz equation is solved, refer to page 52.

3) Intracellular calcium localisation explored in the neutrophil via transmission electron microscopy

Localisation of intracellular calcium is considered a major problem to most workers in the field (personal communication Head of electron microscopy, UP). Localisation of calcium in neutrophils therefore present a severe challenge for the uninitiated as further technical pitfalls such as isolation of neutrophils from blood and proper cell packing should be performed without disturbance of the intracellular composition of the cell. It was therefore considered essential to start the procedure by an attempt to prepare transmission electron microscopy (TEM) preparations after neutrophil isolation, before starting on the Ca^{2+} precipitation technique. Since no local expertise could be identified on the preparation of neutrophil TEM samples, a thorough study of the available literature was conducted. After completion of the literature study several protocols were designed and evaluated in order to find the most suitable for the TEM preparation of neutrophils. The most critical step that determines the success of the preparation procedure is the adequate fixation of the cells. Broken plasma membranes, washed out figures, coagulated-appearing cytoplasm, and swollen or shrunken cells are produced at the time of fixation. (38) To preserve such cellular detail glutaraldehyde has gained superiority over osmium tetroxide as a primary fixative. It seems likely that this superiority of glutaraldehyde over osmium tetroxide as a primary fixative, particularly for non-membranous components of the cytoplasm is partly due to the faster rate of reaction of glutaraldehyde with cellular components resulting in fixation before any noticeable extraction of cellular components by the buffer. As mentioned osmium tetroxide is not the primary fixative of choice but post-fixation with osmium tetroxide can result in emphasising of membrane structures since osmium tetroxide reacts with those lipids that are not fixed by glutaraldehyde and osmium tetroxide

also acts as a stain. (39) It is conceivable that the ideal composition of the primary fixative should match the physiological environment of the cell with respect to pH, osmolarity and ionic constitution. An unsuitable fixative composition during primary fixation, before the cellular components have become adequately preserved, may result in extraction of cellular materials and/or deposition of fixative compounds. The properties required for the vehicle of the fixative include

- Ability to maintain a constant pH during fixation. Nevertheless, there is little evidence that the pH is at all critical within the range 6.5 – 8.0.
- Suitable osmolarity (when mixed with the fixing agent) so that cells and organelles neither swell nor shrink. Many prefer a somewhat hyperosmotic solution.

Alcohol dehydration is generally preferred to acetone dehydration since acetone extracts membrane lipids resulting in inadequate preservation of the membranes. However, most methods for calcium localisation seem to employ acetone. In the neutrophil, as in most other cell types, intracellular calcium is functionally distributed between the various subcellular compartments. These compartments include: binding of calcium to the cytosolic side of the plasma membrane either to phospholipids or plasma membrane associated proteins, binding of calcium to macromolecules including proteins free in the cytosol and sequestration of calcium in various membranous organelles. (40) In order to study the distribution of intracellular calcium ions between various subcellular compartments, the transmission electron microscopy preparation procedure employed should preserve and present the *in vivo* distribution pattern of intracellular calcium. Depletion and translocation of any unbound calcium ions may occur during tissue fixation and in some instances the process of fixation may release calcium ions that were previously bound to organic macromolecules. To circumvent this problem two methods have been described to localise

and precipitate calcium ions in situ. In order to bind and retain calcium in these various subcellular compartments, either inorganic phosphates or oxalate anions can be employed. The methods are based on a) stabilisation and/or trapping of calcium during the primary fixation step in glutaraldehyde by the phosphate or oxalate anions, (theoretically phosphate and oxalate anions target different calcium compartments) b) the subsequent wash-out of all non-trapped cations such as sodium, potassium and magnesium and c) the conversion of the complexed or trapped calcium into an electron-dense and insoluble calcium pyroantimonate salt during post-fixation. This distribution of electron-dense particles can subsequently be explored by transmission electron microscopy. (41) Omission of pyroantimonate from the post-fixative solution results in the complete absence of deposits in both procedures. (42) The precipitation of the calcium ions as part of a pyroantimonate complex is said to assure only negligible loss of calcium ions since the Ca-Sb precipitate displays very low solubility and translocation of precipitates is unlikely because of their crystalline nature. The basis of this reaction is that under the appropriate fixation conditions potassium-antimonate infiltrates the neutrophil, followed by the replacement of potassium ions by calcium ions to form an insoluble electron dense calcium-pyroantimonate precipitate. (42 - 44) The employment of phosphate in combination with pyroantimonate is said to result in the visualisation of acidic phospholipid-bound calcium by formation of a stable complex between inorganic phosphate, calcium and acidic phospholipids. The acidic phospholipids are retained on the cytoplasmic side of the plasma membrane, therefore this phosphate-based procedure principally localises calcium on the cytoplasmic side of the plasma membrane. The subsequent addition of antimonate results in the conversion of the preformed complex into an insoluble calcium-pyroantimonate precipitate so that during the subsequent preparatory steps dissolution or displacement of the complex is prevented. (42, 45)

The oxalate anion is relatively specific for calcium in the presence of physiologic concentrations of other biological cations and therefore can be employed to retain calcium during primary fixation. With the addition of potassium-pyroantimonate during post-fixation the electron-dense precipitate is formed. (40)

Free cellular calcium is not likely to be responsible for a significant fraction of the precipitates subsequently formed as a result of antimonate binding. The low concentration of free calcium in most cells being at or below 10^{-6} M is at or below the threshold for antimonate precipitation, especially when phosphate buffer is employed and most is expected to be lost with other soluble cations during aqueous processing steps. The remaining calcium making up the vast bulk of the cell's total calcium exists in subcellular compartments with differing calcium affinities and degrees of exchangeability. Some of this calcium is unable to react with antimonate. One such form of calcium that probably does not react with antimonate is the insoluble amorphous tricalcium phosphate $Ca_3(PO_4)_2$ found in mitochondria. This has led to the suggestion that the antimonate reaction product represents only more loosely bound exchangeable calcium populations. (41)

It is known that antimonate has a high affinity for various intracellular cations, and that changes in reaction parameters strongly influence the retention and precipitation of physiological cations relative to each other. Nevertheless, the careful choice of reaction conditions can make the phosphate-antimonate technique highly selective for calcium in comparison to other cations that are capable of precipitation. (41) Following are a few important findings from investigators concerning in situ calcium precipitation by the antimonate anion.

- Theoretically, glutaraldehyde fixation preserves more of the cytoplasmic proteins than the other available fixatives, therefore a

greater amount of calcium ions should be retained since many cytoplasmic proteins bind calcium.

- The importance of employing glutaraldehyde as the primary fixative followed by osmium tetroxide post-fixation was discussed in the previous section. In many intracellular calcium localisation studies, cells fixed directly in the potassium-pyroantimonate/osmium reagent usually are poorly preserved when compared to those exposed to glutaraldehyde prior to the potassium-pyroantimonate/osmium reagent. (46) In addition to the morphological preservation superior to that seen with osmium-antimonate or antimonate alone, glutaraldehyde does not affect the antimonate-calcium precipitation thresholds. (41)
- Employing the preferred above-mentioned regime; primary fixation with glutaraldehyde followed by osmium-antimonate post-fixation only results in the precipitation of calcium during the osmium tetroxide post-fixation step and therefore can result in the translocation of calcium during the prior preparatory steps. Nevertheless, there are indications of almost complete retention of calcium during a 0.1 M phosphate-buffered pH 7.2-8.0 glutaraldehyde fixation, although antimonate was used only during osmium post-fixation. This suggests that in the absence of antimonate, phosphate-buffered glutaraldehyde effectively retains calcium in situ until it can be precipitated and made visible by osmium-antimonate. Fixation with unbuffered glutaraldehyde totally eliminated the subsequent formation of antimonate deposits, thus buffer components and not glutaraldehyde appear to be responsible for retaining calcium ions during primary fixation. (41)
- To accomplish maximisation of the precipitation and retention of calcium versus other cations, thus circumventing the non-specific nature of the antimonate precipitation technique the phosphate anion can aid in the precipitation of mobile calcium ions and therefore can enhance the specific localisation of calcium. This possibly by decreasing the sensitivity of antimonate for sodium and potassium ions. It is indicated

that phosphate inhibits the reaction with sodium, potassium and calcium both *in vitro* and in situ but apparently does so differentially, severely restricting precipitation of monovalent ions while only slightly reducing formation of calcium-antimonate precipitates. The employment of a phosphate buffer therefore results in the relative specificity for precipitation of the cell's divalent cations. Thus, while total numbers of intracellular deposits may be lower due to reduction in potassium and sodium precipitation, the precipitates present predominantly contains calcium.

- If antimonate-reactive cations are mainly those bound loosely enough to cellular components to allow antimonate to displace the component we might expect the bound half of total magnesium to precipitate, however in most instances it appears that nearly all magnesium is mobilised and lost during fixation. (41, 47)
- Even if a significant amount of these interfering cations are retained they form relatively soluble sodium, potassium and magnesium antimonate salts which can be removed by rinsing the neutrophil pellet in a non-antimonate containing solution prior to dehydration. (42)

3.1) Experimental evaluation of different neutrophil preparation procedures for TEM suitable for intracellular calcium localisation

In search of a method

a) which would produce acceptable neutrophil micrographs
b) which could be used on Hanks isolated neutrophils (the medium in which the neutrophils were suspended after isolation for the fluorescence techniques)
c) which could be used as basis for the subsequent intracellular calcium localisation.

3.1.1) Methods

- **Isolation of the neutrophils**

The neutrophils were isolated from ACD-anticoagulated (acid citrate dextrose) whole blood according to a modification of the method of Böyum. (26, 27) In short, ACD-anticoagulated whole blood was loaded onto a layer of hypaque-ficoll. The various blood cells were separated into different layers according to densities upon centrifugation. The polymorphonuclear leucocytes formed a layer on top of the red cell mass in the bottom of the tube. The supernate was discarded. The contaminating red cells were lysed with a 0.25 mM EDTA containing ammonium chloride solution. The polymorphonuclear leucocyte layer contained the neutrophils, eosinophils and basophils. Since eosinophils contribute only 0.5-6% and basophils only 0-1% of the total amount of circulating white blood cells (28) no further separation of the neutrophils were performed from the other polymorphonuclear leucocytes. Finally the neutrophils were washed and suspended in Hanks balanced salt solution.

- **Formation of the neutrophil pellet suitable for preparation for TEM**

A volume of 1 ml of the neutrophil cell suspension was pipetted into an eppendorff. A neutrophil pellet was subsequently formed by centrifugation at 2000 r.p.m. for 5 minutes. The supernatant was discarded.

- **TEM preparation procedure**

Four different protocols were designed and tested – (refer to figure 29)

In an effort to determine the most successful neutrophil preparation procedure for transmission electron microscopy suitable for intracellular calcium localisation, primary fixation by 2.5% glutaraldehyde followed by 1% osmium tetroxide post-fixation was employed in all the variations

Neutrophil EM Adaptation 1a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in 0.1 M PBS 1 hr (room temp)
Rinse 3x 0.1 M PBS
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x 0.1 M PBS

Dehydration: 15 min/concentration
1st alcohol=50% ethanol
2nd alcohol=50% ethanol + 1.5% uranyl acetate
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 1b

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in 0.1 M PBS 1 hr (room temp)
Rinse 3x 0.1 M PBS
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x 0.1 M PBS

Dehydration: 15 min/concentration
1st alcohol=50% ethanol + 1.5% uranyl acetate
2nd alcohol=50% ethanol
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 2a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in Sabatini's solution (0.1 M PBS with 6.8% sucrose) 1 hr (room temp)
Rinse 3x Sabatini's solution
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x Sabatini's solution

Dehydration: 15 min/concentration
1st alcohol=50% ethanol
2nd alcohol=50% ethanol + 1.5% uranyl acetate
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 3a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in Hanks 1 hr (room temp)
Rinse 3x Hanks
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x Hanks

Dehydration: 15 min/concentration
1st alcohol=50% ethanol + 1.5% uranyl acetate
2nd alcohol=50% ethanol
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Figure 29. Protocols for the neutrophil transmission electron microscopy preparation procedures

of the preparation procedure. Within this framework different adaptations were made concerning the vehicle for the primary fixative. A volume of 1 ml of the various fixatives was pipetted into the eppendorff containing the neutrophil pellet. Primary fixation of the neutrophil pellet was carried out for 1 hour, whereafter the neutrophil pellet was washed 3 times in the appropriate buffer. The supernate was discarded and a volume of 1 ml of the post-fixative was pipetted into the eppendorff containing the neutrophil pellet. Post-fixation of the neutrophil pellet was carried out for 1 hour, whereafter the neutrophil pellet was washed 3 times in the appropriate buffer. The supernate was discarded and the dehydration procedure started. Dehydration was performed in a range of ethanol solutions. The range included; 50%, 70%, 95% and 100% ethanol solutions. Dehydration in the various ethanol solutions was carried out for 10 minutes. After the final dehydration step the 100% ethanol solution was changed with fresh 100% ethanol solution. The dehydrated neutrophil pellets were left in this 100% ethanol and transported to the Electron Microscopy Unit, UP. The rest of the preparation procedure was conducted at the EM unit. Following dehydration the neutrophil pellet was embedded in a Quetol epoxy resin. (48) An equal volume of Quetol was added to the 100% ethanol solution in the eppendorff containing the neutrophil pellet. The neutrophil pellet was left in this 50:50 mixture of 100% ethanol and Quetol for a period of 1 hour. The supernate was discarded and replaced with a solution of Quetol only and left overnight. The following morning the supernate was discarded and replaced with fresh Quetol. The eppendorff containing the neutrophil pellet in Quetol was placed in an oven at 60°C for 6 hours. The thermal energy allowed the Quetol to polymerise and form a hard resin suitable for sectioning. The resin blocks containing the neutrophils were sectioned, stained and viewed. The sections were stained with both uranyl acetate and lead citrate. Uranyl acetate staining enhances the general contrast of the sections

especially structures that contain nucleic acids and ribosomes. (38) Whereas, lead citrate staining emphasises the cell membrane and glycogen particles most effectively. (38)

3.1.2) Results

Examples of the micrographs obtained can be seen in Figure 30.

1) General

- All 4 methods resulted in preservation of the ultrastructural features adequate to allow continuation of the establishment of the calcium localisation method.

2) Characteristic features of the neutrophil as visualised with the transmission electron microscope

The neutrophil displayed various characteristics, which distinguished the neutrophil from other blood cells.

- The nucleus is lobulated, and these segmented lobules are held together by thin filaments. These filaments are made up of dense chromatin and they are enclosed, like the rest of the nucleus, by the different membranes of the nuclear envelope. As a general rule the more segmented the nucleus, the more mature the neutrophil. The chromatin is very dense and made up of very dark masses of heterochromatin separated by smaller bands of paler euchromatin. These extremely condensed patches of heterochromatin are often discontinuous at the nuclear membrane at points that correspond to the nuclear pores. No trace of a nucleolus remains, which seems to indicate that protein synthesis has ceased. (49)
- Ribosomes are scant and little or no endoplasmic reticulum is to be found.

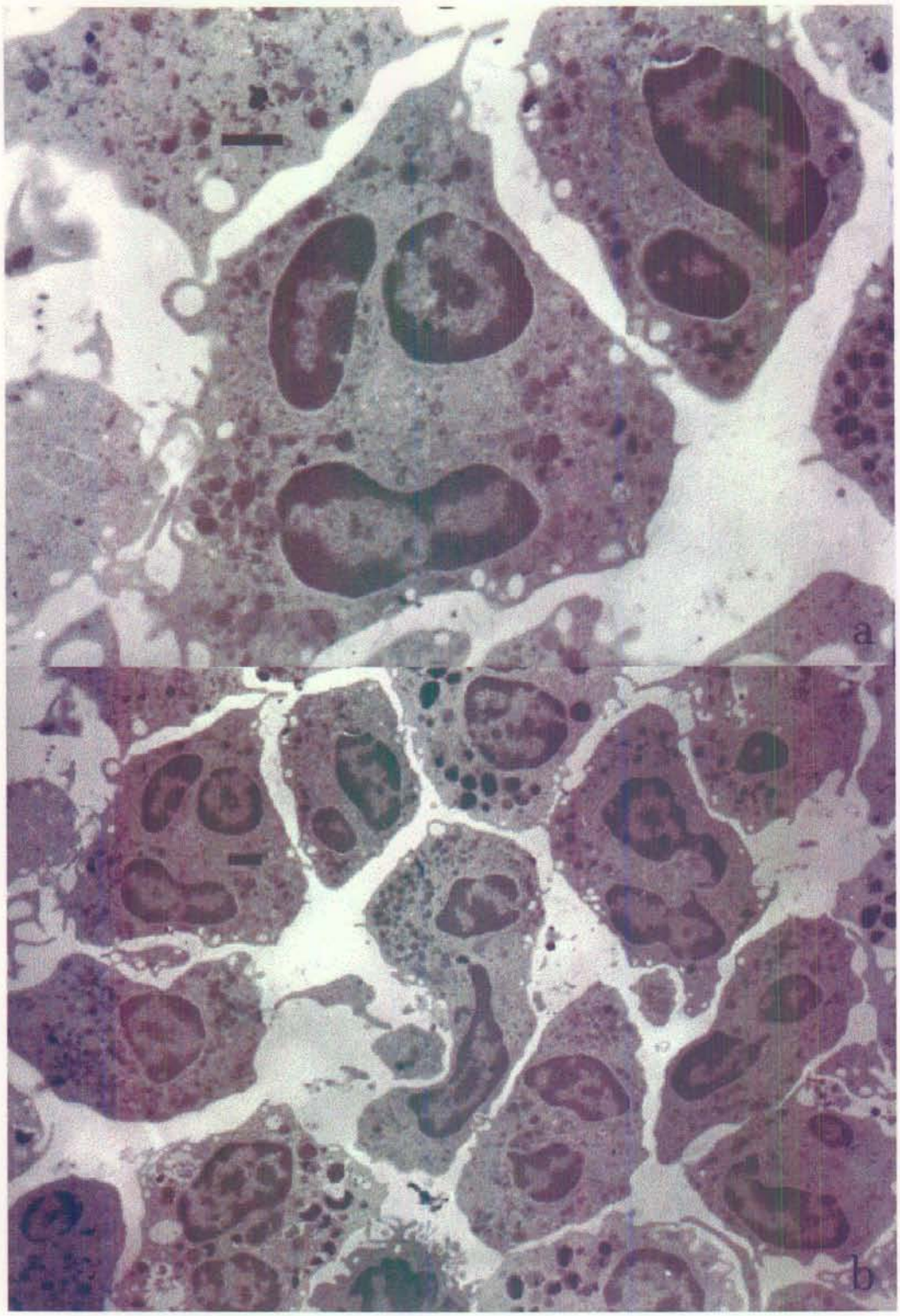

Figure 30 a and b. Electron microscopy photographs indicating suitable TEM neutrophil preparations for intracellular calcium localisation studies. Scale bar 1 μm.

- The Golgi body is inactive and forms a small sphere in the neutrophil center.
- The neutrophil center contains the two centrioles from which many microtubules emanate.
- Mitochondria are few, small and often elongated.
- A large number of glycogen particles, often quite large in size are present which serve as the major energy source for active anaerobic metabolism during phagocytosis.
- Distinctive cytoplasmic granules are present in the cytoplasm of the neutrophil. These granules include: primary granules or azurophilic granules which are large and round, secondary granules or neutrophilic granules which are elongated with an internal crystal structure, and tertiary granules which are small and often dumbbell shaped. The number of granules were variable, even in the strictly normal state, one may see neutrophils with few or no granules and others with numbers of granules which appeared to be distinctly more than in a average cell. (49)
- Fatty vacuoles are round and grayish in color. (39, 49, 50)

3.1.3) Discussion

There are contradictory indications in the literature concerning the employment of glutaraldehyde as primary fixative. On the one hand investigators claim that the effect of differences in the composition of the vehicle are more apparent in aldehyde fixatives. On the other hand since the reaction rate of glutaraldehyde with the cellular constituents is so fast the choice of the composition of the vehicle are not so important. Employment of glutaraldehyde as a primary fixative in the above mentioned vehicles with different compositions concerning osmotically active constituents, resulted in the adequate preservation of ultrastructural detail in all situations demonstrating that the choice of

the composition of the vehicle within limits during glutaraldehyde fixation is not so critical. (39, 49, 50) The morphology of granules are said to be influenced by a variety of technical factors, it is not uncommon for the water-soluble contents of the granules to be lost during primary fixation. The degree of granule content extraction can be influenced by factors including the nature and concentration of the fixative, the duration of the fixation, section thickness, and the method of staining of the sections. Considerable variation was seen in granule content extraction between these various preparation procedures.

3.2) Experimental evaluation of the calcium precipitation reaction: phosphate-pyroantimonate and oxalate-pyroantimonate reactions

3.2.1) Methods

- **Neutrophil isolation**

The neutrophils were isolated as previously described. The only difference being the omitting of the 0.25 mM EDTA from the ammonium chloride solution in some of the procedures. This in order to determine if the addition of EDTA during the isolation procedure could affect the calcium distribution patterns.

- **Formation of the neutrophil pellet suitable for preparation for TEM**

The neutrophil pellet was prepared for TEM as previously described.

- **TEM preparation procedure for the localisation of intracellular calcium**

Six adapted protocols were designed and tested for the localisation of intracellular calcium – (refer to figure 31).

Calcium localisation Adaptation 1a

Neutrophil isolation
+ EDTA

Fix 2.5% GA in 0.1 M NaPBS 1 hr (room temp)
Rinse 3x 0.1 M NaPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M NaPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 1b

Neutrophil isolation
- EDTA

Fix 2.5% GA in 0.1 M NaPBS 1 hr (room temp)
Rinse 3x 0.1 M NaPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M NaPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 2a

Neutrophil isolation
+ EDTA

Fix 2.5% GA in 0.1 M KPBS 1 hr (room temp)
Rinse 3x 0.1 M KPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M KPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 2b

Neutrophil isolation
- EDTA

Fix 2.5% GA in 0.1 M KPBS 1 hr (room temp)
Rinse 3x 0.1 M KPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M KPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Calcium localisation Adaptation 3a

Neutrophil isolation
+ EDTA

Fix 2.5% GA, 90 mM potassium oxalate 1 hr (room temp)
Rinse 3x 90 mM potassium oxalate, 7.5% sucrose
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x alkalinised H_2O pH 10

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Calcium localisation Adaptation 3b

Neutrophil isolation
- EDTA

Fix 2.5% GA, 90 mM potassium oxalate 1 hr (room temp)
Rinse 3x 90 mM potassium oxalate, 7.5% sucrose
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x alkalinised H_2O pH 10

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Figure 31. Protocols for the intracellular calcium localisation procedures

The first part of the evaluation procedure was conducted in order to obtain a suitable preparation procedure for neutrophils for TEM. The most suitable preparation procedure is now employed for the localisation of intracellular calcium via TEM. As discussed all 4 the tested preparation procedures gave satisfactory neutrophil micrographs. Nevertheless, the preparation procedure containing phosphate buffered saline (Adaptation 1a or 1b) was the method of choice. The reason being that phosphate ions influence the pyroantimonate reaction; possibly making the reaction more specific for calcium. The optimal reaction conditions for the pyroantimonate reaction was discussed previously.

- **Control reactions**

In order to determine the composition of the formed pyroantimonate precipitates; whether the precipitates contain calcium or another cation, the sections were left in the presence of 0.2 M EDTA pH 8.4 at 60°C. Dissolution of the precipitates would indicate calcium as the major cation contained in the precipitates.

3.2.2) Results

Examples of the obtained micrographs for the intracellular calcium localisation in the neutrophil can be seen in Figure 32.

1) General

There are indications that the use of EGTA or EDTA prior to or during fixation results in the loss or diminution of deposits that had been shown by X-ray analysis to contain calcium. A 0.25 mM EDTA ammonium chloride solution is employed during neutrophil isolation therefore the effect of the use of EDTA on the magnitude of calcium precipitate formation was investigated. Refer to the various protocols, however no difference was found in the magnitude of precipitate formation in the absence or presence of EDTA.

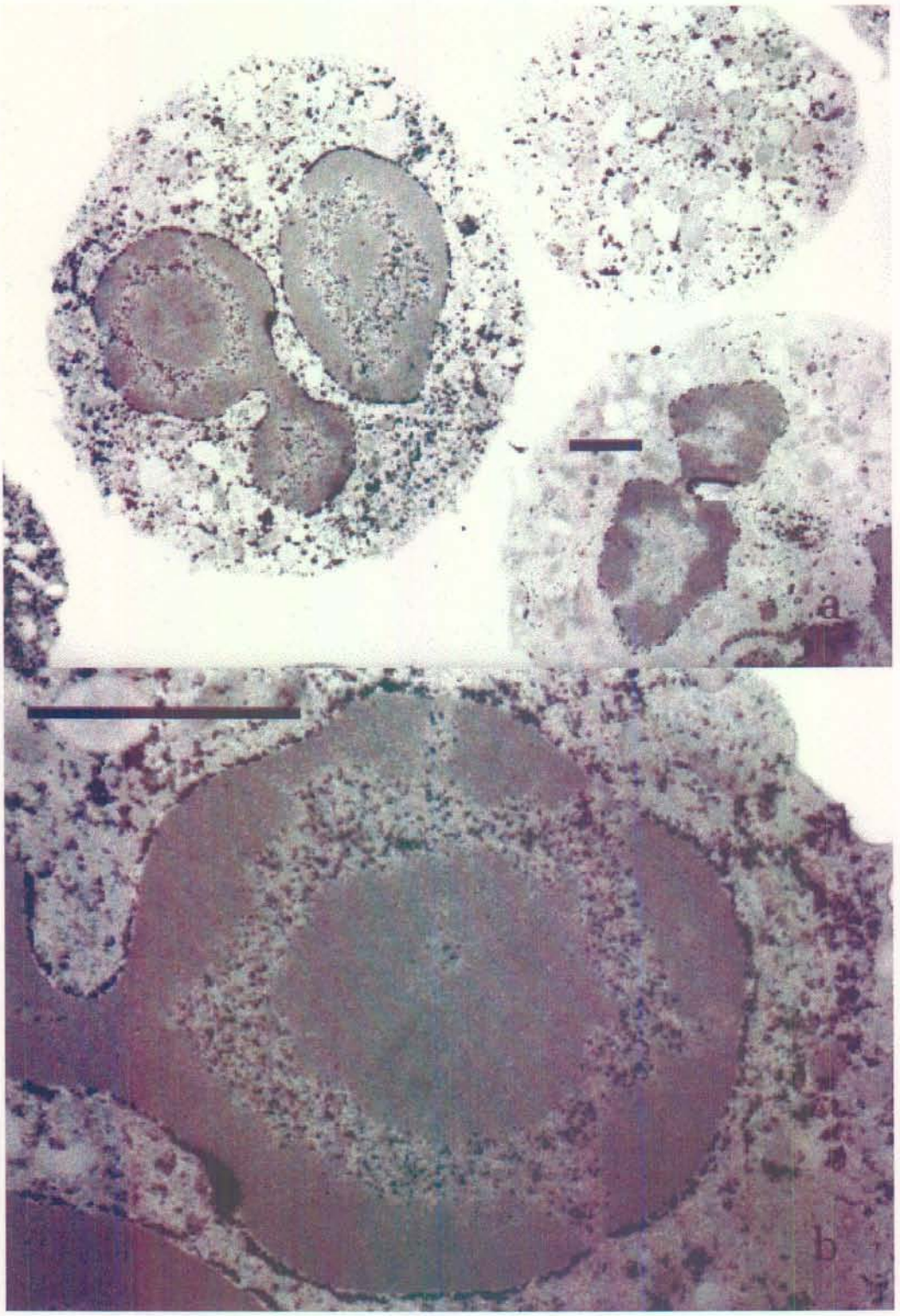

Figure 32 a and b. Electron microscopy photographs indicating calcium-pyroantimonate precipitate formation in neutrophils. Scale bar 1 μm.

2) Calcium distribution patterns obtained with phosphate-pyroantimonate and oxalate-pyroantimonate calcium precipitation reactions

Three different antimonate precipitation protocols were investigated. Refer to figure 31. In adaptation 1 and 2 the phosphate anion was employed to retain calcium, whereas in adaptation 3 the oxalate anion was employed to retain calcium. Only a few precipitates were noticed with the employment of the oxalate anion, indicating unsatisfactory reaction conditions during primary fixation. With the employment of the phosphate-pyroantimonate procedure various calcium precipitation patterns were obtained, and a significant difference was noticed between the employment of either the sodium or potassium phosphate salts. Employment of the sodium phosphate salt resulted in fewer calcium precipitates compared to the employment of the potassium phosphate salt.

3) Control reactions

During a control reaction, a 0.2 M EDTA solution pH 8.4 was employed to demonstrate the presence of calcium in the antimonate precipitation products. Sections were left in the presence of this EDTA solution for 1 hour at 60°C. This resulted in the complete disintegration of the precipitates and the formation of voids, indicating that mostly calcium was present in the antimonate-precipitates. Examples of the micrographs obtained after the removal of the precipitates can be seen in Figure 33.

3.2.3) Discussion

Various calcium distribution patterns were obtained for both the phosphate pyroantimonate procedures (potassium and sodium) except that fewer precipitates were detected for the sodium phosphate salt. The difference in the magnitude of precipitate formation between

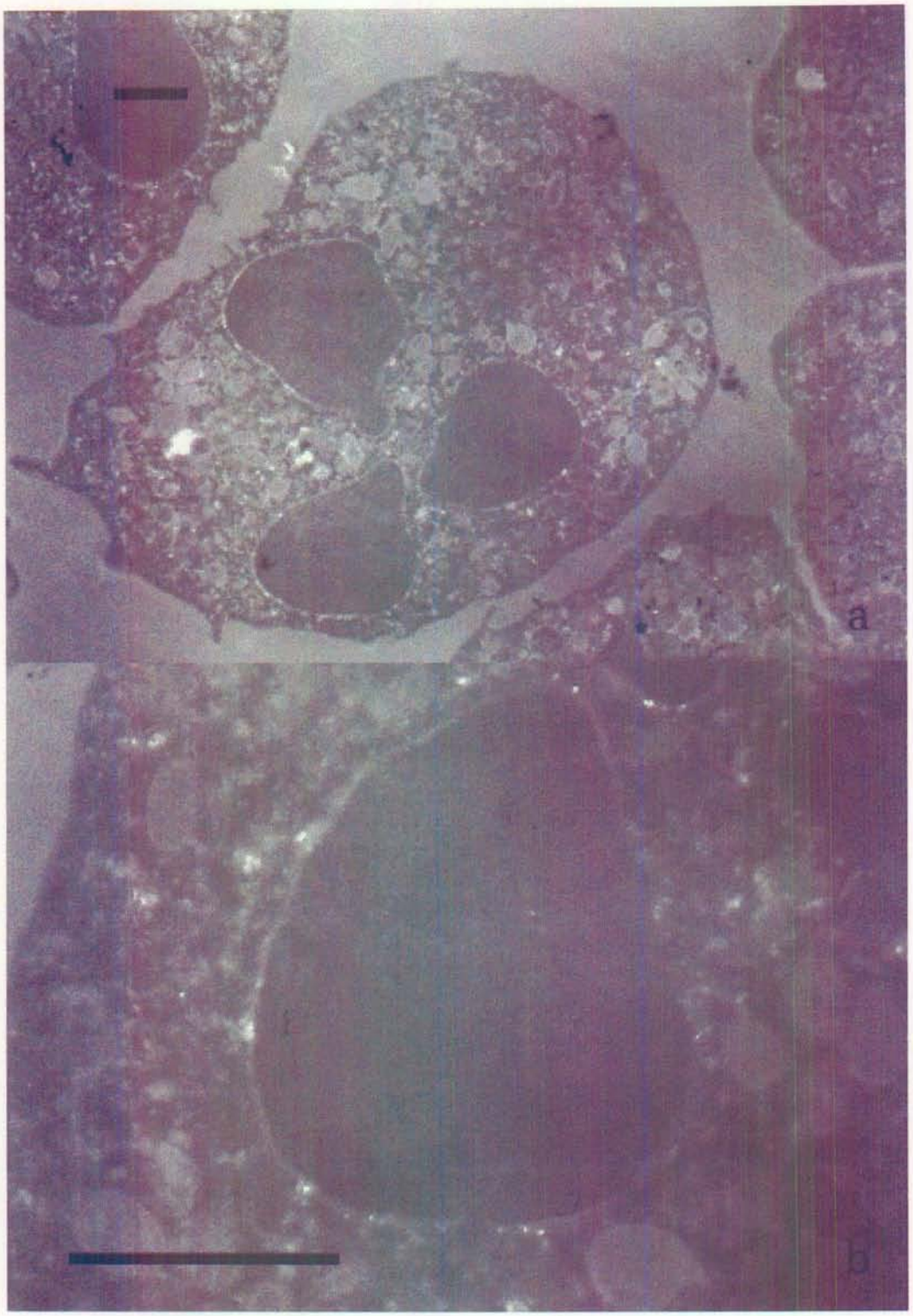

Figure 33 a and b. Electron microscopy photographs indicating the complete disintegration of the calcium-pyroantimonate precipitates and the subsequent formation of voids upon EDTA addition in neutrophils. Scale bar 1 μm.

employment of either the sodium phosphate salt or the potassium phosphate salt might be the result of displacement of calcium by sodium since antimonate has a high affinity for sodium. Various calcium distribution patterns were obtained. Refer to figure 32. These very distinctive calcium precipitation patterns included: 1) pronounced precipitate formation around the nucleus of the neutrophil coinciding with the localisation of the calcium store in the nuclear envelope, 2) precipitate formation in the nucleus as part of the less dense euchromatin, this precipitate pattern projects outward to the nuclear envelope coinciding with the nuclear pores, and 3) throughout the cytoplasm scattered precipitates were noticed. (43, 51) One would expect calcium also to be associated with sites of high DNA content, heterochromatin staining would be attributable to calcium bound to nucleic acids as well as to reactive amino acids on histones. Nevertheless, the present results appear to indicate that the condensed chromatin in the nucleus is largely free of calcium ions. The presence of chelated calcium ions cannot be ruled out since they will not yield insoluble pyroantimonate salts. (45, 46) From these results it was apparent that different neutrophils, even adjacent to one another display variations in the magnitude of calcium precipitate formation. This might be a manifestation of the heterogeneity of calcium homeostasis in different neutrophils and also various neutrophils might be captured at different time-points during various calcium regulated cellular processes.

The chelators EGTA and EDTA are frequently used to demonstrate the presence of calcium in antimonate precipitation products. Monovalent cation-antimonate salts will not be removed by chelators, magnesium-antimonate will remain essentially unaffected by EDTA and will be only slightly diminished by EGTA, while calcium-antimonate deposits will almost totally be abolished by either chelator. (42, 47) With the

employment of a 0.2 M EDTA solution pH 8.4 to demonstrate the presence of calcium in the antimonate precipitation products, complete disintegration of the precipitates resulted.

3.3) Conclusions

The optimised protocol is presented.

Neutrophil isolation

As previously described, refer to page 105.

Calcium localisation for TEM

1) Pipet a volume of 1 ml of the neutrophil suspension into an eppendorff.
2) Centrifuge at 2000 r.p.m. for 5 minutes.
3) Discard the supernatant.
4) Pipet 1 ml of the 2.5% glutaraldehyde, 0.1 M KPBS, room temperature into the eppendorff containing the neutrophil pellet.
5) Incubate 1 hour at room temperature - primary fixation.
6) Discard the supernatant.
7) Wash 3x with 0.1 M KPBS.
8) Discard the supernatant.
9) Pipet 1 ml of the 1% OsO_4, 2% $KSb(OH)_6$, room temperature.
10) Incubate 1 hour at room temperature - post-fixation.
11) Wash 3x with 0.1 M KPBS.
12) Discard the supernatant.
13) Add 1 ml of a 50% ethanol solution.
14) Incubate for 10 minutes, room temperature.
15) Discard the supernatant.
16) Add 1 ml of a 70% ethanol solution.
17) Incubate for 10 minutes, room temperature.
18) Discard the supernatant.

19) Add 1 ml of a 95% ethanol solution.
20) Incubate for 10 minutes, room temperature.
21) Discard the supernatant.
22) Add 1 ml of 100% ethanol.
23) Incubate for 10 minutes, room temperature.
24) Discard the supernatant.
25) Add 1 ml of 100% ethanol.
26) Add 1 ml of a Quetol epoxy resin.
27) Incubate 1 hour at room temperature.
28) Discard the supernatant.
29) Add 1 ml of a Quetol epoxy resin.
30) Incubate overnight at room temperature.
31) Discard the supernatant.
32) Add 1 ml of a Quetol epoxy resin.
33) Incubate 6 hours at 60°C.
34) Section.
35) Stain uranyl acetate and lead citrate.

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