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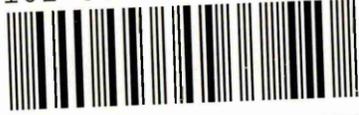
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ProQuest Number: 10701241

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# The effects of zinc compounds on inflammatory and gastric cells

A Thesis

presented by

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Submitted in partial fulfilment of

the requirements for the degree of

Doctor of Philosophy

At

# Sheffield Hallam University

School of Science and Mathematics,

Sheffield Hallam University,

December, 2000

# Abstract

Exogenous zinc has been shown to alter the cellular response to inflammatory conditions. Both common zinc salt and novel zinc compounds have been shown to be therapeutic in various disease states. However, little is known about their action on immune cells in these conditions. This thesis aims to investigate the effectiveness of zinc monoglycerolate (ZMG), a novel zinc compound, in providing bioavailable zinc. This study also aims to examine the effect of zinc on the inflammatory responses of immune and gastric cells.

Using cell culture techniques, fluorescence microscopy and inductively coupled plasma mass spectroscopy (ICP-MS), comparison was made of zinc bioavailability from ZMG, zinc oxide and zinc sulphate. The cellular protein interactions of the zinc provided by these compounds was investigated by autoradiography, Western blot analysis and cytokine assay. As a result of the biological study, chemical analyses were carried out on ZMG using high performance liquid chromatography (HPLC), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and matrix assisted laser desorption ionisation time of flight (MALDI-ToF).

The result of the cell culture study indicated that ZMG and zinc oxide provided significantly more bioavailable zinc than zinc sulphate. Furthermore, these results showed that the different cell lines examined treated the zinc in distinctly different manners. The studies on direct zinc-protein interactions did not conclusively determine whether such interactions occurred between cytoplasmic proteins and exogenous zinc. However, zinc was shown to modulate cytokine secretions *in vitro* in cultured cell lines. The chemical analyses provided novel observations about the polymeric structure of ZMG and its solubility profile.

In conclusion the results showed that ZMG is a highly effective source of bioavailable zinc and that exogenous zinc can modulate the immune response of cultured cell lines to external stimuli.

# Acknowledgements

## Communications and Publications

1. Zeitlin B.D. and Rainsford K.D. (1998), Cellular uptake profile for zinc monoglycerolate (ZMG) in immune and gastric cell lines determined by fluorescence photomicrography and inductively coupled plasma mass spectrometry (ICP-MS), Royal Microscopical Society – Proceedings. 33 (2) p188.

2. International Conference on Inflammopharmacology and VIth Side-Effects of Anti-Inflammatory Drugs Symposium, Braselton, Georgia, USA, 23-26 May, 1999.

   Zinc monoglycerolate (ZMG) and zinc salt cellular uptake profile *in vitro* determined by plasma mass spectrometry.

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# Glossary

| | |
|---|---|
| 2,5-DHB | 2,5 – dihydroxybenzoic acid |
| ANOVA | Analysis Of Variance |
| BCA | Bicinchoninic acid |
| CRIP | Cysteine Rich Intestinal Peptide |
| DCT-1 | Divalent cation transporter - 1 |
| (sf)DME | (serum free) Dulbecco's Modified Eagle's (medium) |
| ELISA | Enzyme Linked Immunosorbant Assay |
| EMSA | Electrophoretic Mobility Shift Assay |
| FITC | Fluorescein Isothiocyanate |
| HBSS | Hank's Buffered Saline Solution |
| HPLC | High Pressure Liquid Chromatography |
| ICP-AES | Inductively Coupled Plasma – Atomic Emission Spectroscopy |
| ICP-MS | Inductively Coupled Plasma – Mass Spectroscopy |
| IFN-$\gamma$ | Interferon-Gamma |
| IL (-1$\beta$, -2) | Interleukin –1beta, -2 |
| I$\kappa$B | NF$\kappa$B inhibitory protein |
| kDa | kiloDalton |
| LPS | Lipopolysaccharide |
| MALDI-ToF | Matrix Assisted Laser Desorption Ionisation – Time of Flight |
| NF$\kappa$B | Nuclear Factor kappa B |
| PAF | Platelet aggregating factor |
| PBM(C) | Peripheral Blood Mononuclear (cells) |
| PBS | Phosphate Buffered Saline |
| PG($E_2$, I) | Prostaglandin $E_2$, I |
| PMA | Phorbol Myristic Acetate |
| ppb | Parts per billion |
| ppm | Parts per million |
| (sf)RPMI | (serum free) Roswell Park Memorial Institute (medium) |
| SDS-PAGE | Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis |
| TBS | Tris Buffered Saline |
| TLC | Thin Layer Chromatography |
| TNF-$\alpha$ | Tumor Necrosis Factor - alpha |
| UV | Ultraviolet |
| WST-1 | Tetrazolium salt used to measure cell proliferation |
| ZMG | Zinc Monoglycerolate |
| ZNT | Zinc transporter |

# A brief history of zinc and its uses in medicine

Zinc has been used since Roman times for metal working where, as a contaminant of copper compounds, it was a constituent of brass alloys however it was not known as a metal in its own right. It appears possible that metallurgists in India, around the 13th century, may have developed metal purification methods that allowed the isolation of the metal itself and it is thought that the knowledge of metal work came from India, via China, to Europe (Encyclopaedia Britannica). Certainly, zinc does not seem to have been known as a specific metal in Europe until it was separated from zinc rich slag metal, a by product of the silver smelting industry, by Erasmus Ebener of Nurnberg in 1509 (Encyclopaedia Britannica). Around the same time, Paracelsus (original name - Theophrastus Bombastus Von Hohenheim) a Swiss physician and alchemist, first used the recorded name of *zinken*, for a metal which he thought was a contaminated form of copper. Paracelsus turned his knowledge of chemicals to the treatment of his patients and he first described the usage of zinc compounds in texts on pharmaceutical preparations (Hoogenraad, 1998). However, it was not until the eighteenth century that zinc was purified and described as a separate element, probably by Johan Friedrich Henckel or Andreas Sigismund Marggraf (Colliers Encyclopaedia). Shortly after the isolation and description of this 'newly' isolated element, a paper was written in 1771 by Gaubius, a Professor of medicine and chemistry in the University of Leiden, which described the effect of zinc oxide in curing convulsions in a child (Hoogenraad, 1998). By the eighteen hundreds, high concentrations of zinc were being used as an anti-epileptic and emetic. Gradually the use of zinc oxide as an anti-epileptic was reduced leaving zinc being used pharmaceutically mainly for emetic purposes (Hoogenraad, 1998).

Interest in zinc revived as in 1869 Raulin showed that zinc was essential for the growth of the fungus Aspergillus Niger (Reviewed Prasad, 1991). This was shown to be true also for higher plants in 1926 by Sommer and Lipman, for rats in 1934 by Todd et al. and in 1958 zinc was also shown to be essential for growth in chickens by O'Dell et al. (Prasad, 1991).

It was not until relatively recently that zinc was discovered to effectively and consistently treat a specific disease state in humans and the first of these was

Wilson's disease. In 1961 Schouwink showed that oral zinc was able to reverse the effects of this genetic defect in copper metabolism (Grüngreiff and Reinhold, 1997). Wilson's disease results in a toxic build up of copper in the patients and it was later shown that zinc administration caused the appearance of large amounts of copper in the faeces indicating a direct cause and effect between zinc administration and excess copper excretion (Grüngreiff and Reinhold, 1997).

Around the same time, in 1963, Prasad and co-workers first described the symptoms of zinc deficiency and attributed them to lack of zinc as opposed to, for example, parasitic infection (Prasad, 1991). They focussed on populations where growth retardation was prevalent, particularly in Egypt, and showed that the general failure to thrive in the patients was due to zinc deficiency as a result of dietary insufficiency. The diet was low in animal protein and high in cereals. Previously O'Dell and Savage (1960) had shown that phytate (inositol hexaphosphate), a constituent of cereal grains, was able to inhibit the absorption of zinc. Supplementation of the diet with zinc reversed many of the symptoms of growth retardation in the Egyptian patients (Prasad, 1991). And so began the study of zinc as an essential micronutrient for humans.

As it was realised that zinc was an integral requirement for nutrition the study of zinc biology expanded enormously. Zinc was found to be associated with an increasing number of biological molecules involved in metabolism. From 1940 and for approximately the following twenty years, only five biological molecules containing zinc were identified (Prasad, 1991). The first of these was carbonic anhydrase which Keilin and Mann showed contained zinc, and who further showed that the zinc was essential for the enzymatic function of the molecule (Prasad, 1991). It would now be difficult to put a precise figure on the number of metalloproteins that require zinc for structure and function as new molecules are still being identified and with the publication of the human genome, it is likely that even more zinc binding proteins will be identified. For enzymatic reactions alone there are now known to be over 300 molecules requiring zinc for function (Kruse-Jarres, 1997).

However, not all protein bound zinc is equal, it was realised that the role of zinc atoms in metalloenzymes could be divided into three general areas, catalytic zinc,

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co-catalytic zinc and structural zinc (Vallee and Falchuk, 1993). Catalytic zinc was shown to have a direct role in the enzyme function and was essential for that function, where zinc was able to enhance or diminish enzymatic activity but was not absolutely required, it was defined as co-catalytic zinc. Structural zinc atoms played no part in the enzymatic reaction itself but were required for structural stability of the enzyme (Vallee and Falchuk, 1993).

In general zinc is found co-ordinated in these metalloenzymes by a few specific ligands. Cysteine and histidine are the two main peptide ligands in zinc metalloenzymes and, in molecules where zinc is catalytic, a water molecule is often bound to the zinc atom.

Molecules other than enzymes bind zinc. Metallothionein, a cysteine rich protein, was identified as a cadmium and zinc-binding molecule in 1957 (Vallee and Falchuk, 1993). The functions and zinc binding activity of the molecule are discussed later in this chapter. Whilst metallothioneins are the main storage molecules for intracellular zinc there is another class of zinc molecule that was only discovered in approximately the last fifteen years. The zinc finger proteins were first proposed by Klug in 1985 as DNA binding molecules that controlled transcription and that required zinc atoms for binding co-ordination (Rhodes and Klug, 1993). The first of these, Transcription Factor III A (TFIIIA), was discovered during a search to understand how a relatively small protein could be associated with a particularly long piece of DNA as it appeared to have a rather large binding site on the DNA strand (Rhodes and Klug, 1993). It was shown that the zinc fingers fitted into the grooves of the DNA double helix and the flexibility of the zinc finger transcription factors allowed them to stretch along the DNA strand with successive zinc fingers making contact along it (Rhodes and Klug, 1993). This was an unknown ability for transcription factors, which had previously been thought to have only one DNA binding site per molecule.

The breadth of action of zinc fingers, and other related zinc co-ordinated peptide structures such as clusters and rings has grown to encompass protein-protein binding in addition to DNA binding. LIM domain proteins contain zinc-binding sequences that can mediate protein complex formation. Schmeichel and Beckerle (1994) showed that the three LIM domains in the cytoskeletal protein

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zyxin were required to bind another cytoskeletal protein cysteine-rich protein (CRP). The protein binding and DNA binding activities of zinc fingers are neatly brought together in the form of the oestrogen receptor molecule. It was shown that the receptor binds DNA as a receptor dimer and that both the DNA binding and the stability of the dimer structure were determined by zinc finger interactions *with* the DNA and *between* the two receptor molecules (Rhodes and Klug, 1993).

The ubiquitous activities of both enzymes, transcription factor and signal transduction molecules indicate the importance of zinc in metabolism in particular and in organism survival in general. As the knowledge of the physiology of zinc is understood it is clear that zinc is essential for all aspects of homeostasis. For example any mechanism requiring cells to proliferate, such as tissue growth and repair, T-cell expansion as a result of immune challenge or just about any aspect of the reproductive cycle will require zinc for proper function. Due to its universal use around the body it has been increasingly apparent that even small changes in zinc levels that do not result in gross symptoms can have measurable and important effects (Sullivan and Cousins, 1997).

Zinc therapy for zinc deficiency and also for various disease states has taken great strides since its importance was realised. Many zinc compounds in which zinc is the active component have been developed, each with different properties, often improving zinc availability to the body by novel mechanisms (Fairlie et al., 1992, Rainsford and Whitehouse, 1992) or adding some additional properties to those of zinc alone (Navarro et al., 1988).

Prevention of zinc deficiency, understanding the role of zinc in health and disease states and designing therapeutic zinc compounds depend heavily on understanding how zinc is taken into the body. This chapter aims to examine and discuss the literature describing zinc uptake by the body, by various tissues and by individual cells. The therapeutic use of zinc in the disease state is then looked at in detail followed by an examination of zinc function in the immune system as it related to the thesis as a whole.

# Absorption and biodisposition of zinc

Little was known about the mechanism of zinc transport at the cellular level until quite recently which might seem unusual as both the dietary and therapeutic value of the element have been known experimentally and clinically for decades. Perhaps the sudden interest and intense investigation of cellular zinc uptake has been driven simply by the massive expansion of known involvement of zinc with protein components of so many biological systems or because of the ability shown for zinc to modulate other mediators of these systems. Another factor could be the increased number of therapeutic applications of zinc and the development of new zinc bearing drugs. It has also relatively recently been understood that both moderate to marginal chronic deficiencies of zinc are widespread in the industrialised countries (Mares-Perlman, 1995) and not only to countries traditionally associated with poor diet in third world countries (Prasad, 1961, Halsted, 1972). This has quite recently increased interest in understanding the biology of nutritional zinc. It should be pointed out that until recently, methods for determination of zinc deficiency were limited to clinical determination which was really only effective in moderate to severe disease and only relatively recent developments have allowed determination of marginal deficiency (King, 1990, Sullivan and Cousins, 1997). Whatever the reasons behind the increased study of zinc transport at the cellular and membrane level, it has led to a greater variety and increased sensitivity of zinc specific detection and labelling techniques.

Vital to the understanding of the functions and mechanics of both dietary and pharmacological zinc is knowledge of how zinc enters the body. The exact mechanism has not been described to date but several possibilities exist and are described in the following section.

## Kinetic Studies

Kinetic studies of zinc uptake using radiolabelled zinc ($^{65}$Zn) have been carried out for several decades and have looked at *in vivo*, *ex vivo* and *in vitro* preparations. These studies almost exclusively have described different kinetics or transport velocities with each method and preparation. They have also shown

that zinc uptake by the gastrointestinal tract is site specific. There is also dispute as to the site of greatest zinc absorption, although there is general consensus that the duodenum, jejunum and ileum absorb more zinc than the colon. This has been backed up by more recent studies of expression patterns for zinc binding molecules such as metallothionein and CRIP (cysteine-rich intestinal protein) in animals consuming zinc deficient and zinc adequate diets.

The majority of the work on zinc kinetics has been carried out on rats, either whole animal or tissue preparations as described in this following section. The kinetic studies have also shown that there are the characteristics of both facilitated (readily saturable) and non-mediated (not readily saturable) components of zinc uptake in the gastrointestinal tract (Davies, 1980, reviewed Albergoni, 1998). Methfessel and Spencer (1973) carried out studies on intact rats and ligated sections of rat intestine to look at both the kinetics of uptake and the sites of uptake. The animals received food and water ad libitum before and throughout the experiment, a factor that should be noted and will be discussed later. Rats were given a dose of $^{65}ZnCl_2$ by intubation. They were then maintained in a metabolic cage, to allow collection of urine and faeces, for varying times before sacrificing and removal of portions of the gastrointestinal tract. In the ligated intestinal sections, the dose of $^{65}Zn$ was placed directly into the sacs. The rats were anaesthetized in all *in vivo* experiments currently discussed. With the *in vivo* ligation the gastric blood supply was uninterrupted by the ligatures, so molecular transfer from gastric lumen via mucosal cytosol to the general circulation was unhindered. Tissue was measured for $^{65}Zn$ uptake by radioassay by gamma-scintillation counter, the samples included liver, heart, lungs, spleen, kidneys, femur, muscle and whole blood sample as well as the gastrointestinal sections. The results indicated that the stomach did not retain label for very long, 8% total $^{65}Zn$ after 4 hours. Very little zinc absorbed during this time was found as label in any of the major organs tested when compared to zinc absorbed by other ligated sections in the small intestine. A similar observation was made after zinc was introduced into the ligated colonic sacs. Although in the whole animal experiment zinc was present far longer in this organ than in either stomach or small intestine, only trace amounts of label was found in either blood or plasma or any of the major organs tested. However in the small intestine, 33% of total $^{65}Zn$ was found after 15 minutes, increasing to a maximal 65% within an hour. In

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addition to this, the ligated sacs of duodenum, mid-jejunum and ileum provided the majority of the percentage total label found in blood, plasma or other organs when compared to the uptake from the other gastrointestinal section. The duodenum provided the greatest percentage of labeled zinc to all organs compared to the mid-jejunum or ileum. What is particularly of note in this study is that non-fasted animals were studied and that the zinc absorption reached a plateau at 30 minutes and was maintained at this level (~25% total $^{65}$Zn) for the following 7.5 hours. The fasted and non-fasted state takes on greater importance with later studies showing both kinetic and intestinal protein differences between these two experimental conditions.

Seven years later the next oft quoted study was performed, which described similar site-specific uptake of isotopic zinc and went on further to describe two distinct phases of zinc uptake (Davies, 1980). In this study, Davies described experiments similar to those described previously by Methfessel and Spencer (1973), but they altered conditions to account for the passage of digesta and consequently zinc binding ligands and dietary zinc. It should be noted that in their study, Methfessel and Spencer (1973) acknowledge this factor. They point out that in the ligated small intestinal segments there were similar amounts of digesta and therefore similar amounts of dietary zinc (and, it may be expected, similar amounts of binding ligands) and that this should not be a variable factor in their uptake observations. Again, all rats were anaesthetised for all procedures.

The first major difference between the two studies is that Davies fasted the test animals 18 hours before the experiment and that additionally they were fed a zinc deficient diet two days prior to surgery, in order to prevent a high dietary zinc concentration from affecting the $^{65}$Zn uptake. During surgery, the rats were opened up and $^{65}$ZnCl$_2$ in saline solution was injected directly into the lumen of the duodenum, jejunum, ileum or caecum. The labelled zinc was also injected intramuscularly. The wounds were then sown up and the animals placed back into clean cages with free access to water and food after total body gamma counting. The total body measurement was carried out on successive days until the end of the experiment. This method allowed the measurement of relative absorption of radiolabel and successive subtraction of initial body count allowed calculation of uptake by the various segments injected with label. Further

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experiments used modified forms of the ligated intestinal loops described in the previous study.    In the first of these, in situ ligated loops were injected with various concentrations of zinc sulphate containing $^{65}ZnSO_4$ and incubated for increasing times before having the contents washed out.    The loop was excised and the loop, the loop contents and the whole body were separately measured for levels of radioactivity.    The second experiment looked at absorption, during the first 15 minutes (a period of rapid uptake), of varying concentrations of labelled zinc sulphate into the duodenal mucosa.    *In situ* ligated duodenal loops were cannulated at either end.    Radiolabelled zinc sulphate was introduced into the loop and incubated for fifteen minutes before the loop was flushed with saline. This was followed by varying incubation periods before the animals were killed and the loop contents and tissue assayed for presence of label.    This is really an extension of the previous experiment to allow more detailed kinetic study of zinc absorption in this particular site.

Results for the intact rat studies indicated that, as was shown by Methfessel and Spencer (1973) that the duodenum provided the highest levels of retained label when the whole body was analysed over time.    After eight days the levels of radioactivity were still distinctly higher than those of any of the other intestinal sites.    The order of decreasing uptake was the duodenum (57.9% total zinc) followed by the ileum (30.1%), the jejunum (8.9%) and the colon with the caecum (3.0%) providing virtually no bioavailable zinc after eight days.    Intramuscular injection of the radiolabelled zinc chloride in the whole animal study showed the highest levels of retained zinc after eight days.    This was an indication perhaps that bypassing the gastrointestinal regulatory barrier for zinc uptake allows greater incorporation of zinc, however this may only reflect the fact that the zinc is readily bound by muscle tissue.    Thus the label was not available to the circulating zinc ligands that are part of the regulatory mechanism for excretion, and the radiolabel retention may not reflect total incorporated zinc.    Due to this, the study would not seem to be a useful comparison for the intestinal absorption and retention of zinc.    However, as the rate of elimination of radiolabelled zinc from all sites, including the intramuscular injection, was very similar (as determined by regression analyses) there may be a comparison made in the manner of handling the zinc whether administered intestinally or intramuscularly. Billiary secretions had no effect on duodenal uptake as shown by uptake

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measurements in duodenally administered [65]Zn with or without ligating the bile duct.

That the labelled zinc introduced into the caecum was very poorly absorbed was an observation interesting in the light of later studies that showed the importance of fermenting digesta in the rat caecum for optimal absorption of zinc at that site (Seal and Mathers, 1989). A further observation from this study noted that the caecum increased in size in rats fed a high fibre diet in the form of increased pectin content. These studies also showed that, in comparison to other studies, the colon did indeed absorb dietarily important amounts of zinc although less than duodenum and ileum and that dietary fibre content which increased size of colon especially at the caecum might therefore increase zinc absorption. The fact is pointed out that as digesta are held for longer in the colon, this may compensate for the lower absorption rates (Seal and Mathers, 1989). It has also been observed that caecal tissue in rats and humans has a high cell proliferation rate (Hall et al., 1992), which might be expected to reduce mucosal zinc transport as zinc absorbing cells would be rapidly sloughed before absorption could occur. Therefore prolonged exposure of that portion of the intestine to readily fermentable digesta containing zinc ligands could enhance the otherwise poor caecal zinc uptake. Indeed the mass of the caecal and other gastric tissue is significantly increased from normal in the presence of fermentable digesta in rat (Seal and Mathers, 1989), pig (Jørgensen et al., 1996a) and chicken (Jørgensen et al., 1996b). This indicates that cell proliferation occurs faster than cell loss, which would aid zinc absorption across the mucosa. This possibility is supported by the fact that most cells, including gastric cells, require zinc to proliferate, zinc being required especially for nucleic acid replication and translation (Albergoni, 1998) and higher rates of proliferation therefore require more zinc. However studies have not shown a high level of zinc transfer from colon or caecum specifically to the circulation or body in general. Zinc absorbed by the caecum could thus play a localised role in the absorption or metabolism of some other nutrients, but studies have yet to look at this. It is clear that the importance of the caecum and the effect of fermenting digesta on zinc uptake cannot be dismissed for animal studies, and the large proportional difference in size of caecum between human and rodent must be considered when comparing studies.

Davies (1980) further showed that in ligated loops of duodenum, absorption of zinc showed rapid onset, defined as greater than 1% uptake one minute after dosing.   This may effectively be due to binding to the mucous layer at this point rather than absorption into the cells, which one might expect to take a little longer, as the dose penetrates or saturates the potential zinc ligands in the mucus.   Binding to mucus in effect could not be considered absorption, instead it would be surface retention, and indeed 2 minutes after dosing, the rate of apparent zinc absorption increased rapidly as, it could be suggested, the true mucosal absorption began.   Davies (1980) then made the observation that has directed research into zinc uptake right up to date.   Zinc is absorbed over sixty minutes in a curvilinear pattern, but if this is divided into the first fifteen minutes and the following 45 minutes, two distinct patterns emerge.   The first is a saturable phase characterised by a linear relationship for dose versus absorption when plotted as double reciprocals (Lineweaver Burk graph).   The second uptake phase increases linearly with time and is not saturable within normal physiological limits for zinc concentration.   Davies (1980) concluded that zinc is initially absorbed in the rapid uptake phase by an enzyme or carrier-mediated process that is predominant with lower zinc concentrations.   However, at higher concentrations of luminal zinc ($50\mu g$ and above) a non-specific binding and uptake mechanism predominates.   A specific uptake process was consistent with observations of competitive inhibition of copper uptake by zinc (Brewer et al, 1979) which it appeared must share at least one specific uptake mechanism.

It should be noted when considering these two studies that neither used any more than descriptive statistics, mean and standard error and that the conclusions, especially about sites of greatest absorption, were not specifically stated as being statistically significant.   In addition, both studies use measures of integrity for the mucosal barrier to ensure that tissue degradation did not account for zinc efflux from the lumen.

These two studies, amongst the earliest of the comprehensive studies looking at absorptive differences along the gastrointestinal tract headed a series of studies examining this in more detail.   As mentioned in association with the two previous studies there are distinct differences in absorption of zinc associated with dietary zinc status.   Jackson et al. (1981) carried out a series of experiments that

described this dietary modulation of zinc uptake in rats. The experiments carried out in this study were similar to the whole animal preparations of the previous studies. They showed that zinc depleted diets could cause an increase in zinc absorption when radiolabelled zinc was administered acutely, compared to animals fed zinc adequate diets. They also differentiated zinc bound to the mucosa and zinc taken up into the whole body. They showed that the levels of zinc bound to the intestinal wall did not change in acute administration to either zinc-adequate or zinc-deficient rats, even though total body zinc was significantly higher in the zinc deficient rats. This observation would, at first sight, seem to argue against dietary modulation of a proposed regulatory zinc binding molecule in the mucosa, in turn being the cause of the dietary regulation of zinc uptake and this is what Jackson et al. (1981) proposed. However as all the rats were fasted 24 hours prior to the experiment, this may easily have had a large influence on expression of such a regulatory protein, so such a conclusion is not entirely valid.

Whilst examination of zinc uptake over isolated portions of the intestine can produce a great deal of information it has been argued that this does not provide a complete or even accurate picture of actual zinc uptake from the gastrointestinal tract (House and Wastney, 1997). This is because the exact sites of specific zinc uptake or even the sites of variations in non-specific uptake are simply not known with any certainty. Further studies were carried out, under varying conditions, that analysed zinc transport over the whole intestine (House and Wastney, 1997, Steel and Cousins, 1985, Gisbert-Gonzalez and Torres-Molinas, 1996) and as a result distinctly different results were obtained from them, all claiming to be an accurate picture of zinc uptake in the gastrointestinal tract.

The major site of zinc uptake in the gastrointestinal tract was and still is a matter for great debate. Logic would decree that if a positive result is obtained in a well controlled study, then it should have practical predominance over a negative result in an equally well controlled study. Thus, as has been proposed (Steel and Cousins, 1985), probably all parts of the intestine participate in absorption of zinc, despite observations of no uptake from various gastrointestinal sites. The occurrence and the extent of this activity is dependant on luminal, dietary and physiological conditions (Gisbert-Gonzales and Torres-Molinas, 1996, House and Wastney, 1997). A comparison of the three whole intestine studies is again

difficult, as one uses a modelling system based on intravenous administration of radiolabelled zinc chloride from which tissular zinc uptake and transfer between organs is determined (House and Wastney 1997). In this study uptake from the gastric lumen can only be calculated indirectly, as zinc is not taken directly from the gut, instead transport is inferred from calculation of tissue zinc transfer over time. In this study, House and Wastney (1997) make the claim that zinc transferred from the gastrointestinal tissue to the plasma is greatest from the caecum followed by the jejunum and colon with very minimal transfer from the duodenum. Another group, Gisbert-Gonzales and Torres-Molina (1996), looked at zinc uptake and secretion in whole animals but used zinc acexamate (Esplugues et al., 1985) as the zinc source and as representative of zinc. However, zinc acexamate has been shown to have properties quite distinct from simple zinc salts and whilst it may prove an indication of true zinc uptake kinetics, it is difficult to claim it as a definitive study. This study concluded that zinc is absorbed most rapidly from the jejunum closely followed by the ileum and colon absorbing at approximately the same rate and finally the duodenum with a rate of absorbance roughly three fifths that of the jejunum. The third study (Steel and Cousins, 1985) examined the whole intestine and set out to look at the effect of dietary zinc status on zinc uptake after acute zinc administration and not specifically to identify the sites of zinc uptake. They used whole perfused intestine to look at zinc uptake kinetics in zinc-adequate and zinc-deficient rats from perfused lumen to perfused vasculature, vascular perfusate contained albumin as a major circulating zinc-binding ligand, to better simulate the normal vascular compartment. Their results backed up those of Davies (1980) who showed an uptake profile for zinc in ligated intestinal sacs that was biphasic, expressing saturable and effectively non-saturable kinetics. Steel and Cousins (1985) measured both absorbed radiolabelled zinc in the vascular perfusate and mucosally bound zinc in the gut. They showed that not only was zinc bound within the whole mucosal cytosol in greater concentration in zinc-deficient rats compared with zinc-adequate rats (control), but that after an hour there was a five fold increase in zinc concentration in the deficient rats' vascular perfusate fractions compared to that of the zinc adequate rats. However both groups of rats displayed the biphasic uptake profile although the control intestines saturated at around 100$\mu$M zinc whereas the deficient rat intestines were not saturated at the concentrations used in the experiment. Assuming that non-specific sites were

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not increased in number, which would be difficult unless the intestine grew substantially in area, the study's claim is that there is an upregulation of the specific component of zinc uptake in zinc deficient rats allowing them to maintain normal zinc levels with less available dietary zinc.

It seems therefore that only limited consensus exists over the exact site of zinc uptake, but what is generally agreed is that dietary constituents within the lumen may play an important role in zinc uptake. These include specific ligands which may aid or prevent zinc uptake, anion coupling which may also affect absorption and other divalent ions which may compete with zinc for uptake pathways.

## Factors effecting zinc uptake mechanisms

### Luminal ligands

Ligands for zinc within the lumen can be divided conveniently into two major groups. Dietary ligands found to varying degrees in different foods (Peres et al., 1998, Hansen et al., 1997, Lonnerdal, 1988), and secreted endogenous ligands (Prasad and Oberleas, 1970, Seal and Heaton, 1983).

Dietary ligands are often the major dietary sources of zinc but may also act as inhibitors of uptake (Reviewed Lonnerdal, 1997) where the ligand is part of an indigestible food source (Lonnerdal, 1997). As discussed previously zinc in humans is obtained proportionally in the main from red meat and to a lesser extent from other animal sources such as milk and fish (Lonnerdal, 1997, Mares-Perlman, et al 1995).

Zinc is bound very well by several small peptides (Prasad and Oberleas, 1970, Seal and Heaton, 1983) and by combinations of peptides (Prasad and Oberleas, 1970). In vitro studies have shown that histidine binds zinc with greatest affinity, with glutamine, threonine, cysteine and lysine also markedly binding zinc (Seal and Heaton, 1983). Histidine and 2-picolinic acid have been shown to increase zinc absorption *in vivo* but not tryptophan (picolinic acid progenitor) or cysteine (a major intracellular zinc binding ligand) (Seal and Heaton, 1983). Milk proteins, casein, albumin, lactoferrin, and metallothionein in human breast milk have also

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been shown to bind zinc (Michalke et al, 1991) and cow milk equivalents have been proposed as supplements to increase zinc absorption in food rich in natural zinc chelators (Lonnerdal et al, 1984). In addition, milk proteins casein and alpha lactalbumin have been proposed to affect zinc absorption *in vitro* and *in vivo*. Casein specifically has been described as the major individual binding ligand for zinc in cows' milk with the rest being found in the whey fraction by HPLC analysis (Kincaid and Cronrath, 1992). Another study quantified the zinc binding capabilities of whole bovine and human casein (Singh et al., 1989). It approximated 5-8 Zn atoms/mol but showed competition for the binding sites from calcium atoms. Additionally they described the other proposed zinc binding constituents, bovine whey proteins, beta-lactoglobulin, alpha-lactalbumin and lactotransferrin, as having minimal capacity for binding zinc. Interestingly this study also showed that casein binds less zinc with increasing ionic strength, the implication possibly being that in the acidic milieu of the stomach it may release much of the bound zinc, although this was not directly examined in this study. Casein has also been shown to improve zinc absorption in rat intestinal perfused loops (Peres et al., 1998) and in human volunteers casein phosphopeptides increased zinc absorption from rice based meals (Hansen et al., 1997). In primate studies casein and whey hydrolysate based diets increased both zinc and calcium bioavailability when compared to soya based diets (Rudloff and Lonnerdal, 1992).

In the gastric milieu there is also a large bacterial population and many bacterial proteins have been shown to bind zinc or contain it as an active or structural component. These include superantigenic (immune cell activating) proteins which require zinc to bind to the major histocompatibility protein (MHC) on cells, complement, which is part of the non-specific defence mechanism, and several inflammatory modulators (Papageorgiou et al., 1999, Al-Daccak et al., 1998). It might be interesting to look at the contribution of these interactions. These must occur quite regularly on zinc uptake in the intestine, due to the certain ubiquitous presence of antigens and superantigens and the expression of MHC on almost all cells, however no study has yet covered this.

The effect of dietary or gastric secreted ligands for zinc was investigated with several small molecules being proposed as major transport facilitators for the zinc

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ion (Jackson et al., 1981). Jackson *et al.* (1981) carried out one experiment in which the luminal contents of zinc deficient rats were dosed with radiolabelled zinc and replaced in the lumen of zinc-adequate rats to see if the increased absorption was due to an endogenously secreted factor. No increase in absorption was seen compared with that of normal rats. Neither was there a decrease in absorption of labelled zinc when luminal contents of zinc-adequate rats were placed in the lumen of zinc-deficient rats. These results appeared to indicate that secreted inhibitors or enhancers of absorption did not affect zinc absorption. This disagreed with other groups (Seal and Heaton, 1983) and did not at least take into account the effect of secreted endogenous zinc on exogenous zinc uptake. Jackson *et al.* (1981) were however careful not to *exclude* the possibility of specific ligands in the diet and specifically proposed mucosally bound ligands as one possible uptake mechanism. They examined a proposed ligand from the literature, picolinic acid, which was shown not to significantly affect zinc uptake, but another ligand, citrate, did increase the transfer rate of zinc from lumen to carcass.

Seal and Heaton (1983) amongst others (Lonnerdal et al., 1980, Evans and Johnson, 1980) also looked at the effect of anions and organic ligands such as picolinic acid and citrate on zinc uptake. It should be noted that while Jackson *et al.* (1981) used fasted animals, Seal and Heaton (1983) did not fast the animals used. Thus the two studies may not be directly comparable. Seal and Heaton (1983) used everted intestinal sacs, intestinal loop sections or everted gastric sacs that allowed greater control of zinc uptake conditions. Everted tissue, in this case gastrointestinal tissue, is turned inside out so that the mucosal surface is on the exterior, thus allowing transport of material through the mucosa to be examined at constant or specific concentrations. Using these preparations, Seal and Heaton (1983) found that zinc acetate was more effective than zinc chloride at zinc absorption and zinc citrate less so. The latter observation disagreed with the previously discussed study (Jackson et al., 1981) which showed increased zinc uptake with citrate, however this increase, whilst significant was very small and a corresponding loss of mucosally bound zinc makes the effect of citrate appear real. Seal and Heaton (1983) also looked at different anionic partners for zinc and concluded that zinc sulphate was absorbed better than the other anionic forms in the order of zinc sulphate > zinc chloride > zinc phosphate. These

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observations make it even more difficult to compare studies that have used two different anions, normally chloride and sulphate but also citrate, to examine zinc uptake. However when radiolabelled zinc sulphate or chloride are used to determine uptake profiles they tend to be used at such low concentration within a larger amount of cold zinc that they are unlikely to affect the overall uptake. Despite this fact and with the additional observation that zinc chloride uptake is dependant on pH of the medium it is incubated in (Seal and Heaton, 1983), the differences in ionic form of zinc used in higher concentrations in an experiment must affect interpretation of uptake results. In this study picolinic acid (a metabolite of tryptophan) was shown to vary in aiding zinc uptake. This depended on isoform, 2-picolinic acid greatly increasing uptake and 4-picolinic acid reducing it. This supported a possible role of picolinic acid in one isoform aiding zinc uptake as proposed in another study (Evans and Johnson, 1981).

Other than protein, many other substances will chelate zinc and many will reduce its uptake from the lumen. In 1961, Ananda Prasad first observed zinc deficiency in a young male population in Iran where geophagia, consumption of earth, was practised (Prasad et al., 1961). Iron supplementation was used but subsequent studies indicated that zinc deficiency was the prevalent problem in malnourished individuals (Halsted et al., 1972) in this area. Comparison of zinc supplementation with iron supplementation in similar cases in Egypt indicated that it may have been trace zinc in the iron supplement that reversed the symptoms (Prasad, 1963). It was discovered in the course of these studies that the low endogenous zinc levels were causing physical symptoms that were readily reversed by zinc supplementation and cessation of geophagia. It appeared that the clay in the earth was chelating the zinc and was subsequently being excreted with minimal absorption. Also, a study carried out on anorexic patients showed that vegetarian anorexics took in significantly lower levels of zinc than non-vegetarian anorexics (Bakan et al., 1993). However these extreme cases are examples of reduced zinc uptake due to diet. More commonly, if dietary zinc concentrations are adequate then reduced zinc absorption may be due to high fibre content and more specifically the presence of high phytate levels in the diet. Phytate (inositol hexaphosphate) is a highly negatively charged molecule that will bind strongly with divalent cations such as zinc (Lonnerdal, 1997). It is not absorbed by the body (Lonnerdal et al., 1988) and so, like the clay in the previous

case, chelates zinc and prevents its uptake in the gut. Phytate, along with fibre, is found in large amounts in unrefined flour, especially wheat flour and has been shown in vivo to significantly reduce absorption of zinc in rats (Levrat-Vernay et al., 1999). Often marginal and pronounced zinc deficiency may be due to socio-economic conditions where reduced red meat consumption is combined with higher vegetable consumption or simply lower overall consumption of food (Mares-Perlman et al., 1995). Two groups where marginal zinc deficiency may result in marked symptoms are the elderly and the very young. In the former case reduced overall food intake may contribute to significantly lower zinc levels than the normal population, in the latter case reduced overall consumption may be a factor, but also infant formula with high concentrations of phytate, soya based, can chelate significant amounts of zinc. Soya based phytate may be indicated as contributing to the reduced absorption of zinc in animal models (Lonnerdal et al., 1988, Rudloff and Lonnerdal, 1992, Momcilovic et al., 1976), in human infant studies (Chan et al., 1987, Shinwell and Gorodischer, 1982) and in adults exclusively fed infant formula (Lonnerdal et al., 1984). However it is noted in one of the human infant studies that general nutrition was low and this may be a contributing factor. It is interesting to compare this with the *in vivo* studies of zinc uptake in rats fed zinc deficient diets in which they adapted to the low zinc availability by increasing uptake capacity. In these *in vivo* experiments, the kinetics of uptake changed over time to maintain adequate levels of zinc (Davies, 1980). This observation may indicate that dietary zinc-binding constituents themselves could be more important in modulating human zinc deficiency than any effect of dietary composition or metabolic imbalance on the specific and non-specific zinc uptake sites in the gut. It is even possible that the rat model for zinc uptake is not a very good one for drawing conclusions about human zinc deficiency. It is most likely that dietary ligand binding of zinc is both directly and indirectly contributory, but a study that would conclusively compare these two factors is not present in the available literature. Additionally in relation to the infant studies, little is known in general about zinc absorption kinetics in very young children and these may well be distinct from adult absorption kinetics, even in healthy zinc adequate infants, as it is known that at least one proposed major gastric mucosal cytosolic zinc ligand, CRIP, is developmentally regulated (Levenson et al., 1993).

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In vivo studies have also shown directly that plant products in various forms may modulate zinc absorption (Seal and Mathers, 1989), with good evidence that phytate contributes greatly to decrease in zinc absorption (O'Dell and Savage, 1960, House et al., 1982). However Seal and Mathers (1989) carried out studies in everted gut-sac preparations as described previously and showed that dietary fibre, and especially pectin, fermented to a great degree in the caecum, increased size and reduced pH of the large intestine. The high fibre diet also increased zinc intake. Rats absorbed zinc most rapidly from fibre preparations particularly high in pectin, followed by high bran preparations, when compared to normal diets (Seal and Mathers, 1989). The pectin fed rats however grew least of all so it seems the high levels of pectin may have been interfering with other nutritional pathways.

It seems therefore that in general, animal derived ligands *tend* to increase zinc absorption and plant derived ligands *tend* to decrease zinc absorption. However, the available evidence indicates that this is not a hard and fast rule. Whilst dietary ligands obviously play an important role in modulation of zinc uptake, endogenous secreted ligands may also be equally important.

### Gastrointestinal Mucus

One proposed substance with ligand properties and which is ubiquitous throughout the gastrointestinal tract is the mucus coating the gut wall. In porcine gut mucosa it varies in strength and morphology throughout the gastrointestinal tract, with stronger mechanical characteristics in the stomach, duodenum and colon and thinner, weaker mucus in the small intestine typified by a high level of insoluble non-mucus glycoprotein which is removable by centrifugation (Sellers, et al., 1987). Obviously differences in the structure and integrity of a physical barrier could affect passage of material through that barrier. Electron probe microanalysis studies of rat duodenum has shown that zinc is evenly distributed throughout the mucus of that part of the gut in contrast to calcium which is bound unevenly (Coleman and Young, 1979). Oddly, cadmium distribution resembled more the calcium distribution which is unexpected as cadmium is generally a strong competitor for zinc binding ligands (Jaeger, 1990, Khoo and Cousins, 1994) and it should be expected to have a similar distribution to zinc, rather than

calcium, in the mucus. One of the first studies to show zinc binding capacity of the gut mucus layer as distinct from zinc binding by the mucosal cells also showed mucin, the major glycoprotein in mucus, to have a capacity for binding divalent ions including zinc (Coleman and Young, 1979). In agreement with an active zinc-binding role for secreted mucus are results that show a very rapid sequestration of zinc to the gastric wall of rinsed tissue, after just one minute (Davies, 1980). This is unlikely to be due only to cellular uptake, simply because the mucus layer itself must be crossed to reach the gastric epithelium. The mucus is thus probably retaining the zinc to some extent.

Of course the mucus also functions as a matrix within which any of the small amino acids or zinc binding proteins, such as albumin or transferrin which have been secreted by mucosal cells (Larhed, et al., 1998, Alvarez-Hernandez et al., 1998, Marriott, 1989) could be retained. Reabsorption of these molecules could conceivably contribute to zinc uptake. The nature of the zinc binding capacity of mucus and its contents remains to be fully determined.


*Copper*

Zinc uptake is also affected by competition with other ions (Reviewed Folwaczny, 1997); the best characterised competition coming from ionic copper. Although the specific pathways that copper and zinc share have not been completely described, various points on the pathways are known to coincide and there are several studies looking at the competitive uptake kinetics of zinc and copper and which are examined below. Although there appears to be a divalent ion transporter that may be involved in zinc and copper uptake (Gunshin et al., 1997), it is certain that the two ions will compete for binding to metallothionein in enterocytes (Hall et al., 1979). Thus excess of one ion will prevent binding and hence cellular sequestration of the other ion. However the interaction is more complex as described elsewhere, in that zinc will induce metallothionein production. This will itself increase copper binding (Brewer et al., 1990). It should be noted that the role of metallothionein in mucosal to serosal transport of ions, particularly zinc, is still a matter for debate and is examined later in this chapter (p.29). The relationship between zinc, copper and metallothionein is

more complex than simple competition for binding sites.   In treatment of Wilson's disease (Reviewed Ferenci, 1997) which is a congenital inability to excrete copper, zinc has been proposed as a preventative treatment (Brewer, et al., 1990).   The complexity arises in that administration of zinc in pharmacological dosage for Wilson's disease increases production of metallothionein itself over a period of days, so that more copper is bound and held in the enterocyte which is lost by desquamation along with its load of copper.   It is difficult to reconcile the theory behind this treatment with the proposals of metallothionein as both a transferable storage molecule for zinc and a cytosolic transport molecule for zinc (Reviewed Albergoni and Piccinni, 1998).   The reason for this difficulty is that acting as a storage or transport molecule, metallothionein would represent a highly exchangeable pool of either zinc or copper.   Therefore, increasing metallothionein levels may actually increase the availability of copper for uptake into the body rather than locking it into the mucosal cells for excretion.   However the answer to this problem may be that concentration and effect over time are decisive factors for determining the fate of excess copper bound in mucosal cells by the upregulated metallothionein.   The certainty is that at this point in the absorption process there is both direct and indirect interaction and competition for binding by copper and zinc.   In addition, copper has been shown *in vitro* to modulate metallothionein, by competing with zinc for binding to the metal responsive element (MRE) for a transcription factor which in turn activates transcription of metallothionein (Czupryn et al., 1992).   However another study indicated that *in vitro* only zinc activates metallothionein via the MRE and other heavy metals did not (Koizumi et al., 1992, Murata et al., 1999).   It should, however, be pointed out that one particular isoform of metallothionein (MT-IIA) was used for the investigation and the results indicate an even greater complexity of interaction between zinc and copper and putative zinc transport molecules.


*Iron*

Iron has also been proposed as a competitive inhibitor for zinc uptake in vivo however those experiments were carried out under controlled conditions with zinc in aqueous solution and iron at pharmacological concentrations (Craig et al., 1984).   Subsequent studies in infant (Fairweather-Tait et al., 1995) and adult (Davidsson et al., 1995, Lonnerdal et al 1984) humans showed that in fact under

normal dietary conditions iron in food has minimal, if any, effect on the uptake of zinc, although if administered simultaneously there may be some limited interaction.

## Cadmium

Cadmium is another metal that shares protein binding affinities with zinc, however unlike copper, cadmium is rarely found in levels great enough to cause zinc imbalance.    Under conditions of dietary restriction with increased cadmium content, which is mainly ingested from vegetable sources (McKenna and Chaney, 1995), the effects of cadmium may be seen in zinc deficiency when its competitive effects are exaggerated (Nath et al., 1984).    In vitro, the saturable uptake mechanism for zinc is inhibited competitively by cadmium in porcine brush border membrane vesicles, where cuprous ferric and ferrous ions had no effect (Blakeborough and Salter, 1987).    Again the effect of cadmium on zinc uptake may be more complex than simple competitive inhibition but the concentrations of cadmium make it unlikely to have much effect under normal dietary circumstances.

## Calcium

Interactions between zinc and calcium have been mentioned previously in relation to casein and phytate binding from dietary sources (pp 14-19).   The mechanisms of uptake control shared by these two ions are more complex than simple competition for ligand binding with interactions between the ions and transport molecules such as calmodulin (Mills and Johnson, 1985) and lactalbumin (Murakami and Berliner, 1983) being described in detail.   The concept of some mutual regulation was proposed over a decade ago (Brewer et al., 1979). Calmodulin is one of the major binding and transport molecules for calcium and has both functional and structural roles (Brewer et al., 1979).   Zinc, along with other divalent ions, has been shown to bind to calmodulin (Milos et al., 1989) in a non-competitive fashion (Mills and Johnson, 1985) with binding sites that become available only when calcium is bound to its calmodulin binding sites (Mills and Johnson, 1985).   As calmodulin has been proposed as a transport molecule for zinc in spermatozoa (Silvestroni et al., 1989), shown by inhibition of zinc uptake

by calmodulin inhibitors, this molecule may act as a transporter for zinc in other cells as it is present in most tissues. In particular, calmodulin inhibitors have been shown to decrease calcium uptake in duodenal segments of rat intestine indicating a role for calmodulin in gastric absorption of calcium (Pento et al., 1991). It is therefore reasonable to deduce that zinc may also be absorbed to some degree whilst bound to calmodulin in the intestine. Calcium ion permeable kainate channels in cultured cortical neurones have also been shown to facilitate transport of zinc (Sensi et al., 1999) after exposure to kainate. In addition to this, patch clamp analysis of nuclear membrane in hepatocytes has shown zinc and calcium transport through ion channels, with characteristics so similar that it was proposed that they are using the same channel (Longin et al., 1997). Zinc has also been shown to be taken up by submandibular cells via ATP (adenosine triphosphate) sensitive purinergic receptors (Dehaye, 1995). The uptake is inhibited by calcium, nickel and lanthanum (Dehaye, 1995). However of these ions calcium is probably the most dietarily relevant ion due to its widespread availability. The presence of zinc permeable calcium ion channels in salivary gland cells (Dehaye, 1995) and hepatic cell lines (Longin et al., 1997) provide another mechanism whereby zinc uptake *may* be regulated by calcium. Studies of zinc intake in elderly humans with high calcium diets, either as milk or calcium phosphate supplements, have shown that zinc absorption is reduced (Wood and Zheng, 1997). In another study by the same group adults were subject to whole-gut lavage followed by test meals containing normal or supplemented zinc content, which was challenged by acute treatment with high dose calcium carbonate (Wood and Zheng, 1997). Results indicated 50% reduction in zinc absorption when calcium was administered at the same time as the meal. The inhibitory effect was itself partially reversed by co-administration of the higher zinc dosage with the calcium carbonate bolus. It would seem then that whatever the molecular interactions between zinc and calcium at the cellular level, the net absorption of zinc from a meal can be inhibited by dietary calcium.

**Zinc uptake at the cellular level**

Zinc transport at the level of the mucosal cell itself has been only recently looked at in any detail with the advent of molecular techniques. These have facilitated the identification of several zinc specific ion channels (Palmiter and Findley, 1995,

Palmiter et al., 1996, Wenzel et al., 1997, Huang and Gitschier, 1997) and at least one non specific divalent ion channel able to transport zinc (Gunshin et al., 1997). In addition to these channels there is the possibility of zinc uptake by ion channels such as those for calcium discussed previously. Other studies have suggested that zinc uptake may be related to an endocytic mechanism (Fleet et al., 1993, Grider and Vasquez., 1996) which may in turn be related to soluble or membrane-bound zinc ligand uptake (Bax and Bloxam, 1995). These various transport routes are discussed below.

## Non-specific divalent ion channel - DCT-1

Very recently a gene was cloned that coded for membrane bound protein, divalent-cation transporter 1 (DCT1). It was ubiquitously expressed in rat tissue, particularly highly in the duodenum, jejunum and kidney, with very little expression in the ileum or the colon and virtually none in the stomach (Gunshin et al., 1997). DCT1 was shown by *in situ* hybridisation to be highly expressed in enterocytes lining the villus, particularly the crypts and lower portions of the villi. In the same study oocytes expressing DCT1 large inward currents were recorded for zinc, manganese, copper and cadmium ions, with uptake for iron shown to be linked to co-influx of $H^+$ ions. This allows for a direct uptake mechanism of zinc by cells and interestingly, as calcium showed a weak outward current different from the other ions, it was proposed be part of the zinc-calcium uptake regulation mechanism as suggested by previous studies in brush border membrane vesicles (Gunshin et al., 1997).

## Zinc specific ion channels

### *Zinc Transporter –1 (ZNT-1)*

Four zinc specific ion channels have been cloned and characterised to date, and have been placed in a newly emerging class of ion channels called cation diffusion facilitators (Reviewed in Paulsen and Saier, 1997). The first of the zinc transporters to be identified was as recently as 1995. Palmiter and Findley (1995) used a complex method, involving deletion of function by mutagenesis of baby hamster kidney (BHK) cells, to produce mutant cells which did not have the

resistance to accumulated intracellular zinc that the wild type BHK cells had. They then screened an expression library of rat DNA, transfected into and expressed by the mutated BHK cells, for the gene that conferred resistance to excess zinc. Transfected cells that contained rat DNA coding for this gene recovered the ability to tolerate excess zinc. This was successful to varying degrees in different clones. They then extracted the DNA from the successful clones and sequenced the library fragments that contained the gene conferring zinc resistance. The sequences from the DNA library that, when expressed in the zinc sensitive mutant BHK cells, made them zinc resistant if grown in high zinc medium, were identified as coding for a protein of 507 amino acids long designated ZNT-1. They were also identified as being homologous with two yeast genes, one that conferred resistance to zinc (Kamizono et al., 1989) and another identified as a cobalt transporter (Conklin et al., 1992). These two yeast proteins and the newly sequenced mammalian protein from the BHK study were determined, by hydrophobicity plot, to have six membrane spanning regions with both N- and C- terminals intracellular. In addition all three proteins had identical amino acids in position 121 with general similarity being highest in, or adjacent to, the membrane-spanning domains. All had a large intracellular histidine-rich loop between domains IV and V spanning the membrane and finally a long intracellular C-terminal tail. Once the mammalian ZNT1 gene was cloned it was engineered to contain a marker gene at the C- terminal, coding for green fluorescent protein (GFP). It was then transfected back into BHK cells and expression of the ZNT1 protein linked to the fluorescent GFP protein was examined. In the BHK cells ZNT1 was localised to the plasma membrane by fluorescence that was not present in untransfected cells. Additionally, another engineered gene with a different marker, βGeo, on the C-terminus showed that the C-terminal fluorescence was situated in an organelle-rich compartment, supporting the results of the hydrophobicity plot that indicated that the C-terminal end was in the cytoplasmic compartment. These results were modified by later studies (McMahon and Cousins, 1998). Those studies showed a definite localisation of the ZNT-1 protein at the basolateral membrane of the enterocyte cells lining the villi rather than the luminal membrane. This meant that the channel could not be involved in uptake of zinc from the gastrointestinal lumen, but rather affected mainly efflux of zinc from and to the serosa and microcirculation.

Zinc influx was greater than efflux in the transfected BHK cells compared to the wild type (original) cells (Palmiter and Findley, 1995). However, this could have been a function of the cell growth, as the influx was measured by intake of radio-zinc. This could thus be affected by higher cell proliferation, as zinc is essential for adequate cell growth and thus higher intake would itself increase cell growth. The interesting point about this observation is that the transfected cells regulated intracellular zinc levels by increasing zinc *efflux* under conditions of increased zinc concentration, whilst untransfected, wild type cells modulated cellular zinc by reducing *influx* of zinc. ZNT-1 mRNA expression was increased (50%) in the rat intestinal but not hepatic tissue by dietary zinc supplement (McMahon and Cousins, 1998). However the ZNT-1 protein level increased only 10% in the intestinal tissue with the same dietary supplement. Acute administration of zinc increased intestinal mRNA 8-fold but had no effect on the protein. In the liver the acute dose of zinc had no effect on the mRNA expression but caused a 5-fold increase in the protein.

## Zinc Transporter-2 (ZNT-2)

As a continuation of the study that described ZNT-1 further clones, coding for different proteins were identified, that also protected cells against high zinc concentrations. These were examined using the same process as used for ZNT-1 and a second zinc resistance gene was identified that coded for another membrane bound protein with similar characteristics to ZNT-1 (Palmiter et al., 1996). This study showed that ZNT-2 has only a 26% amino acid similarity to ZNT-1 but in structure is rather similar with six presumed membrane spanning domains, a large intracellular histidine-rich loop and a long C-terminal tail. Using zinquin, a zinc specific fluorophore, it was shown that ZNT-2 facilitates zinc uptake into intracellular vesicles and again, use of a GFP fusion protein system showed that the expressed ZNT-2 protein was localised to the membrane of these intracellular vesicles. Mainly, the channel facilitated uptake into the vesicles, however a little zinc appeared to leak back into the cytoplasm from the vesicles. The significance of this vesicular sequestration was emphasised by the fact that only the highest zinc concentrations allowed optimal influx of zinc into the vesicles. Expression of either ZNT-1, the cellular efflux channel, or metallothionein, the major cellular zinc storage/binding protein, suppressed this

vesicular sequestration activity.   This would seem to indicate a lower affinity for zinc by ZNT-2 and subsequently a role perhaps only in preventing toxicity of zinc in the cell, rather than as a major zinc regulatory metabolic pathway.   The low level of leakage back into the cytoplasm could also indicate that ZNT-2 allows these vesicles to act as cellular reservoirs for zinc.   ZNT-2 mRNA in rat tissue was expressed clearly in kidney, intestine, seminal vesicles and testes as shown by reverse transcription polymerase chain reaction (RT-PCR).   The authors did point out that as the transfected gene was expressed very highly using a strong transcriptional CMV promoter in the BHK cells, which they assumed would not normally express the protein, the expression may have been directed to inappropriate cellular compartments.   Thus cellular location of normal ZNT-2 would differ from just that of the vesicles.   Currently only this one original publication exists describing ZNT-2 so a more specific understanding of its role in regulation of intracellular zinc is as yet unknown.

### Zinc Transporter-3 (ZNT-3) and Zinc Transporter-4 (ZNT-4)

With continuing study of the ZNT-2 gene a murine homologous gene, ZNT-3, was identified and cloned.   This had a 53% homology with murine ZNT-2 and coded for another protein with six membrane-spanning domains (Palmiter et al., 1996). Whilst neither ZNT-3 nor ZNT-4 are expressed in the gastrointestinal tract a brief description of their identification and function are included at this point.   The murine ZNT-3 was shown to have 86% homology with the amino acid sequences for human ZNT-3 that was also cloned in this study.   In order to identify and describe ZNT-3, antibodies were raised to the protein, which were then used for immunocytochemistry to examine localisation of the protein.   *In situ* hybridisation was used to examine the corresponding expression of ZNT-3 mRNA in the tissue and in the same manner as used for ZNT-2, GFP fusion proteins were produced to look at the expression of transfected ZNT-3 DNA.   Results of the sequencing showed that ZNT-3 lacked the distinctive large intracellular histidine rich loop of ZNT-1 and ZNT-2.   ZNT-3 mRNA was identified by RT-PCR only in the brain and testes and localised by *in situ* hybridisation to the hippocampus and the cortex. Antibody staining indicated that immunoreactive ZNT-3 protein was spread in granule cell neurones in a pattern that was very similar to that of histochemically reactive zinc.   This pattern was examined further (Wenzel et al., 1997) and ZNT-

3 was found to be localised in the plasma membrane of small, clear, synaptic vesicles, 60-80% of which contained histochemically reactive zinc. Unlike ZNT-2, ZNT-3 did not facilitate uptake of zinc into vesicles when expressed in BHK, cells so it was proposed that other proteins aid ZNT-3 in its function. However, targeted disruption of the ZNT-3 gene in mice produced animals with total zinc levels reduced by 20% in the hippocampus and cortex. Specifically, the disruption abolished the histochemically reactive zinc normally found in the synaptic vesicles to which ZNT-3 was found to be localised (Cole et al., 1999). Disruption of only one allele for ZNT-3 led to less ZNT-3 protein being identified on the synaptic vesicles and a corresponding reduction in amount of zinc being found in the vesicles.

ZNT-4, the most recent mammalian zinc transporter in this series cloned to date (Huang and Gitschier, 1997) was shown to be expressed in mammary glands and coded for a protein homologous to ZNT-2 and ZNT-3. This protein also facilitated sequestration of zinc into endosomal/lysosomal compartments and also synaptic vesicles.

Despite their localisation in regions other than the gut, discussion of the ZNT-3 and ZNT-4 studies has been added for completeness but since the recent identification of ZNT-1 in 1995 the even more recent cloning of subsequent receptors does leave the possibility that other channels remain to be identified. Additionally, clearer understanding of the cellular localisation of ZNT-2 may further elucidate its role in gastrointestinal tissue.

**Vesicular transport**

There are a few publications recently that have suggested that zinc may be transported in association with vesicular activity, endocytosis, or more specifically 'potocytosis' of caveolae (Chang et al., 1998, Grider and Vasquez, 1996). Caveolae are non-clathrin coated, vesicle forming, pits on the surface of cell membranes. These membrane structures have been identified by a resistance to solubilization by Triton X-100 at 4°C, by a light buoyant density and by a richness in glycosphingolipids, cholesterol and lipid anchored membrane proteins (Reviewed by Anderson, 1998). Initially discovered to transport folate into cells,

the transport of a large number of molecules has been shown to be associated with caveolae (Anderson, 1998). Potocytosis is the endocytic mechanism by which the caveolae transport a variety of molecules into the cytoplasm and target them to specific compartments within the cell (Anderson, 1998). Zinc, bound to a variety of anions, has been proposed to be transported along with these ions via a mammalian ion transporter mechanism (Vasquez and Grider, 1995). This transport was shown to be inhibited by an inhibitor of folate transport, probenecid (Chang et al., 1998) and by nystatin an antibiotic and inhibitor of caveolae formation (Grider and Vasquez 1996). These observations indicated that zinc uptake into cells can occur through a potocytic mechanism. However the probenecid study used both healthy fibroblastic cells and *acrodermatitis enteropathica* fibroblasts and showed activity of a secondary uptake mechanism. *Acrodermatitis enteropathica* is a genetic disorder that prevents zinc absorption and manifests as acute and severe zinc deficiency in infancy, it is completely treatable by zinc supplementation. Probenecid did not inhibit the uptake of zinc by the *acrodermatitis enteropathica* cells, which under normal conditions have only a low level of zinc uptake. This observation suggested that there was a secondary uptake mechanism unrelated to the potocytic mechanism and additionally that it indicated the deficiency in the *acrodermatitis enteropathica* fibroblasts might be that of the potocytic mechanism. The exact mechanism by which zinc is transported via the caveolae system has yet to be described in the literature.

**Intracellular ligands involved in zinc uptake - Metallothionein and Cysteine-Rich Intestinal Protein (CRIP)**

Whatever the mechanism by which ionic zinc or loosely ligand-bound zinc enters the cell and specifically the gastrointestinal cell, there exist two major zinc binding molecules that have variously been proposed as initial intracellular storage and transport facilitators for zinc. These are metallothionein and the more recently characterised CRIP.

Metallothionein was first characterised in 1957 as a cadmium binding protein (Margoshes and Vallee, 1957) and shown to be a low molecular weight zinc binding ligand implicated in the homeostasis of zinc and inducible *by* zinc (Pulido

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et al., 1966). Metallothionein has been very well described and a wide range of publications exists on the topic (Albergoni and Piccinni, 1998, Vallee and Falchuk, 1994) so only the area relating to zinc absorption will be considered in this part of the chapter. The role of metallothionein in zinc absorption is still debatable, although its involvement in the process is certain. However, whether it acts as a storage molecule for zinc (Olafson, 1983), a rescue molecule protecting against excess zinc or a zinc transfer molecule (Kruse-Jarres, 1997) has not been decisively determined. In vitro studies have shown metallothionein synthesis is directly proportional to zinc concentration in the human gastric cell line CACO (Rafaniaello, 1991). In fact the metallothionein genes have a transcriptional promoter that is activated by levels of various metals, mainly zinc, cadmium and copper, and these sequences are called metal responsive elements (MRE's) (Murata, 1999). Metallothionein has a number of isoforms some of which are tissue specific and some that are more ubiquitous (Albergoni and Piccinni, 1998). Some isoforms respond to different stimuli (Huber 1993).

Both the hepatic and intestinal metallothioneins are inducible by zinc, although this seems to depend on species and experimental condition. There appears to be a differential response to dietary zinc in various parts of the murine intestine with endogenous metallothionein concentrations decreasing from the stomach down the gastrointestinal tract (Tran et al., 1998). In this recent study a high (400mg/kg food) dietary zinc concentration increased metallothionein levels most in the duodenum, then the jejunum and finally the ileum with no increase in the stomach, caecum or colon. In another study examining mouse intestine, oral zinc doses caused no increase in duodenal metallothionein but did increase hepatic metallothionein indicating that the zinc was being absorbed (Olafson, 1983). However duodenal metallothionein was induced by dietary zinc at exceedingly high dietary concentration (300ppm) and with repetitive dosing (Olafson, 1983) although the biological relevance of this zinc concentration is questionable. Further work has shown intestinal metallothionein to be readily induced, by orally administered zinc, in the jejunum (Ouellette et al., 1982) and gastric mucosa (Starcher et al., 1980). In the latter case, the mice were first injected intra-peritoneally with zinc and then the absorption of radiozinc was examined. This indicated that a high injected concentration of zinc increased metallothionein

levels but decreased intestinal zinc uptake, whilst a ten fold decrease in concentration of the injected dose caused an increase in zinc uptake.

In zinc depleted rats it seems that dietary zinc will increase expression of intestinal metallothionein mRNA and production of protein (Menard et al., 1981). However, in one study (Blalock et al., 1988) a very high zinc dosage was required to stimulate intestinal metallothionein production, whilst stimulation of kidney metallothionein from animals in the same study occurred at lower zinc concentrations. More drastic differences were seen in another study with zinc deficient rats expressing 76% reduction in ileal metallothionein mRNA compared to controls and rats fed a zinc supplement expressing 80% higher levels of metallothionein mRNA than control animals (Levenson et al., 1994). In addition induction of metallothionein in rat intestine by zinc was investigated for its effect on cadmium uptake and increased metallothionein was shown by immunohistochemical staining techniques (Ohta and Cherian, 1991).

As was pointed out in the discussion of zinc uptake kinetics, it is reasonable to say that the studies in rat and mouse varied greatly in protocol and methodology and that these differences in either animal treatment or zinc administration for example could lead to the variation of results. However a general observation can be made that zinc at pharmacological doses seems to induce intestinal metallothionein mRNA and probably protein to some level and that dietary zinc levels *may* induce metallothionein.

In humans the study of zinc induction of intestinal metallothionein is more difficult. It is not easy to get healthy people to undergo gastroscopic biopsy for the sake of science. However several disease states have been studied where analysis of gastrointestinal tissue is necessary and metallothionein has been looked at. In Wilson's disease, for example, copper is not excreted adequately and eventually becomes toxic. Zinc therapy has been used to inhibit the copper uptake (Yuzbasiyan-Gurkan et al., 1992) and does this by increasing metallothionein levels in the gut as calculated from analyses of intestinal biopsies. In this study metallothionein levels in the gut correlated directly with levels of zinc excreted in the urine, a measure of zinc status. In another study, duodenal biopsy

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metallothionein levels were increased 1500% from untreated control patients after oral therapy with zinc sulphate (Sturniolo et al., 1999).

It is clear from all these studies that zinc and metallothionein have a direct and specifically controlled relationship in intestinal processing of zinc, however in 1990 the identification of CRIP (Liebhaber et al., 1990) began to change the understanding of intestinal zinc transport and the function of metallothionein. A subsequent paper showed tissue specificity of CRIP in rat intestinal mucosa and not liver or pancreas and described saturation kinetics for zinc binding to the molecule (Hempe and Cousins, 1991). CRIP was shown not to be directly induced by dietary zinc supplementation and ileal CRIP mRNA was reduced by only 19% in rats fed a zinc deficient diet as opposed to metallothionein in the same study which was highly regulated (Levenson et al., 1994) by dietary zinc. A competitive interaction was suggested for CRIP and metallothionein as radio-zinc was preferentially bound to CRIP in isolated intestinal loops from rats fed low zinc diets whilst in rats fed high zinc diet the proportion of CRIP bound radio-zinc reduced and metallothionein bound zinc proportion increased. Additionally, as luminal zinc concentration increased, CRIP-bound zinc gradually decreased (Hempe and Cousins, 1992). The transport of zinc in these loops was inhibited as metallothionein levels increased. These results suggest a co-operative modulation of zinc transport by CRIP and metallothionein in which high zinc level lead to saturated CRIP zinc binding and consequently greater zinc binding by metallothionein which seems to point to a protective role for metallothionein in preventing toxic levels of cytoplasmic free zinc. In partial disagreement with this theory Fleet et al. (1993) showed that increased metallothionein and reduced CRIP mRNA, in monolayer cultured CACO cells occurred with increased zinc transport across the monolayer. However they went on to show that zinc transport was inhibited by a lysosome disrupting agent in this model and proposed a zinc transport mechanism involving lysosomal sequestration and transport, with metallothionein in a protective role as described above.

The role of CRIP has also yet to be fully understood. Currently two functional roles have been proposed, the first being the involvement of CRIP in zinc transport and the second being a role for CRIP in modulation of immune function and inflammatory response (Halquist et al., 1996, Khoo et al.,1996). CRIP was

shown to be expressed in immune mononuclear cells and macrophages and also in thymus, spleen and plasma to a lesser extent. Furthermore, the levels of CRIP were increased in peritoneal macrophages and mononuclear cells by the presence of lipopolysaccharide (LPS) (Halquist et al., 1996). An inflammatory model of carbon tetrachloride-induced intestinal and liver damage in rats, showed that CRIP mRNA levels were increased in the intestinal and liver tissue but not in immune cells. This suggests differential control of CRIP induction depending on stimulus and tissue type. The common factor between the two studies is the increase of CRIP mRNA levels with cell proliferation, either as immune response or for purposes of tissue repair. In fact recent immunohistochemical studies on the localisation of CRIP in rat intestine indicate a role other than transmucosal zinc trafficking for the molecule (Fernandes et al., 1997). CRIP was highly expressed in the cytoplasmic granules of Paneth cells in rat duodenal sections and to a far lesser extent in the tips of the villi. Paneth cells are derived from pluripotent stem cells, share many features with leukocytes (Bry et al., 1994) and have been shown to contain zinc rich granules (Dinsdale and Biles, 1986). These cells have also been implicated in antimicrobial activity in the intestine (Ouellette et al., 1992). This further reinforces a role for CRIP specifically in the defensive and reparative functions in the intestine, and by association with macrophage and mononuclear cells, in the whole of the body. This does not preclude a subsidiary role in zinc transport for CRIP but certainly brings it into question.

## Zinc binding molecule in the circulation

Whether by ion channel activity, vesicular sequestration or transfer by specific cytoplasmic ligand the collection of zinc into the plasma is the final step for absorption of either dietary or pharmacological zinc and its distribution to the tissues. By far the most important zinc binding plasma component is albumin with most of the circulating zinc (around 80%) loosely bound to the molecule. Around 15% of the plasma zinc is tightly bound to $\alpha2$-macroglobulin, with the remainder bound to diverse small molecules, mainly amino acids and circulating metallothioneins (Reviewed by Albergoni, 1998). In addition to soluble plasma components circulating erythrocytes absorb zinc (Schmetterer, 1978) and

presumably participate in the circulation of zinc. However, a study of zinc supplementation in inflammatory rheumatic disease showed no change in erythrocyte zinc content (Peretz et al., 1993). Thus if erythrocytes are involved in zinc transport, they are unlikely to be part of any response to acute changes in zinc levels. By *in vitro* analyses of serum components stable zinc content was identified mainly in fractions containing albumin but also in those of gamma, beta and alpha globulins. However a distinction was made between exogenous and endogenous zinc (Prasad and Oberleas, 1970). Dialysed serum when incubated with zinc, representing exogenous zinc, showed ready binding to albumin, haptoglobin, ceruloplasmin, $\alpha2$-macroglobulin, transferrin and IgG. Amino acids were shown to compete for zinc bound to albumin, haptoglobin, transferrin and IgG, but not ceruloplasmin or $\alpha2$-macroglobulin (Prasad and Oberleas, 1970). In perfused rat intestine albumin was shown to be the major circulatory binding molecule for zinc absorbed from intestinal mucosal cells (Smith et al., 1979). This agrees with the zinc binding molecules in porcine plasma, in which again, albumin and $\alpha2$-macroglobulin were shown to be the major zinc binding proteins, whilst transferrin, proposed as an important serum zinc binding molecule (Harris and Keen, 1989), appeared to bind zinc but was described as being in too insignificant quantities to affect proportions of bound plasma zinc (Chesters and Will, 1981). In human studies, mainly of disease states with healthy controls, albumin is also shown to be the major zinc-binding molecule in serum (Kiilerich and Christiansen, 1986, Boosalis et al., 1988, Failla et al., 1982). $\alpha2$-Macroglobulin was the next major binding protein, although with only 5-6% of the total serum zinc in one study (Kiilerich and Christiansen, 1986).

**Final destination of absorbed zinc**

Zinc from the circulation may be taken anywhere in the body, but certain organs are particularly rich in zinc. In one whole body analysis of rats, the tissue with the highest concentration of zinc was bone with $217\pm3$ μg/g dry weight, followed by the testes ($165\pm4$μg/g), with spleen, gut, liver, kidney and skeletal muscle having about half that concentration and plasma having the lowest concentration ($1.69\pm0.03$μg/g) (House and Wastney 1997). In terms of percentage body zinc, bone was still highest as it is a large volume of tissue with a high zinc

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concentration, roughly twice as much as any other tissue. Testes tissue was lowest, reflecting tissue volume. The plasma and gut percentage was not determined. The same study indicated that the transfer of zinc from plasma to liver was over twice that of any other tissue transfer of zinc indicating its importance in zinc homeostasis. Presence of a large amount of metallothionein in the liver allows sequestration of a large amount of zinc.

## Summary of zinc uptake

The studies described in this section show that zinc absorption is similar but not identical between species and that it is a highly complex mechanism with a variety of possible pathways. Zinc in dietary or pharmacological form appears to interact with dietary or endogenous ligands on the mucosal surface, in various ways depending on the precise site within the intestine. A practically saturable mechanism at low zinc concentrations and a non-specific mechanism at higher concentrations then moderate the transport of zinc into the mucosa. These mechanisms may involve specific and non-specific zinc ion channels, ligand or cation transport channels or vesicular sequestration, followed by either vesicular transport across the mucosa or transport bound to a cytoplasmic carrier. Transfer from mucosal tissue to circulation may involve further zinc ion channel activity and vesicular exocytosis and collection of absorbed zinc in the plasma is mainly by albumin, which then transports the ion around the body.

# Zinc Pharmacology - Zinc and the Disease State

The role of zinc in disease and treatment of disease is a very broad subject and has been reviewed extensively in the literature, the scope of this section will cover zinc in disease as it relates to pathological inflammatory and immune conditions with particular reference to ulcerative disease.   As zinc is intimately and vitally involved in cell proliferation, any condition that requires a rapid cellular response such as defense or repair will require either increased mobilization of zinc or increased cellular concentration of zinc, most probably both of these.   Generally, pathological states may be divided into those that are caused by zinc deficiency, those that have zinc deficiency as a side effect from some other causative agent and those that respond to zinc treatment in a specific manner.   This is an arbitrary division in that zinc deficiency has been observed and zinc supplementation has been indicated as therapeutic to varying extents in all of these conditions.   In some of these disease states, zinc is the primary treatment and in others, zinc is very much a secondary treatment.   Ulcerative disease, especially in the GI tract, is an area where zinc salts and novel zinc compounds are being found highly effective in treatment of the condition.


## *Zinc Deficiency*

Zinc deficiency can arise due to dietary reasons as described previously where either dietary zinc is in insufficient quantities or it is prevented from being absorbed by other dietary constituents.   Disease is the other main reason for zinc deficiency, where zinc is either prevented from uptake or is used up and excreted excessively.   These cases are discussed in the following sections.   Zinc deficiency in any form has several common aspects, including impaired taste and smell, height and growth retardation, immune dysfunction, flattening of affect, flattening of emotions and other neuropsychiatric symptoms, visual impairment, weight loss, alopecia, dermatitis and delayed sexual development (Agget, 1997). Often mild to moderate deficiency may manifest itself only in a very limited fashion, but improved direct (Hsiung et al., 1997, Wastney et al., 1996) and indirect (Sullivan and Cousins, 1997) detection techniques for variations in zinc

levels have led to increased diagnosis of the condition. Deficiency caused by dietary insufficiency has been observed in several distinct groups including infants, the elderly, strict vegetarians, patients on total parenteral nutrition (TPN) and those with habitually poor diet for socioeconomic reasons.

The effect of the ingredients in infant formula on zinc absorption in young rodents (Momcilovic et al., 1976) and human infants (Cooke and Nichoalds, 1986) has been discussed previously. In practice, deficiency is readily preventable by addition to the formula of ingredients that increase zinc uptake, or reducing the ingredients that are inhibiting the zinc uptake. In breast-fed infants, zinc deficiency, may be due to decreases in zinc levels in the milk and by inference low absorption by the mother (Atkinson et al., 1989). Although this is rare under normal circumstances of nutrition, in areas of poor nutrition breast milk may not contain enough to supply adequate zinc, but infants have been shown to recover readily after weaning away from the breast milk (Bhaskaram and Hemalatha, 1995).

Zinc deficiency in the elderly is most probably due to reduced consumption (Mares-Perlman et al., 1995, Fabris, 1997), but zinc absorption kinetics change with age, so this may be a function of homeostatic changes and can be prevented by zinc supplementation to the normal recommended daily allowance (RDA) (Turnland et al., 1986). One major area of research relating to zinc and aging is the role of zinc in the immune systems of the elderly and has produced conflicting results. Zinc has been documented *in vitro* as having immune modulating effects (See p68 for discussion on zinc immunology). *In vivo*, under some circumstances, the *in vitro* effects can be related to *in vivo* immune function. Thus the efficacy of zinc treatment in a population such as the elderly with, for many reasons, a reduced immune function, has been studied and the treatment shown effective (Prasad et al., 1993, Boukaiba et al., 1993, Bodgen et al., 1990, Girodon et al., 1999) with particular benefit to the humoral immune response (Girodon et al., 1999) and response to some (Girodon et al., 1999) but not all (Johnson and Porter, 1997) infections. In disagreement with this are studies that showed mixed effect, no effect or solely negative effects, of zinc supplementation on immune system function, or hypersensitivity response (Remarque et al., 1993, Bogden et al., 1988, Bogden et al., 1990).

Vegetarians also run the risk of zinc deficiency and it is distinctly due to dietary restriction (Bhattacharya et al., 1985). By definition vegetarians consume larger than normal amounts of plant material which will chelate and remove zinc from the gut without allowing absorption. They also consume fewer foods such as meat, which can contain adequate levels of zinc to compensate for the reduced absorption. Vegetarian infants are particularly at risk of zinc deficiency (Shinwell and Gorodischer, 1982) however, this is treatable with zinc supplementation (Golden and Golden, 1981).

Zinc deficiency in patients undergoing total parenteral nutrition (TPN) has been identified (Chen et al., 1991, Tagaki et al., 1986) but was marginal and completely reversible by zinc supplementation (Chen et al., 1991). However, in post surgical or otherwise recovering patients, even marginal zinc deficiency could be deleterious, as repair mechanisms require amounts of zinc and zinc supplementation has been advised (Alfieri et al., 1998, Younoszai, 1983). In at least one study of TPN usage for children undergoing treatment for severe burns, increased excretion of zinc in the urine (hyperzincuria) and reduced plasma zinc concentration was observed, despite rather high concentrations of zinc being administered (Cunningham, et al., 1991). Close monitoring of zinc metabolism is thus indicated in cases of TPN during recovery, despite zinc supplementation. As there is an increase in urinary protein excretion (proteinuria), including the major zinc binding molecule albumin, in burns patients (Lindquist et al., 1984, Shakespeare et al., 1981) this might account for hyperzincuria in the study by Cunningham et al. (1991).

In general, zinc deficiency as a primary cause of illness is readily treatable by zinc supplementation and normal sources of nutrition and adequate consumption of these sources are enough to compensate and prevent deficiency. However, certain particularly susceptible groups may require additional pharmacological supplementation and monitoring.

## Zinc and ulcer

In order to better understand the actions of zinc in and on gastrointestinal ulceration, where zinc therapy has been shown to be effective, a very brief description of the structure of the gastric tissue and ulcerative process is presented below. A more detailed discussion of gastrointestinal physiology is beyond the scope of this thesis except where it involves zinc.

## Gastrointestinal mucosal structure and causes of experimental ulcer

The gastrointestinal tract consists of a series of four major layers, each layer is composed of a mixture of thinner layered tissue and cell types with one predominant one that characterizes that layer. The inner layer, the mucosa (Fox, 1987), is exposed to the digesta, gastrointestinal secretions and the various flora and fauna of the gut. It is itself coated with various amounts of mucus secreted by goblet cells on the mucosal surface, the strength and coverage area of which varies with position along the gastrointestinal tract (Jentjens and Strous, 1985). The mucosa, the absorptive and major secretory layer, is mainly comprised of columnar epithelium and contains various specialized secretory cells (Beinborn et al., 1993). It is supported by connective tissue, the lamina propria, within which there are lymph nodes. Surrounding the lamina propria are thin layers of muscle, the muscularis mucosa, which create the distinctive mucosal folds increasing the absorptive area of the gut. The folds are named villi and the troughs between these folds are called the Crypts of Lieberkühn. The troughs also contain various gland ducts which cross through the muscularis mucosa into the second major layer of gut tissue, the submucosa. The submucosa contains a large number of blood vessels, branches of which extend from the submucosa to the mucosa and along the villi, and act to receive material absorbed through the epithelium and lamina propria from the lumen of the gut and also to transport endogenous molecules from the circulation back to the gastric mucosa. The submucosa itself is innervated by autonomic nerves in the form of the submucosal plexus. Surrounding the submucosa is the major musculature of the gut, the muscularis, which provides motility to the gastrointestinal tract, it is innervated by nerves in the myenteric plexus. The outer layer of the gastrointestinal tract, the serosa, is

a protective coat of connective tissue covered in simple squamous epithelium (Fox, 1987).

The stomach has a very similar structure to the rest of the intestine but has some specialized cells distinguish it from the rest of the intestine. The 'villi' of the stomach are modified with the villus epithelium consisting mainly of mucous cells that secrete dense protective mucus. The troughs between these microscopic folds in the stomach are called gastric pits. The lower portions of those are comprised of chief cells secreting pepsinogen and parietal cells secreting hydrochloric acid, also argentaffin cells secreting 5-HT and histamine and G cells secreting the hormone gastrin. The folds and troughs and the associated cells comprise as a whole, the gastric gland. The cells themselves form 'tight junctions' with adjoining cells, thus minimizing leakage of material from the lumen around the cells (Iwata et al., 1998). The structure of the stomach in particular is designed to maintain integrity and prevent back-diffusion of gastric acid that would damage the stomach itself. Ulcer preventions and cures, including zinc, have utilized most of these structures and secretagogues (D'Souza and Dhume, 1991).

Peptic ulcers can occur in the stomach or duodenum and occur when some causative agent weakens the factors, such as the mucus, that protect the mucosa. This may happen through a number of mechanisms. The main ones include nonsteroidal anti-inflammatory (NSAID) usage such as aspirin and indomethacin acting via a number of mechanisms (Navarro et al., 1994), stress causing increased gastric acid release mediated by the vagal nerve (Cho and Ogle, 1977) and *Helicobacter pylori*, a bacterium resident in the digestive system of about 50% of the population (Chan et al., 1991). *H. pylori* has been closely associated with peptic ulcer formation (Chan et al., 1991) and stomach cancer incidence (Yoshimura et al., 2000). The basis for zinc therapy in ulcer treatment is twofold. First, significant changes in endogenous tissue zinc occur in ulcerative disease (Troskot et al., 1996) and zinc deficiency has been shown to be associated with impaired gastric ulcer healing (Watanabe et al., 1995). Secondly zinc treatment, if effective, is well tolerated and has few side effects except in overdose.

Within the mucosa, endogenous zinc is associated either structurally or catalytically with a number of important enzymes. These include carbonic anhydrase which regulates secretion of bicarbonate ions that protect the mucosa by neutralizing gastric acid, and also superoxide dismutase a well characterized copper/zinc enzyme which is a scavenger of superoxide (Vallee and Falchuk, 1994). Also associated with zinc are thymidine kinase synthesis, important for nucleic acid synthesis, alcohol dehydrogenase and a large number of enzymes (Kruse-Jarres, 1997) and DNA binding zinc finger transcription factors (Vallee and Falchuk, 1994) that are vital to cell control and proliferation.

Ulcers can form as a result of disruption of pathways involving any of these zinc requiring proteins. Ulcer formation and treatment is well documented by *in vivo* studies and several mechanisms have been used to induce experimental ulceration, each with a specific aetiology, and therapeutic compounds may display different actions on disease modification depending on the model (Cho and Ogle, 1992). Methods for induction of experimental ulceration *in vivo* include application of cold restraint, ethanol (alcohol), exogenous acid, reserpine, cysteamine, NSAID and *Helicobacter pylori* or secreted components of *Helicobacter pylori* as discussed below. In addition other agents may be used such as platelet aggregating factor (PAF) (Escolar and Bulbena, 1989) when a specific mechanism is being examined. Occasionally induction methods are used in conjunction with other factors likely to speed formation or increase duration of ulcers (Desai and Parmar, 1994).

Cold restraint ulceration is induced by immobilizing the animal in a restrictive cage and reducing the temperature (Alarcon de la Lastra, 1996). Tissue damage by hydroxyl radicals after inactivation of gastric peroxidase appears to be involved in cold stress-induced ulceration (Das et al., 1998). Ulceration by this method has been prevented experimentally by the calcium antagonist Verapamil (Koo et al., 1986) which may inhibit histamine production and therefore acid secretion. Also by Cisapride a prokinetic agent that stimulates gut motility (Alarcon de la Lastra, 1996) the involvement in ulceration of which has not been clearly described. Finally, zinc compounds have also been shown effective in ulcer prevention (Escolar et al., 1987). Prostaglandin administration, particularly $PGE_2$, has been shown to be effective against some ulcerogenic agents but may not be so

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effective against cold stress induced ulcer. However, diets that increased $PGE_2$ release into the lumen did show a reduction in stress induced mucosal lesions (Schepp et al., 1988).

Ethanol is a well known ulcerogenic agent however the exact mechanism of the ulcerative process involved is not entirely clear with possible underlying cellular mechanisms still being elucidated (Hiraishi et al., 1999). It is has been shown however that there is a reduction in mucus secretion in rats treated with 40%, 50% and 80% alcohol (Wong et al., 1986). Divalent ions including zinc have been shown to dose-dependently reduce ethanol-induced gastric ulceration (Bandyopadhyay and Bandyopadhyay, 1997, Dupuy and Szabo, 1986) partially by reduction in acid secretion but mainly by stabilizing sulphadryl residues in mucosal proteins (Dupuy and Szabo, 1986) indicating the involvement of these two processes in ulceration. Another action of ethanol in ulceration has recently been suggested to involve oxygen radical production (Hiraishi et al., 1999).

Exogenous acid-induced ulceration simply enhances the endogenous acid effect of gastric erosion. Increases in endogenous acid secretion can lead to tissue erosion and ulcer when stimulated by a variety of agents for example *H.pylori* (Kang and Wee, 1991) or cysteamine (Groves et al., 1974).

Reserpine induced ulceration is thought to involve stimulation of mast cell degranulation which releases histamine and other ulcerogenic agents (Ogle and Cho, 1978). It has been shown that increasing or depleting mast cell populations in the mucosa respectively intensify or prevent ulcerative responses to reserpine (Ogle and Cho, 1978, Räsänen and Taskinen, 1967).

Cysteamine-induced ulcer does not seem to have one major ulcerative action but has a wide range. Those include increased histamine secretion (Man et al., 1990), increased gastrin secretion, increased acid secretion (Kirkegaard et al., 1980), a reduction in the protective $PGE_2$ (Pugh, 1991) and copper/zinc superoxide dismutase levels (Chen et al., 1994) and also alkaline phosphatase (Japundzic et al., 1991). A difference in the ulcerative process has been observed when cysteamine has been injected submucosally or applied topically to the mucosa. These account for a paradoxical damaging and protective effect for

cysteamine on mucosal injury (Abdel-Salam et al., 1996). Subcutaneous injection of the compound decreased mucosal blood flow and inhibited gastric acid secretion. However, cysteamine applied topically to the serosa increased blood flow (Abdel-Salam et al., 1996). The clinical relevance of this is unclear but certainly indicates that cysteamine has a complex effect on gastric tissue function.

NSAID-induced ulceration is probably the most relevant model for clinical application and testing of drugs, as it is probably the most common cause of gastro-duodenal ulceration in the western population. As with ethanol and cold restraint, NSAID induced ulceration has been used commonly in investigations of zinc therapy in ulcerative disease (Bandyopadhyay and Bandyopadhyay, 1997, Rainsford and Whitehouse, 1992). Bolus administration of NSAID in tablet form but not suspension or solution has been shown to produce distinctive ulceration on point of contact with the mucosa (Rainsford, 1978) indicating definite physiochemical differences in the manner of administration for NSAID-induced ulceration. Two NSAIDs commonly used in experimental models are indomethacin (McCafferty et al., 1995, Kasuya et al., 1978) and aspirin (Kasuya et al., 1978, Desai and Parmar, 1994). The major and recently described factor in NSAID induced ulceration is the non-selective inhibition of both cyclooxygenase (COX) isoforms COX-1 and COX-2 (Kawai, 1998). NSAID-induced ulceration occurs mainly by reduction in protective prostaglandin biosynthesis (Vane, 1971). Selectivity in NSAID inhibition of COX isoforms has been suggested to reduce ulcerative effects of the drugs, COX-1 is constitutively expressed in many cells whilst COX-2 is inducible and is expressed especially at sites of inflammation (Warner et al., 1999), so it would seem that drugs that selectively inhibit COX-2 would be preferable. Aspirin and indomethacin have been shown *in vitro* to be COX-1-selective but with COX-2 inhibitory activity (Kawai, 1998) and thus the ulcerative activity they display may be associated with this observation.

*H. pylori*-associated ulceration is still relatively uncharacterized at the molecular and cellular level in ulcer, with much of the work investigating gastric cancer rather than ulcer, but its association with ulcer as a probable causative agent is undoubted and has been well described (Yamaoka et al., 1999).

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## Role of zinc in recovery and treatment of gastric and duodenal ulcerative disease

Healthy endogenous zinc levels are vital to both prevention of ulceration and recovery from either external ulceration such as venous leg ulcer (Wissing et al., 1997) or internal, duodenal and gastric ulceration (Troskot et al., 1996). This has been indicated by studies that showed initially reduced serum zinc concentration after experimental induction of ulceration. This was followed by increased tissue zinc concentration in the ulcerous area and subsequent reversal of this process, and the reduction of tissue zinc which correlated over time with ulcer healing (Troskot et al., 1996). Therapeutic treatment of ulcer with zinc has also indicated a positive effect for the ion on healing of the lesion, in fact this is a very well documented action of zinc as discussed below.

The localization of endogenous zinc in rat gastric and duodenal ulcerous tissue has only fairly recently been studied with only one paper specifically dealing with endogenous tissue zinc levels in ulceration (Troskot et al., 1996). Ulceration does not appear to lead to increased secretion of zinc into the gastric fluid as shown by lack of significant differences in metal content of gastric juice between healthy and peptic ulcer patients (Powell et al., 1992), so the localization of zinc in ulcer would seem to be limited to local tissue activity as opposed to increased secretion. Much of this activity could be associated with the repair process, particularly increased cellular proliferation as the ulcer heals. This is indicated by a study in which zinc deficiency reduced cell proliferation rates (Watanabe et al., 1995). Studies of the effect of zinc deficiency on rat models of ulcer have shown that zinc deficiency produces or aggravates gastric ulceration (Cho et al., 1987). However another study showed that rats that were fed zinc-deficient diets to produce systemic deficiency, then subjected to acid-induced ulceration, had no increased incidence of ulceration but did display a slower healing rate and a lower cell proliferation rate by four days after ulcer induction (Watanabe et al., 1995). The effect of experimental zinc deficiency on the stomach has been observed to produce a significant increase in gastric volume, in acid and in pepsin (Cho et al., 1987). In addition there was a decrease in mast cell count which may adversely

effect ulcer formation (Cho et al., 1987) as mast cells are involved in ulcer formation (Santos et al., 1998) and zinc therapy has been shown to inhibit mast cell degranulation which releases histamine and other ulcer promoting soluble mediators (Ogle and Cho, 1978).

Although simple zinc salts have been shown to be effective in a variety of ulcerative conditions, there are a number of problems associated with their usage. Ionic zinc compounds may not be very well absorbed in the gastric milieu and can have emetic actions, probably due to gastric irritancy (Reviewed Rainsford and Zeitlin, 1998). A number of zinc compounds have been developed to counteract these problems and are discussed individually below. Two of these, zinc acexamate and zinc carnosine (polaprezinc), have been tested experimentally and clinically in ulcerative disease. Several other zinc complexes, zinc monoglycerolate (ZMG), zinc-cimetadine, zinc-aspartate, zinc-glycinate, zinc-indomethacin and zinc-aspirin have been tested variously *in vivo* and *in vitro*. The latter two zinc NSAID complexes were designed specifically to reduce the ulcerative side effects of the NSAIDs but have also been proposed by the authors as making the zinc bioavailable whilst reducing the irritancy of the zinc ion (Singla and Wadhwa, 1994, Singla and Wadhwa, 1995).

## *Zinc acexamate*

Zinc acexamate has been widely described in a number of experimental models and clinical trials (Rainsford and Zeitlin, 1998). Zinc acexamate has been developed now for over thirteen years (Alcala and Santaella, 1985).

Zinc acexamate appears to act at several levels with protective effects on all ulcerative models tested. In cold restraint-induced ulceration, zinc acexamate exhibited dose dependant reduction of ulcerative lesions (Navarro et al., 1988, Pfeiffer et al., 1987, Navarro et al., 1990). In this model mucus secretion and prostaglandin production were increased after zinc acexamate treatment (Navarro et al., 1988), the effects of which were reduced by preadministration of the NSAID, indomethacin. After application of zinc acexamate, with cold restraint-induced ulceration, zinc levels in the tissue corresponded with ulcer healing, however there was no such correlation with serum level of zinc (Navarro et al., 1990).

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Mucosal mast cell count (300mg/kg zinc acexamate) and related ulceration by reserpine administration (75-400 mg/kg zinc acexamate) was dose dependently inhibited by zinc acexamate pretreatment. These results were related to reduction in membrane permeability as indicated by dose dependant inhibitory effects on leakage of hepatic lysosomes induced by Triton X-100 (0.1 - $2x10^4$M zinc acexamate) (Pfeiffer et al., 1987).

Acetic acid-induced ulceration was inhibited by zinc acexamate (200mg/kg) and ranitidine (30mg/kg), with lesions statistically smaller in the zinc acexamate treated animals (Navarro et al., 1990). Other chemical induced ulcerative lesions examined included those caused by platelet aggregation factor (PAF) (Escolar et al., 1989). PAF binding to its receptor is dose dependently inhibited by zinc (Nunez et al., 1989) and the widespread aggressive ulceration induced by PAF in the gastric mucosa was prevented by zinc acexamate by an action that included inhibition of mast cell degranulation (Escolar et al., 1989).

Acetyl salicylic acid (100 mg/kg ASA) induced ulceration was inhibited by zinc acexamate, which increased protective mucus production and PGE2, levels (Bravo et al., 1992). However, the zinc acexamate (100mg/kg zinc acexamate) did not interfere with the anti-inflammatory properties of aspirin in carrageenin induced oedema (Bravo et al., 1990). Zinc acexamate administration over three days (50-150mg/kg/day), also significantly reversed, to varying degrees, $PGE_2$ inhibition by indomethacin, naproxen and piroxicam, but not diclofenac or ketoprofen. Moreover zinc acexamate significantly raised $PGE_2$ levels from control animals without NSAID pretreatment (Navarro et al., 1994). Zinc acexamate in the same study significantly prevented ulcerative lesions induced by ketoprofen, naproxen and piroxicam but not diclofenac and indomethacin (Navarro et al., 1994). The most recent study has described a novel action of zinc acexamate, as an antioxidant, utilizing an *in vitro* superoxide radical-dependant fluorescence technique on rat neutrophil and guinea pig macrophages (Tsutsui et al., 1999). *In vivo* administration of 30mg/kg zinc acexamate significantly inhibited alcohol/acid produced radicals in homogenate preparations of gastric mucosa (Tsutsui et al., 1999), both observations which suggest another level of gastric protection by zinc acexamate.

There have been at least six recent publications on clinical observations of zinc acexamate, however two of the most recent are non-English publications (Guilarte Lopez-Manas et al., 1998, Garcia-Plaza et al., 1996). By 1992 there had been eighteen clinical trials, a meta analysis of which was carried out (Jiminez et al., 1992). This study examined thirteen randomized clinical trials with a sum of 757 patients in total, with placebo control treatments and proton pump antagonist comparison treatments, with zinc acexamate treatments of 300, 900 and 1200 mg/day for 3-6 weeks, concentrations and time period which differed between original studies (Jiminez et al 1992). It might be a consideration when comparing experimental effect and clinical effects that the experimental concentrations were far higher with between 50-200 mg/kg in rats as opposed to 300-1200 mg in patients, assuming body weights around 60-90 kg. This is approximately ten times lower than the experimental concentrations that did not always give complete protection from ulcer. Although the actual clinical effectiveness of zinc acexamate is undoubted, it does make it difficult to draw definite conclusions on the clinical mechanism of action of zinc acexamate. However, the meta study still indicated that the anti-ulcer activity of zinc acexamate was significantly better than placebo, but was not different from the proton receptor antagonists (Jiminez et al., 1992). In the meta study there was a low frequency of reported side effects (less than 3%) and these included nausea, vomiting, constipation, somnolescence, headache and dry mouth (Escolar and Bulbena, 1989).

There has been an acute allergic reaction observed for zinc acexamate taken as an anti-ulcerative and published as a case study (Galindo et al., 1998). This was not seen in the large meta study, however it does indicate a possibly serious reaction based on patch tests representing all the constituents of the proprietary zinc acexamate preparation. The perfume, flavouring and matrix were reactive and so was a 5% solution of zinc acexamate alone indicating a general allergic response to all the constituents of the proprietary compound as well as the other ingredients (Galindo et al., 1998). The importance of this adverse reaction to the wider population is unlikely to be known until wider commercial usage of the product is examined.

It may also be noted that acexamic acid (also called 6-aminocaproic acid and 6-acetamidohexanoic acid) has a pharmacological action of its own including

modulation of the coagulation cascade (Green et al., 1986). In many of the functional *in vitro* and *in vivo* studies, it should be noted that there is no control for acexamic acid itself, although it is possible that these controls were examined in earlier studies published by these authors in Spanish. It would have seemed appropriate to have included acexamic acid alone in the later *in vivo* and *in vitro* studies due to its above mentioned properties.

## Zinc carnosine

The second novel zinc delivery compound developed that has been extensively studied *in vitro* (Furuta et al., 1994), *in vivo* (Yoshikawa et al., 1991a) and in clinical trial (Kashimura et al., 1999) is zinc carnosine (also called polaprezinc and Z-103).

Zinc carnosine transport has been found to follow Michaelis-Menten kinetics in everted gut sac preparations, with transport inhibited by ouabain and low temperatures, indicating a carrier mediated active uptake process (Furuta et al., 1994). Uptake of zinc carnosine in acetic acid-induced ulcer was greater than either zinc sulphate or L-carnosine alone and was localized in higher concentration at the site of lesion (Furuta et al., 1995a). In addition, it was shown that zinc carnosine dissociated in the gastrointestinal tract as a function of time, being found as a complex after 0.5 hours with dissociation occurring by 1 hour (Furuta et al., 1995b). The binding of zinc carnosine to gastric mucosa was increased in the presence of the anti-ulcer drug cimetidine (Furuta et al., 1995b).

In acetic acid-induced ulceration, post treatment with zinc carnosine, 3 and 10 mg/kg two times daily for 14 days, reduced size and depth of ulcerous lesion and promoted mucosal regeneration (Ito et al., 1990). In the same study, a relapse model of ulcer was induced by hydrocortisone administration. Zinc carnosine with the above regime for 20 days, prevented mucosal exfoliation in the regenerated tissue (Ito et al., 1990). These observations indicate the *in vivo* efficacy of zinc carnosine in prophylaxis and therapy of a recurring ulcer model.

Ischaemia and reperfusion injury was used *in vivo* to examine the actions of zinc carnosine on gastric lesions (Yoshikawa et al., 1991a) by application of a clamp to

the celiac artery supplying blood to the stomach. Zinc carnosine significantly inhibited erosion of gastric mucosa produced in this model without affecting blood flow in the blood restricted areas. The mechanism of action in this model appears to include significant inhibition of the lipid peroxide increase in the affected areas of mucosa (Yoshikawa et al., 1991a). Further study of this antioxidant action of zinc carnosine was carried out, comparing it with zinc sulphate and L-carnosine (Yoshikawa et al., 1991b). Both zinc carnosine and zinc sulphate, but not L-carnosine, inhibited superoxide generation from stimulated polymorphonuclear leukocytes. They also inhibited increase of lipid peroxides produced by rat brain homogenates and liver microsomes (Yoshikawa et al., 1991b). The inhibitory effect of zinc carnosine on lipid peroxidation, which occurs with free radical damage of tissue, is proposed in both studies as a major mechanism of the compound in its therapeutic action on gastric mucosal damage (Yoshikawa et al., 1991a. Yoshikawa et al. 1991b). Oxidative damage of gastric mucosal cultured monolayers by hydrogen peroxide was measured by radio-chromium release in a further study. It was shown to be dose-dependently reduced by zinc carnosine by shifting to the right of dose-response curves for the hydrogen peroxide induced chromium release (Hiraishi et al., 1999).

The effect of zinc carnosine on models of gastric ulceration caused by monochloramine was examined *in vivo*. The drug dose-dependently inhibited the ulceration but the healing mechanism was suggested to be different from the anti-ulcerative mechanism of $PGE_2$ (Nishiwaki et al., 1997, Kato et al., 1997).

In clinical trials, zinc carnosine has been shown to be effective against *H.pylori* in combination therapy (Kashimura et al., 1999). A group of 66 patients was divided into two groups, one of 31 received lanzoprazol, amoxycillin and clarithromycin. The other group of 35 received the same drug treatment in addition to zinc carnosine, 150mg twice daily, for one week. Including drop out patients the, patients taking zinc carnosine with combination therapy had a significantly higher percentage recovery, 94%, than those without zinc carnosine, 77% (p<0.05) (Kashimura et al., 1999).

The efficacy of zinc carnosine appears good in the models of ulcer tested but this has not been as complete as those for zinc acexamate, although all appropriate

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controls appear to be included in the *in vitro* experiments that have been carried out. Again, as with zinc acexamate, some of the studies have been published in foreign language journals and may contain information that would further elucidate the actions of zinc carnosine. Certainly, the clinical improvement of zinc carnosine-treated patients infected with *H.pylori* added to the improvement, seen *in* vivo, of ulcer models, is an indication that this therapy may prove valuable in the clinical treatment of ulcer. However a clinical study of this has not yet been published.

## Zinc monoglycerolate

Zinc monoglycerolate has been studied *in vivo* in models of inflammation and ulceration (Whitehouse et al., 1990, Rainsford, 1992, Rainsford and Whitehouse, 1992). It has been shown to be a slow release source of zinc having anti-arthritic properties *in vivo* given parenterally but not orally (Whitehouse et al., 1990) and subsequently shown to have significant antiulcerative properties *in vivo* compared to zinc salts and comparable to traditional antiulcer drugs when taken orally (Rainsford, 1992). Models of ulceration in rats using orally or parenteral administered NSAIDs, aspirin or indomethacin, in animals pre-sensitized by arthritis, were used to examine the anti ulcerative effects of zinc monoglycerolate compared to zinc sulphate as determined by lesion size (Rainsford and Whitehouse, 1992). ZMG was very similar to zinc sulphate in its ability to reduce lesion size at doses from 5mg/kg up to 23 mg/kg, showing significant reduction in both aspirin and indomethacin induced ulceration, but the ZMG displayed none of the mucosal irritancy seen with the zinc sulphate (Rainsford and Whitehouse 1992). In arthritic rats using this same model, ZMG was compared with zinc sulphate and zinc acexamate and showed similar antiulcerative activity in the 100 μmol/kg range for both aspirin and indomethacin induced lesions (Rainsford and Whitehouse, 1992). Effect of ZMG (100mg/kg) on ulceration induced by a variety of NSAIDs and cold stress indicated that ZMG caused reduction in lesion size for indomethacin, piroxicam, diclophenac, phenylbutazone, meclofenamic acid, isoxicam and aspirin, with results similar to cimetadine. ZMG did not seem to provide protection from ulceration by the propionic acid series of NSAIDs in general (Rainsford and Whitehouse, 1992).

In ulcers induced by ethanol with HCl in cold stressed normal or arthritic rats, pretreatment with ZMG was comparable to both zinc sulphate and zinc acexamate in ability to significantly reduce ulceration in the 100-500 µmol/kg range (Rainsford and Whitehouse, 1992). Orally pre-administered ZMG was also effective in significantly reducing reserpine-induced ulceration in rats (Rainsford and Whitehouse, 1992). Of importance in possible clinical usage of ZMG, it was shown to have antiulcerative effects without compromising the anti-inflammatory actions of the NSAID aspirin in rats challenged with cold stress to induce ulceration and subplantar carrageenan induced paw inflammation (Rainsford and Whitehouse, 1992).

Further investigation into the mechanism of ZMG antiulcerative activity was carried out focussing on aspirin and indomethacin-induced ulcer-related biochemical changes and actual ulceration in rats and mice (Rainsford, 1992). ZMG, 50mg/kg, inhibited an aspirin induced increase in mucosal 5-HETE, a component of the 5-lipoxygenase pathway which induces vasoconstriction and contributes to inflammatory damage (Rainsford, 1992). Paradoxically in relation to the antiulcerative actions of ZMG, it was also observed that under the same conditions of aspirin and ZMG as above the aspirin-induced reduction in $PGE_2$ levels was significantly increased by the ZMG treatment (Rainsford, 1992). In the same study ZMG in single dose, 100mg/kg, and three consecutive four hourly doses of 50mg/kg, were shown to significantly reduce lesion size and number however a single dose of 50mg/kg had no significant effect on lesion size or number (Rainsford, 1992).

In addition to the directly antiulcerative actions of zinc monoglycerolate, this compound has recently been shown to inhibit growth and reduce viability of *H. pylori* when administered as a mixture with β-cyclodextrin *in vitro*, with more pronounced effects seen in a growth medium of pH5.0 as opposed to pH7.2 (Rainsford et al., 1997). The inhibition of *H. pylori* growth by ZMG (Rainsford et al., 1997) is likely to be effective in treating ulcer in a similar manner to zinc carnosine (Kashimura et al., 1999).

***Zinc-cimetidine***

Zinc-cimetidine has been shown to have ulcer-healing effects in acetic acid-induced ulcers in rats (Ito et al., 1995). The model described involved injection of 0.05ml 20% acetic acid into the submucosal layer at the junction between the fundus and antrum and the test compounds were given orally twice daily for 14 days, after which measurements of the ulcer size and other parameters were taken. A dose-related reduction (range 15 mg/kg - 60mg/kg p.o.) in ulcer index was observed with zinc cimetidine but not with the molar equivalent of zinc as zinc chloride (Ito et al., 1995). The index of mucosal regeneration was increased with both the complex and the mixture of cimetidine and zinc. The ulcer-healing effect of zinc cimetidine preceded an increase in thiobarbituric acid reactants, suggesting that an anti-oxidant effect of the complex was responsible for this ulcer healing activity. Oddly, the zinc-cimetidine complex did not inhibit acid secretion even though cimetidine did, so it is puzzling to understand why this complex was developed in the first place. This is especially so as the anti-secretory effect of cimetidine would have been expected to have a specific advantage in being incorporated in the complex.

## Zinc-indomethacin and zinc-aspirin

Briefly, neither of these can strictly be classed as compounds inhibiting or treating ulcer as both NSAID groups may actively induce ulcer. However, for this reason and as these NSAID groups are such widely used medications in the non-zinc form, the complexing of zinc to the drugs may be seen to create, in practical terms, antiulcer therapeutic agents (Singla and Wadhwa, 1994a, Singla and Wadhwa, 1994b).

## Zinc-aspartate and zinc-glycinate

The zinc-amino acid complexes, zinc-aspartate and zinc-glycinate, given intraperitoneally reduced the incidence, number and severity of reserpine-induced ulcers in rats (Barbarino et al., 1988). This was paralleled by histochemically-observed increases in PAS+ve mucosubstances in the outer mucous cells, decrease in RNA in chief cells, and increased periglandular capillary ATPase the latter being interpreted as reflecting improved gastric mucosal microcirculation (Barbarino et al., 1988). These observations may reflect the actions of

endogenous zinc-aminoacid complexes from following the oral administration of zinc compounds.

## Inflammatory Bowel diseases

The role of zinc in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis is not entirely clear. It seems certain that a level of zinc depletion occurs with these conditions, but clinical symptoms of zinc deficiency appear rare in the studies described below. Additionally, disruption of zinc absorption *by* the diseased gastric mucosa has been proposed to cause the deficiency rather than the zinc deficiency being a causative factor in the bowel disease (Reviewed Thompson, 1997). As early as 1977, zinc deficiency was observed in patients with Crohn's disease where hair samples contained significantly low zinc levels and significantly low plasma zinc correlated directly with low plasma albumin levels (Solomons et al., 1977). It had been shown in several further studies of inflammatory bowel diseases, such as Crohn's disease, that plasma zinc levels were significantly reduced compared to healthy controls, $p < 0.05$, (Sjogren et al., 1988) and colitis, where serum zinc was highly significantly reduced compared to healthy controls, $p < 0.0007$ (Fernandez-Banarez et al., 1990).

In a study of Crohn's disease and colitis patients superoxide dismutase (SOD) and metallothionein (zinc proteins with involvement in free radical scavenging) levels were measured in inflamed and non-inflamed Crohn's and colitis patients' intestinal mucosa and compared to control mucosa from colorectal cancer patients (Mulder et al., 1991). No difference in SOD levels compared to controls was seen in non-inflamed tissue from inflammatory bowel disease patients. However there was a significant reduction in SOD compared to controls, $p < 0.005$, in inflamed areas of tissue (Mulder et al., 1991). In both inflamed and non-inflamed tissue, metallothionein levels were significantly reduced compared to controls, in samples from the inflammatory bowel disease patients, $p < 0.001$ and $p < 0.02$ respectively (Mulder et al., 1991).

Although zinc uptake and levels in various body compartments appear to be altered, records of overt clinical symptoms of zinc deficiency do not seem to appear often in the literature for inflammatory bowel disease. One such case study describes acrodermatitis enteropathica occurring in conjunction with Crohn's disease whilst on parenteral nutrition, which was subsequently alleviated by intravenous zinc supplementation (Myung et al., 1998). However, as discussed previously parenteral nutrition alone may be a cause of zinc deficiency. Certainly therapy of inflammatory bowel diseases with zinc compounds has not been greatly studied in clinical trial, probably for the very fact that the imbalances of zinc in the body do not seem to lead to zinc deficiency and other specific treatments are available for these disease states (Reviewed Thompson, 1997).

**Inflammatory disease - particular reference to rheumatic disease**

As previously discussed, several inflammatory mechanisms are zinc dependant, due either to zinc atoms integral to the structure of inflammatory mediators, zinc ions vital to enzyme function and possibly zinc as an inflammatory mediator itself. Thus, it would seem from the physiological role for zinc that a pharmacological role for zinc therapy would be indicated for chronic inflammatory diseases, but the experimental and clinical literature does not consistently support this idea. One such example of this is rheumatoid arthritis, in which the involvement of zinc metalloproteins in the degradation of cartilage (Gunther et al., 1994), the high cellular infiltration of the synovium by T-cells, macrophage and activation of articular chondrocytes and subsequent cytokine induction has been described (Bondeson et al., 1999, Gunther et al., 1994). Additionally, *in vivo* experimental data has shown efficacy for zinc for treatment of inflammatory joint models (Whitehouse et al., 1990). It should also be noted, that fluctuation of zinc status in inflammatory disease, including rheumatoid arthritis, has been documented (Svenson et al., 1985, Simkin, 1981). However, clinical evidence for the use of zinc therapy for rheumatoid arthritis is at best unclear with few clinical studies having been carried out.

In rats, zinc plasma levels are reduced and hepatic metallothionein levels are increased in acute experimental inflammatory conditions such as adjuvant (heat killed M. tuberculosis) induced arthritis (Rofe et al., 1992). The peak

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metallothionein levels corresponding with the lowest plasma zinc concentrations at 16 hrs after adjuvant injection and subsequent induction of inflammation. After a recovery of both zinc and metallothionein levels to near normal, zinc levels subsequently fall and metallothionein concentration increases a second time between 8 and 14 days after adjuvant injection (Rofe et al., 1992). Studies of patients with inflammatory diseases, especially arthritis, indicate a significant reduction for mean patient values of plasma zinc compared to healthy controls, $p < 0.001$ (Svenson et al., 1985). This phenomenon has long been recognized and as early as 1981, Simkin (1981) reviewed published reports in which serum zinc concentrations had been determined in patients with rheumatoid arthritis compared with those in control subjects. There was marked geographical variation, with the differences in zinc levels between patients with rheumatoid arthritis and controls appearing greatest in the data derived from studies in India, New Zealand and two locations in southern USA, whereas they were less so in data from Glasgow and Omaha, and not significantly different in groups from Seattle, Rochester and Parma (Simkin, 1981). In addition, there was no consensus on the effect of zinc treatment for rheumatoid arthritis, with some studies showing great improvement with supplementation and some showing no improvement. It was hypothesised that, when it occurred, the improvement of disease parameters was due to a general zinc deficiency as indicated by the serum zinc levels (Simkin, 1981). It is difficult to draw definitive conclusions from the various studies as differences in zinc measurement and sampling and differences in patient profile and the lack of case-matching in some studies, provides an area of great variability. However there would seem to be a trend to lower serum zinc in patients with rheumatoid arthritis that agrees with more recent studies.

Frigo et al. (1989) reported plasma concentrations of zinc determined by atomic absorption spectroscopy to be reduced in patients with rheumatoid- but not osteo-arthritis compared with controls. In addition, the plasma zinc status was found to be correlated with duration of illness, number of swollen joints, ESR and $\alpha 2$-globulins (Frigo et al. 1989). Urinary elimination of zinc over a 24 hr period was not shown to be reduced in patients with rheumatoid arthritis. This was also confirmed in later studies (Dore-Duffy et al., 1990).

Possible differences between the earlier studies, many of which were from assays of serum samples compared with those observations in plasma samples, could be related to the fact that serum samples are often obtained under variable conditions of collection and the red blood cells can serve as a source of zinc sequestered during formation of serum. Furthermore, plasma zinc levels correlate with albumin (Dore-Duffy et al., 1990) which is the principal ligand for zinc in the circulation. Since plasma albumin is often reduced in rheumatoid arthritis it is important to establish if the reduction in plasma zinc is related to the reduced mass of circulating albumin. Thus, in their carefully-controlled analysis of zinc profiles in blood components in patients with rheumatoid compared with osteoarthritis, Dore-Duffy and co-workers (1990) showed that the statistically significant reduction in both plasma and serum zinc concentrations in the former but not the latter patients was also observed in the fraction of albumin binding zinc (g Zn albumin/g albumin), as well as in the plasma protein fraction. This suggests that either zinc affinity for albumin is somehow reduced in rheumatoid arthritis or that zinc levels are lower than a saturation concentration for albumin. No such changes were reflected in the patients with osteoarthritis. Furthermore, no changes were observed in the zinc concentrations in red blood cells or in white blood cells (Dore-Duffy et al., 1990). In contrast to the Dore-Duffy group which analysed zinc levels by atomic absorption spectroscopy, Svenson and co-workers (1985) utilised X-ray emission nuclear microprobe and, in contrast to Dore-Duffy et al. (1990), were able to show marked reductions in zinc concentrations in the granulocyte, platelets and erythrocytes of patients with rheumatoid arthritis, seronegative spondylo-arthropathies and scleroderma. Thus, the more sensitive and specific nuclear microprobe technique probably accounts for the differences observed in the studies by Svenson et al. (1985) compared with those from the studies by Dore-Duffy et al. (1990). The former authors also confirmed the reduction in plasma zinc concentrations in patients with rheumatoid arthritis, again using the more sensitive methodology of X-ray fluorescence spectrophotometry. Negative correlation of plasma zinc concentrations with disease activity (ESR, serum orosomucoid and other biochemical parameters) was again observed (Svenson et al. 1985). This feature has been observed by others in serum samples in rheumatoid arthritis patients assayed by atomic absorption spectroscopy (Mussalo-Rauhamaa et al., 1988) as well as in plasma samples from patients with psoriatic arthritis analysed by this technique (Frigo et al., 1989).

In contrast to the observed reduction in zinc in plasma and blood leukocytes there is an apparent increase in zinc concentration in the synovial tissues of patients with rheumatoid arthritis (Simkin, 1981). This could be expected from the observations that the reduced levels of zinc in serum and plasma are not necessarily predictive of zinc deficiency but instead may be indicative of zinc redistribution related to the inflammatory disease progression. Furthermore the disappearance of zinc from this compartment could be related to a redistribution of high zinc bearing cells, especially immune cells, to the inflamed synovium and can also be directly correlated with metallothionein induction in the liver (Rofe et al., 1992). The role of metallothioneins in inflammation has not been distinctly explained, possibly because of the complex induction mechanisms of metallothionein which include the metals it binds, zinc and copper (Blalock et al., 1988), glucocorticoid hormones and cytokines (Hempe et al., 1991), all of which are possible components of the inflammatory process itself. It seems clear therefore that zinc is involved in mechanisms active during acute inflammatory insult, as shown particularly with the adjuvant arthritis studies but also in chronic inflammatory states such as rheumatoid arthritis, and further that low plasma and serum levels correlate to disease severity.

It is with the background of these observations that zinc therapy for inflammatory conditions such as rheumatoid arthritis was investigated.

Simkin (1981) was the first to report studies of zinc supplementation on the progress of patients with rheumatoid arthritis. It was reasoned that since leg ulcers are a manifestation of rheumatoid disease, a trial of oral zinc for this complication seemed appropriate after the efficacy of oral zinc sulphate in treatment of leg ulcers had been observed in an unrelated study (Serjeant et al., 1970). Furthermore, the apparent zinc deficiency in this disease was another reason to study the effects of zinc treatment. Thus, the study involved observing the effects of 250 mg zinc sulphate heptahydrate (50 mg elemental zinc) in capsule form given three times daily with meals for 12 weeks under double-blind cross-over (to placebo) in 24 patients. Evaluations included grip strength, time to walk 50 ft, sum of points for swelling and tenderness in 68 joints patients' "global" assessment of their conditions, severity of morning stiffness, Westergren

sedimentation rate, and various laboratory parameters determined initially then at 2 and 4 week intervals. All the clinical parameters improved in the $ZnSO_4$ treated groups and the mean serum zinc levels rose from 84 μg/dl to 116 μg/dl whilst the serum histidine concentration declined from 1.57 to 1.36 mg/dl. Upon cessation of therapy most patients qualitatively considered the disease had worsened without zinc and improved upon reinstitution of zinc therapy. While no patients were cured by zinc supplementation in this study and some did not show any beneficial effects at all, there was a number who did see an improvement in the disease state when taking zinc.

Despite the reported effects from the Simkin study, subsequent studies have shown no beneficial effect for oral supplement zinc in rheumatoid arthritis (Job et al., 1980, Rasker and Kardaun, 1982, Mattingly and Mowat, 1982). One study appears to have directly repeated the Simpkin study (Menkes et al., 1981). Although not published in English and with no translation available, the abstract reports a regime of zinc sulphate 600 mg/day split into three doses given to 35 patients over a period of months which is close to the conditions used by Simkin. This study showed no improvement of rheumatoid arthritis in either clinical or laboratory indices.

Although the efficacy of zinc as a specific treatment for inflammatory conditions was shown in gastric lesions and external ulcer and wounding, it seems that for internal inflammatory diseases, zinc is generally not effective in reducing symptoms other than to return plasma and serum zinc levels to normal. It could be argued that this is expected to happen as ingestion and excretion of zinc is tightly regulated as previously discussed. Therefore pharmacological doses of zinc taken orally do not result in greater than normal plasma or serum levels of zinc. Consequently localised areas of inflammation at organs or joints do not receive the high concentrations of zinc that are seen to be present at external wounds or ulcers treated with topical zinc. Even zinc concentrations in the gastrointestinal tract after swallowing a pharmacological dose of zinc would be generally much higher than normal, with dilution gradually occurring with passage of the bolus through the gut. This could explain the disparity between the positive clinical effects of zinc in inflammatory conditions such as peptic ulcer and epidermal lesion and the lack of effect of zinc treatment in 'internal' inflammatory

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conditions such as rheumatoid arthritis. This would suggest that if local zinc levels at inflamed joints for example, could be increased to above normal concentration, then the therapeutic properties of zinc could be seen, but zinc salts are water soluble and the skin acts as a very effective barrier to them. Topically-applied drugs have the additional advantage in reducing the burden of toxic effects on the gastrointestinal tract, as might happen if taken orally, or on metabolism by the liver. Furthermore, topical application of drugs has the advantage in being a controllable means of drug delivery and this is particularly the case for lipophilic metal complexes (Fairlie and Whitehouse, 1991). Fairlie and Whitehouse (1991) have considered the theoretical and practical issues of a number of metal complexes applied in transdermal systems in animal model systems and achieved significant success with ZMG. In fact, a search of the literature reveals no other studies into the efficacy of topically applied zinc in models of arthritis.

ZMG, when applied to the skin has been found effective in controlling polyarthritis in rodent models of arthritis. The preparation, physical properties and crystalline morphology of ZMG have been previously described (Whitehouse et al., 1990, Hambley and Snow, 1983, Radoslovich et al., 1970). This lipophilic, highly lubricious, polymeric slow-releasing zinc complex has been found to be highly effective when applied topically or subcutaneously in, e.g. DMSO/glycerol mixtures, or rubbed on the skin as the powder in the therapeutic or prophylactic models of treatment for controlling fore- and hind-paw swelling of adjuvant-polyarthritic rats (Fairlie et al., 1992). Topically applied ZMG was found to be non-irritant to the skin and indeed when administered subcutaneously (in DMSO/glycerol) does not elicit inflammatory responses even though there is a localised encapsulation at the injection site (Fairlie et al., 1992). When injected subcutaneously in the polyarthritic rat model, ZMG dispersed in saline acted dose dependently to reduce hind- and fore-paw inflammation which reappeared after cessation of the treatment. ZMG was ineffective in controlling this chronic inflammatory disease model when given *orally* and also in carrageenan induced rat paw oedema models of acute inflammation when injected subcutaneously (Fairlie et al., 1992).

The latter observation may seem contradictory to other studies in which intraperitoneal zinc chloride (in saline) has been found to reduce the carrageenan-induced pleurisy in rats as evidenced by reduction in pleural exudation and neutrophil accumulation (Yatsuyanagi et al., 1987). In contrast, the lack of effect of ZMG on the hind-paw inflammation induced by carrageenan (Fairlie et al., 1992) may be due to insufficient zinc ions reaching the inflamed site in the short time period of 1-3hrs used. In the studies by Yatsuyanagi et al. (1987) the injection of zinc into the peritoneal cavity would presumably have enabled this ion to move rapidly into the pleural cavity where the inflammation was induced. This twin-compartment model of inflammation, separated by the well vascularized smooth muscle of the diaphragm, is a useful means of examining the direct functions of zinc, and indeed other agents, in vivo without the confounding problem of the putative anti-inflammatory agent coming into direct contact with the inflammogen. Other investigations by Yatsuyanagi et al. (1987) showed that i.p. zinc chloride (16.4mg Zn/kg) markedly reduced neutrophil chemotaxis, phagocytosis and superoxide production in the pleural inflammation model in vivo; these effects being corroborated from studies in vitro in which these neutrophil (rat) responses where shown to be significantly inhibited by 100-300$\mu$M zinc (Yatsuyanagi et al., 1987). Thus control of both the vascular permeability and neutrophil accumulation/activation by zinc may account in part for the acute anti-inflammatory properties of this metal ion.

## Zinc and liver disease

Zinc deficiency has long been linked to liver disease, as long ago as 1956 hypozincaemia was described, by Valee et al., (1956) in patients with alcoholic hepatic cirrhosis (Valee et al., 1956). Subsequently it has been shown that the observed hypozincaemia is not dependant on cause of cirrhosis with hepatitis B patients also displaying reduced serum zinc in addition to increased urinary zinc (zinc excretion) (Gusau et al., 1990). In addition to this, the course of experimental liver disease in rats has been shown to affect (Khan and Ozeran, 1957) and be affected by (Anttinen et al., 1984) zinc status. Both observations are mutually compatible and led to studies about the effect of zinc

supplementation on both liver tissue and serum zinc status in liver disease patients and also of the effect of the supplementation on disease state.

Zinc has been implicated in the modulation of hepatic nitrogen metabolism with an inverse relationship between serum zinc and ammonia being observed in cirrhotic patients (Grungreiff et al., 1988). Investigations using experimental liver damage in rats have reinforced this observation, with zinc supplementation reducing the plasma ammonia levels to near normal in cirrhotic rats when compared to healthy animals (Riggio et al., 1992). This was accompanied by an increase in the mean ornithine transcarbamylase (OTC) concentration of the livers of cirrhotic animals that had been treated with zinc compared to untreated cirrhotic animals (Riggio et al., 1992). OTC is a component of the body's ammonia detoxification process and improvement of this aspect of liver disease may indicate a role for zinc treatment cirrhosis.

Studies of zinc levels, zinc supplementation and liver diseases have indicated more general aspects of the disease that are affected. Collagen accumulation may occur in areas of tissue inflammation and the carbon tetrachloride (CCL) model of liver damage in rats displays such accumulation (Anttinen et al., 1984). In this study, oral zinc supplementation inhibited CCL-induced collagen accumulation, although this was not disease or tissue specific as collagen levels were also lower in liver and skin of healthy animals given zinc (Anttinen et al., 1984).

Zinc has also been shown to be involved in apoptosis (programmed cell death) in liver tissue and cultured liver cells to varying effects. *In vivo* studies using the CCL induced rat liver damage model have shown that a zinc supplemented diet produces an inhibition of DNA fragmentation (an accepted measure of apoptosis) which was proportional to the amount of zinc in the tissue, when compared to diseased controls (Cabre et al., 1999). However it should be noted that various studies from another laboratory using in vitro culture of human hepatoma cell line HuH-7, have indicated that zinc induction of the c-myc gene leads to cell death (Xu et al., 1996). This cell death appears apoptotic under light, fluorescent and electron microscopic observation, but does not have the characteristic DNA fragmentation normally displayed during apoptosis (Xu et al., 1996). The cell

death resulting from zinc induction of c-myc also appeared to be related to increased intracellular hydrogen peroxide ($H_2O_2$) levels and reduced intracellular glutathione (a free radical scavenger) levels (Xu et al., 1997).

It would seem that zinc therapy could be effective in liver disease, in preventing liver tissue damage and liver cell apoptosis, and it seems certain that maintenance of normal zinc levels would be beneficial. However further research, both clinical and experimental, needs to be carried out to determine the therapeutic actions of zinc in this disease state.

## Zinc and Bacterial Infection

The effects of zinc on *H.pylori* infection have been discussed previously with antibacterial activity being seen for certain zinc compounds, however zinc therapy has been studied for a number of other bacterial infections.

Studies on the effect of zinc and bacterial infections appear to divide generally into several distinct groups, the effect of bacterial infection on zinc metabolism, the effect of zinc administration on bacterial infection, the effect of zinc on oral bacteria (especially in studies on dental protection) and the effect of zinc on wound infection, this last being dealt with in a separate section discussing zinc and wound healing.

The effect of bacterial infection on circulating zinc levels is to reduce the concentration in either plasma or serum as indicated in both animal (Tufft et al., 1988, Erskine and Bartlett, 1993, Barton and Embury, 1987) and human (Heese et al., 1985, Taneja, 1990, Ray et al., 1998) studies. As the zinc levels are reduced it appears that the zinc is being redistributed around the body with corresponding increases in splenic and hepatic zinc levels after infection, as indicated in at least one poultry study (Tufft et al., 1988) but which is consistent with many studies of serum zinc in disease states some of which have been discussed previously (Svenson et al., 1985). A two year randomized, double-blind, placebo controlled trial investigating the effect of mineral (zinc and selenium) and vitamin supplementation on incidence of respiratory and urogenital infection in institutionalized elderly individuals was carried out (Johnson and

Porter, 1997). A significant reduction in mean number of infections was seen in those receiving the minerals ($p < 0.01$) but no significant reduction was observed as a result of the vitamin supplementation (Johnson and Porter, 1997). The amount of supplements administered was less than twice the recommended daily allowance (RDA). This means that the results show the effect of maintaining normal body levels of zinc and selenium rather than showing a pharmacological effect. Nonetheless, as it is increasingly apparent that modern diets lead to low grade mineral deficiencies (Sullivan and Cousins, 1997) and taking into account that selenium is required in very minute amounts compared to zinc, it maybe understood, from the Johnson and Porter (1997) study, that zinc supplementation has an important prophylactic effect for prevention of infections.

More specifically, there are two areas in particular where zinc has been used as an antibacterial agent, dental hygiene and prevention of wound infection. Zinc-acetate containing preparations have been shown to be effective in inhibiting oral bacterial plaque accretion and gingivitis when compared to untreated controls (Hefti and Huber, 1987). Zinc preparations generally appear to be simple salts, either sulphate or citrate, which are administered in conjunction with mainly triclosan, although some research was carried out on plant extracts of sanguinaria, a folk remedy containing benzophenanthridine alkaloids. Zinc and triclosan mouthwash preparations have been shown to reduce gingivitis (Schaeken et al., 1996) and in a double blind parallel group study of 544 individuals, to reduce the formation of dental calculus (Fairbrother et al., 1997). Furthermore the zinc and triclosan preparations have been shown to have additive effects on plaque growth inhibition (Cummins, 1991) which may be due to the anti bacterial effects of both agents but also to the effect of zinc on triclosan clearance. In one study, varying the concentrations of either zinc or triclosan to optimize the formulation increased the retention of both agents in the plaque (Creeth et al., 1993). This would directly increase antiplaque and antibacterial activity over time, zinc levels in particular increased by up to seven fold over controls (Creeth et al., 1993).

Other than the zinc triclosan combination, zinc and sanguinaria have been investigated as possible co-candidates for oral hygiene products. Whilst several studies were carried out in the late 1980s and early 1990s, little work appears to

have been published recently on this preparation. In fact, whilst a review of the preparation in 1995 indicated that co-use of zinc and sanguinaria mouthwash and toothpastes is widespread in Europe and America (Grenby, 1995). The most recent publication looking at its clinical effectiveness indicated that after thorough dental cleansing, post use of a sanguinaria/zinc chloride mouthwash gave no significant protection against bacterial plaque reoccurrence or gingival bleeding (Cullinan et al., 1997).

A potentially interesting action of zinc has also been reported for zinc in dental hygiene use and that is the protective action of zinc on the gums which are exposed to the irritant sodium lauryl sulphate (sodium dodecyl sulphate) a component of many toothpastes (Skaare et al., 1997). However, only this study appears to have been published on the subject.

Whilst many studies have been carried out on the antibacterial action of zinc in toothpaste others exist that examine the antibacterial action of zinc in other hygiene products. It would seem that zinc has antibacterial properties but is most effective in combination with a second antibacterial compound. This is another area where the novel zinc compounds have not been examined where they may prove more effective than the commonly used zinc salts.

**Zinc and skin lesions (burns, ulcers and incisions)**

It is as a topical treatment for wounds that zinc paste has been used for millennia, for example, in calamine paste used during the Egyptian Empire (Lancet editorial, 1975). The application of topical zinc either in compound preparation or in impregnated bandage is still used particularly for burns (Hallmans, 1978) and venous ulcer (Mayrovitz et al., 1998) and has been shown effective in incision wound healing (Agren et al., 1991b). Certainly *endogenous* zinc has an integral role in epidermal wound healing with zinc concentrations increased at the site of an incision wound (Iwata et al., 1999), burn (Berger et al., 1992) and venous ulcer (Ackerman et al., 1990) as a result of the lesion itself.

The zinc loss directly from burnt areas has been measured in humans by determining the zinc concentration of the burn exudate obtained from the textile

dressing which, in one study of ten people, averaged 14.4mg per day (Berger et al., 1992). This loss compares in magnitude with the 15mg zinc required as the average daily dietary intake (Vallee and Falchuk, 1994) suggesting that this is a major route for zinc loss in burns. In a study of lower limb venous ulcer, zinc concentration was found to be raised at the site of ulcer compared to healthy tissue in the same leg and also the unaffected leg ($p > 0.05$) (Ackerman et al., 1990). However the same study indicated significantly raised zinc concentration in skin from the arms of the leg ulcer patients when compared to that from the arms of the control group (Ackerman et al., 1990). Whilst the first observation indicates an accumulation of zinc to the wound site, authors suggest that the second observation indicates an imbalance of general zinc distribution in the leg ulcer patients (Ackerman et al., 1990). *In vivo* studies of wound healing in mice have shown that zinc localisation and accumulation was an early stage event in the healing mechanism of incision wounds and was associated with an increase in wound metallothionein concentration which occurred immediately prior to the increase in zinc (Iwata et al., 1999). So it is apparent that in all these epidermal lesion states zinc may be localised to the wound site in greater than usual concentration and that, whilst some of this increase may be due to increased fluid loss at the wound site of the epidermis, much of it is localised to the tissue and so may be a function of the wound healing process.

Reinforcing this idea, nutritional zinc status has been suggested to affect wound healing rates in all three types of epidermal lesion with low serum zinc levels observed in a number of studies of venous ulcer (Wissing et al., 1997, Rojas 1999, Cruse et al., 2000). Similar observations of low serum zinc is observed with burns patients especially receiving TPN where zinc excretion is raised and the normal uptake of zinc through the gastric system is bypassed by intravenous drip (Cunningham et al., 1991). *In vivo* studies using rodent models of post-surgery wound repair, in which animals receive TPN, have further indicated the importance of zinc supplementation for improved wound zinc concentration and concomitant increase in wound strength (Nezu et al., 1999). These studies indicate that zinc supplementation is beneficial both for general nutritional reasons alone and also for improvement of the wound lesions. It should be noted however that in at least one study looking specifically at burns patients, intravenous administration of large doses of trace elements, including two and a

half times the RDA of zinc, reduced hospitalisation time but had no effect on wound severity. It thus appeared to indicate improvement of general well being but had no special effect on the specific healing process (Berger et al., 1994). This study however used a large dose of copper as part of this supplementation and this might have affected zinc uptake into cells altering normal processing of zinc. So it seems that a normal level of zinc intake may improve healing rate and wound strength and that low body levels of zinc may be a cause of delayed healing.

Direct topical application of zinc to incision wounds has been shown to promote wound healing in several studies when applied for example as zinc oxide, in vivo (Kietzmann, 1999, Agren et al., 1991b) and on human patients as zinc chloride spray (Pastorfide et al., 1989). In regular practice, compression bandages impregnated with zinc oxide, Unna's sleeves, are used to treat venous leg ulcers and as a supplement to other venous ulcer therapies (McCulloch et al., 1994). Additionally, burns requiring graft are often treated with Unna's paste or graft surgery followed by application of an Unna sleeve which aids wound repair and improves graft take (Grube et al., 1992).

The action of zinc at the cellular level is complex during the tissue remodelling and re-epithelialisation activity of wound repair. *In vivo* studies showed that application of zinc oxide but not zinc sulphate increased re-epithelialisation significantly more than controls ($p<0.05$) in a porcine model of partial thickness wounds (Agren et al., 1991a). This exogenous zinc may act at many levels of the repair process. Studies in mice have indicated that metallothionein mRNA is expressed within 12hrs in the proliferating region of the epidermis surrounding the wound and that this is followed by an increase of the zinc content of the wound (Iwata et al., 1999). The endogenous zinc absorbed by the epidermal cells within the lesion may well act as a source of zinc for the newly formed metalloprotein, which itself acts as a storage or sink for zinc exchange with other metalloproteins such as zinc finger transcription factors (Kruse-Jarres, 1997). Recently, Huang et al. (1999) showed *in vitro* that endogenous zinc and high concentrations of calcium, such as those found in the dermal layer of the skin, act synergistically to promote DNA synthesis in fibroblasts (Huang et al., 1999). Studies on

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keratinocytes, the main cells comprising the epidermis, have implicated zinc in a rather complex involvement in the mechanisms of wound healing, both alone (Tenaud et al., 1999) but also as an active component of various molecules such as the matrix metalloproteinase (MMP) enzymes (Pilcher et al., 1997). *In vitro,* zinc gluconate induced the expression of α2, α3, αV and α6 integrins, external cellular adhesion proteins responsible for cellular migration and proliferation in wound healing (Tenaud et al., 1999). As an integral component of the enzyme MMP-1, zinc is vital for another aspect of keratinocyte migration, in this case through collagen-I the main structural molecule of the extracellular matrix in the dermis (Pilcher et al., 1997). On contact with collagen the keratinocyte is induced to produce MMP-1 (Sudbeck et al., 1994), this may occur through interaction of the collagen with the α2 integrin (Pilcher et al., 1997). Then after synthesis and secretion the MMP-1 cleaves the collagen and the cells are able to migrate through the matrix (Pilcher et al., 1997). This mechanism is a good example of one in which zinc both modulates and participates leading to possible cell progression to the wound site.

In summary, these studies collectively indicate that clinical application of zinc is positively therapeutic for dermal incision, burn and ulcer lesions and that at the cellular level, exogenous zinc may exert a direct modulatory effect on some aspects of cellular motility integral to wound healing. It should be noted that in most medical studies, zinc oxide alone was used as a zinc source, none of the newer zinc compounds such as ZMG, polarprezinc or zinc acexamate appear to have been studied or tested clinically for possible usage in wound healing.

# Zinc immunology

Zinc is involved at almost every level of the immune system however its precise role has only begun to be elucidated in the last ten years. Barnes and Moynahan (1973) first described genetic zinc deficiency and subsequent investigation of this disease indicated that patients displayed immune dysfunction. Immune symptoms of zinc deficiency related to T-cell function alone include thymic atrophy, alteration in lymphocyte subpopulations, and reduced proliferation and differentiation of immature T-cells, reduced thymulin production and reduced mitogen responsiveness (Reviewed Fabris, 1997). These symptoms of deficiency indicate the importance of zinc to healthy immune function and in fact zinc has a very wide role in the function and immune response of other cell types such as macrophage, neutrophils and natural killer cells however it is beyond the scope of this chapter to discuss all of these. Instead, only aspects of zinc modulation of immune cells will be discussed, which relate to the studies in this thesis. These include zinc modulation of T-cell maturation and function, modulation of macrophage function, effect of zinc on cytokine production and on apoptosis.

## *Zinc modulation of cytokine levels in vivo*

Studies of cytokine modulation *in vitro*, specifically by zinc, have only relatively recently been carried out mainly by two or three groups, and so the subject is still very much under examination. Analysis of cytokine levels in patients and healthy volunteers has been examined more widely. However, in inflammatory and immune disease states it may be more difficult to attribute cytokine modulation to variations in zinc levels as the diseases tend to involve other factors that may affect cytokine production. Marginal zinc deficiency has been shown to be very prevalent, as previously discussed, and is also often found in head and neck cancer patients (Prasad et al., 1997). In this study Prasad et al. (1997) observed cytokine production in three groups, head and neck cancer patient, healthy volunteers with mild zinc deficiency and in healthy volunteers with adequate zinc levels. The healthy and zinc replete groups had zinc deficiency induced by

dietary restriction. All groups then received dietary zinc supplementation and the cytokine levels were measured again. It was shown that the cytokines secreted by TH1 helper T- cells, IL-2 and interferon gamma (IFN-$\gamma$) were sensitive to zinc status and reduced in concentration. In the cancer patients and zinc deficient healthy volunteers IL-2 was significantly lower than zinc replete healthy controls, p=0.003 and p=0.023 respectively. IFN-$\gamma$ production by PBM cells was found to be significantly reduced in zinc deplete healthy volunteers compared to zinc replete volunteers, p=0.05. TNF-$\alpha$ and IL-1$\beta$ were found to be significantly lower in zinc deplete individuals compared to healthy controls, respectively p=0.0001 and p=0.0056 for cancer patients and p=0.005 and p=0.037 for healthy zinc deplete volunteers. The TH2 T-cell cytokines, IL-4, IL-5 and IL-6 appeared unaffected by marginal zinc deficiency (Prasad et al., 1997).

In a study of cytokine production from PBM cells from chronic liver disease patients the effect of zinc supplementation was observed (Reinhold et al., 1996). The investigation was carried out in light of the fact that chronic liver disease often gives rise to immune dysfunction, indicated by abnormal circulating cytokine levels, and also to reduced serum zinc levels. The effect of zinc supplementation was observed in cytokine production of isolated PBM cells after mitogen stimulation. At 0.1mM no effect was seen on cytokine production however at 0.2mM there was a significant decrease in IL-2, IL-6 and IL-10 production compared to controls without zinc (p<0.05 in each case) (Reinhold et al., 1996). Although a high zinc concentration, 0.2mM was well below the toxic limit in this study where cell viability was shown by trypan blue stain and MTT assay to be greater than 90% up to 0.4mM zinc (Reinhold et al., 1996).

In overdose zinc has been shown to affect cytokine levels. Zinc oxide fume inhalation which may lead to metal fume fever in high enough dose and which is common in welders, was experimentally induced in volunteers (Kuschner et al., 1997). Level of cytokines were measured in exudate obtained from bronchioalveolar lavage before and, over a period of time, after zinc oxide inhalation. TNF-$\alpha$, IL-6 and IL-8 were shown to be increased significantly (p=0.02, 0.009 and 0.02 respectively). The TNF-$\alpha$ response occurred before the

other increases at 3 hrs and all cytokine changes were dose related to zinc concentration inhaled.

It is clear therefore that evidence exists of a role for zinc in the modulation of cytokine levels and that supplementation with zinc may in some cases return the cytokine levels to normal. Additionally, cytokine levels are altered specifically by zinc in overdose. Further studies have investigated the functions of zinc in cytokine modulation *in vitro*.

### *Zinc modulation of cytokine levels in vitro*

*In vitro* studies using enzyme linked immunoassay (ELISA) or whole cell assays have allowed the investigation of zinc modulation of cytokines at the cellular level. Human peripheral blood leukocytes incubated in whole medium (culture medium with foetal calf serum and growth supplements) zinc was shown to stimulate TNF and IL-1$\beta$ in a concentration dependent manner (Scuderi, 1990). Addition of lipopolysaccharide (LPS, a bacterial surface protein), along with zinc, to the cell medium, caused a synergistic and concentration dependant increase in TNF release (Scuderi, 1990). The action of zinc with LPS is interesting in that LPS may be used to induce both inflammation and immune response *in vivo*. The synergistic activity of zinc and LPS was the subject of a later study by one group in Germany, who additionally have done most of the work on zinc and cytokine stimulation *in vitro* (Wellinghausen et al., 1996a). They showed that zinc is able to potentiate TNF-$\alpha$ and IL-1$\beta$ release from PBM cells stimulated with LPS from different bacterial sources and that this ability was specific to zinc and not similar cations such as cobalt and nickel (Wellinghausen et al., 1996a). Additionally, the study indicated that the synergy was a specific action of zinc on the LPS and not as a result of action on the target cells. It was shown that zinc altered the structural state of the LPS making the structure more fluid when zinc-bound and that this enhanced the cytokine stimulation abilities of the LPS molecule (Wellinghausen et al., 1996a). The same group showed that zinc stimulation of cytokine release *in vitro* was dose dependent on protein concentration of the culture medium. Although zinc alone in LPS and protein free medium stimulated IL-1$\beta$ release from PBM cells it was shown that proteins such as transferrin and

albumin greatly potentiated this action in a dose dependant manner although maximal cytokine release appeared to be independent of protein type (Driessen et al., 1995). From previous discussion of zinc uptake it may be remembered that both albumin and transferrin are major transport molecules of zinc. Therefore, it may be that the increased cytokine release seen in this study was due to an action of the increased bioavailable zinc bound to the transport proteins and not to any action of the proteins themselves (Driessen et al., 1995). The same study showed an increase in IFN-$\gamma$ by PBM cells incubated with zinc and also a decrease in superantigen induced cytokine production when in the presence of zinc (Driessen et al., 1995). It should be noted however that no statistics are presented in this study so, although the values appear dramatically different between cytokine stimulation by zinc alone and zinc plus protein supplement, there is no measure of validity for the results and so they should be accepted with caution.

### Control pathways for zinc modulation of cytokine stimulation

The mechanism by which zinc alters cytokine levels has not been completely described. A number of possible points of control have been described. Continuing the studies on modulation of cytokines by zinc, Wellinghausen et al. (1996b) showed that zinc added to PBM cell preparations, in serum free and LPS free medium, led to an increase in expression of TNF-$\alpha$ and IL-1$\beta$ mRNA (Wellinghausen et al., 1996b). Interestingly, they reported that IL-1$\beta$ mRNA was induced by zinc but also by the related cations cobalt, nickel and mercury whilst TNF-$\alpha$ mRNA was induced only by zinc (Wellinghausen et al., 1996b). These results agreed in part with the earlier study by Scuderi (1990) who also reported an increase in TNF mRNA when cells were incubated with zinc in endotoxin (including LPS) free culture medium.

The same group further published a report describing the inhibition of zinc induced cytokine stimulation by inhibitors of cellular signal transduction (Wellinghausen et al., 1996c). Herbimycin A, an inhibitor of the signal messenger protein tyrosine kinases, significantly inhibited the secretion of IL-1 (p=0.005) as did HA 1004, an inhibitor of cAMP and cGMP, also signal

messenger (p=0.005) (Wellinghausen et al., 1996c). The authors do not specify at what level or manner zinc participates other than it may act synergistically with these second messengers of cellular signal transduction. Within the literature there appears to be no other studies published on the effect of zinc on the signal transduction system during cytokine production so the subject is still very much open to investigation.

Whilst a putative link between zinc stimulation of tyrosine kinases, cAMP and cGMP may exist, there are more widely studied possible mechanisms for intervention by zinc. Zinc, as a constituent of polymerases or zinc finger molecules, is vital for transcription of any DNA sequence to RNA and subsequent upregulation of release of the final protein however it is beyond the scope of this thesis to examine all such zinc finger molecules. Instead, a transcription factor, nuclear factor kappa B (NF-κB), common to control of several cytokines, immune molecules and inflammatory mediators, will be discussed as it is involved in rapid modulation of cytokine production with particular relevance to TNF- production (Reviewed Chen et al., 1999). NF-κB and TNF participate in a rather complicated control mechanism whereby the nuclear factor is integral to, although not solely responsible for, TNF gene transcription (Bergmann et al., 1998, Swantek et al., 1999). Conversely, the cytokine can downregulate NF-κB activity by inhibiting its release from IκB a control molecule to which it binds in its inactive form (Ghosh and Baltimore, 1990). The cytokine modulation of NF-κB may occur, in part, via a cytokine responsive kinase IKK (IκB Kinase), that phosphorylates IκB thus allowing its degradation and therefore release of NF-κB (DiDonato et al., 1997). The transcription factor is then released to travel from cytoplasm to nucleus where it may bind DNA and promote transcription.

Whilst zinc, in the form of zinc finger-bearing signal transduction molecules, metalloenzymes, transport molecules or transcription factors other than NF-κB, is integral to cytokine production NF-κB links many of the inflammatory molecules that are controlled by endogenous administration of zinc. Thus investigation of zinc modulation of NF-κB was one of the aims of this thesis. In this regard NF-κB is examined further in the Discussion Chapter.

Of all cells involved in immune function, T-cells appear most intricately involved with zinc, development and maturation of these cells are in fact dependant on zinc whilst the T-cell related organ, the thymus relies on adequate zinc levels for healthy structure and function.

Zinc has been described as the simplest mitogen; it induces blast cell formation and immune cell growth including pre T-cells. T-cells derive from bone marrow pluripotent stem cells that mature to lymphoid progenitor cells, which migrate to the thymus. The preT-cells then mature in the thymus under control of thymic hormones and surface markers are changed as the T-cell differentiate to either cytotoxic, helper or supressor T-cell which is then released to circulate until required for host defence. The surface markers or surface antigen are membrane proteins that both distinguish the type of T-cell and allow its function as the particular T-cell subtype. In lymphocytes isolated from zinc deficient individuals and compared to those from zinc sufficient individuals, a clear difference has been shown in these proteins (Prasad et al., 1997). In this study it was shown that zinc deficiency led to a significant reduction in the numbers of immature helper T-cells, designated by surface antigen as CD4+ CD45RA+ cells, as compared to mature cells of this type CD4+ CD45RO+ cells (p=0.021) (Prasad et al., 1997). These helper T-cells are vital to the function of CD8+ T-cells which are involved in immune defence from viral attack. The ratio of CD4+/CD8+ was seen to decrease and the authors suggest that this was due to the decreased numbers of immature CD4+CD45RA+ cells.

There was also a significant decrease seen in the numbers of the CD8+ subset CD8+ CD73+ CD11b- cells from zinc deficient patients compared to those from zinc sufficient individuals (p=0.0001) (Prasad et al., 1997). This subclass are precursors to the cytotoxic T-cells involved in combating viral attack and so it may be seen, between maintaining helper T-cell numbers and function and numbers of cytotoxic T-cells, that zinc plays an integral role in healthy T-cell mediated host defence.

In partial agreement with these results Oksel and Taneli (1996) looked at T-cell levels in children with and without upper respiratory tract infection and in which the zinc deficient infected patients received zinc supplement. They showed that zinc deficiency in these patients caused a significant decrease (p<0.013) in the CD4+/CD8+ ratio but that this was due to an increase in the CD8+ T-cell numbers rather than a decrease in any of the CD4+ subsets (Oksel and Taneli, 1996). Certainly though, in both cases, zinc supplementation normalised the altered T-cell subset levels.

## Zinc and T-cell maturation

Changes in plasma zinc levels affect thymic hormone levels such as thymulin, a hormone that controls T-cell maturation and which binds one zinc atom per molecule (Reviewed Fabris, 1997). Either illness related or artificially induced zinc deficiency causes a decrease in active thymulin in humans and animals (Brignola et al., 1993, Mocchegiani et al., 1998). Thymulin is required to bind zinc in its active form and disease related increase in molecules with high affinity for zinc, such as $\alpha_2$ macroglobulin, can compete for zinc binding, thus causing a decrease in active thymulin and consequently impairment of immune response (Mocchegiani et al., 1999). The authors suggest here that zinc supplementation in, for example, cervical carcinoma patients where zinc may be bound by increased high affinity zinc binding molecules such as $\alpha_2$ macroglobulin, may restore immune activity (Mocchegiani et al., 1999).

Again, as with the cytokine modulation via NF-$\kappa$B, zinc is involved in a complex control mechanism related to T-cell maturation. As has been previously mentioned, zinc can modulate IL-1 production and it can stimulate metallothionein production via the metal responsive element on the metallothionein gene. In thymic epithelial cells IL-1 has been shown to regulate secretion of the zinc thymulin complex and additionally, in conjunction with free zinc, to induce metallothionein production in this cell line (Coto et al., 1992). It is thought that metallothionein is the donor that transfers zinc to the inactive thymulin molecule (Savino et al., 1984). So zinc can upregulate IL-1 production that, along with zinc can upregulate metallothionein synthesis, promote formation of the zinc thymulin complex and therefore promote T-cell maturation. Coto et al. (1992) also show

that IL-1 and zinc thymulin, but not IL-1 alone, greatly stimulate protein kinase C (PKC) activity. PKC is a second messenger in the cellular signalling cascade that leads to IL-2 production and IL-2 is a strong promoter of T-cell proliferation. Zinc thereforeappears to be exquisitely bound up with the thymulin based control of T-cell maturation.

As was mentioned earlier, zinc status appears to significantly effect IL-2 levels, possibly dependant on zinc concentration, zinc lowers IL-2 secretion in both deficiency or in high concentration (Prasad et al., 1997, Beck et al., 1997, Reinhold et al., 1996). Zinc has also been shown to increase IL-2 receptor (IL-2r) synthesis in a dose dependant manner (Driessen et al., 1994). IL-2 is the main proliferative stimulus on T-cells at all points in the life time of the immune cell and as such, modulation of the cytokine and its receptor would appear to be a highly significant control point for zinc on T-cell activity.

There are several zinc finger molecules that are vital specifically to the T-cell maturation events, however as zinc finger control in general is not specific to T-cell maturation it is not discussed here in detail. Mutations of the δEF1 gene have been shown to specifically and drastically reduce the numbers of mature T-cells in a murine model (Higashi et al., 1997). δEF1 is a transcription factor with two zinc fingers and a homeodomain, mutation of the gene causes the thymus, medulla and cortex to develop poorly and whilst drastically altering the profile of mature T-cells, the mutation has no effect on other cells of the haematopoietic lineage (Higashi et al., 1997). Another nuclear transcription factor vital to the development of the T-cell lineage, as well as other lymphoid cell types, is the early growth response-1 gene product (Egr-1) (Shao et al., 1997). Egr-1, and possibly the other members of the Egr family, is involved with the maturation of T-cells from the double negative CD4-/CD8- cells to the double positive CD4+/CD8+ more mature cells (Miyazaki, 1997). δEF1 and Egr-1 are examples of just some of the many zinc finger transcription factors required for T-cell maturation.

Zinc is also vital to the signalling mechanisms that allow the T-cell to function as well as mature. Light chain kinase (Lck, also called p56$^{lck}$) is a T-cell specific cytoplasmic kinase that is anchored to the inner membrane of the cell (Pernelle et

al., 1991). Lck is stimulated to autophosphorylation by micromolar amounts of zinc and is involved in T-cell activation (Pernelle et al., 1991). As previously mentioned, CD4 and CD8 are cell surface markers for T-cells and are required for recognition of target cells by the T-cell, binding of Lck to CD4 or CD8 is necessary for their function (Lin et al., 1998). Zinc, but not calcium, nickel, iron or copper is essential for binding of Lck to CD4 and CD8$\alpha$ (Lin et al., 1998). In CD4 at least, binding of the zinc ion has been shown to be co-ordinated by thiol binding of the ion by cysteine residues on the cytoplasmic tail of CD4 and the N-terminal region of Lck (Huse et al., 1998). Addition of zinc chelators in isolated binding reactions disrupts the CD4/Lck or CD8/Lck complexes (Huse et al., 1998, Lin et al., 1998). The same was also shown to be true for these same complexes in cells pre-treated with the chelators (Lin et al., 1998).

In summary, the maturation, proliferation and direct immune function of the T-cell lineage is intimately linked to the zinc ion and by extension, general zinc status. In light of these relatively recently understood roles for endogenous zinc in T-cell activity, examination of the actions of exogenous zinc is an important area for research.

**Zinc and cells of the monocyte/macrophage lineage**

Whilst these cell types are not as intimately bound with zinc for development and function as T-cells are, zinc still plays an important role in immune activity associated with these lines. Cells of the monocyte/macrophage lineage and neutrophils from the granulocyte lineage are phagocytic, they recognise non-self material and particles that have been coated with secreted markers such as antibody and complement, attach to these particles and engulf them.

As with T-cells, the zinc finger transcription factor Egr-1 is involved in macrophage differentiation (Krishnaraju et al., 1995), directing the differentiation of some granulocyte/macrophage colony stimulating factor (G/MCSF) stimulated cell lines specifically along the macrophage lineage. This example being one of the many nuclear factors, including NF$\kappa$B (Chen et al., 1999), that are involved in macrophage activation.

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An important aspect of zinc modulation of macrophage activity has been observed in cases of zinc deficiency where cells isolated from deficient individuals have reduced phagocytic ability *in vitro* (Reviewed Shankar and Prasad, 1998). Phagocytosis was reconstituted by zinc supplementation *in vitro* and also in macrophages isolated from zinc deficient individuals given dietary zinc supplementation. Zinc at higher concentrations has been shown inhibit some macrophage functions *in vitro* (Shankar and Prasad, 1998). Bronchiolar macrophages have been shown to secrete high levels of inflammatory cytokines in response to excess zinc in cases of metal fume fever (Kuschner et al., 1997).

There is little more general data for the action of exogenous zinc on macrophage function in the literature. Much of what has been published in relation to macrophage and zinc has looked at zinc metalloprotein function and very little work appears to have been carried out on the interaction of macrophage and exogenous free zinc.

# Chemical analysis of Zinc Monoglycerolate (ZMG)

## Chapter Introduction

In the course of this investigation the nature of ZMG was investigated, both in solid form and in preparative solutions. As differences were seen in cellular uptake between ZMG in solution and suspension and as the ZMG solution acted in a very similar fashion to that of free zinc ion solution, it was thus to determine the state of ZMG in the preparations used. However, until recently, there has been no published analytical method that has been directly able to examine ZMG preparations in media. Described in this chapter is a series of experiments that were developed to give an insight into the nature of ZMG in solid form, suspension and solution and which add to the published data on ZMG chemistry (Fairlie et al., 1992, Tiekink et al., 1996). In addition to analyses of ZMG preparations, it was necessary to develop a preparation of ZMG compatible with cell culture conditions and non-toxic to the cell lines.

## 2.0.1  High Pressure Liquid Chromatography (HPLC)

HPLC is an analytical technique that has been developed for applications across the disciplines in the chemical and biological sciences (LKB Handbook, 1982). HPLC requires a suitable liquid mobile phase to carry an analyte through a solid phase packed in a column varying in length, but normally between 0.75-2.5cm wide (LKB Handbook, 1982).   The column is the most important functional component of the HPLC system.   Column packing beads have been developed for use with most solvents and the beads may be cross linked to functional groups that can 'add' an affinity for a specific compound of interest.   In this project, size exclusion chromatography and metal chelate chromatography are used for analysis of glycerol and zinc respectively.

## 2.0.2  Degradation analysis

The two constituent ingredients of ZMG are zinc and glycerol, both of which have well described methods of analysis.   It was preferable to have an analytical method for study of ZMG that could be applied to both constituents and HPLC was selected due to the convenience and adaptability of the technique.   It was decided to analyse initially aqueous preparations of glycerol, zinc and ZMG for the following reasons.   For cell culture experimentation the ZMG and other compounds would be administered to the cells which are grown in a balanced aqueous salt solution.   Also *in vivo* the ZMG, administered orally, would encounter first saliva and ultimately gastric juices, both of which are aqueous based.   In all three media mentioned above there is a significant protein content, and it has been shown that ZMG has higher solubility in such biological solutions (Fairlie et al., 1992).   However, dissolution in these solutions is incomplete and other simpler reactions may be assumed to occur between the ZMG and the aqueous and salt constituents of these media.   However, for construction of an HPLC method for ZMG analyses it was preferable initially to keep the system as

simple as possible. A search of the available literature provided very little on methods of glycerol analysis by HPLC other than with a refractive index detector (See for example Parpinello and Versari, 2000). At the beginning of the study a refractive index detector was not available and a method using UV detection was developed.

## 2.0.3 Determination of zinc in solution by HPLC and Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) analyses

Poros MC™ is a chromatographic column packing material with a bimodal pore size with large pores (6000-8000 Å) and smaller pores (800-1500 Å) which allow a high flow rate. It consists of polystyrene/divinylbenzene particles (2μm in diameter) cross-linked by a polyhydroxylated polymer with imidodiacetate functional groups, which bind transition metals with varying affinity. The Poros MC™ material, used in this study, is suitable for use over · pH range, pH 1-14. It was developed and is marketed as a protein purification material such that transition metals could be preparatively loaded onto the column and metal binding proteins run through the column, binding to the metal ions and consequently, the column. The commercial protocol suggested that the metals be loaded onto the column in weakly acidic conditions, most of the ZMG preparations were either neutral or slightly acidic.

All of these properties made Poros™ an ideal material for developing a method for analysis of the soluble zinc content of ZMG preparations. The free chelatable zinc from the preparations would be bound and other soluble material would pass through. There were two possibilities as to the fate of the zinc in the ZMG solution; either it will be free ionic zinc and will bind directly to the column or it will be zinc in the form of a soluble ZMG fraction. In the latter case it will either pass through unbound to the column and be detected in the load eluent fractions or it will be bound to the column along with the free zinc. If bound to the column, the zinc can then be eluted from the column using a chelator solution as indicated for removing metal bound proteins in the original method. It was thought that free ionic zinc might be eluted from the column in a fraction different from that of zinc in the form of soluble ZMG. Fractions collected from the HPLC were analysed for zinc by ICP-AES; copper was also analysed as a reference metal.

ICP-AES is an analytical technique that allows the detection and measurement of several elements at one reading from one specimen. It is very commonly used for elemental analysis of biological, environmental and geological materials. The method works as a sample aerosol is introduced into a stream of super heated gas, normally argon plasma, which is able to vaporise, atomise, excite and ionise the sample. In this form the sample constituent elements may be measured by a number of detector types including atomic emission spectrometer (AES), mass spectrometer (MS) and atomic fluorescence spectrometer (AFS) (Reviewed Montaser et al.,1998). In this study an AES detector was used. This utilises a photomultiplier array to measure the unique wavelengths of radiation emitted by the analyte as it is passed through the argon plasma flame (Montaser et al., 1998).

It is a relatively rapid technique, with approximately one sample per minute, or slightly longer, being processed depending on the number of elements to be measured in each sample. A computer attached to the ICP-AES collated results.

## 2.0.4 ZMG synthesis

Radiolabelled ZMG was required for cell uptake experiments but no data was available to compare ZMG synthesis techniques for product recovery and purity of recovered product. These experiments were carried out to determine the most appropriate method for production of radiolabelled ZMG. In addition to this, experiments were undertaken to investigate the possibility of labelling ZMG with the fluorescent marker fluorescein isothiocyanate (FITC).

In its most basic form, ZMG is synthesised by heating zinc salt with glycerol to between 240°C and 290°C at which point the zinc and glycerol spontaneously combine. Above 300°C the ZMG complex begins to degrade. In order to examine the uptake pathways of ZMG in cultured cells, ZMG was synthesised with a radioactive tracer, $^{65}ZnCl_2$. This could be detectable by autoradiography. Prior to using the radioactive tracer the most efficient method for ZMG synthesis had to be developed.

The choice of zinc compound was arbitrary and the decision was based, in this study, on availability, mechanical characteristics and eventual product recovery. Initially, zinc oxide was used as it was the first compound available but its lack of solubility meant that determining complete reaction was difficult as both zinc oxide and ZMG look rather similar when in suspension. In addition to this, zinc oxide, even when finely crushed, did not form a very efficient suspension, specifically, as the glycerol was heated and its density decreased the zinc oxide powder remained resolutely on the base of the reaction vessel.

The next method carried out, utilised zinc chloride. Zinc chloride is water-soluble and the solution is miscible with glycerol. This removed the problem of differentiating product from source material during the reaction and reduced requirement for constant stirring. Zinc chloride was also attractive as a zinc source as it matched the radioactive tracer, $^{65}ZnCl_2$, and so should react exactly as the main body of zinc salt. The tracer had very high specific activity and so represented an exceedingly small fraction of the total volume. The method incorporating zinc chloride was taken from the original paper describing the properties of ZMG (Fairlie et al., 1992).

ZMG was also synthesised from zinc acetate. The method was described in a personal communication (Personal communication from Dr. Reg Taylor, Adelaide, South Australia via Professor Kim Rainsford).

The synthesis of ZMG with an FITC label was a novel concept and whilst the results were only preliminary they are included for completeness and for reference in future work. It was observed that the structure of the fluorescent marker used for cellular endocytosis, FITC-dextran conjugate, was a long chain polymer (Sigma Technical Notes for FITC-dextran). From the commercial literature it was seen that the FITC fluorescent label on the dextran was attached to a hydroxyl residue at random points along the dextran polymer chain. As ZMG is theoretically able to present free hydroxyl residues at the end of the ZMG polymer it was decided to investigate the possibility of conjugating ZMG with FITC. The benefit of this would be the possibility of observing the progress of ZMG uptake by cells directly or even observing uptake in conjunction with a zinc

specific fluorophore. Specifically, FITC fluorescence is pH sensitive with neutral pH being the optimum. By co-staining cells with zinquin, a fluorescent ZMG-FITC conjugate would allow the tracking of ZMG uptake through acidic or neutral cell vesicles. This is discussed further in the following chapter dealing with cellular zinc uptake.

### 2.0.5 ZMG preparations for use in cell culture

Data has been published on the physicochemical properties of ZMG and its solubility (Fairlie et al., 1992). However Fairlie et al. (1992) did not especially consider preparations for *in vitro* studies and did not set out to establish a particular preparation as preferable. The study only documented a solubility profile for ZMG. To enable to the investigation of the effects of ZMG for *in vitro* cell culture systems it was important to prepare the compound so that it was compatible with cell culture. The most important consideration was the toxicity of the preparation; the pharmacological effects of the vehicle itself must be taken into account in order that any overall effect may be attributed directly to the ZMG. Ease and rapidity of formulation was also important and, with very little data on the effect of ZMG in suspension or solution, it was prudent to keep the preparation time as short as possible in order to reduce the probability of molecular degradation.

### 2.0.6 Matrix Associated Laser Desorption/Ionisation – Time of Flight (MALDI-ToF)

MALDI-ToF is a relatively new analytical technique, available to the biochemical and pharmaceutical industries for approximately a decade (Reviewed by Cotter, 1997). The widespread use of this instrument has occurred even more recently with the manufacture of bench-top MALDI-ToF analysers. The technique uses a target slide, bearing a matrix-coated sample, at which a laser is fired. The laser-hit causes desorption of the sample from the target slide and simultaneous ionisation of the sample matrix coating the particles. The energy from the laser accelerates the sample particles towards the detector array of a mass spectrometer. The time-of-flight between target slide and detector is dependent

on the velocity of the sample particle, which in turn is inversely proportional to the square root of the particle mass.   A computer attached to the mass spectrometer analyses the time-of-flight, relative to an internally calibrated set of masses, and produces a mass/charge versus intensity graph of particles detected by the mass spectrometer.   This method has been used with great success in analysing large biological molecules and polymeric compounds (Cotter, 1997).   For this reason it seemed ideal for analysing the state of ZMG in various preparations.

## Experimental

### Reagents

Solvents acetonitrile, methanol and dichloromethane, were all HPLC grade from either BDH, Fischer or Fisons. Hydrochloric acid, sodium hydroxide, potassium hexacyanoferrate and citric acid monohydrate were Analar grade and obtained from BDH. Laboratory grade glycerol, EDTA (Ethylenediamine tetraacetic acid, disodium salt) and sodium chloride (NaCl) were also obtained from BDH, as were the zinc and copper-standard stock calibration solutions for ICP-AES (ICP analytical grade). ZMG was obtained from Glyzinc (Bellara Pharmaceuticals). All other zinc compounds, tris-HCl, glycerol (SigmaUltra grade), dimethyl sulphoxide (DMSO), 2,5-Dihydroxybenzoic acid, Hydroxypropyl-$\beta$–cyclodextrin, $\gamma$–cyclodextrin and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich. Ultrafine cobalt metal powder was obtained from Shimadzu. Helium and argon, for degassing solutions, were obtained from BOC. Poros™ HPLC column packing material was obtained from Boeringer Mannheim. Absolute ethanol was obtained from Hayman Ltd. $^{65}ZnCl_2$ was obtained from Amersham. Disposable plasticware was obtained from Falcon or Nunc. Roswell Park Memorial Institute (RPMI) cell culture medium and Hank's Buffered Saline Solution (HBSS) were obtained from GIBCO BRL. Bovine Serum Albumin (BSA) supplement, PEG300 and Tween 20 were unbranded laboratory reagents. Ultrapure deionised water was obtained from Millipore Milli-Q$^{PLUS}$ PF water system.

### 2.1.1 HPLC Analysis of ZMG - Determination of glycerol in solution and analyses of ZMG degradation

**Equipment**

The initial method development experiments were carried out on an integrated Waters HPLC system. A Waters 510 HPLC pump was attached to a Waters 717 plus autosampler, with 20-50µl injection volume, coupled to a Waters 486 Tuneable Absorbance Detector. The whole system was controlled from a Digital Venturis computer, running Waters Millennium Chromatography Manager software, which both remotely managed the hardware control and recorded data received from the detector. When refractive index detection was carried out, the above system was used replacing the Waters 486 Tuneable Absorbance Detector with an unbranded refractive index detector.

Due to reallocation of resources, further method development and subsequent analyses were carried out on a different system. This consisted of a Gilson Model 302 pump attached via a Gilson Model 802 Pressure module to a Waters Model 480 Lambda Max LC Spectrophotometer. Manual injection of sample was carried out through a Rheodyne switching valve Model 7125 with a 20µl injection loop. Data from this second system was recorded on a Western Systems computer running DUO interface software.

The HPLC column used for glycerol analysis was a Spherisorb $NH_2$ 5µm column with length 250mm and diameter 4.6mm, for size exclusion chromatography. The column was obtained from Alltech.

ZMG and glycerol analyses were carried out using 80% acetonitrile:water degassed under helium or nitrogen. Laboratory grade glycerol was used for initial method development experiments and was replaced by SigmaUltra grade glycerol for the later analytical experiments. All solutions used were filtered through Whatmans filter paper.

## Method

ZMG was initially undetectable by the HPLC methods used and subsequent detection problems were encountered with the citric acid Tris buffered preparations initially used to dissolve ZMG. It was therefore decided to concentrate on glycerol detection alone and then to move on to ZMG analysis. This further allowed development of a method for quantification of glycerol release from degraded ZMG

### *Adaptation and validation of HPLC method for glycerol detection*

Initial adaptation of the HPLC method was necessary, as a working refractive index detector, recommended for glycerol detection, was unavailable. The Waters automated system was set up with a UV spectrophotometer detecting at 210nm.

Dilutions were made of glycerol (2mg/ml) in ultrapure water and injected using the Waters autosampler.

It was found that glycerol was not detectable at wavelength 210nm and so 190nm was tried instead. Glycerol was diluted in double distilled water to make a standard curve, starting with 100mM glycerol which was serially diluted 1 in 2 down to a concentration of 1.56mM glycerol. A volume of 50 µl from each concentration was run at 1ml/min and the results recorded on computer.

### *Detection of glycerol by HPLC*

For the continuation of the study the Gilson/Waters hplc apparatus was used and the validation was repeated on this apparatus to determine reproducibility.

Glycerol in aqueous solution was analysed as follows. Dilutions were made of glycerol in distilled water, in the concentration range 1mM-800mM. Samples were filtered through a 0.22µm syringe tip filter into clean tubes. Samples to be

loaded onto HPLC were drawn into a polypropylene syringe before injecting into a 20μl loading coil attached to a Rheodyne™ injection valve. Conditions used on all HPLC runs in this method were 1000bar column pressure, 1ml/min flow rate, 80% acetonitrile/double distilled water mobile phase. The column used for the separation was a Spherisorb $NH_2$ affinity chromatography column.

After every third or fourth glycerol injection the system was flushed with dilute (0.1mM) hydrochloric acid as it was found that repeated sample injections caused deposition of glycerol and build up of pressure. Following the acid cleaning, the system was allowed to flush with mobile phase for approximately 30 mins or until the baseline stabilised.

Glycerol, eluted from the column, was measured by a UV spectrophotometer set at 190nm wavelength. A computer linked to the spectrophotometer and running DUO data recording system recorded absorbance.

### Analysis by HPLC of glycerol released from ZMG in suspension

ZMG (1-1.25g) was weighed out into 50ml plastic centrifuge tubes. Ultrapure water was added to give a final ZMG concentration of 100mg/ml. Immediately after the water was added the tubes were vortexed for 15 seconds and then placed on a rotating wheel to incubate at room temperature with continual inversion for increasing periods of time. At the end of the incubation period the suspension was drawn into a syringe and immediately filtered through a 0.22μm syringe tip filter. Samples were then treated as described above for the analysis of glycerol.

The filtrate was also stored for increasing periods of time, 18hr, 42hr, 66hr and 1 week and rerun on the HPLC as described above.

## 2.1.2 HPLC Analysis of ZMG - Determination of zinc in solution and analyses of ZMG degradation

**Equipment**

The HPLC system used in this series of studies was based on the Waters/Gilson system used for the glycerol analyses although a WatersTM 510 HPLC pump later replaced the Gilson pump, as it required repair. Data was captured as before using the DUO software. The analytical column used was 50mm in length by 8mm in diameter, packed with Poros MC™ polystyrene-divinylbenzene beads with particle diameter 20μm. The beads were coated with a cross-linked hydroxypolymer with imidodiacetate functional groups as the immobilised metal chelating ligands. The guard column, 30mm in length by 8mm in diameter, was packed with Poros MC™ as with the analytical column. Sample injection was carried out through an Omnifit valve attached to a 2ml reservoir. Fraction collection was carried out manually and fractions were collected into clean borosilicate test tubes.

All solutions were tested on ICP-AES for zinc contamination before use and subsequently throughout the study periodic analysis of solutions was carried out to ensure mobile phase (water) and eluent (EDTA:NaCl solution) contained no zinc contamination.

**Method**

*Detection of zinc in solution by HPLC and ICP-AES - Sample preparation and separation by HPLC*

Zinc released into solution by either zinc salt, dissolved ZMG or ZMG in aqueous suspension was analysed by HPLC using a Poros MC™ column. Zinc sulphate in aqueous solution was initially used to construct a standard method for extraction of zinc from a solution and measurement of zinc in eluted fractions.

The mobile phase was distilled water filtered through Whatman's coarse filter paper and degassed under helium for a minimum of 15 mins with flow rate 2.9ml/min (1mM zinc analysis) and 6ml/min (10mM zinc analysis) at room temperature. Zinc sulphate was dissolved in distilled water to a final concentration of either 1mM or 10mM. ZMG was prepared in citric acid (10mM) to the same concentrations of ionic zinc and balanced to a pH of ~6.6 by sodium hydroxide. Before loading, the sample was filtered through a 0.22$\mu$m syringe tip filter to remove any particulate matter. The sample loading was timed from the instant the flow started and a computer using DUO data capture system monitored the whole procedure. The sample was eluted through guard and sample columns packed with Poros MC$^{TM}$. Fractions were sometimes collected during the load and were started at 1min after loading and subsequently every 30 seconds until the baseline returned to normal. The mobile phase was allowed to elute for a further 5-10 mins.

Elution was carried out with an aqueous mixture of EDTA (50mM), NaCl (500mM), filtered through Whatman's coarse filter paper and degassed under helium for at least 15 mins. Elution was carried out under continuous flow at 2.9ml/min (1mM samples) and 6ml/min (10mM samples) at room temperature and was timed from the start of eluent flow. For analysis of 1mM zinc, 2.9ml fractions were collected from 30 seconds after the start of eluent flow and subsequently for every minute during elution. For analysis of 10mM zinc, 2.9ml fractions were collected from 30 seconds after the start of elution and subsequently every 30 seconds during elution. Eluent was allowed to flow for a further 5-10 minutes to completely remove trace zinc after which distilled water was run through the system until the baseline returned to normal. Fractions collected from both stages were either run immediately on the ICP-AES or stored at 4°C until ready for analysis.

***Analysis of zinc released from ZMG in suspension by HPLC and ICP-AES***

To quantify ZMG degradation in aqueous suspension zinc, a breakdown product of ZMG, was measured. Further studies were carried out on the filtrate from ZMG suspension. The assay allowed detection of released zinc that either bound or did not bind to the Poros MC™ HPLC column packing

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ZMG samples were treated with the same protocol as used for the analysis of glycerol released by ZMG suspension.

ZMG (1-1.25g) was weighed out into 50ml plastic centrifuge tubes. Ultrapure water was added to give a final ZMG concentration of 100mg/ml. Immediately after the water was added the tubes were vortexed for 15 seconds and then placed on a rotating wheel to incubate at room temperature with continual inversion. At the end of the incubation period the suspension was drawn into a syringe and rapidly filtered through a 0.22μm syringe tip filter. The filtrate (2ml) was loaded immediately onto the HPLC following the method described for analysis of zinc sulphate solution (pp.90-91). Fractions collected from both stages were either run immediately on the ICP-AES or stored at 4°C until ready for analysis.

### Sample analysis by ICP-AES

Samples for ICP-AES analysis were prepared by 1:1 dilution with distilled water to give a final volume of 2.9ml. The ICP-AES was calibrated, before every analysis, with zinc and copper solutions as follows. Zinc and copper salt stock standard solutions (both 1000ppm) were diluted serially to give two standard solutions containing 100ppm zinc/copper and 10ppm zinc/copper using zinc free distilled water as reference. These solutions were run through the ICP-AES and the instrument automatically calibrated for each concentration of metal. Samples were then run through the calibrated instrument and the zinc was detected as parts per million per fraction (ppm/fraction).

### 2.1.3 Analysis of ZMG by Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-ToF)

**Equipment**

MALDI-ToF analyses were carried out on a Laser Tof 1500 (SAI Ltd. Manchester). Analysis of ZMG using MALDI-ToF initially followed a protocol using a standard matrix and a solution of ZMG. Further analyses included a suspension of ZMG and different matrices.

**Method**

*Analysis of ZMG in solution using 2,5-dihydroxybenzoic acid (2,5-DBH) as matrix*

Initially ZMG was dissolved in 1M hydrochloric acid to a final concentration of 1M ZMG. This was then diluted 1:10 in distilled water to give a 0.1M ZMG solution in 0.1M HCl. Controls used were HCl solutions of 1M and 0.1M. From each preparation or control 10ml was mixed with 10ml of a saturated solution of 2,5-DHB in methanol. Approximately 2µl of each prepared sample or control was immediately spotted onto a clean, steel MALDI-ToF slide and allowed to evaporate at room temperature to complete dryness. The slide was then placed into the MALDI-ToF for analyses of the samples. The results were obtained in the form of a fragment spectrum given by signal intensity against fragment mass.

*Analysis of ZMG in suspension using 2,5-DBH as matrix*

The media used for suspension of ZMG were water, glycerol and DMSO. ZMG was suspended in distilled water, 100% glycerol or 100% DMSO to a final concentration of 100mg/ml ZMG. The DMSO preparation was also further diluted with 100% DMSO as required. Each preparation was then mixed 1:1 with 2,5-DHB. Approximately 2µl of each prepared sample or control was immediately

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spotted onto a clean, steel MALDI-ToF slide and allowed to evaporate at room temperature to complete dryness with the exception of DMSO. The DMSO did not dry in air but instead was allowed to stand for between 10 and 20 minutes and run whilst still wet. The slide was then placed into the MALDI-ToF for analyses of the samples. The results were obtained in the form of a fragment spectrum given by signal intensity against fragment mass.


## Analysis of ZMG in suspension using potassium hexacyanoferrate (II) as matrix

Results for each of the previous sample preparations were not, at first, consistent and the possibility that the MALDI-ToF matrix, which is acidic, could be degrading the ZMG was considered. Most matrices for MALDI-ToF analysis are acids but there are some far less commonly used matrices that are not acidic including potassium hexacyanoferrate (II) and cobalt. The matrix used in the following experiment was potassium hexacyanoferrate (II) and was carried out as described in the literature (Zollner et al., 1997).

A saturated solution of potassium hexacyanoferrate (II) was prepared in methanol and glycerol was added to a final concentration of 5% (v/v). This was then mixed vigorously by repeated vortex and inversion for 5min and then centrifuged at $1500_g$ for 1min. A slightly opaque suspension with a large pellet consisting of excess compound was produced after centrifugation. The opaque supernatant (the matrix) was pipetted into a clean tube and 0.5µl then spotted onto a clean target slide where the methanol evaporated rapidly leaving a colourless coating of matrix on the target slide. It should be noted that in the published methods (Zöllner et al., 1997) the matrix is not directly mixed with the sample, instead the sample is layered on top of the matrix. For the sample preparation ZMG was vigorously suspended, by vortex and manual inversion in 100% DMSO to a final concentration of 100mg/ml that was diluted 1:100 to give 1mg/ml. Approximately 2µl of each dilution was spotted onto target slides precoated with matrix.

ZMG was also prepared, without DMSO, in potassium hexacyanoferrate (II) solution alone as follows. ZMG was mixed with saturated potassium

hexacyanoferrate (II) solution to a final concentration of 100mg/ml, which was diluted further by 1:10 to give 20mg/ml ZMG. From both sample concentrations ~2µl was spotted onto a clean target slide not pre-coated with matrix.

### Analysis of ZMG suspension using cobalt matrix

Ultrafine cobalt metal powder was also tested for suitability as a non-acidic matrix for ZMG analysis. A suspension of cobalt powder was made in methanol. The ZMG sample was prepared as before by suspension in 100% DMSO. The sample was then sonicated for 5 minutes with an equal volume of cobalt suspension. Sample preparations (~2µl) were then spotted onto the target slide.

As with the potassium hexacyanoferrate (II) matrix, ZMG without DMSO, was also mixed directly with the cobalt matrix for analysis. The preparation (~2µl) was spotted onto a clean target slide.

### 2.1.4 Synthesis of ZMG

**Equipment**

The glassware and Buchner funnel were washed in 1M HCl, then detergent and rinsed thoroughly in tap water and then distilled water both before and after use. Polypropylene centrifuge tubes were clean sterile plastic.

**Method**

***ZMG Synthesis using Zinc Oxide***

In a round bottomed glass flask zinc oxide (81mg, 1mmole) was mixed manually with excess glycerol (9.2g, 100mmoles). The flask was then placed in a heated sandbath and stirred continuously for 15 minutes maintained at between 260°C and 280°C. At this point the opaque zinc oxide suspension became flocculent and the reaction was stopped. A high temperature mercury thermometer placed directly into the glycerol/ zinc mixture monitored the temperature of the preparation.

After heating the mixture was allowed to cool to room temperature and then filtered through Whatman's coarse filter. The precipitate was then washed with copious amounts of methanol and allowed to air dry. A sample was then collected into a sample vial and sent for microanalysis to a commercial analytical company, Medac LTD.

The synthesis was repeated exactly as described above, with ten times more glycerol, (92g, 1mole) in order to examine the effect of increasing the excess glycerol level on product recovery efficiency. This sample was not sent for further microanalysis.

## ZMG synthesis using Zinc Chloride

This method was taken from the one published method for ZMG synthesis (Fairlie et al., 1992). In a polypropylene centrifuge tube zinc chloride (219mg) was dissolved in 160ml distilled water. The solution was adjusted to pH7.5 with 1M sodium hydroxide, at which point a precipitate formed. The precipitate was spun in a centrifuge at 1500g for 5 minutes and the supernatant liquid poured off. The pellet was washed in distilled water and methanol by resuspension and centrifugation as described above. The resulting gelatinous pellet was finally resuspended in 10ml methanol and then mixed with 20ml glycerol.

The mixture was heated very gradually to 180°C, the methanol and water visibly boiling off at 110°C and below. Gradual heating was continued until 248-250°C was reached and continued for a total of 90 minutes from the start of heating. A flocculent precipitate formed during the heating after which it was allowed to cool to 30°C, a temperature cool enough to filter but still warm enough to lower the viscosity of the glycerol, allowing easier pouring and washing of the sample from the flask. The precipitate was filtered off under reduced pressure, washed with copious amounts of distilled water and methanol and allowed to air dry. The product was then sent for microanalysis.

## ZMG synthesis using zinc acetate

In a round bottomed flask zinc acetate, 447.6mg, was dissolved in 20ml distilled water. To this was added 39ml glycerol, which was mixed manually. This mixture was heated gradually to 190°C and maintained at this temperature for 15 minutes until a flocculent precipitate formed. The sample was then allowed to cool and the precipitate filtered off, washed with a large amount of water and absolute ethanol before drying in an oven at 65°C. The dry product was then sent for microanalysis.

One change was made to this protocol in the light of the synthesis of radiolabelled ZMG and that was to repeat the above method but with the inclusion of $ZnCl_2$, the form in which $^{65}Zn$ was purchased. For this $ZnCl_2$, 10µl of a 10mM solution, was

added prior to the addition of the glycerol. The sample was processed exactly as described above for the ZMG synthesis from zinc acetate alone. The resulting product was also sent for microanalysis.

### Synthesis of radiolabelled ZMG from zinc acetate and $^{65}ZnCl_2$

The basic method for synthesis of ZMG from zinc acetate and glycerol was used as it required lower temperatures and had least handling stages. In a round bottomed flask zinc acetate, 455.1mg, was dissolved in 20ml distilled water and into this was mixed 39ml glycerol (Ultrapure grade). To this mixture $^{65}ZnCl_2$, 2μl, was added and mixed by gently swirling the flask. The mixture was heated on a sand bath to between 170C°-185°C and kept at this temperature for 20min. The preparation was then allowed to cool to 40°C at which point it was mixed with 200ml distilled water and the precipitate was filtered off. The resulting product was then used for in vitro studies of ZMG uptake by cells in culture.

### Synthesis of a ZMG Fluorescein isothiocyanate (FITC) conjugate

Two methods were used to try and conjugate ZMG to FITC, short periods of heating or long term incubation with constant agitation. In the first method, ZMG, (30mg) and (FITC), 10mg, were mixed with DMSO, (6ml). The mixture was heated until almost boiling and then allowed to cool. The suspended material was recovered by filtration and washing in water and methanol.

The process was difficult to work up, as the mixture was highly viscous and filtration took a long time, even under reduced pressure from a vacuum pump. In addition, although the heating process was slow and boiling was avoided, charring still appeared to occur during the heating process where the mixture was in contact with the glass of the flask. A red powder was recovered but experimentation with this method was not continued.

As the first results had been obtained from the MALDI-ToF analysis of ZMG in DMSO it was seen that ZMG in this preparation appeared to exist as individual

polymeric chains.   The hydroxyl residues should thus be readily available for reaction.

It was decided to try incubation without heating.   ZMG (1.7mg) and FITC (17mg) were mixed by brief vortex in DMSO and 5 minute incubation at room temperature in a sonicating waterbath.   The preparation was incubated at room temperature on a rotating mixer for 24 hours.   The preparation was then compared with an equal amount of ZMG in DMSO without FITC for apparent dissolution.   The ZMG FITC preparation was clear with apparently effective dissolution whilst the ZMG alone was completely opaque.   After several attempts, it was discovered that a roughly 1:10 or 1:20 ratio of ZMG to FITC allowed greatest dissolution.   Whilst, after the first experiment, dissolution with this technique was never complete, it was always clearly greater than controls without FITC.

Preparations were then vortexed to remove undissolved material and the supernate was analysed by ICP-AES in order to determine zinc concentration. Results from the ICP-AES were obtained as parts per million and presented graphically.

Some preparations were also analysed by thin layer chromatography (TLC). Typically, methanol or acetonitrile/water in varying concentrations were used as a mobile phase for the TLC analysis.   ZMG FITC preparations were spotted onto plastic backed silica TLC plates and the plates were placed in a TLC jar containing mobile phase solvent either methanol or acetonitrile/water.   The plates were left in the TLC jars until the mobile phase had risen to within 1cm from the top of the plate then they were removed from the jar, air dried and exposed to UV light.   The fluorescent spots were marked with a pencil for later analysis without UV light.

As the products should be fluorescent and TLC was a rapid and relatively low cost procedure this was thought to be the most suitable analytical method.   In addition to this, it was thought that TLC might prove a useful method to separate FITC-conjugated ZMG from the free FITC that was in great excess in the preparations.

## 2.1.5 ZMG Dissolution

ZMG is an unusual compound in that it is almost completely insoluble in most common solvents and only dissolves completely, in aqueous solution, in direct proportion to the free hydrogen ion concentration. In practice then, ZMG will only dissolve in acid but this is suspected to result in the degradation of ZMG to glycerol and free zinc rather than producing a solution of ZMG. Two papers are available on the dissolution of ZMG, in the first, Fairlie et al. (1992) described the relative solubility of ZMG in a variety of common solvents and tissue preparations. In the second paper Tiekink et al. (1996) described the dissolution of ZMG in acid and they formulated a mathematical model for this dissolution.

Of interest in the present investigation into the effects of ZMG for *in vitro* cell culture systems was the preparation of the compound in a vehicle compatible with cell culture. The most important consideration was the toxicity of the preparation; the pharmacological effects of the vehicle itself had to be taken into account in order that any overall effect may be attributed directly to the ZMG. Ease and rapidity of formulation was also important, and with very little data on the effect of ZMG in suspension or solution it was prudent to keep the preparation time as short as possible in order to reduce the probability of molecular degradation. Examined were commercial solvents commonly used in cell culture techniques, and cyclodextrin carrier molecules. Carrier systems are widely used in pharmaceutical preparations and of these systems some, including cyclodextrin molecules with various side chains, are particularly suited to encapsulation and administration of hydrophobic insoluble material.

**Method**

*Dissolution of ZMG in cell culture compatible solvents*

Cell culture media tested were balanced salt solutions, HBSS and RPMI, the RPMI was also tested with 10% BSA supplement. Distilled water was also tested with or without 10% BSA. PEG300 and TWEEN 20, both detergents were tested

for ability to dissolve or disperse ZMG as they have been used in cell culture preparations. Glycerol, DMSO and ethanol were tested for comparison purposes although they had been tested in the literature.

ZMG, (15.5mg), was placed in a 50ml polypropylene centrifuge tube and was vortexed in 10 ml of the vehicle to be tested. Results were judged immediately by visually comparing black lines on a white background through the diameter of the 50ml Falcon tube containing the various liquids plus ZMG powder. Results were marked by an arbitrary rating system based on the clarity of the lines seen through the liquid, as no method was available for direct measurement of dissolution at this stage. Dissolution was graded as follows; '-' meaning no change in clarity, '-/+' as just perceptible increase in clarity, '+' as definite increase in clarity and '++' as complete clarity. Increasing dissolution was determined as increasing clarity of preparation immediately after mixing.

As no non-acidic solvent was found for ZMG, a rapid preparation for ZMG solution in citric acid was used for experiments as required. A fraction of ZMG administered orally as described in the literature (Whitehouse et al, 1990, Rainsford, 1992) is likely to be dissolved rapidly in the stomach due to the low pH as indicated by Fairlie et al (1992). Whilst this group used a variety of solvents to dissolve ZMG, a simple and rapid preparation for ZMG was sought that could be administered to cells. ZMG was shown to dissolve well in citrate (Fairlie et al., 1992) and as it is a ligand that is found commonly in biological tissue it seemed a good solvent for preparations of ZMG solution. For all ZMG acid solutions presented in these studies ZMG (31mg) was dissolved in citric acid solution (10ml, 10mM) and made up to 20mls with water to give a final ZMG concentration of 5mM.

### ZMG preparations with cyclodextrin carrier systems

Aqueous solutions were made of cyclodextrins at varying concentrations. ZMG was then either added directly to the cyclodextrin solution or was first suspended in water or DMSO before addition. Sonication was carried out to both reduce particle size and aid reaction.

# Results

## 2.2.1 HPLC analysis of glycerol

The initial results from the experiments on the Waters system, validating the glycerol analyses, are shown here for completeness and to show that the method was repeatable after transfer between HPLC systems. As may be seen in figure 2.1 thereisapeak that corresponds to glycerol, and which is absent in the profile obtained from injection of water alone. The baseline is not level in this particular trace but the results were consistent over at least four runs with the glycerol peak present at 6 minutes in all.

The method was successfully transferred to the older Gilson HPLC system and figure 2.2 shows the dose dependent nature of the area under the curve for the increasing glycerol concentrations. This is the standard curve from which glycerol release from ZMG was calculated as described in the next section. There is a slight decrease in sensitivity on the older system with a minimum detection limit, on the linear curve, of 8-10mM. These results however, confirmed that glycerol could be analysed and quantified using a UV detector despite an apparent absence of published protocols for this detector type. The chart response scales differ between system as both computer systems used differing analytical programs.

It should be noted that in each of the series of traces, of which two are presented (Figure 2.1), a peak corresponding with water was visible but it did not increase as the glycerol peaks increased. Additionally, it should be noted that there were no peaks eluted, other than glycerol and water, up to 35mins after sample injection.
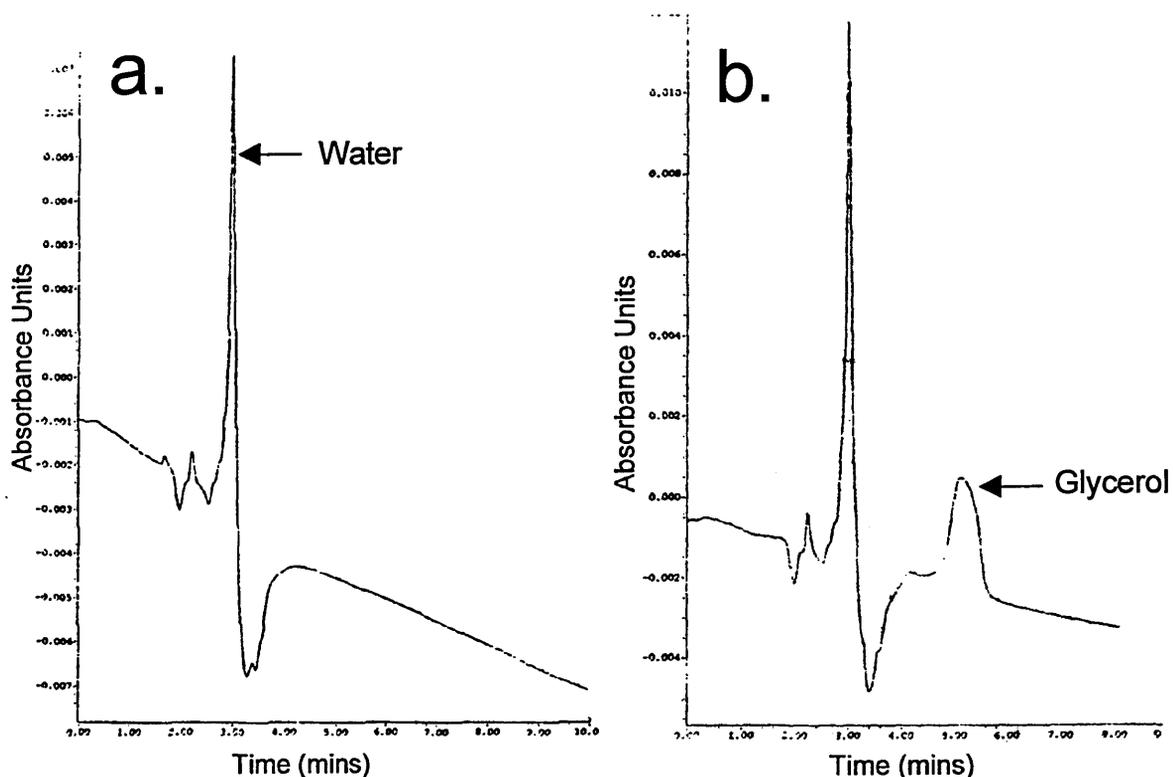
**Figure 2.1.** **HPLC analysis of glycerol:** Analysis of glycerol using Waters HPLC with a Spherisorb NH$_2$ column and a UV detector at 190nm wavelength. Figure 2.1a shows the trace of water alone with a peak around 3 mins. Figure 2.1b shows the trace of 2.5% glycerol in aqueous solution (342mM). The water peak is apparent at 3 mins and a further peak may be seen, at 6 mins after injection, that represents glycerol in solution. These traces are representative of two experiments carried out on the Waters HPLC, each containing 4-5 concentrations of glycerol displaying a peak eluting at the same time point.
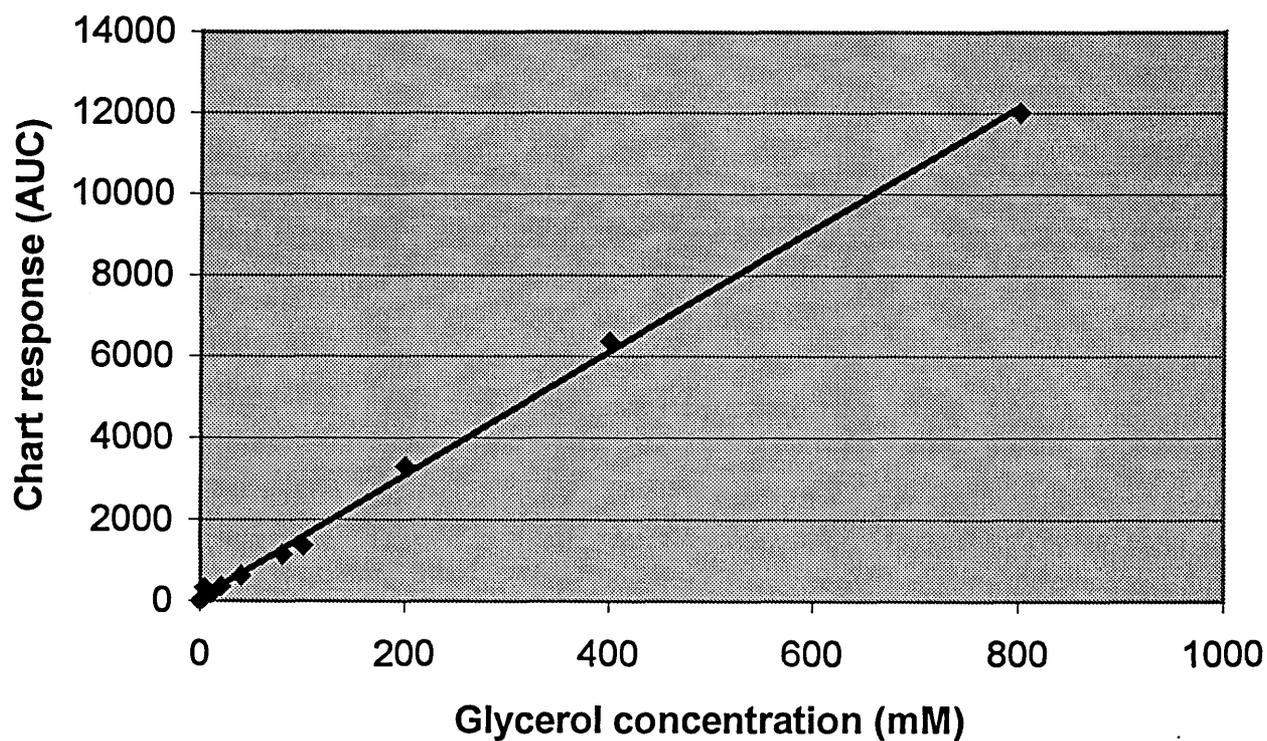
**Figure 2.2** **Analysis of glycerol on Gilson HPLC:** Varying concentrations of glycerol were analysed by HPLC using Gilson apparatus and UV detector. As with the previous standard curve obtained on the Waters HPLC, the linear nature of the glycerol detection system is displayed in this graph. This standard curve of glycerol concentration is representative of three separate experiments. Equation of curve: $y = 15.099x + 77.24$

## 2.2.2 HPLC analysis of ZMG degradation by detection of glycerol release

Glycerol release into solution, from ZMG incubated in neutral pH aqueous suspension, was quantified by HPLC. A peak was eluted at 6 minutes after injection corresponding to that of glycerol. The area under the curve of the peak was analysed by DUO HPLC software. These results were converted to equivalent millimolar glycerol by correlation with known glycerol concentrations from the standard curve, (Figure 2.2). The results indicated that glycerol is gradually released from ZMG 0-240 minutes after suspension in distilled water (Figure 2.3, black circles).

From the ZMG in suspension (4g = 12.9mmol/40ml), the maximum theoretical mass of glycerol available was 25.8mmol glycerol. The maximum amount of glycerol detected in solution, after 240 minutes suspension, was approximately 19.04mmol per 40ml sample. This indicated that after 4 hours in aqueous suspension approximately 74% of the glycerol had been released from the ZMG. After filtration of suspensions there was a large amount of white insoluble material that, these results indicate, was likely to be predominantly zinc oxide. As syringe tip filters were used to obtain the supernate it was not possible to recover the insoluble residue for analysis. The results obtained from these glycerol analyses are discussed, with relevance to the actions of ZMG in biological systems, in chapter 5.

In addition to the expected glycerol and water peaks, a broad peak was eluted from 10 to 16 minutes after sample injection (Figure 2.4). Like the glycerol peak, the unknown peak increased in size in conjunction with increasing suspension time (Figure 2.4 A-E, black arrows). As the glycerol peak eluted consistently at 6 minutes the only other compounds that might have been in solution could be a soluble fraction of ZMG or dissolved zinc oxide. If this represented soluble ZMG then over time it might be expected to release more glycerol which would be detected as an increase in the glycerol peak. In order to examine this possibility, the original filtrate samples were incubated at room temperature and re-analysed by HPLC after approximately 24, 48, 66 and 168 hours. As may be seen in figure 2.3, incubation of the filtrates did not result in increase of glycerol release. There

was no graded increase in glycerol concentration with increasing filtrate incubation time from 24-168hrs, any variations from the original 0hr filtrate appeared random. So it seemed that the unknown peak did not appear to contribute to the total glycerol concentration.

Although no additional glycerol appeared to be released, Figure 2.4 shows that the unknown peak partially resolved over time into two peaks. It would seem unlikely that putative molecules of soluble zinc oxide would react in this manner, eluting as one peak that consisted of two separate peaks. This seems to be a more complex component of the solution than zinc oxide or a zinc salt. A relatively stable, soluble fraction of ZMG would seem to be the only apparent candidate for the unknown filtrate component. Contamination of the original ZMG source may be discounted as indicated by the microanalysis of the proprietary stock ZMG carried out during the ZMG synthesis experiments (Table 2.1, p.127). Contamination of individual suspensions during preparation may be discounted as each suspension was made up separately plus the fact that the size of the unknown peak increases with time in suspension. Further work was started to resolve the two peaks by alteration of the HPLC mobile phase. In addition to the new information describing the nature of ZMG in suspension and solution, this study also provides a novel, simple and quantitative analytical method for examining ZMG degradation.

Some analyses were carried out on the first Waters HPLC system to determine glycerol content of ZMG in citric acid solution and pH buffered acid solution. Some glycerol was detected without the appearance of an unknown peak but time constraints prevented a more thorough examination of these preparations. In the light of the MALDI-ToF analyses described later, it is unlikely that the acid preparation of ZMG would contain a soluble ZMG component. The MALDI-ToF spectrograph shown in Figure 2.14 indicates the presence of a peak corresponding only to glycerol in the ZMG acidic solution.
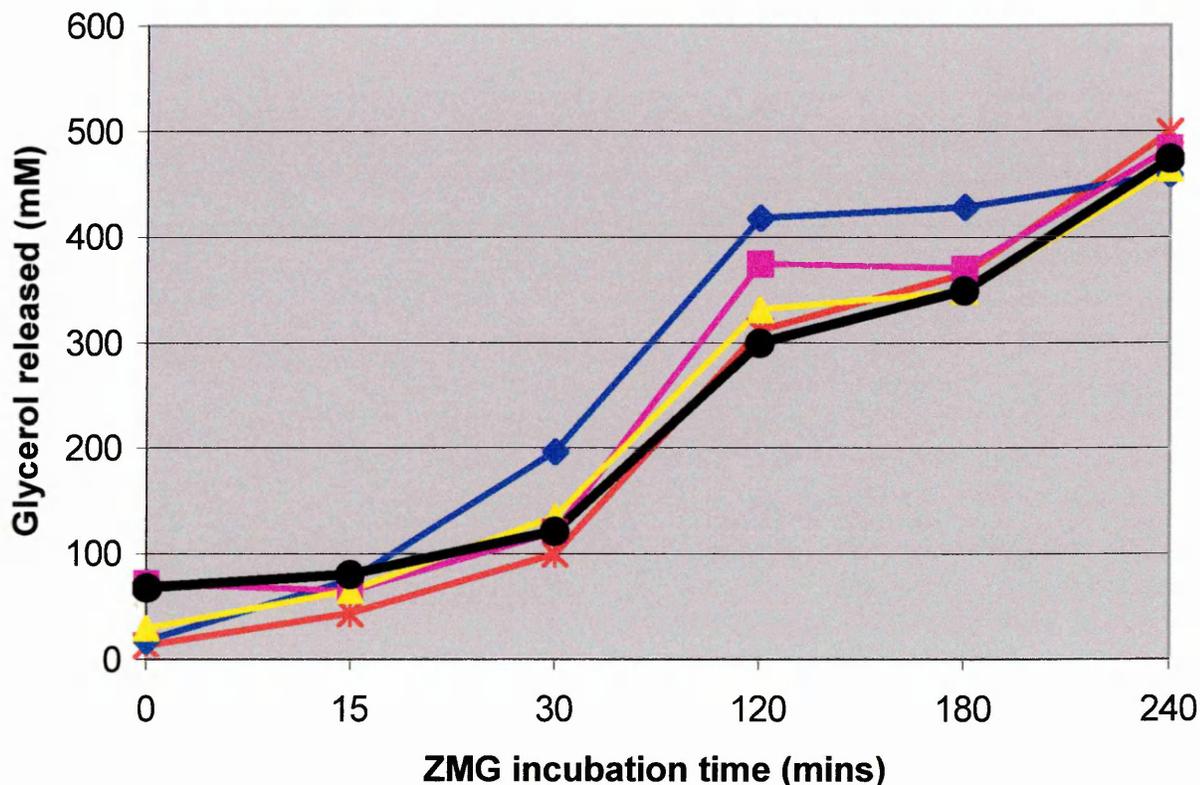
**Figure 2.3     Glycerol released by ZMG in aqueous suspension:** ZMG, 1g/ml, was incubated from 0 to 240 minutes at room temperature in distilled water with constant, gentle mixing.   The suspension was filtered and the glycerol concentration of the filtrates was determined by HPLC analysis on a Spherisorb $NH_2$ column.   Glycerol was released into solution with time of incubation (0hrs, indicated by black circles on graph). Samples, 0-240 minutes, were incubated at room temperature a further 24-168 hours *after* filtration, and analysed by HPLC as before. There was no significant difference between the glycerol release trend of filtrates incubated 24 (yellow triangle), 48 (pink square), 66 (blue diamond) or 168 (red star) hours after filtration and that of the sample immediately after filtration, 0hrs.   It is clear that variation in glycerol release is random with no apparent pattern related to increasing filtrate incubation time from 0-168 hours.
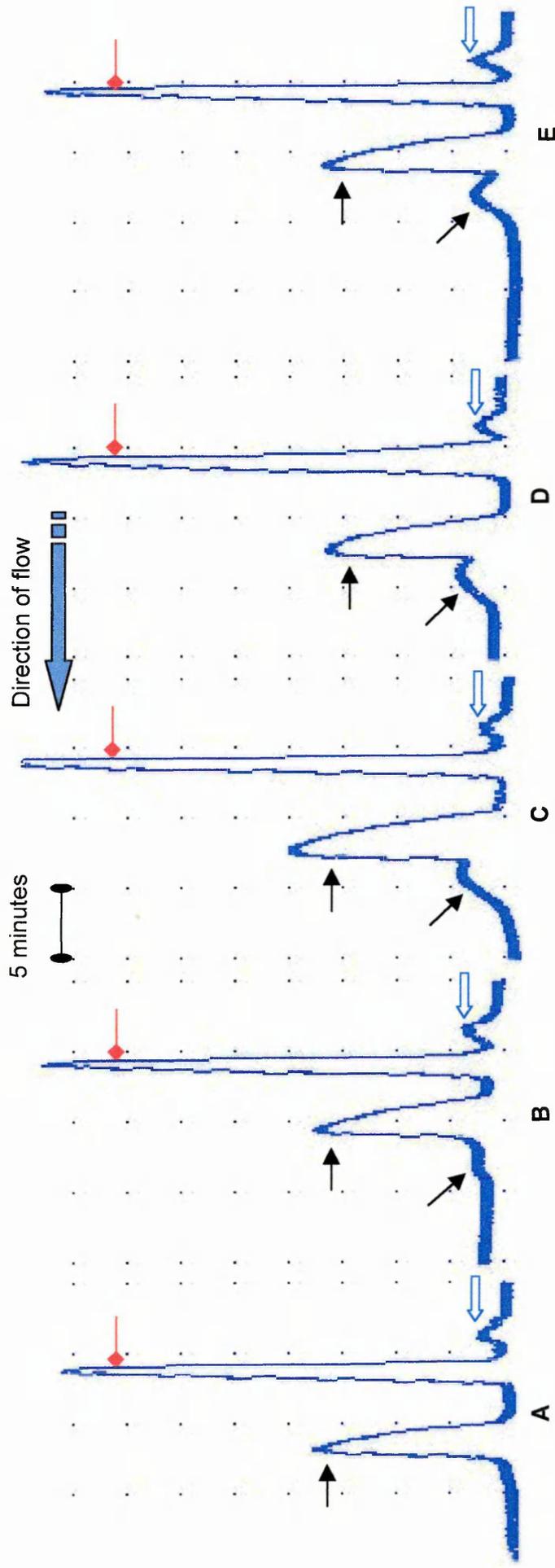
**Figure 2.4    HPLC trace of ZMG filtrate:** ZMG in aqueous suspension was incubated for 120 minutes before filtration.   The filtrate was analysed at 0hrs by HPLC on a Spherisorb $NH_2$ column at 1ml/min, figure 2.4A.   The same filtrate was then incubated for 24, 48, 66 and 168 hours (figures 2.4 B, C, D and E respectively) and reanalysed by HPLC.   Both glycerol peak (red, diamond headed arrow) and unknown peak (upper black arrow) maintain approximately the same sizes, as indicated by area under curve.   However the unknown peak resolves, over time, into two peaks (both black arrows).   Water peak does not change over time (blue, open arrow).

### 2.2.3 HPLC analysis of ZMG degradation by detection of zinc release from ZMG solution

In the fractions eluted from the Poros column after loading with 1mM zinc sulphate solution, zinc is detected in fractions 4-9 (2.9ml/fraction) with a peak at fraction 5 (n=5 for each point), (Figure 2.5). The fractions eluted after addition of 1mM zinc as ZMG solution, prepared in citric acid, also showed zinc eluting between fractions 4-9 with the peak also at fraction 5 (n=4 for each point), (Figure 2.5). Statistical analysis of the two sets indicated no significant difference in zinc elution between the two samples loaded.

This pattern is mirrored by fractions eluted from the HPLC system after loading with zinc sulphate and ZMG solutions at 10mM, (Figure 2.6). No significant differences were observed at this concentration between fractions from the two preparations. ZMG in citric acid solution therefore appears to act like zinc sulphate solution in this system suggesting that both contain zinc in free ionic form.

When comparing 1mM and 10mM results it should be noted that elution conditions were changed. A faster flow rate was used for elution of the 10mM samples and more frequent collection of fractions. This did not affect any comparison between results from ZMG and zinc sulphate at each individual load concentration.
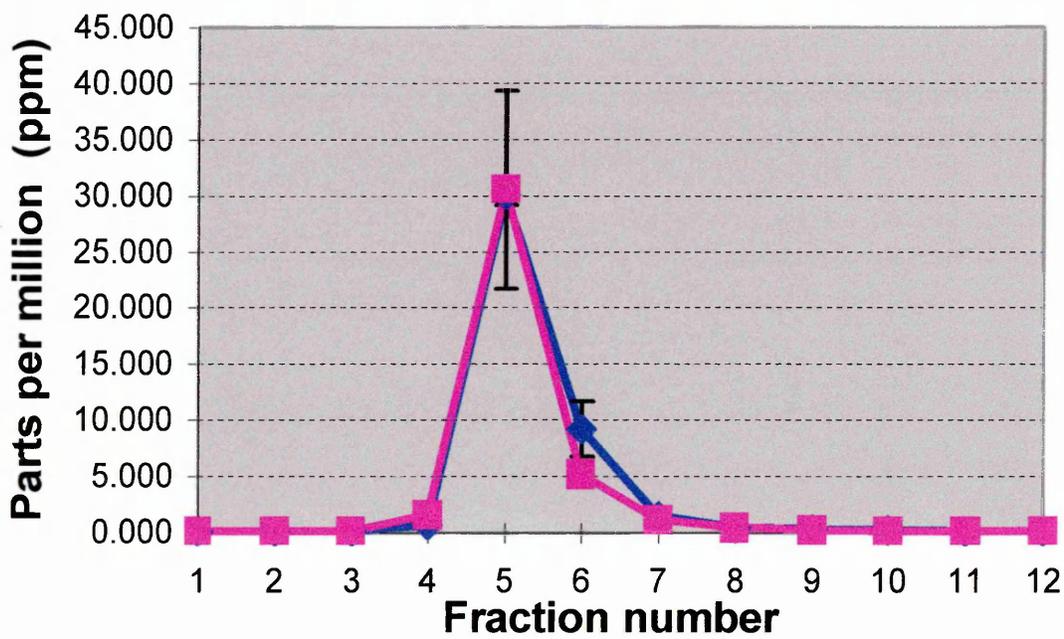
**Figure 2.5    Elution of zinc in fractions from Poros MC$^{TM}$ column:** Zinc, 1mM, was loaded as either zinc sulphate solution or ZMG solution (prepared immediately before use).    Fractions were analysed by HPLC on a Poros MC column (50mmX8mm column, 20$\mu$m bead size and water as mobile phase), eluted with EDTA/NaCl at 2.9ml/min and analysed using ICP-AES.    Fraction volume was 2.9ml.    No significant difference was seen between ZMG (n=4) and ZnSO$_4$ (n=5) at any point, data presented as mean ±SD.    Zinc concentration was plotted as parts per million (ppm) per fraction.  ━ = ZMG, ━ = ZnSO$_4$.
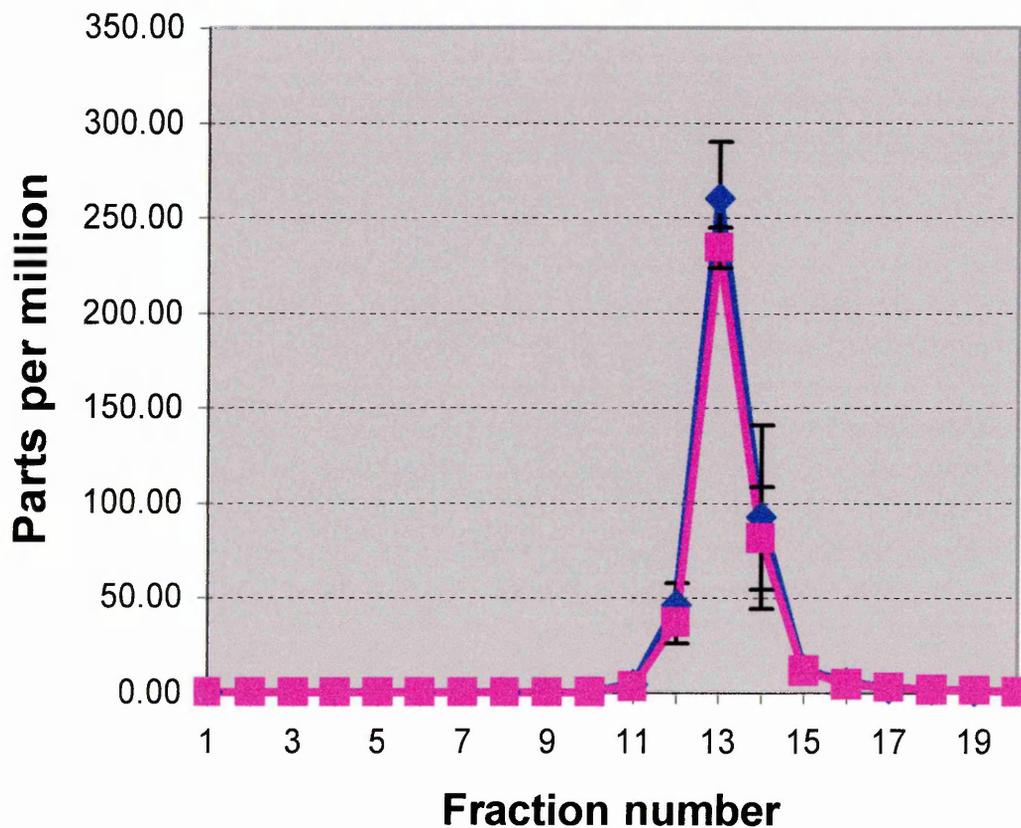
**Figure 2.6** **Elution of zinc in fractions from Poros column:** Zinc, 10mM, was loaded as either zinc sulphate solution or ZMG solution. Fractions were analysed by HPLC on a Poros MC column (50mmX8mm column, 20µm bead size and water as mobile phase) eluted with EDTA/NaCl at 6ml/min and analysed using ICP-AES. Fraction volume was 3ml. No significant difference was seen between ZMG (n=4) and $ZnSO_4$ (n=5) at any point, data presented as mean ±SD. Zinc concentration was plotted as parts per million (ppm) per fraction. —— = ZMG, —— = $ZnSO_4$.

### 2.2.4 HPLC analysis of ZMG degradation by detection of zinc release from ZMG suspension

As may be seen from figure 2.7, when ZMG was suspended in distilled water, filtered and analysed by HPLC as described for the zinc solutions, zinc is detected in some fractions. No zinc is eluted from filtrates obtained from ZMG incubated from 1-15 minutes in water however, zinc appears in the fractions of filtrates of longer incubations (50-90 minutes). Of those samples that contain zinc the maximum concentration is at fraction 5 as it was with the 1mM zinc solutions eluted in an identical manner. ZMG incubated for 50 minutes (n=1) produced a peak with maximum of 45.28ppm. At 60 minutes (n=1) a maximum of 180.8ppm gives the greatest concentration of zinc released by the ZMG filtrate. Filtrates obtained after a 90 minute incubation (n=3) had an average maximum of 126.9ppm. Although based on a single experiment, the results indicate a time-dependent increase of free zinc, peaking at 60 minutes. This would agree, in part, with the results obtained from the analysis of glycerol release, which also gradually increased over time.
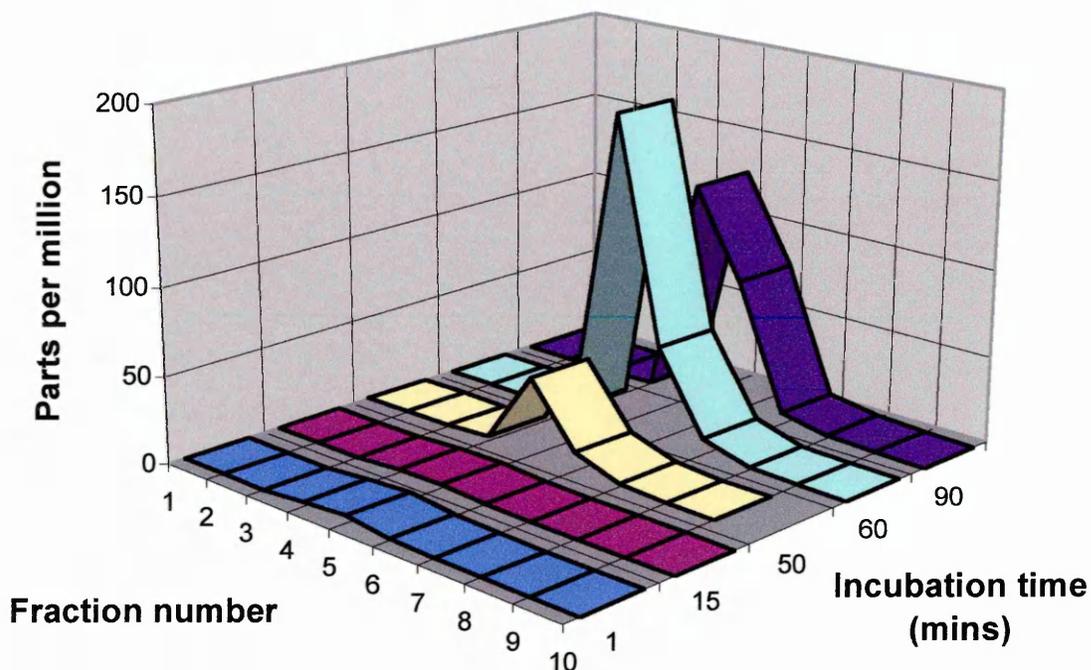
**Figure 2.7    Elution of zinc in fractions from Poros column:** ZMG was incubated in water for increasing periods of time then filtered. The filtrate was loaded onto the Poros MC column (50mm X 8mm column, 20μm bead size and water as mobile phase), fractions were eluted in EDTA/NaCl at 2.9ml/min, and zinc in the fractions was measured by ICP-AES. Zinc does not appear to be released into the filtrate by 15mins but did appear in the filtrate after longer incubations. Zinc concentration peaks at fraction 5 for all incubation times. For values n>1 data are presented as means, n=3 for 1 and 90 minutes, n=2 for 15 minutes and n=1 for 50 and 60 minutes.

In addition to measuring the amount of zinc bound to the Poros column after loading with the ZMG filtrate, fractions were collected during the load to measure any zinc that did not bind to the column. The results obtained from this, figure 2.8, show that almost none of the zinc was lost during the loading process. The zinc levels detected in these fractions were of concentrations at, or near, the threshold of the detection limits for the ICP-AES. Some zinc was detected in fractions at 1, 10, 50 and 90 minutes with distinct peaks in the 10 and 50 minute samples. These results are limited by the detection threshold but do indicate that not all zinc in the ZMG filtrate may be in a form that is able to bind to the Poros column. Fractions collected after loading with zinc sulphate solution did not show any zinc lost from the column (data not shown) but due to time constraints only a few of the zinc sulphate 'load' fractions were tested.
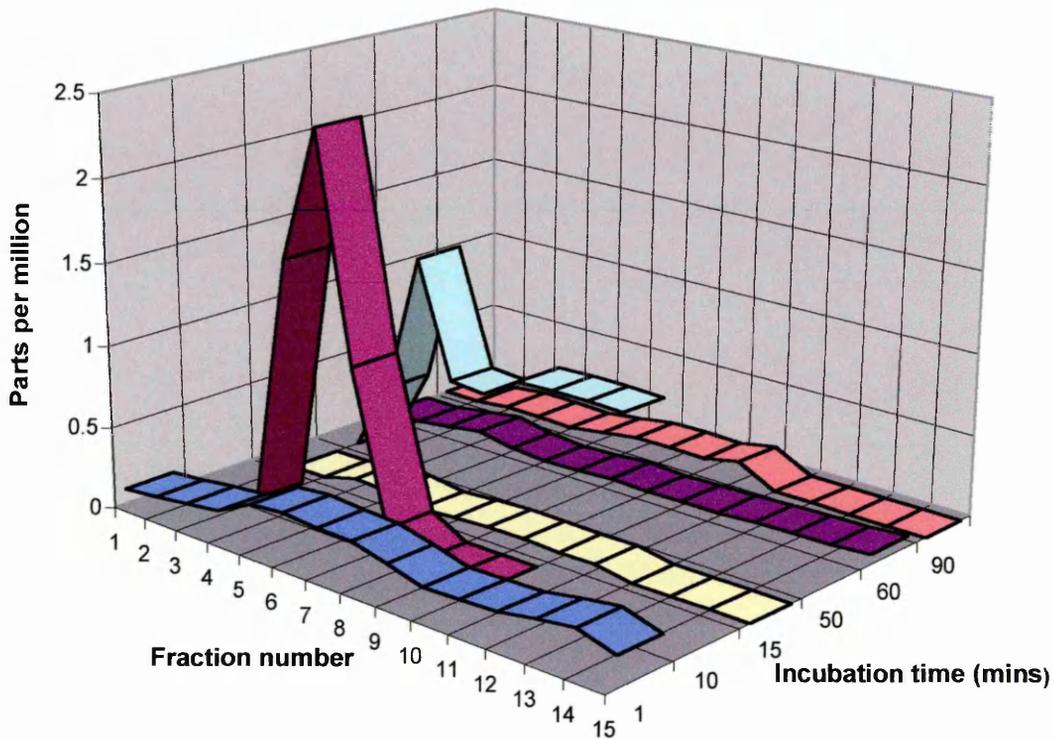
**Figure 2.8 Zinc collected in fraction during loading of Poros MC<sup>TM</sup> column:**
Fractions were collected as the Poros column was loaded with filtrate from filtered ZMG suspension. Zinc concentration was measured by ICP-AES. Very low zinc concentration was found in most samples tested. The n values are n=1-2 for this figure.

### 2.2.5 Analyses of ZMG by Matrix Associated Laser Desorption/Ionisation – Time of Flight (MALDI-ToF)

Analyses of ZMG in HCl solution displayed no peaks that corresponded to an apparent breakdown product of ZMG. A representative trace is seen in figure 2.9. When compared with a profile from the control, HCl alone (not shown), the trace from the ZMG in HCl (Fig. 2.9) showed no difference in the peak other than the very broad mass band stretching from approximately 135, through the 137.1 matrix peak to the peak at 176.9 which is due to an impurity in the HCl solution. Whilst this broad band encompasses the mass of a putative ZMG subunit, ~150, the fact that it is so broad plus the lack of a defined peak or logical correlation to ZMG breakdown products, suggests that this is an artefact of the matrix rather than a true result. For comparison with later results it was found that no peak was found of mass larger than 411.3 as shown in figure 2.9.

The focus was turned on ZMG suspensions for the following studies. Figure 2.10 shows a representative trace of ZMG in DMSO with 2,5-DHB as matrix. As seen here there was a series of regular peaks stretching from masses of approximately 646 up to 4000 in increments of ~65 and ~220. This was one trace from a series of traces showing increasing ZMG concentration from 1-100mg/ml and the series of peaks was found in each one down to a ZMG concentration of 1mg/ml below which the regular repeating pattern was not seen. This profile was also seen in a sample of ZMG, without DMSO, suspended directly into 2,5-DHB (Figure. 2.11). Within the limits of the MALDI-ToF system, these results may be considered identical. The peak separation became more defined, and peak height increased, with increasing ZMG concentration (not shown). This resulted in the sharp and discrete peaks of figure 2.10 which were not present in the control matrix alone (Fig. 2.12). Analyses of the peak trace in Figure 2.10 is shown in Figure 2.14 and Figure 2.15 at the end of the MALDI-ToF traces.
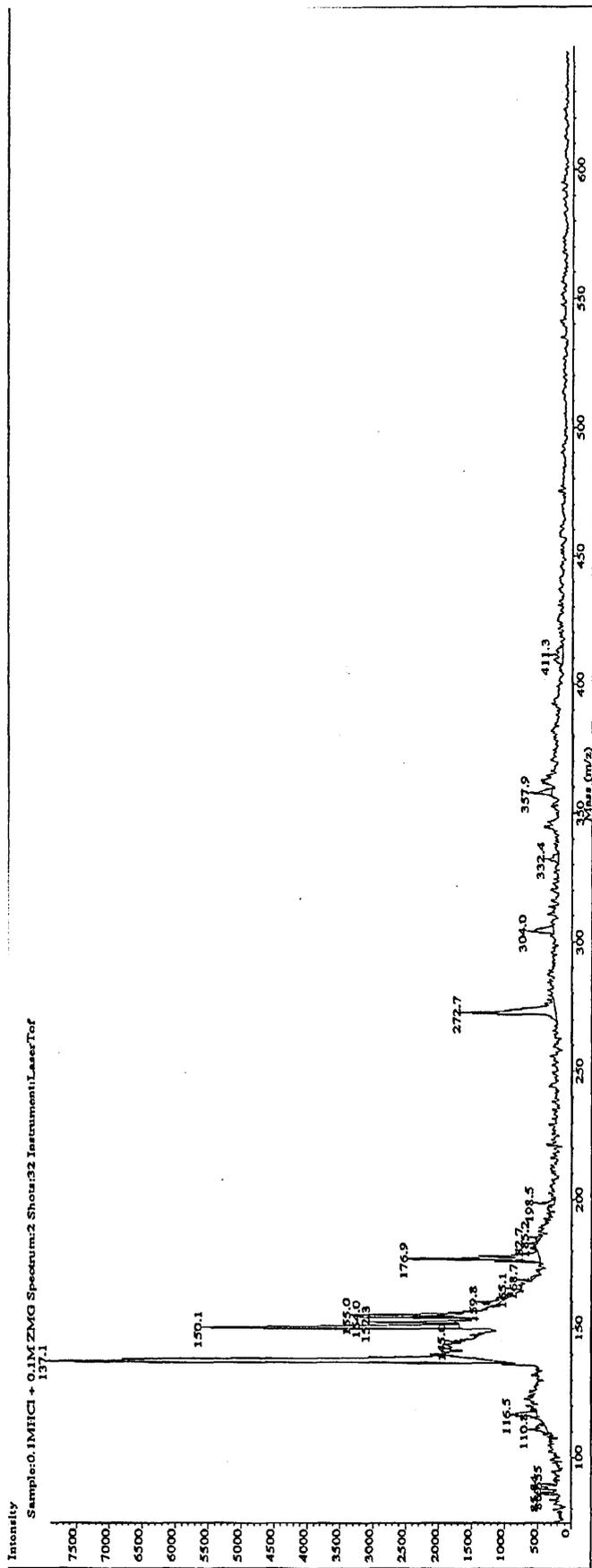
**Figure 2.9** **MALDI-ToF analysis of ZMG solution:** ZMG was dissolved in 0.1M HCl, the solution was mixed 1:1 with 2,5-DHB matrix and spotted onto a MALDI target slide. This representative trace shows no polymeric structure present in the solution. Equally there were no defined peaks corresponding to the molecular weight of a breakdown product of ZMG that were not also present in the control sample, HCl, alone.
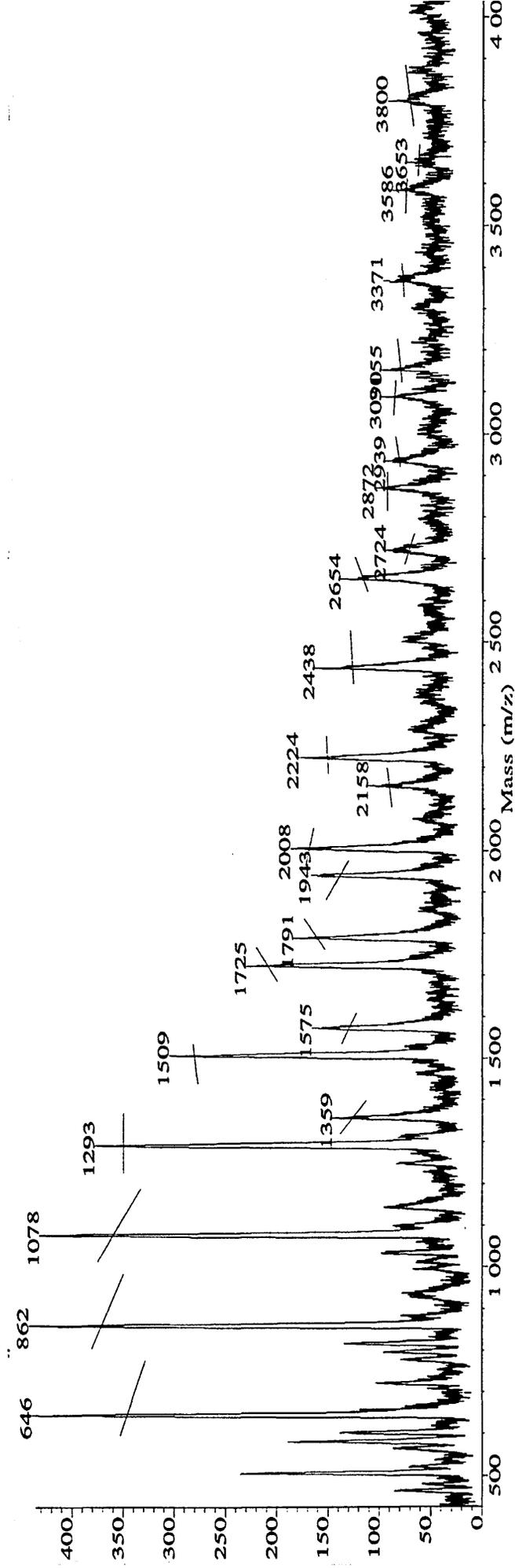
**Figure 2.10  MALDI-ToF analysis of ZMG suspension in DMSO:** ZMG was suspended in DMSO to 100μg/ml and mixed 1:1 with 2,5-DHB, this was then spotted onto a MALDI target slide.  This representative trace indicates a polymeric structure as defined by the peaks corresponding to regularly increasing molecular masses in the ZMG preparation.  The peaks appear as doublets separated by a mass of ~220, and the two peaks forming a doublet are separated by a mass of ~65.  The major peak of lowest mass was at 646 and the highest discernible peak was around a mass of 4000.

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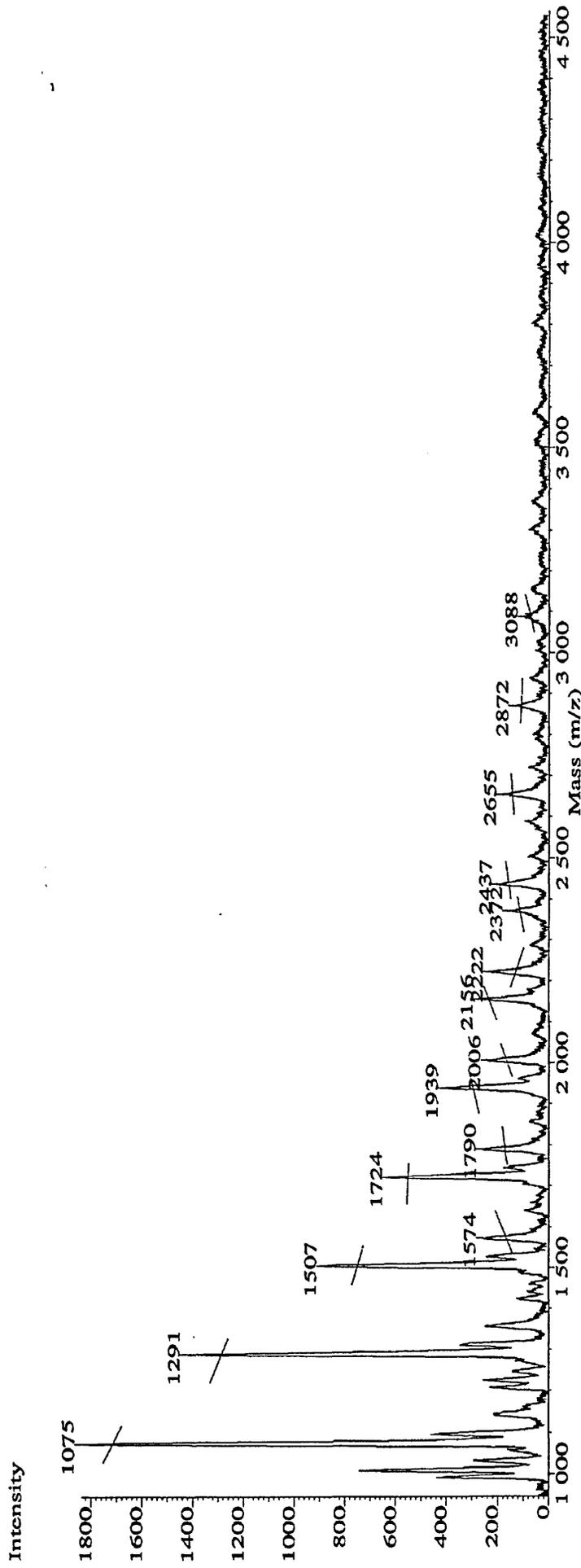
**Figure 2.11  MALDI-ToF analysis of ZMG suspension 2,3-DHB matrix alone:** ZMG was suspended directly into 2,5-DHB saturated methanol solution This representative trace indicates a polymeric structure as defined by the peaks corresponding to regularly increasing molecular masses in the ZMG preparation. The peaks ranged from mass of approximately 645 to approximately 3500 although the trace shows peaks of mass1000 onwards. The peaks appear as doublets separated by a mass of ~220, the two peaks forming a doublet are separated by a mass of ~65. The control blank for this experiment is shown in figure 2.12.

119
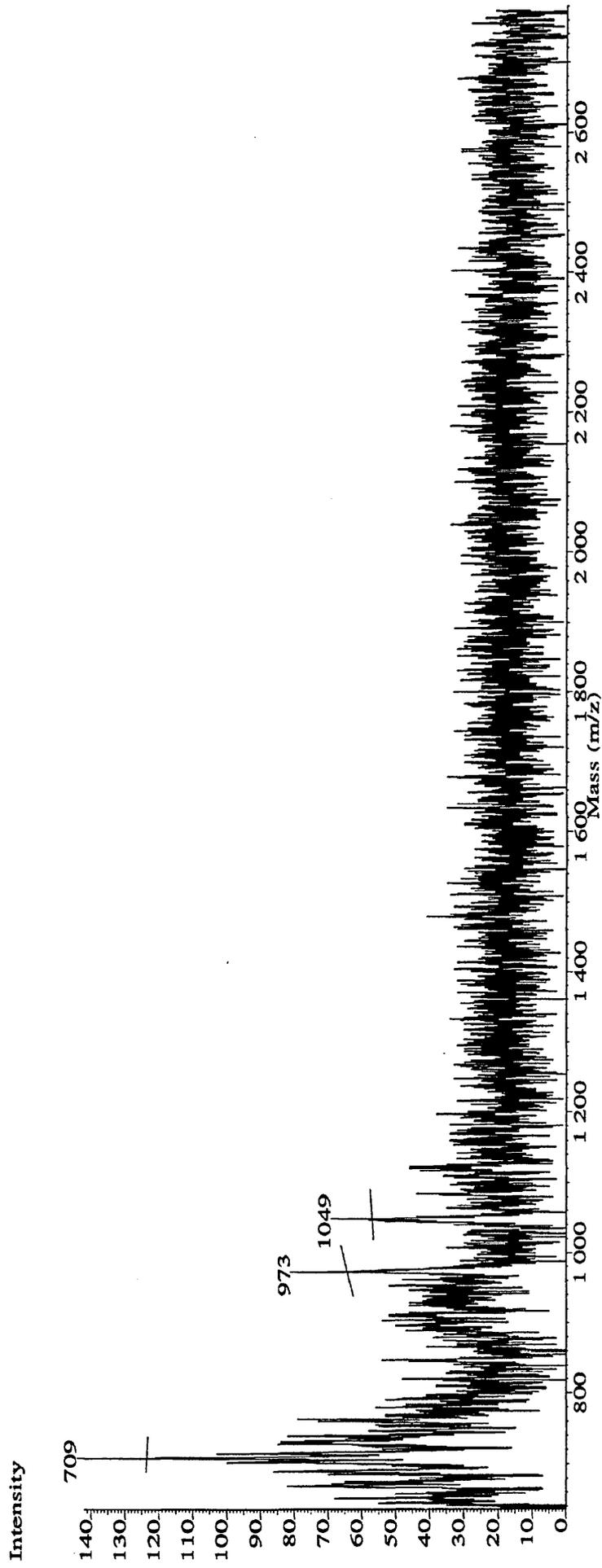
**Figure 2.12    MALDI-ToF matrix control:** A saturated solution of 2,5-DHB in methanol was spotted onto a target slide.    No peaks of any size were found in the mass range that the ZMG polymeric trace presented peaks.    Note the very low intensity levels recorded compared to the traces obtained from test samples.

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Results obtained from MALDI-ToF analysis of ZMG using non-acidic matrices (potassium hexacyanoferrate and cobalt) indicated that, under the conditions used, ZMG was not detected as either polymer, monomer or breakdown product. ZMG suspended in DMSO and analysed using potassium hexacyanoferrate is shown in figure 2.13a. The peaks that may be seen in this trace were also present in the control sample of matrix without ZMG. This experiment was repeated several times without detecting ZMG in any form.

Figure 2.13b shows the analysis of ZMG suspended in DMSO and analysed using ultrafine cobalt metal powder as matrix. No peaks representing polymeric structures or breakdown products of ZMG were observed using cobalt as a matrix. This experiment was repeated without detecting ZMG in suspension. Peaks that are present in figure 2.13b were also present in the control sample, matrix without ZMG.

These results indicate that cobalt and potassium hexacyanoferrate are unsuitable for analysis of ZMG suspension under the conditions used in these experiments. The results from the 2,5-DHB experiments show that it is a suitable matrix for future MALDI-ToF analysis of ZMG. These results also appear to confirm that ZMG in suspension is a polymer with a wide range of molecular weight fragments from 646-4000 whilst ZMG in solution does not appear to have a higher structure, suggesting that it is completely degraded.
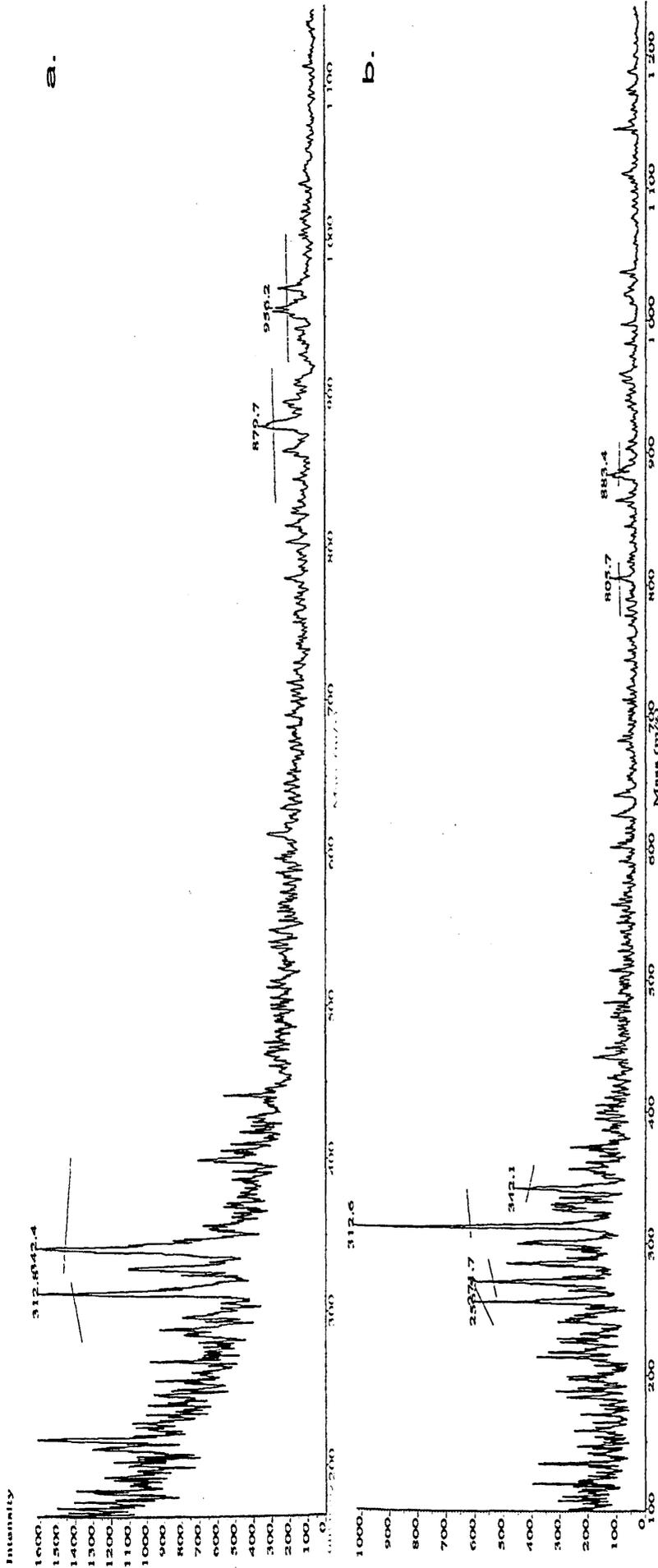
**Figure 2.13   MALDI-ToF analysis of ZMG using non-acidic matrices:** ZMG was analysed using a. potassium hexacyanoferrate as a matrix and b. cobalt as a matrix. No peaks were observed in either instance that corresponded with apparent breakdown products of ZMG. Mass peaks that were observed, such as that of mass 879.7 in trace a. or 805.7 in trace b., were found in the respective controls, matrix alone.

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Figures 2.14 and 2.15 show possible structures of the ZMG polymer range based on the MALDI-ToF analysis presented in Figure 2.10. The basic molecular structure of the ZMG monomer was taken from that described by Hambley and Snow (1984) although the bond lengths and bond angles are not drawn to scale here.

When the masses detected by the MALDI-ToF were first analysed they did not appear to fit any form of ZMG polymer with any accuracy. However, a very common artefact of MALDI-ToF analysis is the association of a sodium ion with the molecule being analysed. When the mass of sodium (23) was removed from that of each peak shown in Figure 2.10, the resulting masses closely matched those of putative ZMG polymers. For example, within the detection error limits of the MALDI-ToF, the first peak with a mass of 646, did not easily fit with any structure based on the ZMG dimer of approximate mass 620. However removing the mass of sodium gave a possible ZMG polymer of mass 623, which closely matched the expected mass of 620 for the dimer.

The structures in Figures 2.14 and 2.15 were derived by logical addition of atoms to the closest whole polymer whether it was the dimer, trimer or higher 'n'-mer. The structures were drawn with the aid of Isis™/Draw (version 2.1.1), which allowed accurate calculation of molecular mass. This further allowed the structures of mass closest to those of the relevant peaks in Figure 2.10 to be determined. Structures B and C in Figure 2.14 show two alternative forms of ZMG of mass 862 and 1078 respectively however all other structures in Figures 2.14 and 2.15 show only the structure of mass nearest to the observed mass.
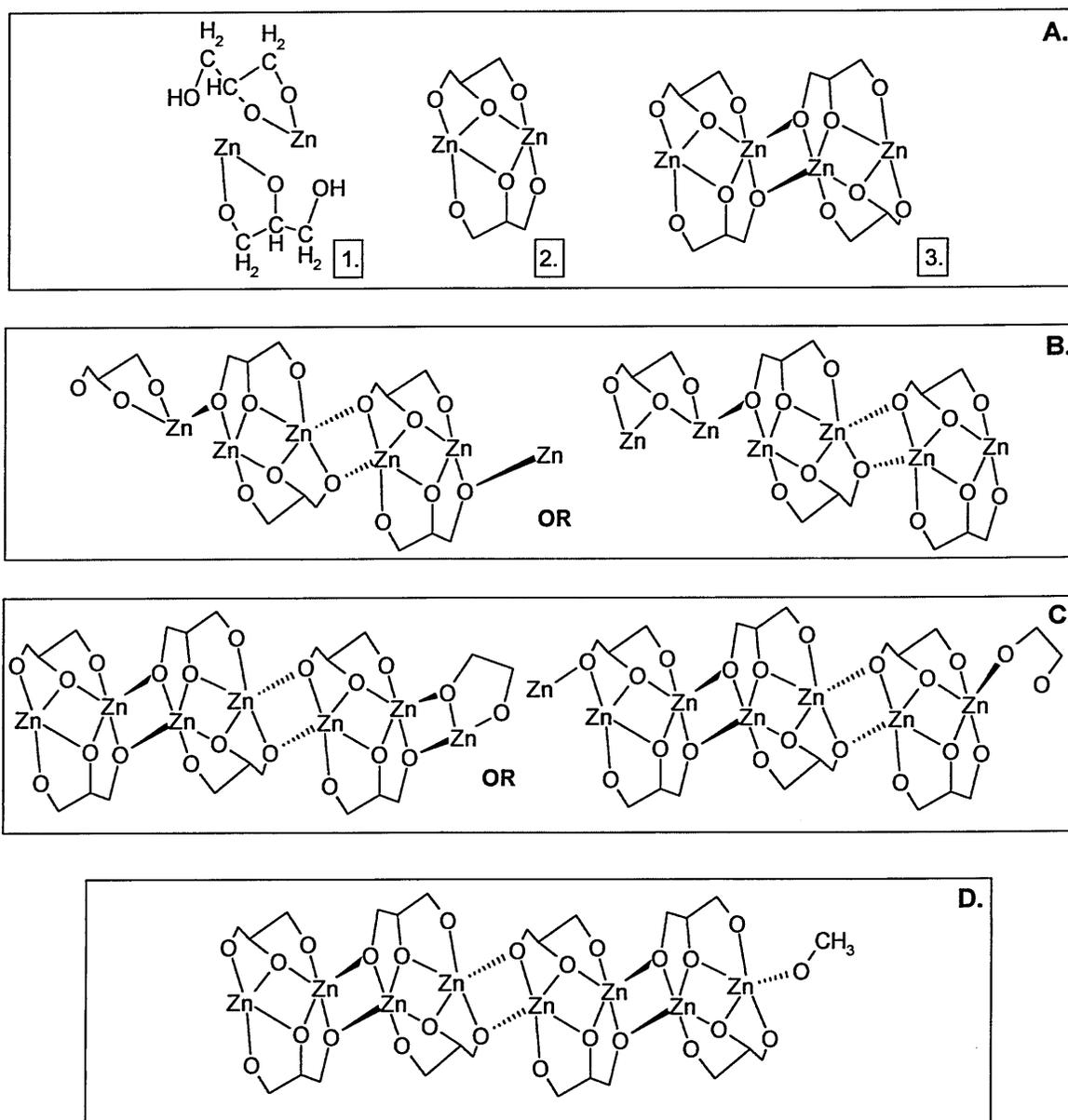
Figure 2.14    Suggested structure of ZMG polymer range derived from MALDI-ToF analysis (Fig. 2.10) mass peaks 646-1293.    Structure A1. represents the exploded structure of A2. the ZMG monomer, it shows the two glycerol molecules and two zinc atoms that form the monomer.    A1. also shows the carbon and hydrogen atoms hidden in the other structures shown above and in Figure 2.15.    Structure A3. is the ZMG dimer and corresponds to the 646 mass peak on the MALDI-ToF trace. Structure B. represents two possible structures calculated for the 862 peak and structure C. represents two possible structures for the 1078 peak.    Structure D. represents the possible structure for the 1293 peak.    For clarity, the carbon and hydrogen atoms shown in structure A1 have been omitted in all other structures.
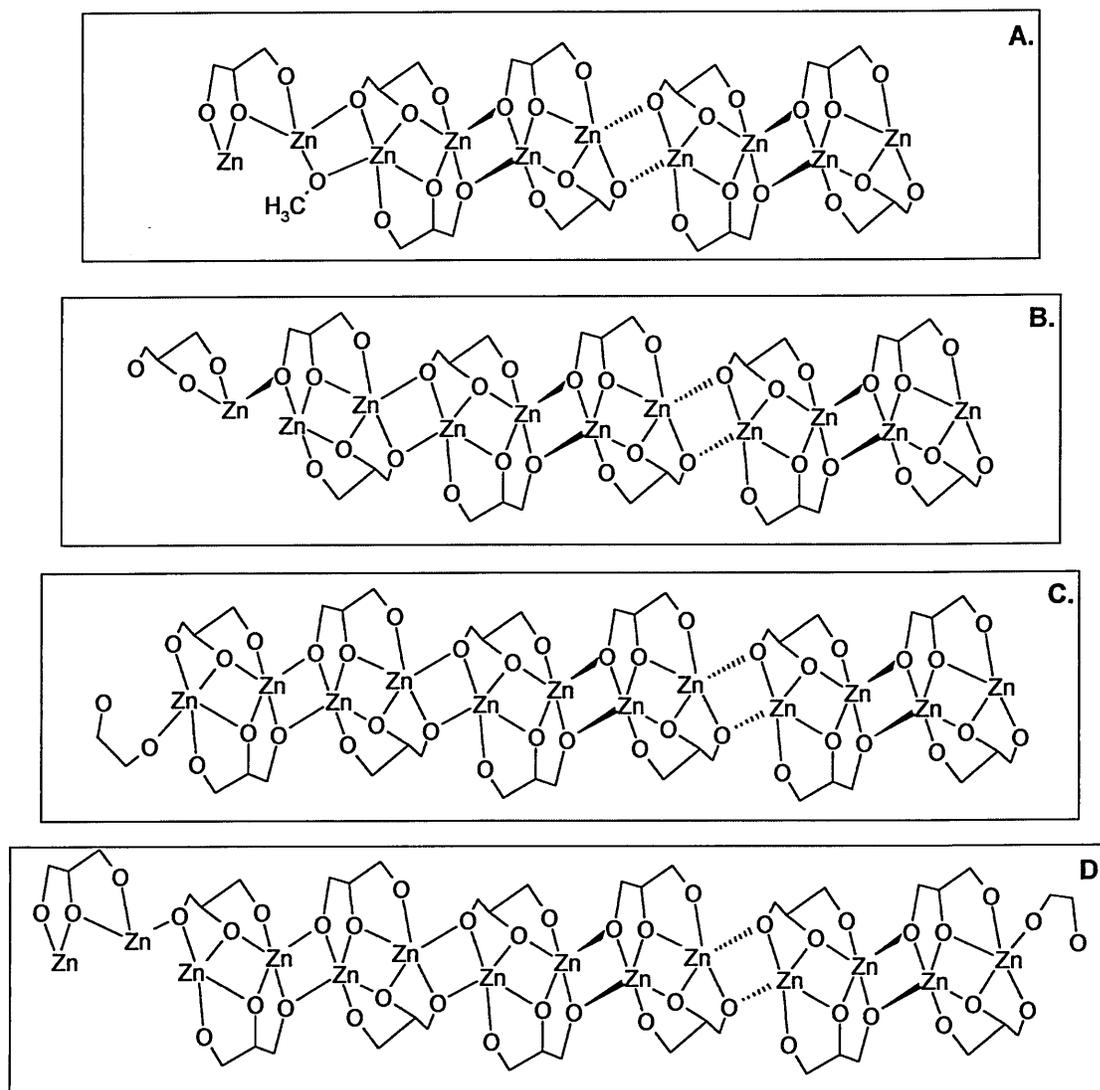
**Figure 2.15 Suggested structure of ZMG polymer range derived from MALDI-ToF analysis (Fig. 2.10) mass peaks 1509-2158.** Possible structures for ZMG polymers of mass peaks 1509, 1725, 1943 and 2158 are shown above in structures A-D respectively. Where several structures are possible for a peak within the limits of the MALDI-ToF error margin, only the structure providing the closest matching mass to the observed MALDI-ToF mass peak has been presented.

## 2.2.6 Synthesis of ZMG and ZMG conjugates

**Synthesis of ZMG**

Results from the microanalyses of the synthesised ZMG preparations are presented in table 2.1. The elements analysed were carbon (C), zinc (Zn) and hydrogen (H); it was not possible to obtain oxygen content from the microanalysis so values for oxygen were calculated by difference. In addition to the newly synthesised ZMG preparations, one sample of commercially prepared ZMG was analysed in conjunction with the samples. As may be seen, most preparations are very similar to both the theoretical proportions for ZMG and the proprietary content but the preparations made with zinc acetate as zinc source are closest to both the proprietary material and the theoretical results. The zinc acetate was also easiest to work with as it required no special processing like the zinc chloride and produced the ZMG precipitate at lower temperatures than the other zinc sources thus avoiding the possibility of over heating. One problem with the synthesis of ZMG was that temperatures between approximately 240°C and 260°C were required for reaction but above 300°C the newly synthesised ZMG decomposes. For this reason good temperature control and constant mixing was required. Zinc oxide and zinc chloride appeared to require temperatures nearer 260°C for ZMG synthesis. However the zinc acetate reacted with the glycerol to form flocculent ZMG precipitate at temperatures nearer 240°C. This reduced likelihood of breakdown and charring of the product. The product from reaction of zinc chloride especially appeared to singe near 260°C instead of near the breakdown temperature of 300°C and so was particularly unsuitable for use. Additionally, there was a high product yield from the zinc acetate method, the product zinc content being 66% of the initial zinc (87.8mg product zinc from 132.6mg initial zinc). This compared well with the published method for synthesis of radiolabelled ZMG using zinc chloride, which achieved 44% yield by weight (Fairlie et al., 1992). The zinc acetate method was used for synthesis of the radiolabelled ZMG.

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| Zinc Source | Zn% | C% | H% | O% by difference | Comment |
|---|---|---|---|---|---|
| Zinc Oxide | 43.45 | 21.01 | 3.57 | 31.88 | Very low yield (<10%) |
| Zinc Chloride | 43.04 | 22.50 | 4.73 | 29.73 | Low yield and charring (<10%) |
| Zinc Acetate (1) | 42.14 | 23.00 | 3.84 | 31.02 | High yield (66%) |
| Zinc Acetate (2) | 42.20 | 23.20 | 3.89 | 30.71 | Repeat of zinc acetate (1) |
| | | | | | |
| Proprietary ZMG | 42.26 | 23.29 | 3.87 | 30.58 | Commercially supplied |
| Theoretical Proportions | 41.94 | 23.23 | 3.87 | 30.97 | Calculated proportions |

**Table 2.1.** **Summary of ZMG synthesis results:** ZMG was synthesised by different methods using different sources of zinc. Products of each method were analysed for carbon (C), hydrogen (H) and zinc (Zn) content and results reported as percentages. Oxygen content was calculated from the other three percentages. The proprietary ZMG was obtained from Bellara Pharmaceuticals.

## Synthesis of ZMG FITC Conjugate

Figure 2.16 shows the dissolution of zinc in the supernatant liquid recovered from ZMG incubated with FITC in DMSO as a percentage of zinc in the initial preparation. The results represent only one experiment but it indicated a large increase of approximately 10 times of dissolved free zinc in the ZMG FITC preparation as opposed to intact ZMG suspended alone in DMSO (DMSO+ZMG). As a percentage of the total zinc added to the preparation, 0.713mg zinc (1.7mg ZMG), approximately 16% of that was in the supernatant liquid of the ZMG plus FITC sample.

Figure 2.17 shows the separation of constituents in ZMG-FITC DMSO preparations. There appeared to be no difference between ZMG FITC preparations and FITC alone. In figure 2.17 it is clear that constituent parts of the FITC in DMSO were separated by 70% acetonitrile/water (lane 1) but there is virtually no difference between the FITC alone and the ZMG FITC preparation (lane 2). This was found for all mobile phases used although different separation patterns were seen in each case. Lane 2, comprising the ZMG plus FITC, appeared more intensely coloured than the FITC alone: this is probably due to differences in concentration of FITC. The powder was highly electrostatic and, despite washing of spatulas and watchglasses (used in the preparation process) in the solvent used to make the sample, some powder may have been lost to surfaces in the electronic balance and to the container surfaces. Due to the small amounts being used this may have affected the concentrations separated to the limited degree visible in the TLC results. As a whole, the TLC results appear to indicate that conjugation had not taken place and that the zinc in solution is free ionic zinc.
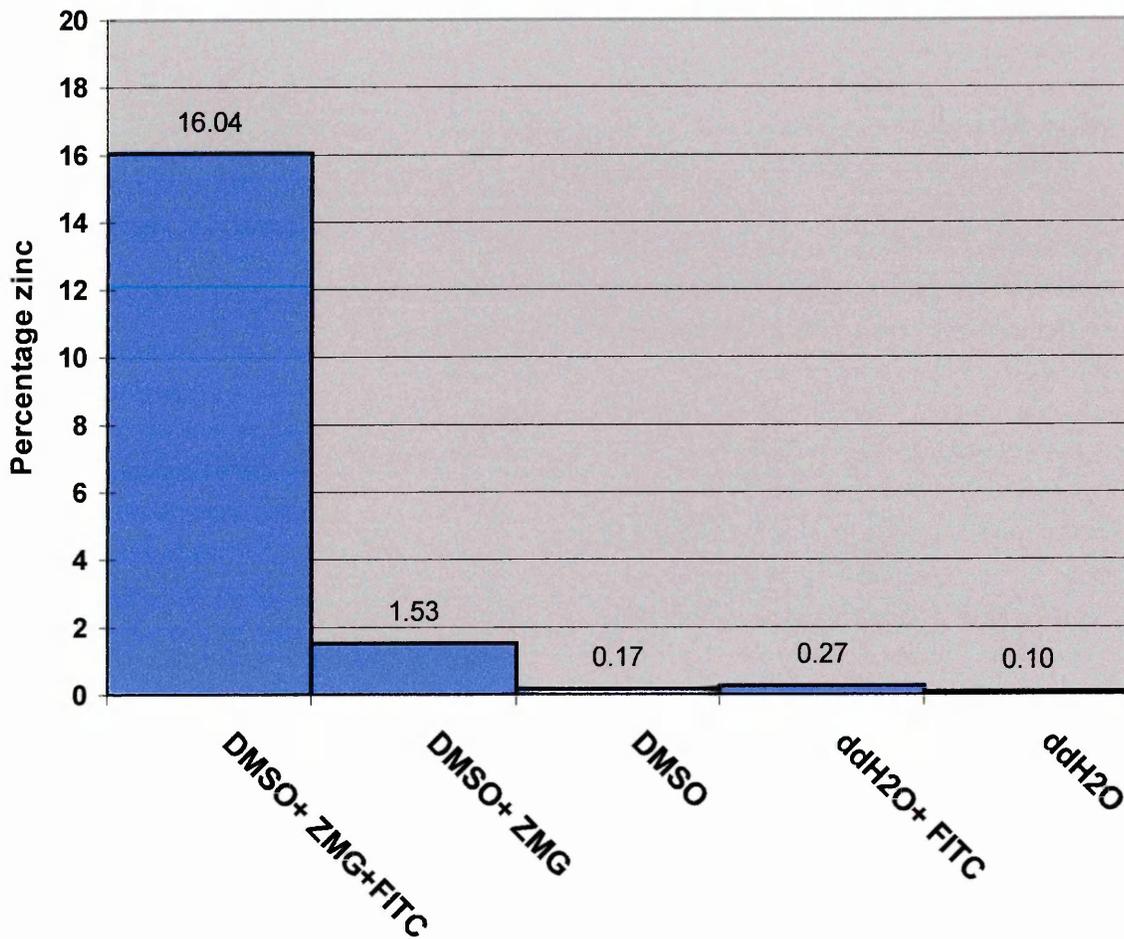
**Figure 2.16 ICP-AES analysis of ZMG and FITC preparations:** ZMG, with or without FITC, was incubated for 24 hours in DMSO then centrifuged to removed remaining particulate matter. The supernatant liquid was analysed by ICP-AES and results obtained as parts per million which was converted to mass of dissolved zinc as a percentage of the initial mass of zinc in the preparation. The ZMG preparation containing FITC had approximately 10 times the amount of zinc than ZMG without FITC. Virtually no zinc was present in either FITC aqueous solution or the DMSO solvent.

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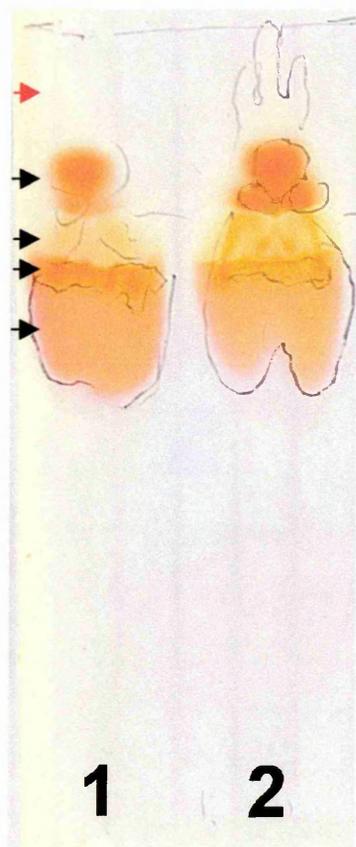
**Figure 2.17   TLC analysis of ZMG FITC preparation:** ZMG and FITC were incubated in DMSO and centrifuged.   The supernatant liquid was analysed by TLC (solvent was 70% v/v acetonitrile/water) and visualised under UV light, pencil marks indicating fluorescent regions.   FITC alone is shown in lane 1 and at least 4 visible fractions, indicated by black arrows, appear to have been separated by the chromatography with a further far fainter fraction, visible under UV light, indicated by the red arrow.   The ZMG plus FITC sample run in lane 2 displays an almost identical fraction pattern although slightly more intense.   These differences may be due to variations in the loaded samples. This figure is representative of three experiments in which at least four plates were run, per experiment, with varying solvent conditions.

## 2.2.7 ZMG Dissolution

**ZMG preparation for cell culture**

Table 2.2 shows a summary of the results from the test of cell culture compatible media for solubilization of ZMG.   When these experiments were initially performed the aim was to find a solvent for ZMG rather than simply a carrier medium.   Consequently, most media were tested for their ability to dissolve ZMG.   ZMG was not found to dissolve completely in any of the culture media tested.   Some media showed a slight apparent dissolution of ZMG, for example water or HBSS when bovine serum albumin was present.   This is supported by published material on ZMG (Fairlie et al 1992) and zinc in general (Wellinghausen et al., 1996a).   ZMG was shown to dissolve to some extent in a BSA saline solution as determined by release of radiolabelled zinc into solution (Fairlie et al., 1992).   Glycerol also appeared to dissolve the ZMG powder fractionally (Table 2.2).

It was observed in the uptake experiments (Chapter 3), that the presence of BSA in the medium caused false positive staining of the cells.   It was surmised that either the BSA itself had a high zinc content, or it was acting as an enhancer of ZMG or zinc uptake into the cells.   In light of the intensity of the stained cells and the ability for media containing BSA to dissolve ZMG, it seemed that BSA in the solutions acted as a zinc carrier and would therefore be detrimental to examining cell uptake of ZMG itself.   In fact, the ability of BSA to act as a cell uptake carrier for zinc was later described in the literature (Driessen et al., 1995) thus vindicating the exclusion of BSA in all experimental cell culture preparations in this thesis.   Driessen et al. (1995) made a point of emphasising the significant effect of the standard cell culture media supplement, BSA, on cell culture experiments examining the actions of zinc.

More media appeared more effective as dispersants than solvents of ZMG, especially Tween 20, glycerol and the salt solution containing BSA.   DMSO gave a poor suspension but a very fine particle size.   Water alone also gave a

relatively fine suspension but again the ZMG was not well suspended and instead precipitates to the bottom of the tube. In the absence of a good solvent, DMSO or water was used as the vehicle for most experiments requiring ZMG suspension. However before use the suspension was always well mixed by vortex and then by rapid manual inversion immediately before use. Water was preferred, as it was the main component of most experimental media used in these studies.

## ZMG Cyclodextrin preparations

Previously, no quantitative methods had been devised to determine solubility of ZMG and results were determined by eye. Cyclodextrins, especially hydroxypropyl $\beta$-cyclodextrin, are soluble in both water and DMSO so it was assumed that any particulate matter visible was ZMG.

No dissolution was seen for ZMG in the presence of hydroxypropyl $\beta$-cyclodextrin at relative concentrations from 1:1 ZMG to cyclodextrin to 1:1000. Suspension of approximately 1:200 ZMG to hydroxypropyl cyclodextrin in water followed by freezing and thawing also had no effect on dissolution of the ZMG.

Suspension of the same preparation in a 50% solution of DMSO/water produced an exceedingly fine suspension but no apparent dissolution. Addition of 20mg $\gamma$-cyclodextrin to this preparation resulted in some apparent dissolution. Repetition of this with the addition of the 20mg $\gamma$-cyclodextrin to the ZMG (1mg) and hydroxypropyl $\beta$-cyclodextrin (200mg) mixture and subsequent suspension in 80% DMSO/water (rather than 50% DMSO) gave slightly more dissolution. Dissolution was not more than approximately 25%.

ZMG did not appreciably dissolve in any other combination of cyclodextrins.

| Vehicle | Dissolved |
|---|---|
| ddH$_2$O | - |
| ddH$_2$O + 10% BSA | -/+ |
| HBSS | - |
| Glycerol, 100% | -/+ |
| TWEEN 20, 100% | - |
| PEG 300, 100% | - |
| Ethanol, 100% | - |
| DMSO, 100% | - |
| RPMI | - |
| RPMI + 10% BSA | -/+ |
| HBSS/glycerol 1:1 | -/+ |

**Table 2.2    Dissolution of ZMG powder in cell culture compatible media:** ZMG, 15.5mg was added to 10mls of various media, at room temperature, and vortexed to mix.  Dissolution was estimated visually, immediately after mixing, by comparing black lines on a white background through the diameter of the 50ml Falcon tube containing the various liquids plus ZMG powder.  Clarity was rated as described previously (Methods section, p.101), starting from complete dissolution, '++, +, -/+' with '-' meaning no dissolution.

# Summary

These results have provided information on ZMG that is relevant to both the phenomenon of ZMG uptake by cells described later in this thesis and, on a wider level, to the study of ZMG in further experimentation and clinical use. HPLC analyses reported that ZMG was degraded in aqueous suspension and solution whilst MALDI-ToF analyses described the nature of ZMG structure in suspension and solution. The dissolution experiments provided information that may be useful to future examination of ZMG in cell culture and biological systems.

HPLC analysis using the Poros column of ZMG dissolved in citric acid, pH6.6, indicated that the zinc in solution appeared to be in the form of ionic free zinc. The Poros MC$^{TM}$ matrix works both by binding zinc to imidodiacetate functional groups and by allowing non-bound molecules to undergo size exclusion through the Poros beads. It would be reasonable to expect that the large ZMG polymers would either remain unbound, to pass through the system, or would bind to the Poros material and be eluted in different fractions to the free zinc salt due to size exclusion. For this reason, the similarity of ZMG solution to zinc sulphate solution in this system would seem to favour the idea that ZMG is completely and rapidly degraded by mild acidic conditions to give a solution of free glycerol and zinc ions.

The complex nature of ZMG as a zinc source is suggested by the results of the ZMG suspension studies. These showed that, zinc is released gradually over time from a ZMG aqueous suspension. The results obtained from analysis of ZMG suspension on the Poros system indicated that maximum zinc released occurred after an hour in suspension. Although the results in figure 2.7 (p.113) show that zinc release appeared to reach a maximum after 60 minutes (Area under the curve=246) and fell again up to 90 minutes (Area under the curve=212), this is most likely an artefact that would even out to a plateau with increased repetition. An explanation for this is that once the zinc release

reaches a maximum, in this case after 60 minutes, it must remain in solution as there is nowhere for it to be lost and so any variation, after 90 minutes, is not likely to be significant. The studies of glycerol release showed that the glycerol release from ZMG in suspension was, like zinc, also gradual over time. However, a maximum of glycerol release was achieved at the longest time point for incubation, 4 hours, not the 2 hour incubation of the ZMG. This would initially appear to conflict with the results from the Poros data but no definite plateau was observed with the zinc release from ZMG suspension and so further repetition may have shown even more zinc release up to 4 hours as seen with the glycerol. The zinc analysis was time dependent as the eluent for the zinc had a relatively high salt content requiring very lengthy washing after each individual run to return the baseline to zero and this limited the number of repetitions carried out in the available time.

In addition, the analysis of glycerol release revealed that this was accompanied by release of another soluble component. As explained in the Results this may only be derived from the ZMG polymer in some form. Precisely what form this unknown component took is difficult to determine.

The fact that 74% of the glycerol detected in the longest incubations (4 hours) was in the form of glycerol, added to the possibility that the maximum glycerol release may not have been achieved, indicates that whatever the unknown peak represents, it can only contain a small fraction of the available glycerol. This argues in favour of small ZMG subunits being the unknown soluble component, as they would represent only a small amount of the total glycerol whereas larger ZMG polymers in solution would theoretically sequester a large proportion of the glycerol. As the unknown peak resolves over time into two peaks this could represent further degradation of, for example, a soluble ZMG trimer into a dimer or dimer into a monomer. This area certainly warrants further investigation, as it will have direct bearing on the nature of ZMG in aqueous experimental media and biological fluids.

The MALDI-ToF results indicated that ZMG naturally takes the form of a series of polymeric molecules from approximately a trimer to a dodecamer, shown by analysing ZMG in 2,5-DHB matrix alone. Polymers consisting of more than 12 subunits may exist but were in such low abundance that the instrument could not readily detect them. The suspension of ZMG in DMSO suggested the same polymeric structure. Suspension of ZMG in glycerol and water did not give as conclusive results but this may have been due to mechanical reasons. The MALDI-ToF requires that the ionising laser hit a sample of the compound to be analysed. In solution this is not a problem as the compound is equally distributed, however in suspension the material is, by its nature, in particulate form and not evenly distributed. ZMG forms a very fine suspension in DMSO unlike glycerol and water and this may also be the case for the matrix solution 2,5-DHB. If this is so then DMSO and 2,5-DHB may simply distribute ZMG more evenly and so provide better targets for the laser to hit.

The MALDI-ToF results are highly interesting as they indicate that ZMG polymers of different lengths are probably asymmetrical, having different terminal structures at either end. In addition the results indicate not only that polymers of one length have two different end structures but that the uneven end structures are different for polymers of different lengths.

If ZMG was synthesised by sequential, equal addition of monomeric subunits then peak differences would equal the mass of the monomer $(C_3H_6O_3Zn)_2$, 310 or possibly the half monomer $(C_3H_6O_3Zn)$ of mass 155 or even multiples of these masses (Figure 2.14 structures A 1-3). However the current results indicate that ZMG polymers are separated in size by masses of 65, 150 and 220. When the mass differences were examined in the context of possible molecular structures that could result from addition to the basic ZMG subunit it seems clear that consecutive polymeric chains must have distinctly different end moieties.

It should be understood that these polymeric chains probably do not represent cleavage patterns that would occur during degradation and zinc release but rather are an indication of the polymeric structure of the undegraded ZMG

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polymers.    This is because the ZMG, particularly when analysed in the methanol solution of 2,5-DHB matrix alone, was suspended in the liquid for a very brief time before application to the MALDI-ToF target slide after which the methanol rapidly evaporated.    This left the dry ZMG/2,5-DHB residue on the target slide.    It is reasonable to assume that degradation cannot readily occur after that point therefor the polymeric mass trace obtained from this ZMG preparation must be very close to the polymeric mass distribution of the native compound.    By extension, the polymeric structures deduced from this particular preparation (Figures 2.10, 2.14-2.15) are likely to be the same as those of the native compound before any degradation occurs.    Use of MALDI-ToF to determine a degradation or cleavage pattern is discussed in the Future Work section.

These polymeric traces were repeatedly obtained several times over a period of four years and on two different MALDI-ToF instruments.    The polymeric trace was found when ZMG was prepared by suspension in DMSO and then in 2,5-DHB matrix or when suspended directly in the matrix without DMSO.    For these reasons the results are unlikely to be artefactual.

The results from the dissolution and synthesis studies of ZMG relate mainly to preparations used in the biological experiments and where relevant with the synthesised radiolabelled ZMG for example, are discussed in the following chapters.    However it should be noted that the synthesis data provides some comparison of ZMG synthetic techniques that does not appear at any point in the literature.

# Uptake of Zinc into Inflammatory and Gastrointestinal Cells

## Chapter Introduction

This chapter discusses the toxicity of ZMG and zinc salts on a variety of cell types followed by a description and discussion of zinc uptake into selected cell lines using a number of techniques, both qualitative and quantitative.

## Background

### 3.0.1 Effect of zinc compounds on cell viability

In order to investigate and quantify cellular zinc uptake it was first necessary to determine the effect of zinc on the cell viability. The cell lines studied included the THP-1 monocyte/macrophage cell line (Auwerx, 1991), Jurkat T-cell line (Gillis and Watson, 1980) and the HGT-1 human intestinal epithelial cell line (Cheret et al., 1985, Sandle et al., 1990). As different cells have different tolerances to zinc and other micronutrients and as zinc was to be administered in higher than physiological concentrations, toxicity was an important consideration. The highest non-toxic dose suitable for all cell lines had to be determined before comparative uptake experiments could be undertaken. To this end, the cell lines that have been used in all the following assays and experiments were tested along with other available cell lines for comparison. At the time that these tests were carried out, there had been little indication that the ZMG solution might contain free zinc ion instead of dissolved molecular ZMG and as a result this preparation, as described in the literature, was chosen for the first experiment. ZMG suspension was tested on the THP-1 monocyte/macrophage cell line only; this experiment is described after the main toxicity experiments. Equimolar zinc sulphate solution was used for the purposes of comparison.

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### 3.0.2 Analysis of cellular zinc uptake by spectrofluorimetry and fluorescence photomicrography

The following set of experiments describes the first attempts to quantify zinc in cells. The concept was described in a paper by Zalewski et al. (1993) who used zinquin to quantify labile zinc within cells. As explained in the introductory chapter, the zinquin staining method was originally developed to be used as both a qualitative and quantitative method for determining labile zinc content in cells. To summarise the published methods, for fluorescent quantification using zinquin a calibration curve of ionic zinc, zinc sulphate, in an excess of zinquin was first constructed. This was followed in the published study by an estimation of cellular zinc ions in cells maximally loaded with zinc in excess zinquin and finally by the determination of labile zinc concentration in the unknown sample. In the current experiments however, the quantitative study was not repeatable, using identical reagents, and the cause was determined. However, the staining procedure (Zalewski et al., 1993) proved very effective in the current study for the qualitative analysis of zinc uptake. Cells incubated with or without zinc were generally well stained by zinquin and this allowed observations to be made on the manner of zinc uptake using photomicrographic techniques.

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### 3.0.3 Analysis of cellular zinc uptake by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Quantitation of zinc from inorganic and biological samples is well documented in many standard techniques differing mainly in sensitivity and specific requirements for sample preparation. Analytical chemical techniques are well established for analyses of trace metals such as zinc and are selected for use by the nature of the sample to be analysed and requirements for sensitivity and speed (Reviewed Vallee and Falchuk, 1993, Kruse-Jarres, 1997).

In the present study, as quantification of cellular labile zinc using the fluorescence spectrophotometer was not possible, the alternative was to quantify *total* zinc in these cells using, in this case, ICP-MS. ICP-MS was chosen as it is a relatively rapid and simple technique that also allows simultaneous measurement of other elements, for example copper, if required. The cellular zinc uptake experiments, using both fluorescence photomicrography and ICP-MS analysis, share a common protocol to begin with and so are incorporated into the same section. The fluorescence staining is derived from the method of Zalewski et al. (1993), as discussed above and the method for the ICP analysis was constructed for this study.

## Reagents

Jurkat, THP-1 and U937 cell lines were obtained from the ATCC. The HGT-1 gastric cell line (Laboisse et al., 1982) was obtained from C. Laboisse (CJF INSERM 94-04, Faculte de Medecin, France). Hanks buffered salt solution (HBSS), RPMI and DME cell culture media were obtained from GIBCO BRL. Zinc sulphate (cell culture grade), zinc oxide, glycerol, 1,4-diazabicyclo[2.2.2]octane (DABCO) and trypan blue solution (4%) were obtained from Sigma-Aldrich. Zinquin acid and ester were a gift from P. Zalewski (Dept. of Chemistry, University of Adelaide, South Australia, Australia). ICP-MS standard solutions (Spectrosol 1000ppm stock solutions) and Analar grade zinc sulphate (used for spectrofluorimetry) were obtained from BDH Chemicals LTD. Analytical grade distilled water was used for the spectrofluorimetry, in all other procedures water was ultrapure deionised water, obtained from Millipore Milli-Q$^{PLUS}$ PF water system.

### 3.1.1  Effect of zinc compounds on cell viability

## Equipment

Cell incubations were carried out using an ICN Flow model 320 Automatic $CO_2$ Incubator and experimental procedures were carried out under a cell culture hood (Gelaire Flow Laboratories TC48 model). Haemocytometers were Improved Neubauer Haemocytometers. Glass coverslips were obtained from Chance Propper LTD.

**Method**

### Cells incubated with zinc salts or ZMG dissolved in citric acid

Cells were cultured in 96 well microtitre plates in media appropriate to the cell line. Jurkat, THP-1 and U937 cell lines were plated out in complete RPMI medium (RPMI+ 5% Foetal Calf Serum (FCS)) to a final concentration in the wells of $5 \times 10^5$ cells/ml, with a 100μl final volume. HGT-1 cell line was plated out in complete DME medium (DME+ 5% FCS) to a final concentration of $5 \times 10^5$ cells/ml, with 100μl final volume and allowed to adhere overnight at 37°C, 5% $CO_2$.

Serial dilutions were made of the ZMG solution (citric acid preparation as described in previous chapter) and zinc sulphate solution as follows. ZMG (5mM stock solution) was diluted in distilled water to give 1200μM, 600μM, 120μM, 30μM and 10μM ZMG solutions, with zinc ion concentrations of 2400μM, 1200μM, 240μM, 60μM and 20μM respectively as one mole of ZMG contains two moles of zinc. Zinc sulphate (100mM stock solution) was diluted in distilled water to give solutions of 2400μM, 1200μM, 240μM, 60μM and 20μM ionic zinc. To each well of every 8 well column of the 96 well plates was added 100μl of the appropriate dilution. Additions to successive columns were separated by 10 minutes to allow time for harvesting and assay by the trypan blue method. The appropriate medium alone (100μl) was added as the 'No Zinc Compound' control, although as indicated on the graphs the media contained approximately 0.5μM residual zinc. The cells' plates were then incubated either 24 or 48 hours at 37°C in a 5% $CO_2$ humidified incubator.

Cell number and viability was then determined for each cell line used, by trypan blue exclusion assay (See Sigma technical notes for trypan blue, product number T8154). Routinely, 20-30μl of cells was removed from the cell suspension by pipetting up and down 10 times, very gently in order to maintain cell integrity. The sample was removed to another clean 96 well plate and carefully mixed with an equal volume of 4% trypan blue. The suspension was then pipetted onto a haemocytometer, a glass slide accurately etched with gradations within an indentation on the slide, to produce a cross hair pattern.

With a glass coverslip fixed above the indentation by gentle pressure, each major etched square has a volume of $0.1mm^3$ from which may be calculated the number of cells within that given volume and hence, by multiplication, the concentration of cells in the sample.   Cells which excluded trypan blue (viable cells) were counted and then cells containing the blue dye (dead cells) allowing a calculation of total number and viability as follows:-

1.      Cell sample concentration $= Cell_{tot} \times 10^4$ cells/ml

2.      Cell Viability $= (Cell_{via} / Cell_{tot}) \times 100\%$

($_{via}$ = viable cells counted, $_{tot}$ = total cells counted)

This assay was also used to determine cell number for all other experiments requiring a known concentration of cells.   Generally, those cell cultures with less than 90% viability were excluded from any experimentation throughout the study unless specifically stated.

Percentage viability for the cells incubated in varying zinc concentration were plotted as line charts.

### Cells incubated with ZMG suspension

THP-1 and U937 cells were washed in serum-free RPMI and prepared as described in the previous protocol but were plated out at $1 \times 10^6$ cells/ml, 500µl/well, into a 24 well plate.   ZMG was suspended in complete RPMI by rapid vortexing to a final concentration of 2mM (0.62mg/ml) and placed in front of a UV lamp in a cell culture hood for ten minutes in order to sterilise the suspension. The ZMG stock was then serially diluted 1:10 to a concentration of 200nM.   To relevant wells containing cells was added 500µl from each ZMG concentration to give final concentrations of ZMG suspension from 1mM to 100nM.   Cells were then incubated for 24, 48 and 72 hours.   Viability was determined as described above using the trypan blue exclusion method.

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### 3.1.2  Analyses of cellular zinc uptake by spectrofluorimetry

**Equipment**

Spectrofluorimetry was performed on a Hitachi Fluorescence Spectrophotometer model F-2000.

**Methods**

An aqueous zinc sulphate stock solution was prepared (6mM), and further diluted in HBSS.   It was found that concentrations of zinc salt, either sulphate or chloride, above approximately 1mM caused visible precipitation of salt out of solution.    Zinquin acid stock solution was prepared in DMSO to a final concentration of 2.5mM (1mg/ml).

The fluorescence profile of zinquin acid was determined to obtain values of the optimum excitation wavelength for induction of fluorescence and the emission wavelength for detection of fluorescence.   Zinquin acid prepared in HBSS, $5\mu M$ final concentration, was mixed in an acid washed, clean UV cuvette with zinc sulphate solution, 440nM final concentration, and immediately run through a fluorescence scan.

A standard curve of zinc sulphate was then constructed by serial dilution in HBSS (the medium used to suspend cells for fluorescence analysis) from $22\mu M$ to 1.99pM.   The zinc solutions were then diluted 1:2 with zinquin solution, $6\mu M$, mixed rapidly in the cuvette, excited in the spectrofluorimeter at 360nm and the fluorescence determined at 490nm.   Results were obtained as fluorescence units and plotted graphically.

### 3.1.3  Analyses of cellular zinc uptake by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and fluorescence photomicrography

**Equipment**

Adherent cell culture chamber slides were obtained from Nalgene Nunc International.  Centrifugation was carried out with an MSE Centaur 2.  ICP-MS analyses were carried out using a Hewlett Packard 4500 series Shield Torch System.

**Methods**

In order to remove exogenous zinc either secreted from the cells or present as protein bound metal, cells were washed and prepared in serum-free media. Suspension cell lines, Jurkat and THP-1, were washed twice in serum-free RPMI culture medium (sfRPMI) by centrifugation in conical tubes, appropriate to the required volume either 15ml or 50ml, at 1000 rcf for 5min.  Cells were finally resuspended to a concentration of $1 \times 10^6$ cells/ml in sfRPMI.

Adherent HGT-1 cells were plated out in DME cell culture medium, with 5% foetal bovine serum supplement, to a concentration of $8 \times 10^6$ cells/flask (for ICP-MS analysis) or $5 \times 10^5$ - 10 $\times 10^5$ cells/chamber slide (for fluorescence analysis) and allowed to adhere overnight.  The HGT-1 cells were then washed twice in the flask with serum-free DME (sfDME) medium and finally covered with 12 mls sfDME.

In some experiments cells were pre-stimulated with lipopolysaccharide, LPS (Sigma-Aldrich).  If so, after the washing process and before the addition of zinc, the cells were allowed to incubate in the presence of LPS ($1\mu$g/ml or $10\mu$g/ml) diluted in culture medium appropriate for the cells.  The cells were incubated at 37°C in a 5% $CO_2$ cell incubator.

The following procedure was routinely used to prepare the zinc compounds for administration to the cell lines.  Prior to their use, aliquots of both zinc oxide and

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ZMG were prepared and sterilised by either heating to 120°C for one hour or incubated under ultraviolet light for thirty minutes. The zinc compounds and glycerol were prepared, immediately prior to addition, in distilled water.

Aqueous standard solutions of zinc sulphate (100mM) and glycerol (1M stock) were diluted to 10mM. Zinc sulphate was always prepared first as the zinc was already in the free ionic form and not subject to any further degradation. To produce zinc sulphate or glycerol solutions each stock solution was diluted in distilled water to give final concentrations of 5mM. To give a zinc sulphate and glycerol mixture, both stock solutions were diluted to 10mM and mixing in equal volumes gave a final solution of zinc sulphate and glycerol (5mM). Zinc oxide suspension was prepared in distilled water as follows. Routinely, zinc oxide (8mg) was suspended in distilled water (10ml) by briefly vigorously shaking and then vortexing for thirty seconds to produce a fine suspension. The suspension was then mixed immediately in equal volume with either 10mM glycerol solution or distilled water alone to give a molar equivalent of 5mM zinc oxide plus glycerol and 5mM zinc oxide suspension. The final zinc oxide preparation, 0.4mg/ml w/v with either glycerol or water, was equivalent to 5mM free zinc. ZMG was prepared in distilled water immediately before use as follows.

ZMG (31mg) was suspended by inversion in 20ml distilled water and mixed by vortexing for thirty seconds. The ZMG preparation was then immediately added to the cells to a final concentration equivalent to 12.5$\mu$M (3.9$\mu$g/ml w/v). Both zinc oxide preparations were then added to a final concentration equivalent to 25$\mu$M (2.1 $\mu$g/ml w/v zinc oxide) and then the zinc sulphate preparations were added to a final concentration of 25$\mu$M. Both ZMG and zinc oxide preparations were inverted vigorously immediately before pipetting into the cells.

Controls were run simultaneously as follows, glycerol was run alone to a final concentration of 25$\mu$M. Vehicle, deionised analytical grade water, was also run as a control, in this case an equal volume of deionised water was added when administering the zinc samples. The final control consisted of untreated cells, i.e. no addition of sample or vehicle.

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The cells were incubated for periods of 2, 4 and 24 hours at 37°C in a 5% $CO_2$ cell incubator.   Up to this stage cells for both ICP-MS analysis and fluorescence staining were treated identically and after this point the divergent treatments and results obtained are described below.   Any variations to this protocol are described in the appropriate related text and figure in the Results section.

*Sample treatment for ICP-MS analyses*

Cells to be analysed using ICP-MS were thoroughly washed in serum free media and counted as follows.   Suspension cells were transferred to 15ml centrifuge tubes and centrifuged at 1500g   for five minutes and the supernatant was discarded.   The pellets were re-suspended in 10ml sfRPMI medium and inverted to mix, and the centrifuge step was repeated.   After discarding the supernatant and re-suspending in 5ml sfRPMI, each sample was counted and the cell concentration noted, in order to normalise the ICP-MS assay values to a given cell number.   The samples were centrifuged to pellet a final time, after which the supernatant was discarded and the pellet air dried by inverting the tubes on paper towelling.   Adherent cells, HGT-1 cell line in particular, were washed simply by removing the medium containing the sample and adding fresh sfDME and agitating the flask gently, removing the wash and repeating.   After the second wash 5mls of sfDME was added to the flask and the cells scraped off the surface using a rubber tipped cell scraper, and transferred into a centrifuge tube.   The flask was washed with a further 5ml of sfDME and the cells were counted as described previously.   From hereon, the adherent cell lines were treated in the same manner as the suspension cell lines.

The pellets were normally either stored overnight at 4°C or immediately processed for analysis.

Before analysis each cell pellet was resuspended, in 1ml 63% nitric acid and transferred by careful pippeting to acid washed 25ml borosilicate bottles for the digestion process.   Pellets were then digested for 1-2 hours at 60°C, or until complete digestion was observed.   The digests were then diluted with 1ml distilled water to reduce acidity for the ICP-MS.

Cell culture media with and without serum were analysed by ICP-MS to determine zinc concentrations before use in the above experiments.

The ICP-MS was always calibrated before use. The instrument itself being calibrated against a standard solution of ions as an internal instrument calibration and then with zinc and copper at standard concentrations to correct for the respective ions being measured. The samples are drawn into the ICP-MS by peristaltic pump action, ionized by the argon plasma and a fraction of the ionized sample being drawn into the mass spectrometer and the zinc and copper contents measured. Values are given as parts-per-billion which are equivalent to nanogrammes per millilitre (ng/ml). The values are then corrected for dilution and cell number as follows:-

Corrected figure F = (2 X Measured value (ng/ml)) / cell number in
                                                                    sample

This gives ng          of zinc per million cells in that sample. These figures were then plotted as bar charts.

*Sample treatment for fluorescence photomicrography*

Fluorescence imaging using zinquin is not well documented and only one procedure for quite specific conditions had been published at the beginning of this study (Zalewski et al., 1993) and although subsequent studies have been published, most use this or very similar methods. This technique was adapted to suit the cells and the equipment used in this study as described below.

Suspension cells for analysis by photomicrography were washed after incubation with the zinc samples, as described for harvest for ICP-MS analysis. However, instead of air drying the pellet at the last stage it was resuspended to a cell concentration of $1x10^6$ cells/ml in sfRPMI. To the cell suspension was added zinquin to a final concentration of $6\mu M$ (from 5mM stock in DMSO). The samples were then incubated for a further 35-45 minutes at 37°C in a 5% $CO_2$ incubator.

After incubation the cells were washed as described above for the ICP-MS assay harvest, but only briefly air-dried to remove most of the liquid without completely drying the cell pellet. The cell pellets were then resuspended in mounting medium, either glycerol/PBS 3:1 or the commercial proprietary Glycerol/PBS mounting medium (Sigma-Aldrich) with either ~2μg DABCO or Immunofluor (ICN) proprietary mounting medium added to enhance fluorescence.

Adherent cells, grown on chamber slides, were treated as described above but centrifugation was not necessary as the chamber slide allowed washing of the cells whilst still adhered. Then sfDME (2ml) was carefully pipetted into the well and the slide was gently agitated before returning a fresh 2ml aliquot of sfDME to the chamber.

One drop of cell suspension (~10μl volume) was dropped onto a glass coverslip which was then carefully placed onto the surface of a glass slide. The coverslip was allowed to settle and any air bubbles gently pressed out using a plastic pipette tip. The coverslip was then sealed to the slide with either black or colourless rapid drying (<1 min) commercial nail varnish.

Slides prepared for photomicrography were kept out of direct light. If not immediately photographed, slides were stored at 4°C for short periods as unfixed cells will, it was found, swell over long periods, lose morphology and even perhaps continue some low level metabolism.

Slides were examined using an Olympus BX60 fluorescence microscope. Fluorescence was observed with a UV dichroic mirror and photomicrographs were taken either from computer images, or directly with a still camera. Video-captured photomicrographs were printed on a Kodak colour printer, whilst still camera photomicrographs were captured on 35mm colour film ASA 100 with an exposure time of 1 min.

A procedure that was employed for all photomicrographs, was that of selecting areas to be photographed, by observation under white light but not ultraviolet. This meant that cells were not selected for photographing by any measure of fluorescence. Selection under these non-fluorescent conditions was intentionally

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biased to some degree by cell number, in that fields were selected that contained as large a number of cells as possible. However, as this was done without any indication of the fluorescence level of these cells, the selection was therefore random from the standpoint of fluorescence.

*Results*

### 3.2.1 Effect of zinc compounds on cell viability

Three separate statistical comparisons were made for cell viabilities;

1) Comparison of differences in cell viability between cells incubated without zinc and those with increasing zinc concentration, calculated by one-way ANOVA and Dunnett's post-test comparison with a control. This describes concentration effects of zinc, (Figures 3.1-3.6 and Table 3.1).

2) Comparison of differences between ZMG or zinc sulphate on cell viability in different cell types, calculated by two-way ANOVA. This describes the effects of zinc source (Figures 3.1-3.6).

3) Comparison between viabilities of different cell lines at each time point with each zinc source, calculated by ANOVA and Tukey-Kramer Multiple Comparisons post-test. This describes the variation in the sensitivity to zinc of different cell types (Figures 3.7-3.10).

Figure 3.1 shows the effects of increasing zinc concentration on THP-1 cells after 24 hour incubation with either ZMG or zinc sulphate. Statistics for the effect of zinc concentration on cell viability is shown in table 3.1. As indicated in table 3.1, there was no significant effect of zinc on viability up to $120\mu M$ zinc in the form of either ZMG or zinc sulphate solution. At the highest zinc concentration both ZMG and zinc sulphate caused a significant decrease in cell viability ($p<0.01$) whilst at $600\mu M$ zinc, only ZMG solution caused a significant decrease in cell viability compared to the zero zinc control ($p<0.01$) (Figure 3.1, table 3.1). There were no significant differences between the effects on cell viability of ZMG solution or of zinc sulphate solution.

| Treatments → ↓ | | 10μM | 30μM | 120μM | 600μM | 1200μM |
|---|---|---|---|---|---|---|
| THP-1 24 hours | ZMG | | | | P<0.01 | P<0.01 |
| Fig. 3.1 | ZnSO₄ | | | | | P<0.01 |
| THP-1 48 hours | ZMG | | | | P<0.01 | P<0.01 |
| Fig. 3.2 | ZnSO₄ | | | | P<0.01 | P<0.01 |
| Jurkat 24 hours | ZMG | | | | P<0.01 | P<0.01 |
| Fig. 3.3 | ZnSO₄ | | | | P<0.01 | P<0.01 |
| Jurkat 48 hours | ZMG | | | | P<0.01 | P<0.01 |
| Fig. 3.4 | ZnSO₄ | | | | P<0.01 | P<0.01 |
| HGT-1 24 hours | ZMG | | | | P<0.01 | P<0.01 |
| Fig. 3.5 | ZnSO₄ | P<0.05 | | P<0.01 | P<0.01 | P<0.01 |
| HGT-1 48 hours | ZMG | P<0.05 | P<0.05 | | P<0.01 | P<0.01 |
| Fig. 3.6 | ZnSO₄ | P<0.01 | P<0.01 | P<0.01 | P<0.01 | P<0.01 |

**Table 3.1    Statistics for effect of zinc concentration in Figures 3.1-3.6:** Comparison of the effect of culture media zinc concentration (compared to zinc free controls) on viabilities of three different cell lines incubated with either ZMG solution or zinc sulphate solution.   The figure numbers indicate the figures to which each set of statistics corresponds.     Statistics were obtained using ANOVA and Dunnet's comparisons of values versus control (cells with no exogenous zinc in culture medium). Green squares indicate viability significantly greater than the "no zinc" control (See Fig 3.6).  Red squares indicate viability significantly lower than the "no zinc" control (See Figs 3.1-3.6).

After 48 hours incubation with varying zinc concentrations (Figure 3.2) both ZMG solution and zinc sulphate solution caused a significant decrease in THP-1 cell viability at 600 $\mu$M and 1200$\mu$M zinc ion when compared with zero zinc control (p<0,01).    There was no significant difference between the overall effect on viability of ZMG solution and that of zinc sulphate solution.    There was no significant difference between cells incubated for 24 hours compared with those cells incubated for 48 hours.

Viability of Jurkat T-cells incubated 24 and 48 hours in the presence of increasing concentrations of zinc is shown in figures 3.3 and 3.4.    Significant reduction in viability was seen in Jurkat cells after 24 and 48 hours for both ZMG and zinc sulphate solutions at zinc concentrations of 600 $\mu$M and 1200$\mu$M when compared to zero zinc controls, p<0.01 for all (Figures 3.3-3.4 and table 3.1).    There was no significant difference between the overall effect on viability of ZMG solution and that of zinc sulphate solution.    As with the THP-1 cells, there is no significant difference between cells incubated 24 hours and 48 hours with either zinc compound.

Thus for both THP-1 cells and Jurkat T-cells there was no toxicity displayed by any zinc compound up to a zinc concentration of 120$\mu$M.    Additionally, there appeared no difference in either cell line whether the zinc source was ZMG solution or whether it was zinc sulphate solution.
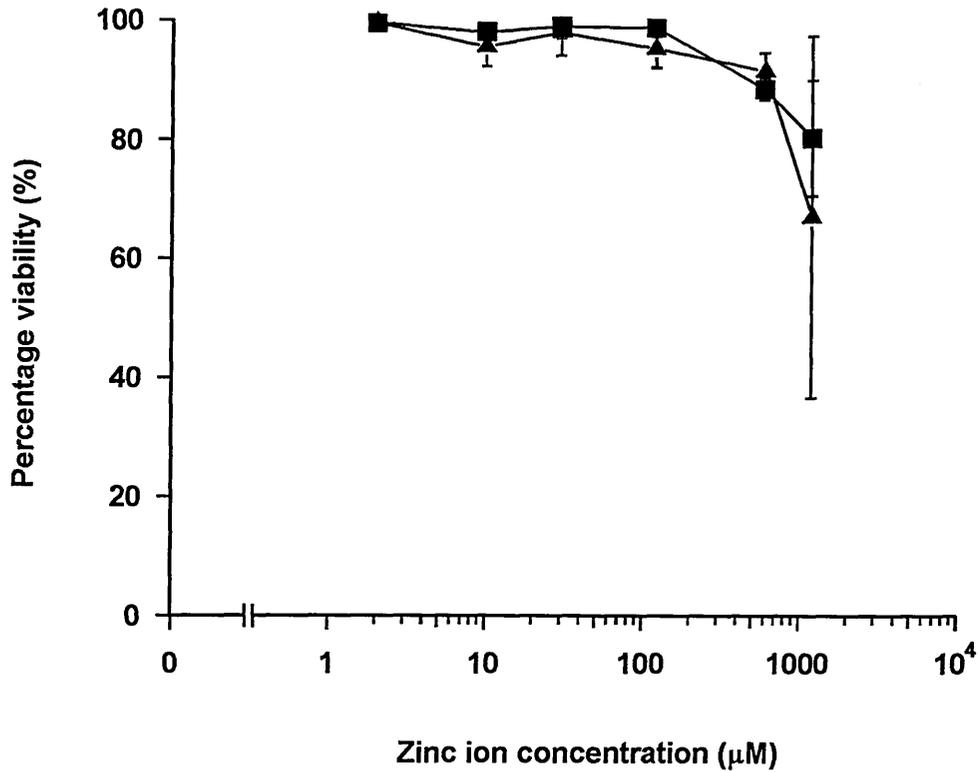
**Figure 3.1.  Change in viability of THP-1 monocytic cells:** Cells were incubated for 24 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, p>0.05).    Values shown as mean and standard deviation, n=4 for each point.    In this and subsequent graphs, x-axis title 'Percentage viability' is taken to mean 'Viable cells as percentage of total cell number in each sample'.
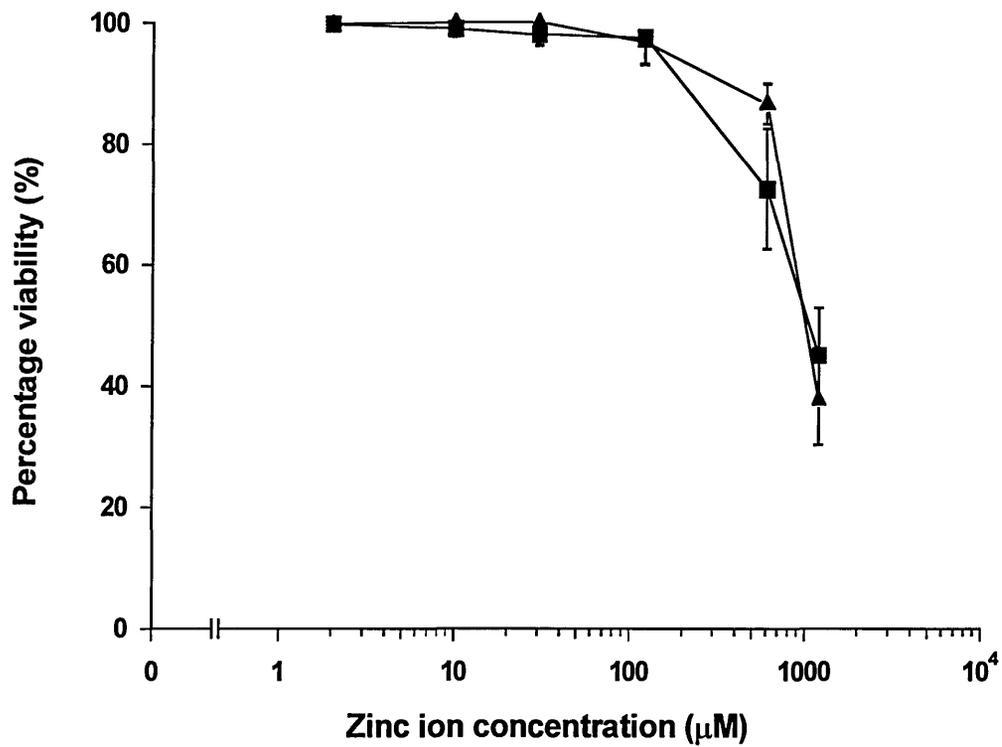
**Figure 3.2. Change in viability of THP-1 monocytic cells:** Cells were incubated for 48 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, $p > 0.05$). Values shown as mean and standard deviation, n=4 for each point.
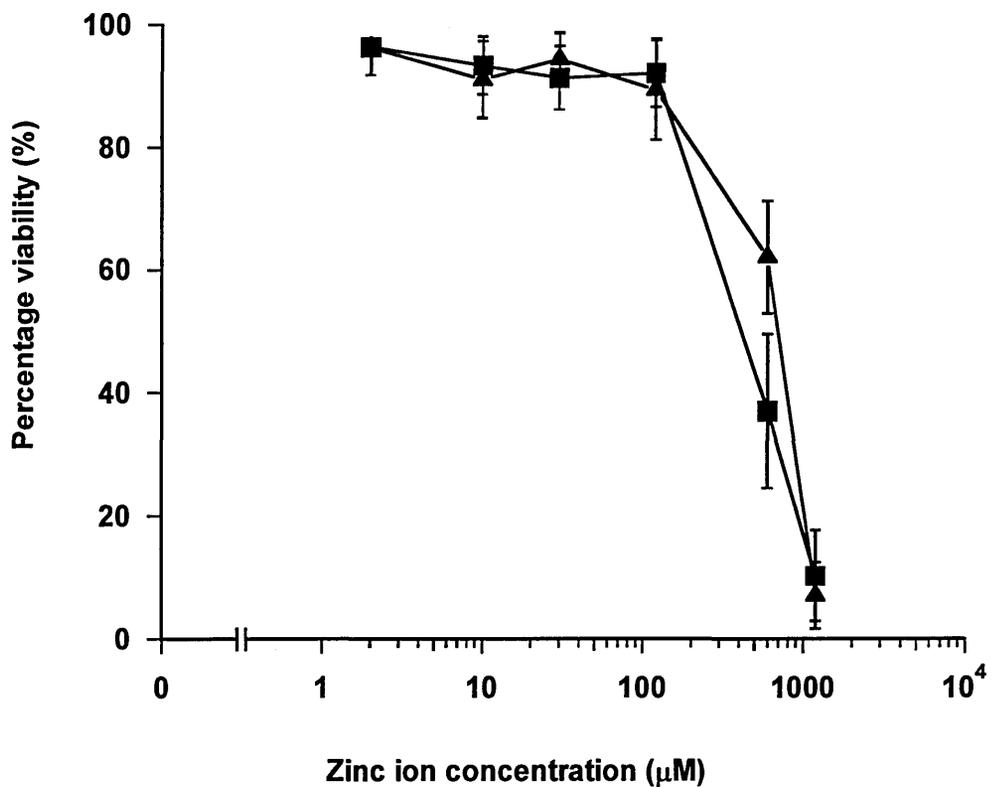
**Figure 3.3. Change in viability of Jurkat T-cells:** Cells were incubated for 24 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, $p > 0.05$). Values shown as mean and standard deviation, n=4 for each point.
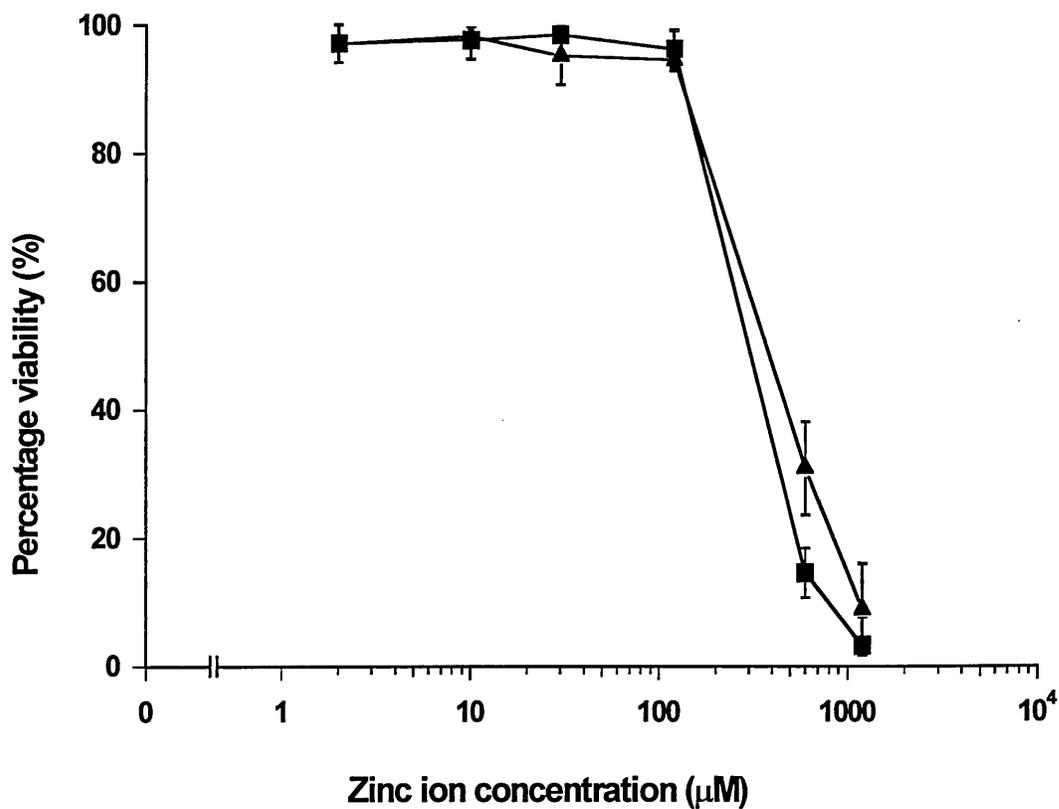
**Figure 3.4.** **Change in viability of Jurkat T-cells:** Cells were incubated for 48 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, p>0.05). Values shown as mean and standard deviation, n=4 for each point.

The viability of HGT-1 human gastric cells incubated 24 and 48 hours in the presence of increasing concentrations of zinc is shown in figures 3.5 and 3.6. The HGT-1 cells appear to have a very narrow window for zinc toxicity as no significant cell death is seen for either zinc compound on the gastric cells up to 120$\mu$M, after which complete cell death was seen at zinc concentrations of 600$\mu$M and 1200$\mu$M. The sensitivity of HGT-1 cells to zinc was emphasised by the significant increase in viability from zero zinc to 10$\mu$M, 30$\mu$M and 120$\mu$M zinc as both ZMG and zinc sulphate solution after 48 hours incubation (Figure 3.6 and Table 3.1). So HGT-1 cells appeared to die without zinc over time periods that do not appear to affect the viabilities of THP-1 and Jurkat cells. In addition, there was no significant difference in overall HGT-1 viabilities whether ZMG or zinc sulphate solution was used as zinc source. There was also no significant difference over time between HGT-1 cells at 24 hours and 48 hours for either zinc source.

Figures 3.7-3.10 figures show a statistical comparison of the effects of zinc concentrations between cell types. In figure 3.7 it can be seen that there is no significant difference between the effect of ZMG on THP-1, Jurkat or HGT-1 cell lines from 0-120$\mu$M zinc. At 600$\mu$M zinc, Jurkat and HGT-1 cells have significantly lower viabilities than THP-1 cells ($p < 0.001$). At this concentration HGT-1 cell viability is also significantly lower than that of the Jurkat cells ($p < 0.001$). At the highest zinc concentration, 1200$\mu$M zinc, there is no significant difference between HGT-1 and Jurkat viabilities, but both lines had significantly lower viabilities than THP-1 cells. Figure 3.8 shows that 24-hour incubation with zinc sulphate instead of ZMG solution gives an identical difference in viabilities between cell lines.

Incubation of the three cell lines with or without zinc for 48 hours shows that the cell viability in the zinc free HGT-1 cells is significantly lower than that of the zinc free THP-1 ($p < 0.001$ for figure 3.9 and 3.10) or Jurkat T-cells ($p < 0.05$ for figure 3.9 and $p < 0.001$ for figure 3.10). The zero zinc points for figures 3.9 and 3.10 are effectively the same conditions but repeated for the two separate sets of experiments.

There was no significant difference between the viabilities of cells incubated 24 hours with either ZMG suspension or zinc sulphate solution at any concentration from 100% viability to 0% viability (Figure 3.11). Figure 3.12 shows the viabilities of THP-1 cells incubated 48 hours with either ZMG suspension or zinc sulphate solution of equivalent zinc ion concentration. There is no significant difference between zinc compounds for the effect on cell viability as determined by two-way ANOVA.
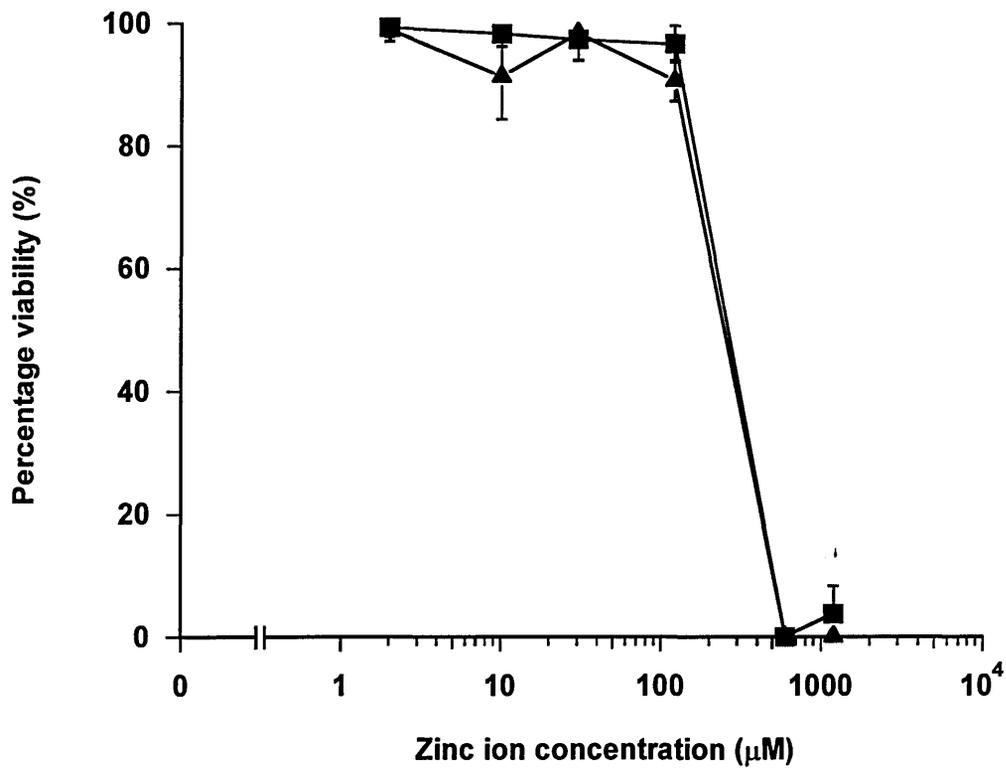
**Figure 3.5. Change in viability of HGT-1 epithelial cells:** Cells were incubated for 24 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, $p>0.05$). Values shown as mean and standard deviation, n=4 for each point.
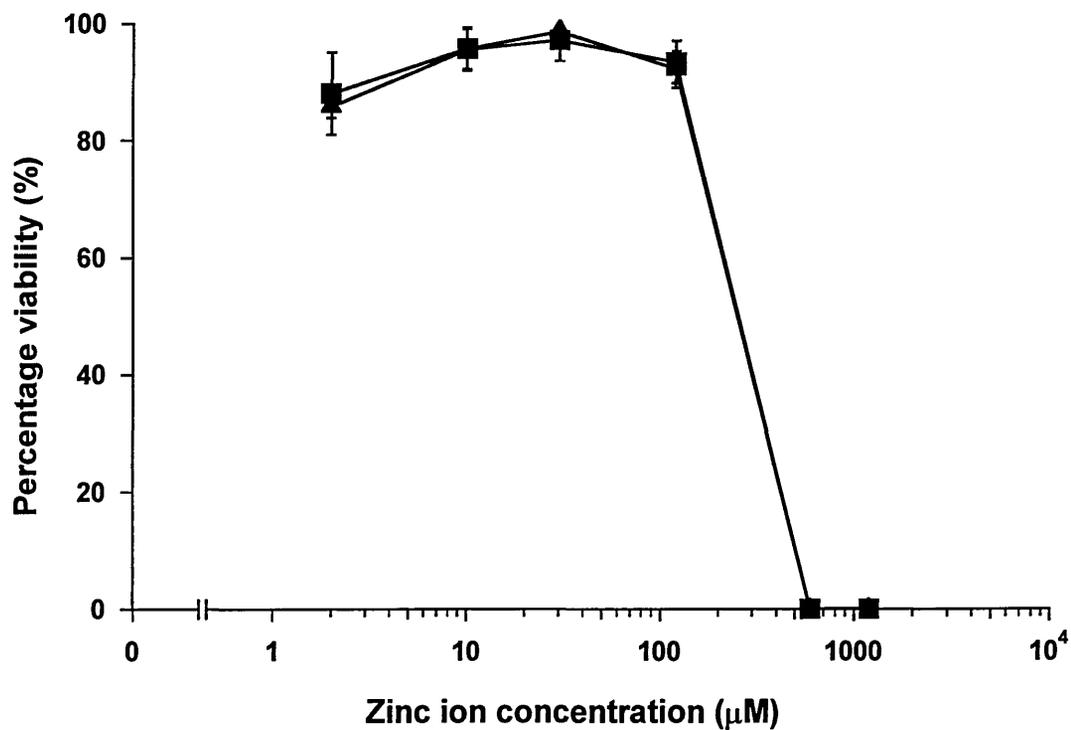
**Figure 3.6. Change in viability of HGT-1 epithelial cells:** Cells were incubated for 48 hours with either ZMG solution (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, p>0.05). Values shown as mean and standard deviation, n=4 for each point.
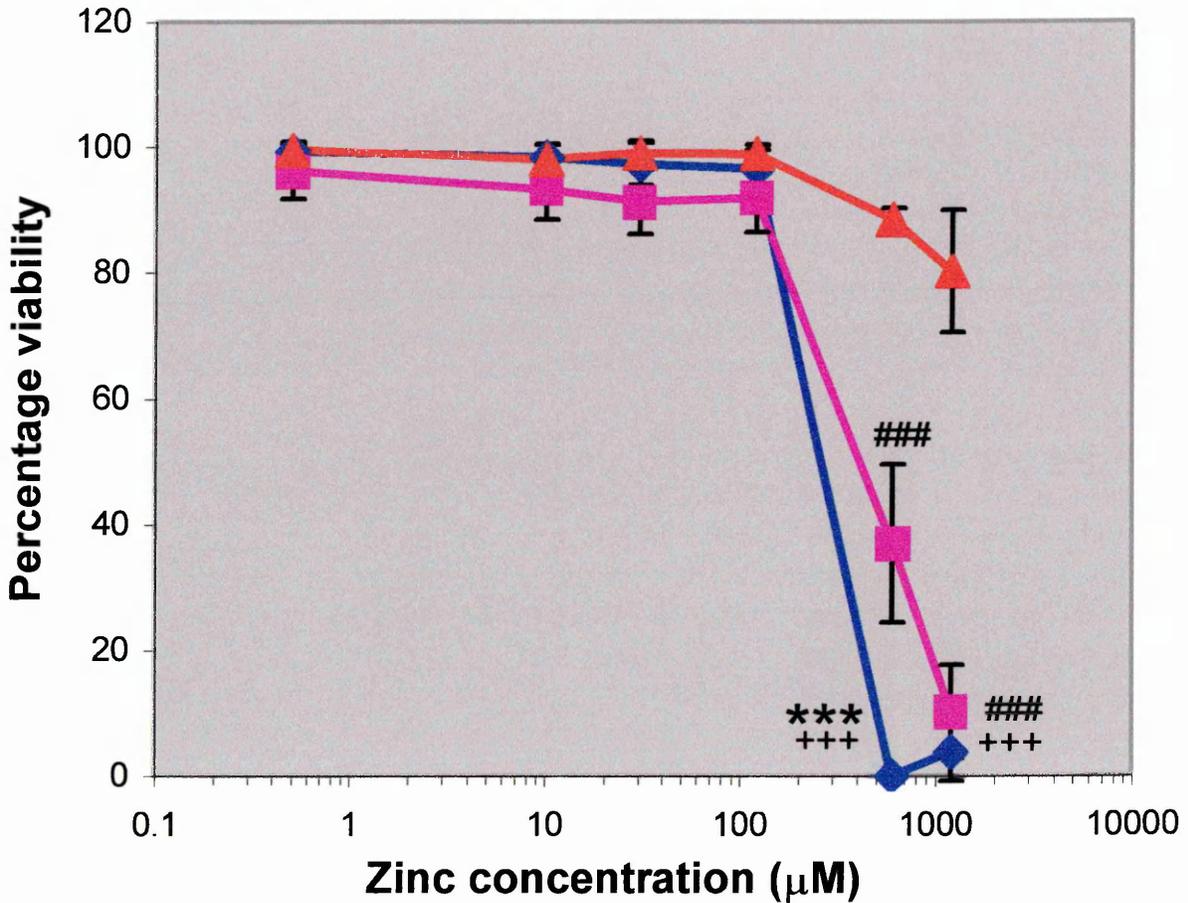
**Figure 3.7. Comparison of viabilities of various cell lines:** Compiled from previous figures, cells incubated 24 hours with ZMG solution. Cell lines were compared for variations in response to ZMG solution. HGT-1 cells (diamond) at 600$\mu$M had significantly lower viability than THP-1 cells (triangle) or Jurkat T-cells (square) (+++=p<0.001 and ***=p<0.001 respectively) and had significantly lower viability than THP-1 cells at 1200$\mu$M (+++=p<0.001). At both 600$\mu$M and 1200$\mu$M Jurkat cells had lower viability than THP-1 cells (###=p<0.001). First point on each graph is 0.5$\mu$M zinc, which represents the residual zinc in the complete media before addition of zinc compounds.
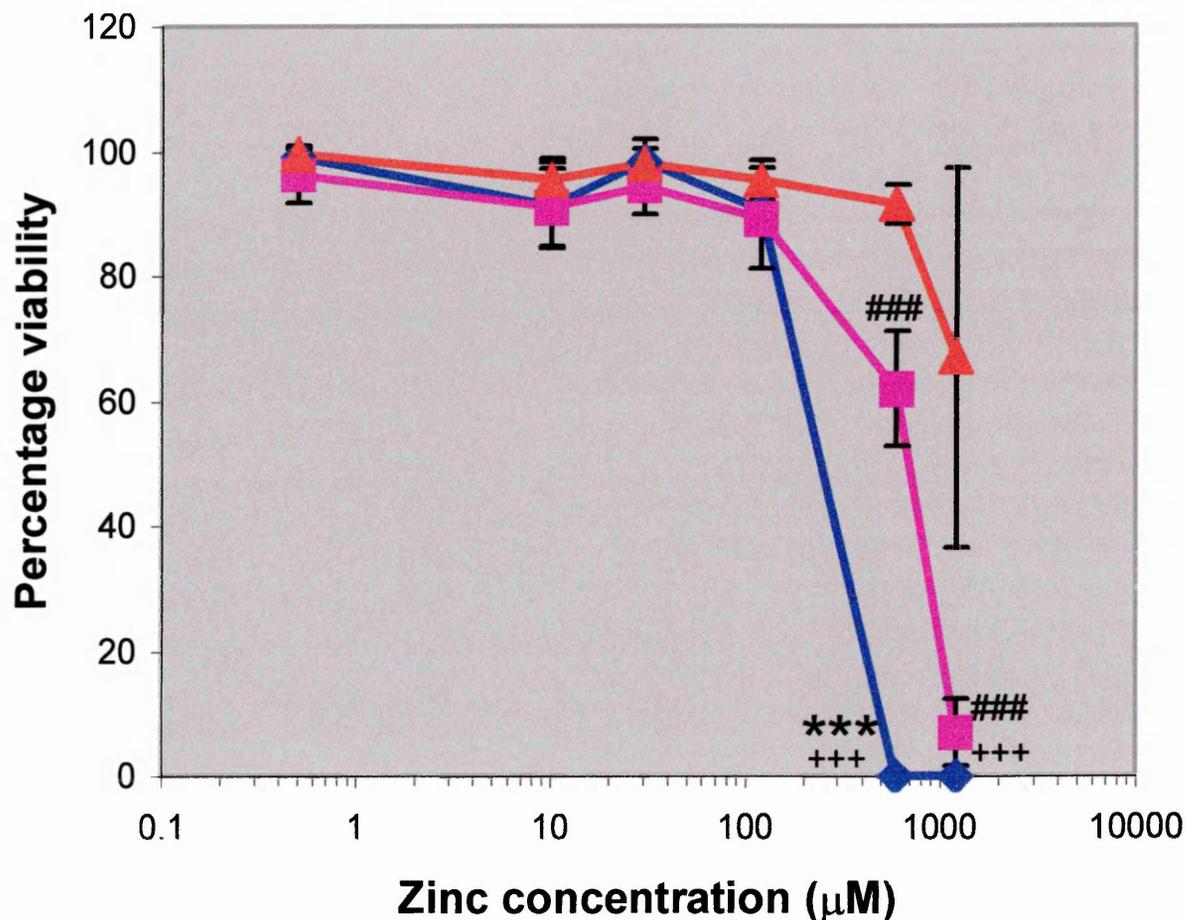
**Figure 3.8. Comparison of viabilities of various cell lines:** Compiled from previous figures, cells incubated 24 hours with zinc sulphate solution. Cell lines were compared for variations in response to zinc sulphate solution. HGT-1 cells (diamond) at 600µM had significantly lower viability than THP-1 cells (triangle) or Jurkat T-cells (square) (+++=p<0.001 and ***=p<0.001 respectively) and had significantly lower viability than THP-1 cells at 1200µM (+++=p<0.001). At both 600µM and 1200µM Jurkat cells had lower viability than THP-1 cells (###=p<0.001). First point on each graph is 0.5µM zinc, which represents the residual zinc in the complete media before addition of zinc compounds.
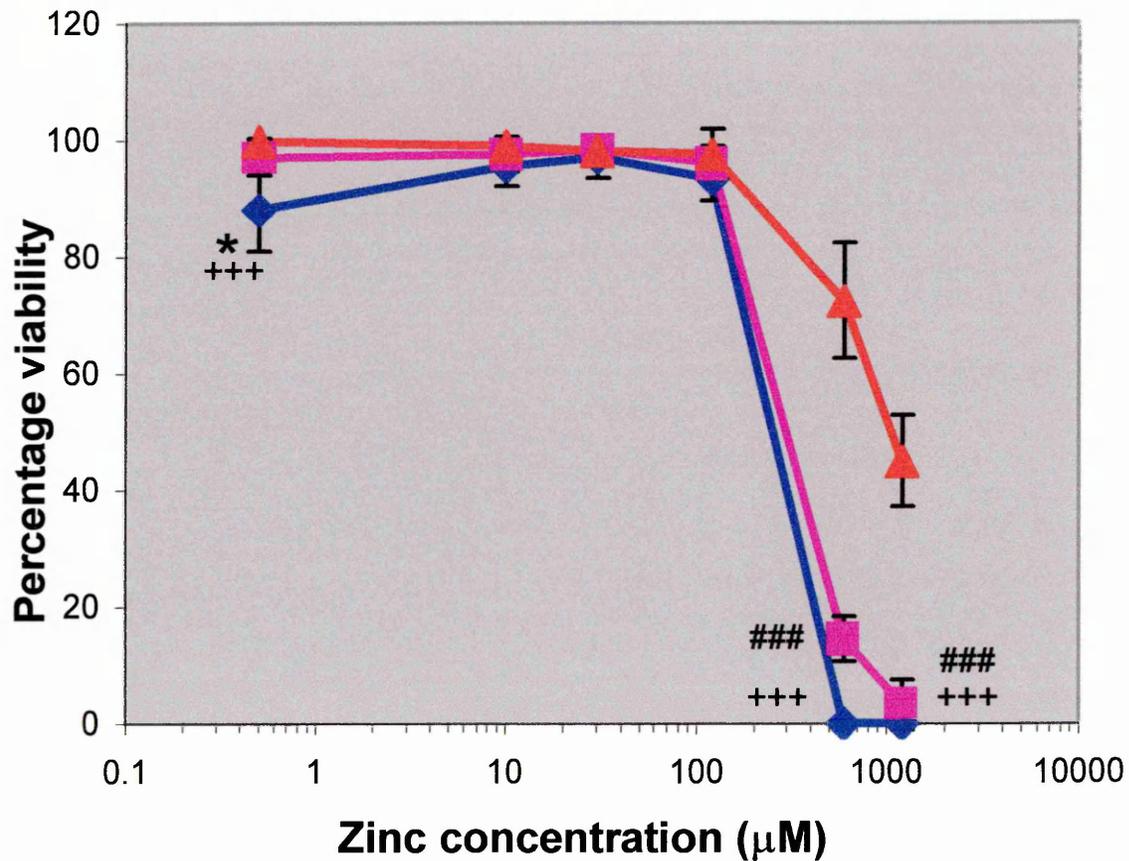
**Figure 3.9. Comparison of viabilities of various cell lines:** Compiled from previous figures, cells incubated 48 hours with ZMG solution. Cell lines were compared for variations in response to ZMG solution. HGT-1 cells (diamond) in zinc free medium, 600μM zinc and 1200μM zinc had significantly lower viability than THP-1 cells (triangle) (+++=p<0.001) and significantly lower viability than Jurkat T-cells (square) in zinc free medium (*=p<0.05). At both 600μM and 1200μM Jurkat cells had lower viability than THP-1 cells (###=p<0.001). First point on each graph is 0.5μM zinc, which represents the residual zinc in the complete media before addition of zinc compounds.
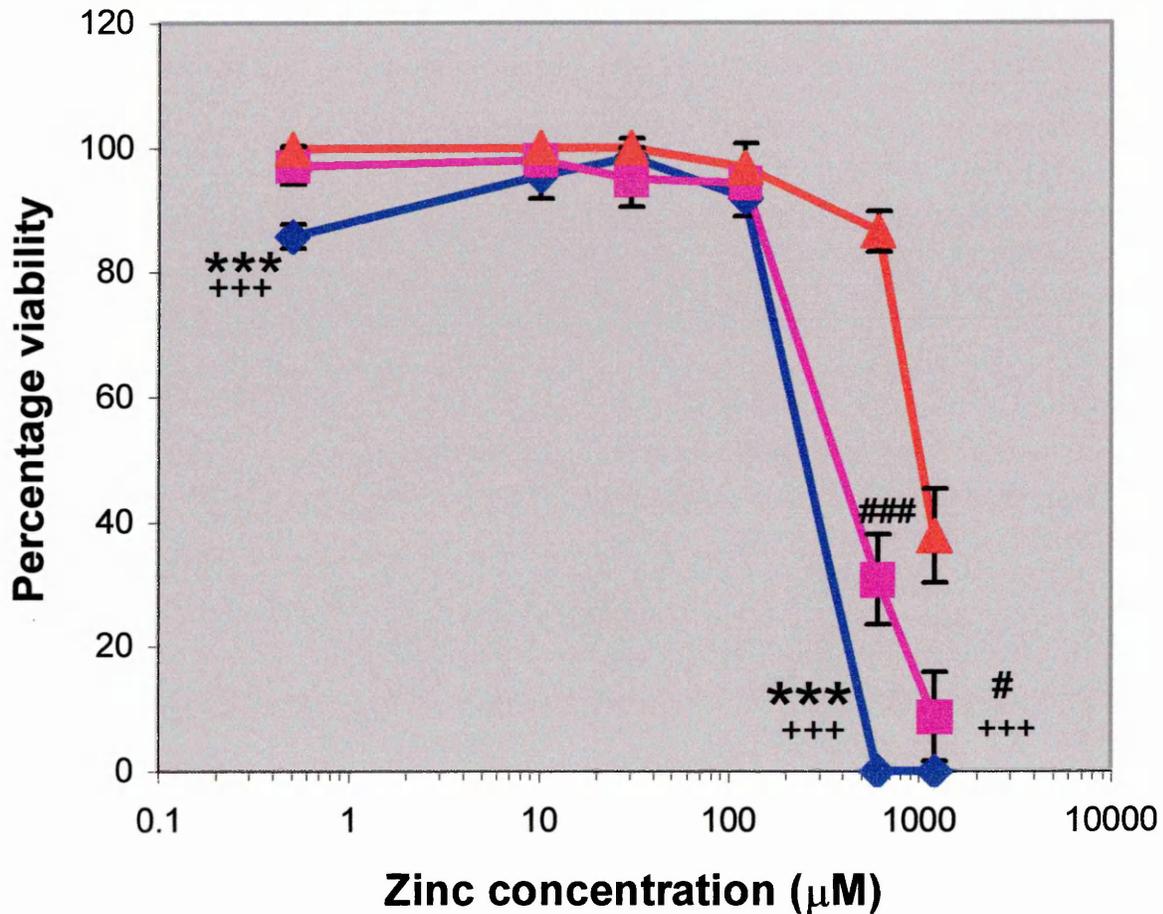
**Figure 3.10. Comparison of viabilities of various cell lines:** Compiled from previous figures, cells incubated 48 hours with zinc sulphate solution. Cell lines were compared for variations in response to zinc sulphate solution. HGT-1 cells (diamond) in zinc free medium, 600μM zinc and 1200μM zinc had significantly lower viability than THP-1 cells (triangle) (+++=p<0.001) and significantly lower viability than Jurkat T-cells (square) in zinc free medium and 600μM zinc (***=p<0.001). At both 600μM and 1200μM Jurkat cells had significantly lower viability than THP-1 cells (###=p<0.001 and #=p<0.05 respectively). First point on each graph is 0.5μM zinc, which represents the residual zinc in the complete media before addition of zinc compounds.
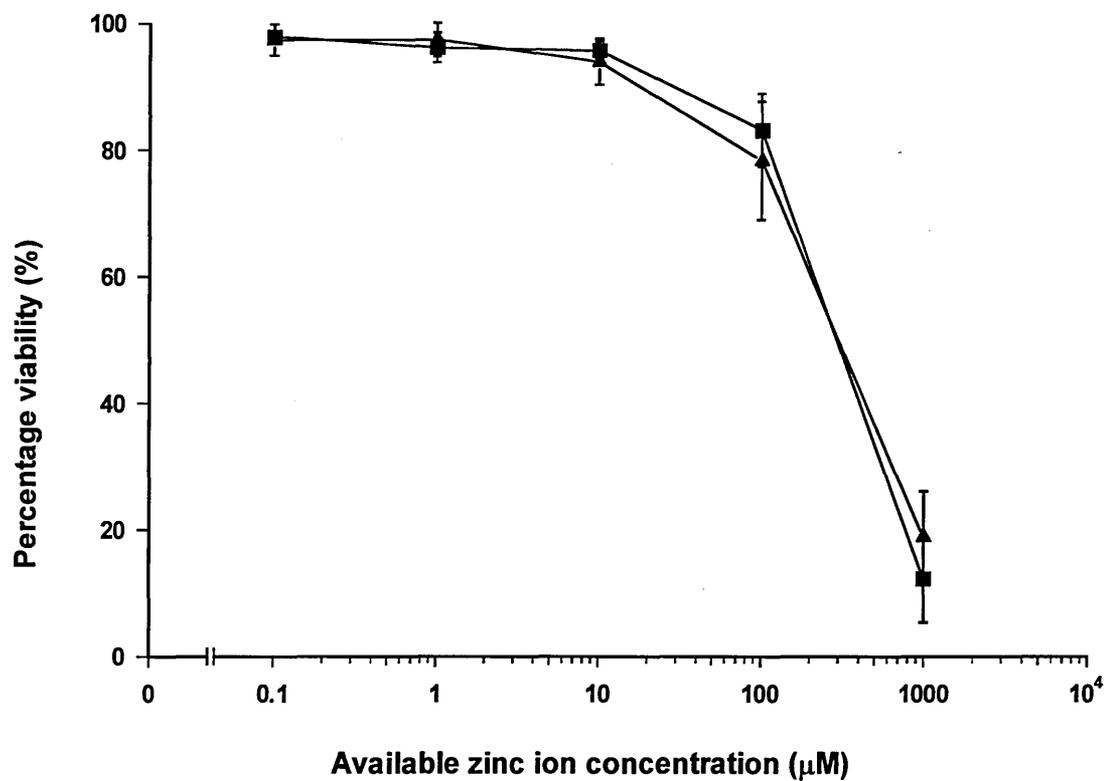
**Figure 3.11.    Change in viability of THP-1 monocytic cells incubated 24 hours with either ZMG suspension (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration:** Zinc concentration given as available ion concentration from zinc sulphate or an equivalent amount of ZMG suspension. There is no significant difference between the effects of ZMG and zinc sulphate (ANOVA, $p > 0.05$).    Values shown as mean and standard deviation, n=6 for each point.
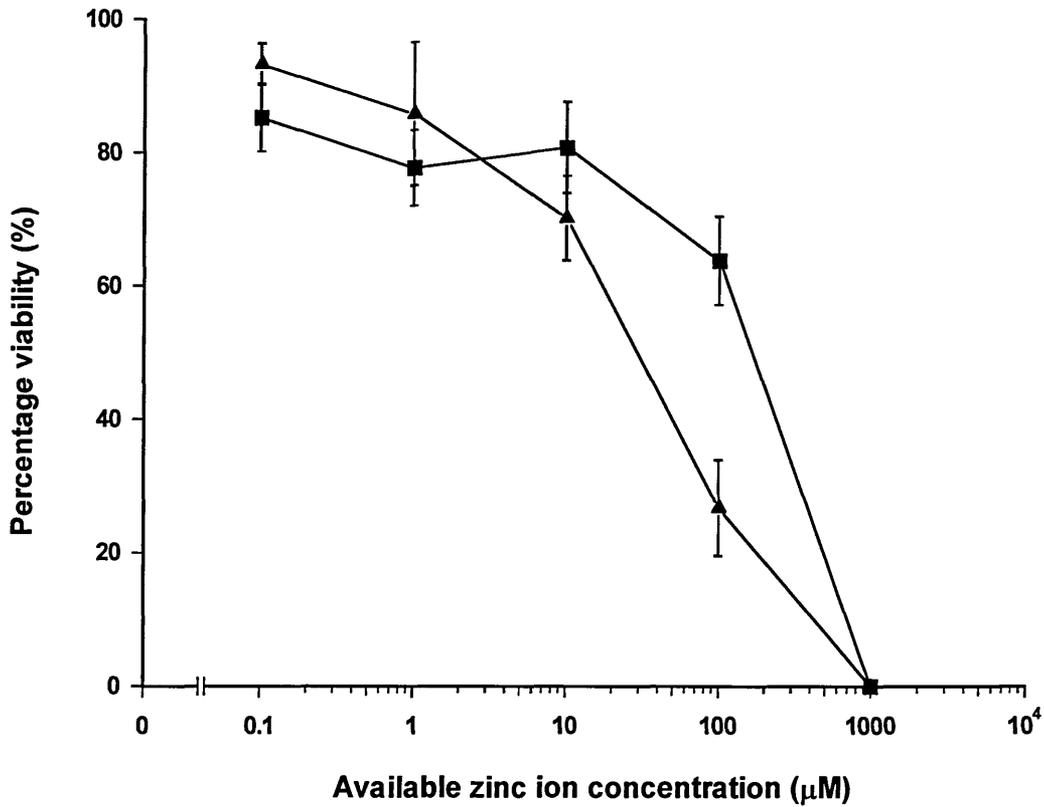
**Figure 3.12. Change in viability of THP-1 monocytic cells incubated 48 hours with either ZMG suspension (square) or zinc sulphate solution (triangle) of increasing zinc ion concentration:** Cell viabilities of cells incubated with ZMG suspension are not significantly different from those of cells incubated with zinc sulphate (ANOVA, $p > 0.05$). Values are shown as mean and standard deviation, n=6 for each point.

### 3.2.2 Analyses of zinc uptake by spectrofluorimetry

Figure 3.13 shows a signature trace of zinquin as a fluorescence wavelength scan. This showed that zinquin acid was maximally excited to fluorescence at 360nm (Fig. 3.13a) as opposed to 370nm which was indicated in the published protocol. This may be due to batch differences in the zinquin acid production or mechanical error with instrumentation differences. The emission wavelength of 488nm determined by this test agreed very closely with the published data at 490nm (Fig. 3.13b). These observations have significance only in spectrofluorimeter studies and not the fluorescence photomicrographic studies where broad band wavelength filters covering the UV spectrum are used to excite to fluorescence the zinquin localised within the cells.

Figure 3.14 shows increasing zinquin acid fluorescence with increasing zinc concentration as zinc sulphate or ZMG solution. The functional portion of the curve lies between $\mu$M–mM zinc ion concentration.

It was observed as these experiments were carried out, that the fluorescence of zinquin acid in the cuvette started high but was reduced very rapidly when exposed to the excitation beam in the fluorescence spectrometer.

(a)
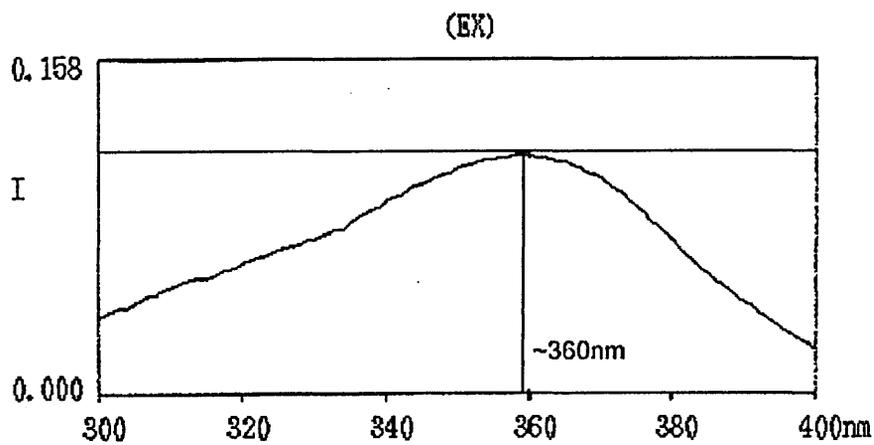


(b)
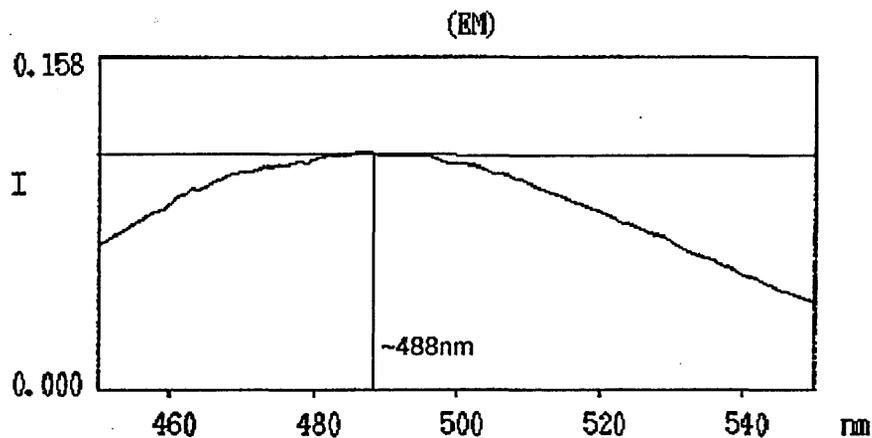


Figure 3.13. Determination of excitation and emission wavelengths for zinquin acid (5μM) in the presence of zinc sulphate (440nM) in HBSS: Zinc sulphate was dissolved in distilled water and, immediately prior to measurement, zinquin in aqueous solution was added. The scan was carried out immediately on a Hitachi Fluorescence Spectrophotometer. Traces were obtained showing maximum zinquin fluorescence at (a) 360nm excitation wavelength and (b) 488nm emission wavelength.
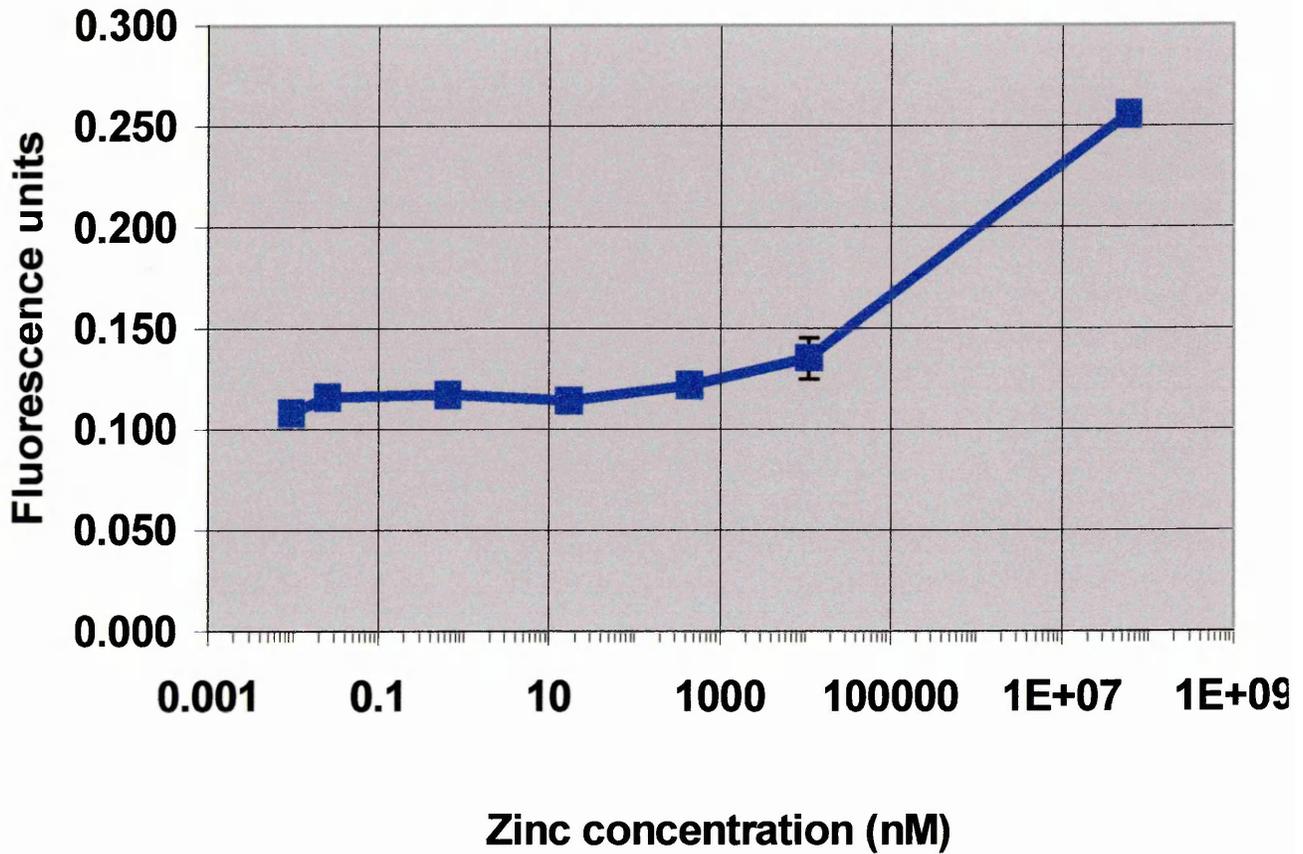
**Figure 3.14   Standard curve of fluorescence of zinquin acid in zinc sulphate solution:** Zinquin acid $6\mu M$ was diluted 1:1 with varying concentrations of zinc sulphate solution, inverted thoroughly and immediately placed in a spectrofluorimeter.   The results were obtained as fluorescence units.   This graph is representative of the standard curves obtained with zinquin acid and zinc solution using the conditions described by Zalewski et al (1993) (data shown as mean $\pm$SD, n=3 for each point).

### 3.2.3  Analyses of cellular zinc uptake by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The following series of figures, (Figures 3.15-3.19) show the uptake of zinc into the various test cell lines.   Some of these figures show comparison of zinc uptake over time and others show cross comparison of uptake of a compound in each cell line.   Statistics have been displayed on the graph or, when space has not been available, they have been described in the text.

The statistics used were ANOVA and the Dunnet Multiple Comparison test that compares sample values with a control value.    Uptake of zinc by THP-1 monocytic cells is shown in figures 3.15 and 3.16.   Figure 3.15 shows the zinc uptake by cells after 2 hours incubation with various zinc compounds.   Cells incubated with ZMG suspension and zinc oxide plus glycerol had a highly significant increase in zinc concentration compared with control cells ($p<0.01$). Cells incubated with zinc oxide alone also had a significant increase compared to control cells ($p<0.05$).   Cells incubated with zinc sulphate with or without glycerol did not show a significant increase in zinc concentration after 2 hours incubation.

After 4 hours incubation with zinc compounds a similar pattern is observed compared to the 2 hour incubation (Figure 3.16).   However, only cells incubated with ZMG contain significantly more zinc than untreated control cells ($p<0.05$). Zinc oxide alone and zinc sulphate alone or zinc sulphate plus glycerol show very little apparent difference from controls, although cellular zinc concentrations in general are lower than the values from the 2 hour incubations.   Values for THP-1 cells incubated for 24 hours are not shown as no significant increase in zinc concentration was seen for cells incubated with any zinc compound.
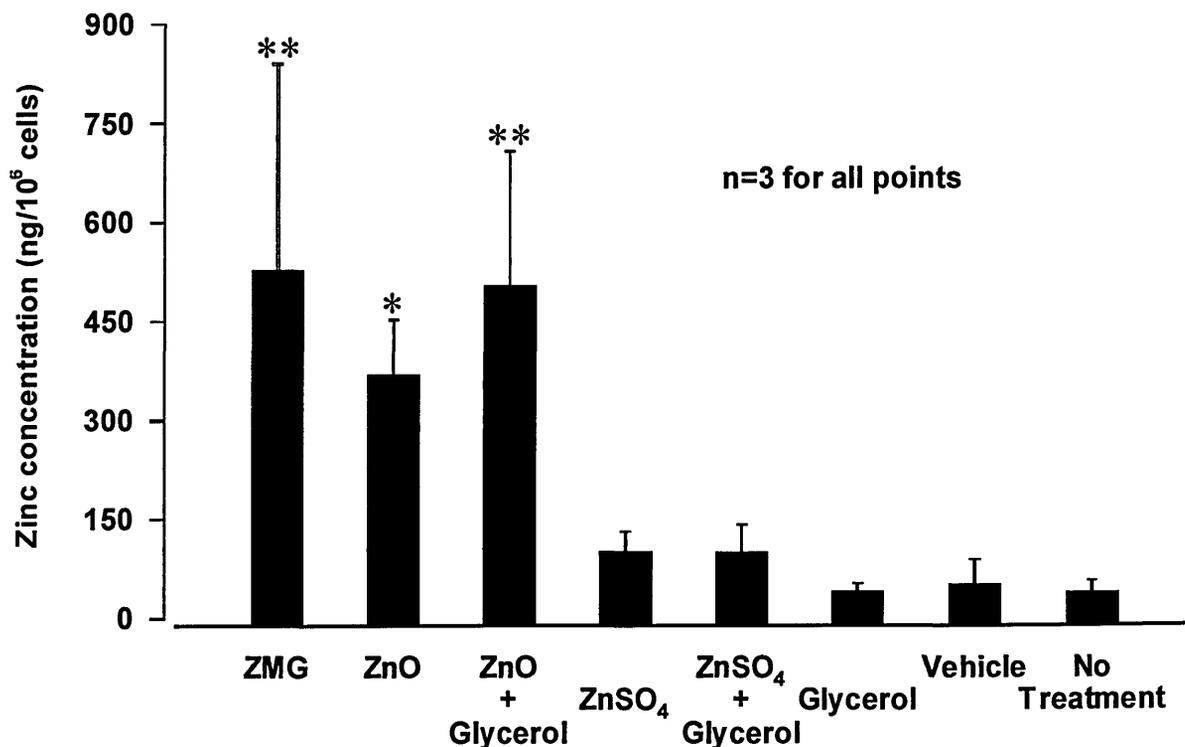
**Figure 3.15. Zinc uptake by THP-1 monocytic cells, 2 hrs incubation with or without zinc compounds, as determined by ICP-MS (mean ±SD):** Statistically significant uptake was seen in ZMG, zinc oxide and zinc oxide + glycerol suspension when compared to untreated cells. All zinc compounds were $25\mu$M zinc molar equivalent. Values are expressed as ng (zinc) per million cells (ng/$10^6$ cells). Statistical significance (Dunnet's multiple comparison with control): (*) = $p \leq 0.05$, (**) = $p \leq 0.01$.
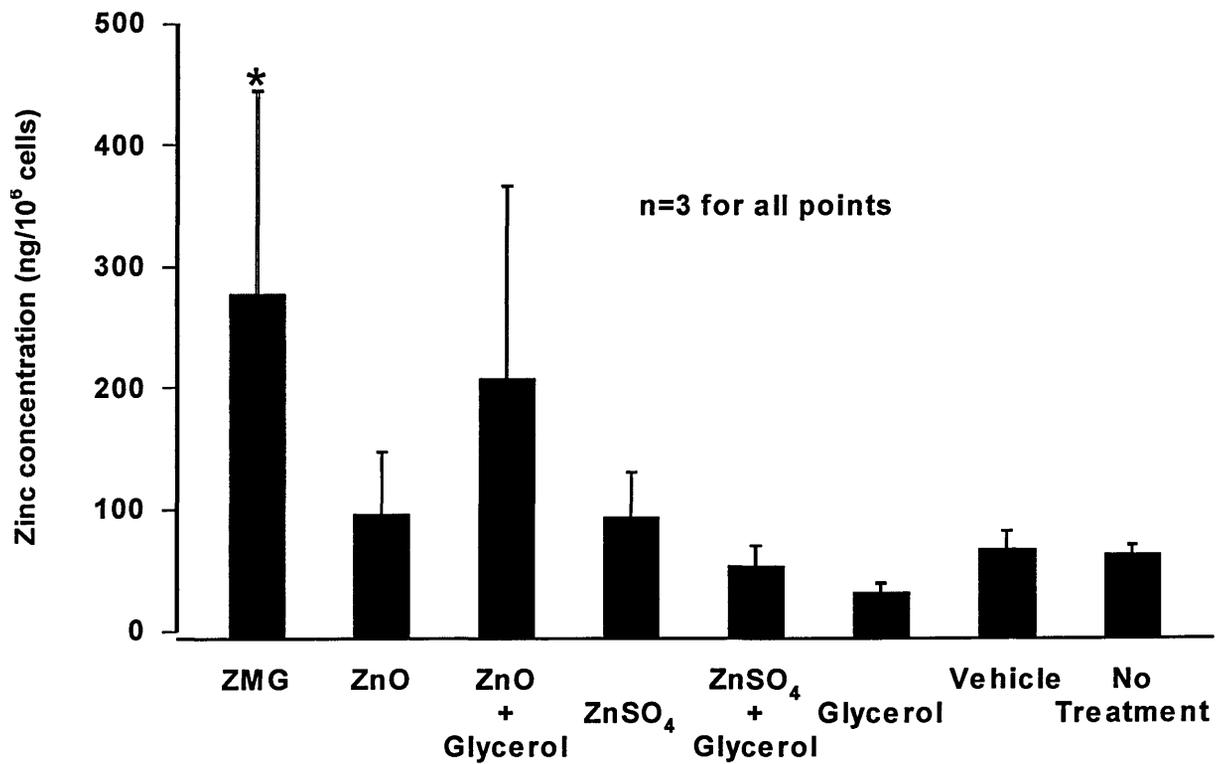
**Figure 3.16. Zinc uptake by THP-1 monocytic cells, 4 hrs incubation with or without zinc compounds, as determined by ICP-MS (mean ±SD):** Statistically significant uptake was seen only in ZMG suspension when compared to untreated cells. Values are expressed as ng (zinc) per million cells ($ng/10^6$ cells). Statistical significance (Dunnet's multiple comparison with control): (*) = Statistical significance of $p \leq 0.05$.

Figures 3.17 and 3.18 show zinc uptake over 4 and 24 hours by Jurkat T-cells incubated with different zinc compounds or zinc free controls. Unlike the THP-1 monocytic cells, the Jurkat T-cells had no significantly increased absorption of zinc in the shortest time period (2hrs not shown), but significant uptake was seen by cells incubated with both ZMG and zinc oxide suspensions for 4 hours ($p < 0.01$ and $p < 0.05$ respectively) (Figure 3.17). At 24 hours, cells incubated with ZMG suspension contained a significantly higher concentration of zinc than controls ($p < 0.01$) (Fig. 3.18) although the overall cellular zinc was far lower than at 2 or 4 hours.

HGT-1 cells did not absorb zinc at all when incubated at any time point with any zinc compound. Data is shown graphically for a 24 hour incubation in Fig. 3.19. Compared with the controls there was not even an apparent non-significant increase in cellular zinc content in cells incubated with zinc compounds and most seem to have mean values below that of the "no treatment" control.

Figures 3.20 and 3.21 show comparisons of cellular zinc concentration trends over time in THP-1 cells and Jurkat T-cells respectively where cells were incubated with or without zinc. HGT-1 cells are not shown as they did not display any level of zinc uptake from the zinc compounds tested and additionally, only two time points (2 and 24 hours) were carried out for the uptake from these cells. Each point, in figures 3.20 and 3.21, is representative of three experiments. Comparisons were made by two-way ANOVA between cellular zinc concentration trends of cells incubated with zinc compounds and the 'No Treatment' control cells for each cell line. In both THP-1 cells and Jurkat T-cells, incubation with ZMG, zinc oxide, zinc oxide plus glycerol or zinc sulphate resulted in significantly greater zinc concentrations over time than the cells incubated without zinc (significance values indicated in Figures 3.20 and 3.21).

It may be seen from figures 3.20 and 3.21 that zinc concentration for both THP-1 and Jurkat cells have similar overall trends, initially high and decreasing with increasing incubation time.
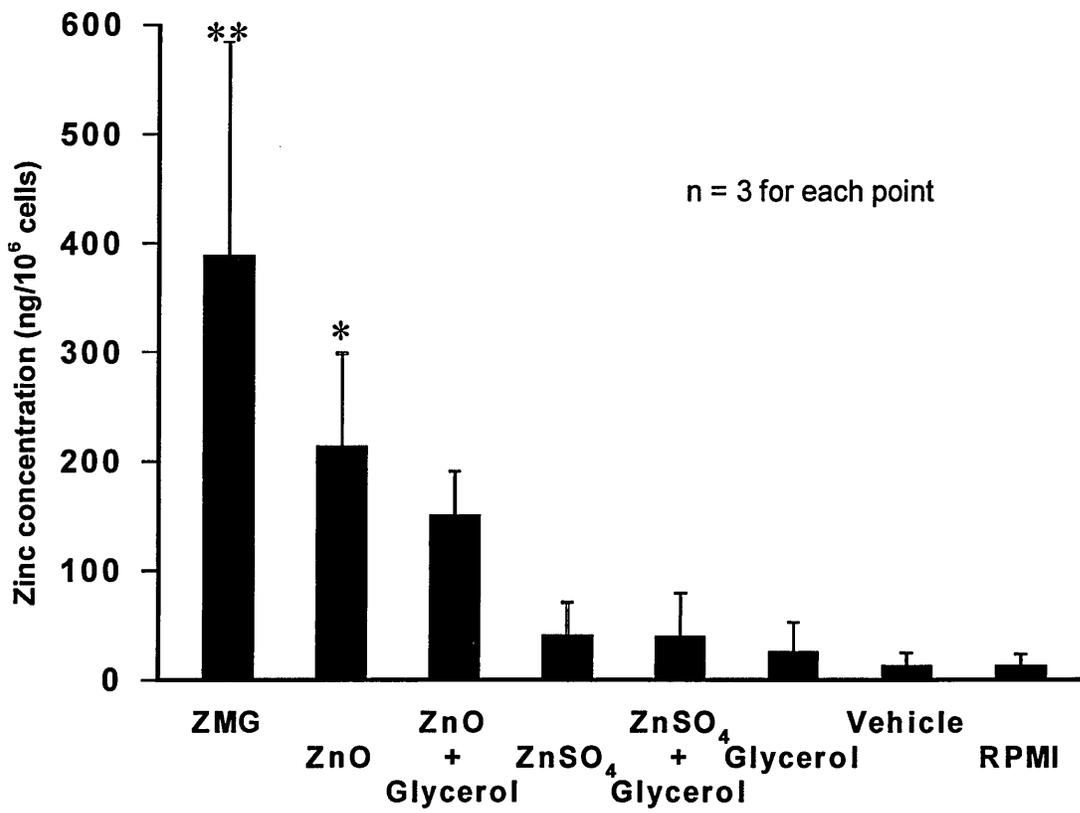
**Figure 3.17.** **Zinc uptake by Jurkat T-cells, 4 hrs incubation with or without zinc compounds, as determined by ICP-MS (mean ±SD):** Statistically significant zinc uptake was seen in cells incubated with ZMG suspension and zinc oxide when compared to untreated cells. Values are expressed as ng (zinc) per million cells (ng/$10^6$ cells). Statistical significance (Dunnet's multiple comparison with control): ** = p<0.01, *=p<0.05.
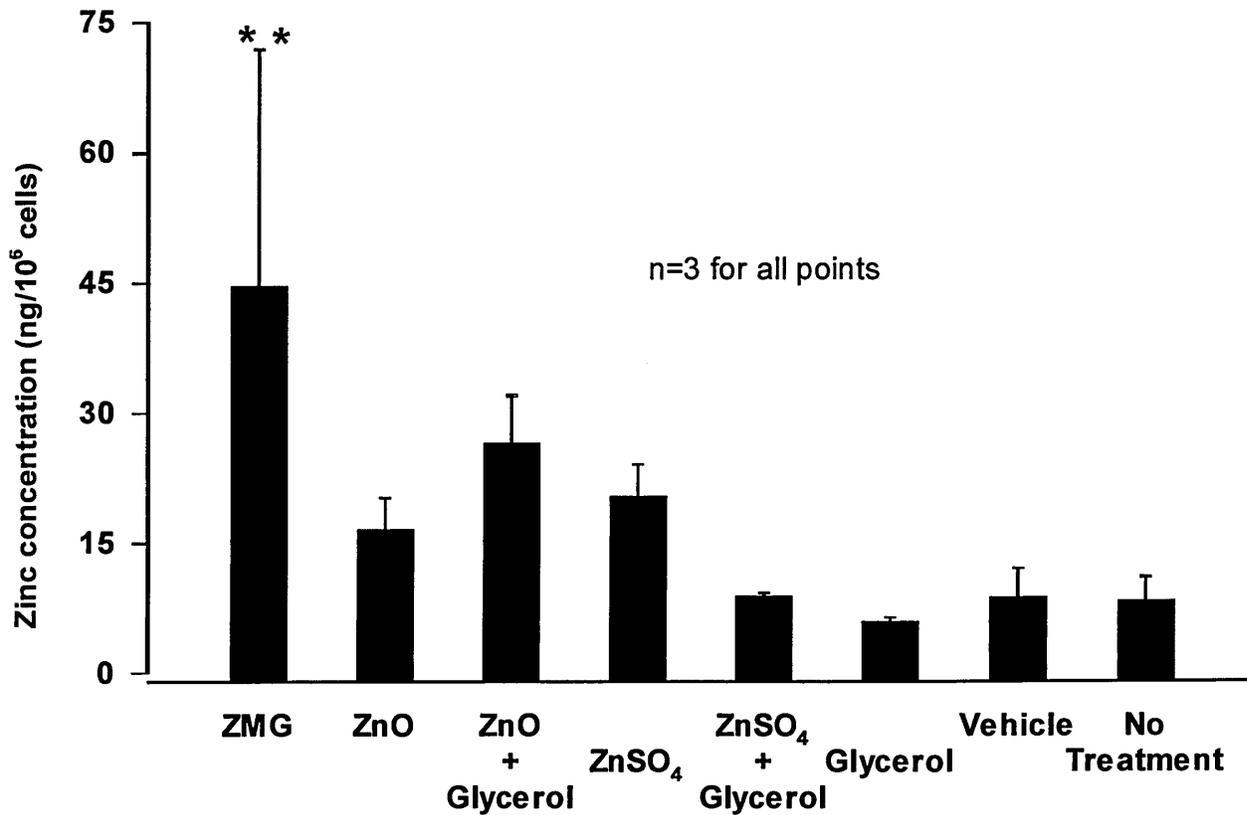
Figure 3.18. Zinc uptake by Jurkat cells, 24 hrs incubation with or without zinc compounds, as determined by ICP-MS (mean ±SD): Statistically significant uptake was seen only in cells incubated with ZMG suspension for 24 hrs. Values are expressed as ng (zinc) per million cells (ng/10⁶ cells). Statistical significance (Dunnet's multiple comparison with control): (**) = p≤0.01.
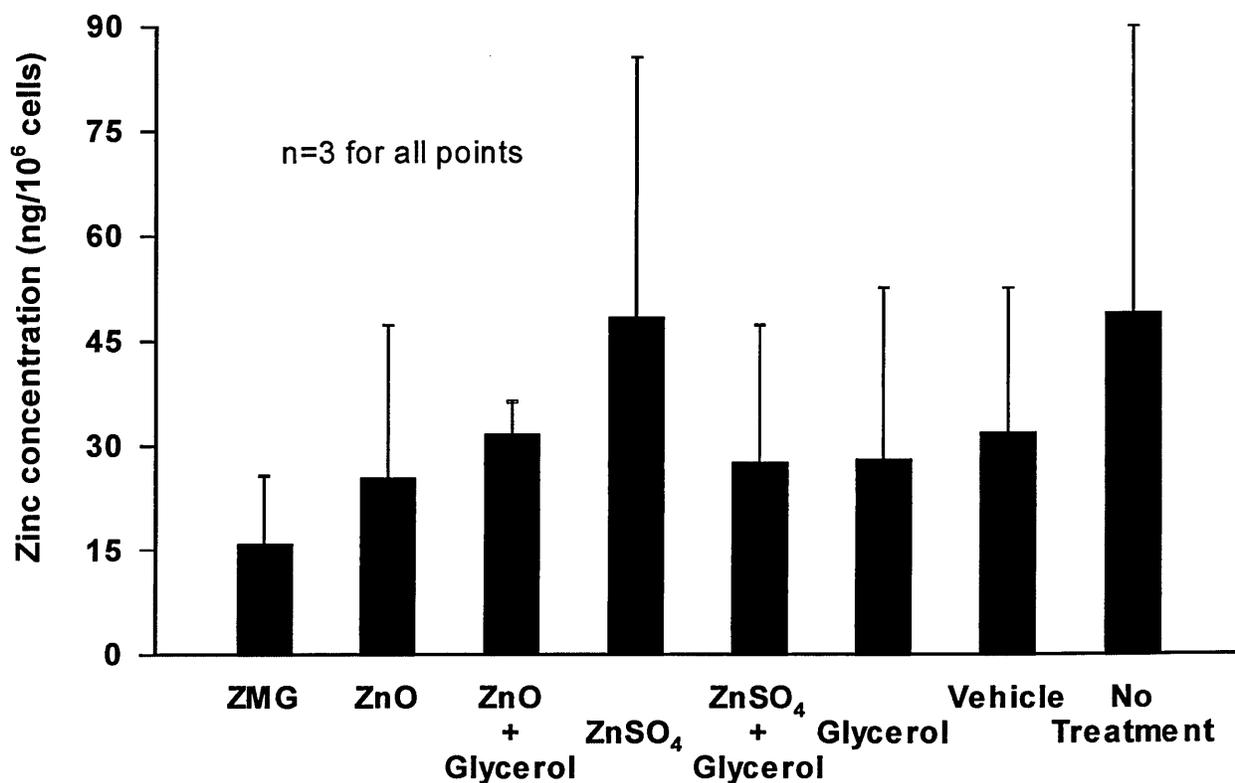
**Figure 3.19. Zinc uptake by HGT-1 cells, 24hr incubation, as determined by ICP-MS (mean ±SD):** No significant difference from control for any zinc preparation after 24hr or after 2hr (not shown). Values are expressed as ng (zinc) per million cells (ng/$10^6$ cells).
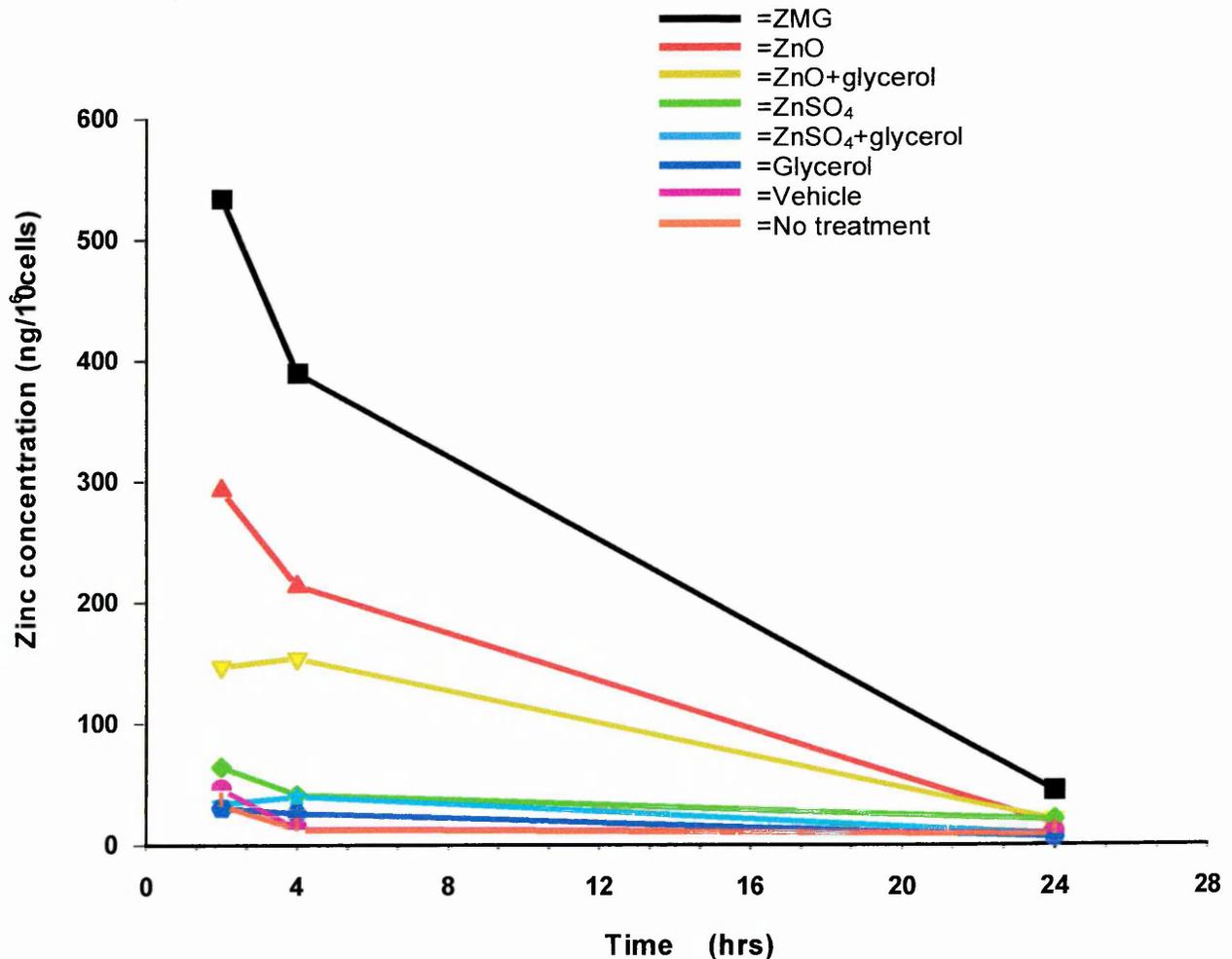
**Figure 3.20.** **Amalgamated time course for cellular zinc concentration of Jurkat T-cells incubated with or without zinc compounds over three time points 2, 4 and 24 hours:** This figure shows the trend of cellular zinc concentration for Jurkat T-cells incubated with various zinc compounds. Compared to the 'No treatment' control, cells incubated with ZMG, ZnO, ZnO+glycerol or $ZnSO_4$ had significantly higher cellular zinc concentration trends over time (By ANOVA: p=0.02, p=0.02, p=0.002 and p=0.01 respectively). Data represented as means, standard deviations have been omitted for clarity, n=3 for each point.
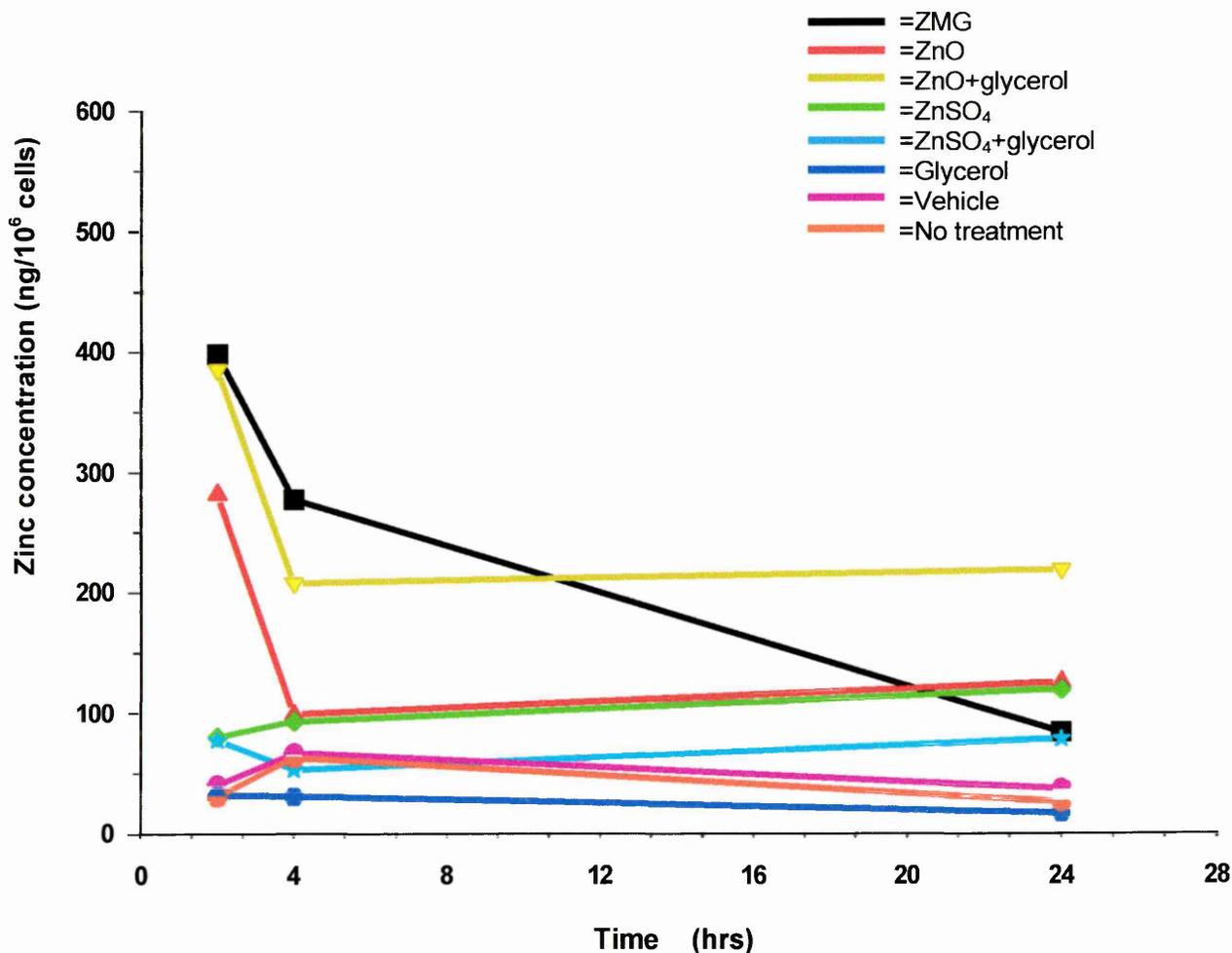
**Figure 3.21.** **Amalgamated time course for cellular zinc concentration of THP-1 cells incubated with or without zinc compounds over three time points 2, 4 and 24 hours:** This figure shows the trend of cellular zinc concentration for THP-1 monocytic cells incubated with various zinc compounds. Compared to the 'No treatment' control, cells incubated with ZMG, ZnO, ZnO+glycerol or $ZnSO_4$ had significantly higher cellular zinc concentration trends over time (By ANOVA: $p=0.02$, $p=0.01$, $p=0.01$ and $p=0.05$ respectively). Data represented as means, standard deviations have been omitted for clarity, n=3 for each point.

Further studies were carried out to examine the effect, if any, of external stimulation or intervention on the uptake of the various cell lines. THP-1 cells, Jurkat T-cells and HGT-1 human gastric carcinoma cells were independently stimulated with LPS to simulate an inflammatory response and zinc uptake was determined as before. HGT-1 cells were incubated with a mucolytic agent to determine whether the lack of zinc uptake was a mechanical effect of a mucus barrier.

The effect of LPS appears to be to reduce zinc uptake in THP-1 cells. However under these conditions the decrease is not significant when comparison is made between cells with and without LPS (Figure 3.22). The trend to lower zinc content with increasing LPS concentration is not significant either. However the trend appears to be decreasing and it is possible that with more repetition and a wider range of LPS concentration that a clearer picture would be seen of the effects of LPS on zinc uptake. This is reflected by the results from the effect of LPS on Jurkat T-cells (Figure 3.23). The trend appears to be that increasing LPS concentration results in an increase in cellular zinc concentration although whether this is a real affect can only be determined with greater 'n' values for each sample.

It should be noted that due to lack of cell counts for one set of the Jurkat results (Figure 3.23) the overall results were not corrected for cell number and so are presented as parts per billion. This does not invalidate the results, as correction for cell number is not integral to interpretation of results. Correction of cellular zinc concentration for sample cell number was used to increase the probability of obtaining an accurate result. However the correction did not tend to change the general pattern of uptake, rather it corrected individual points that might be skew due to harvesting inaccuracies.
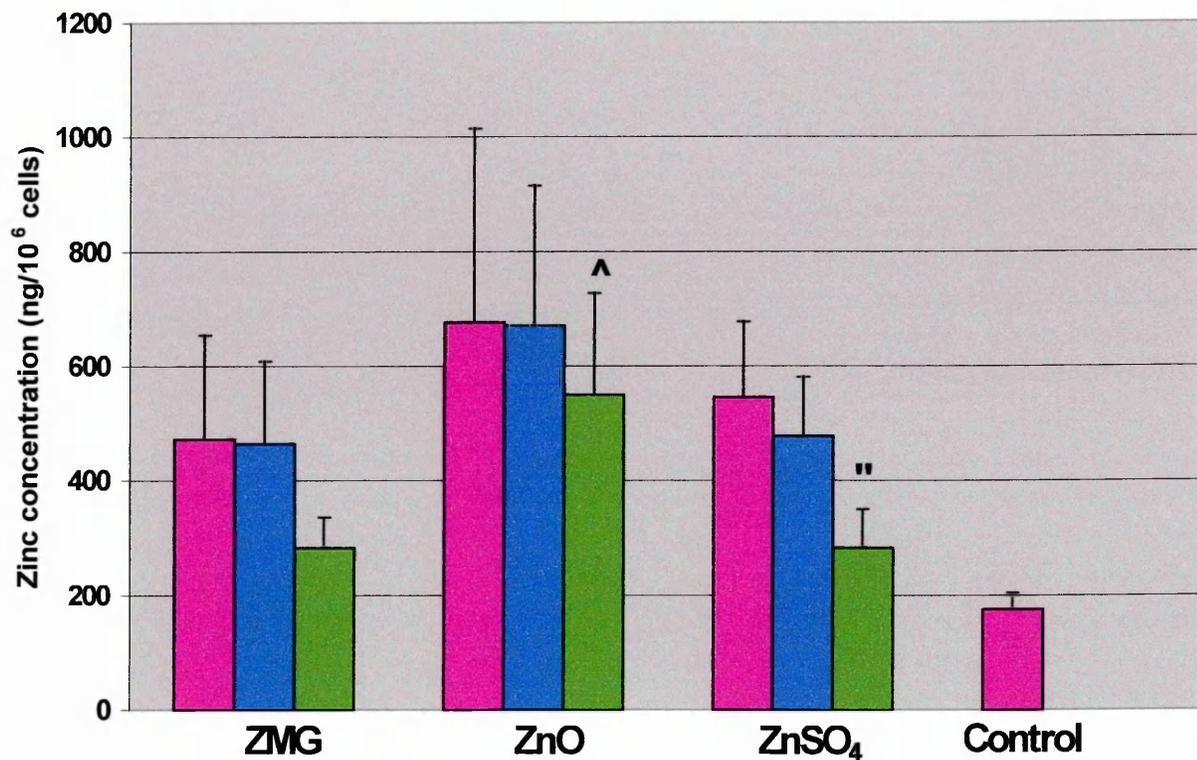
**Figure 3.22   Zinc analysis of THP-1 cells incubated with or without LPS and zinc compounds:** Cells were incubated 24 hours with 0, 1 and 10ng/ml LPS ( ■ , ■ and ■ respectively) and then incubated a further 24 hours with or without zinc compounds (25µM molar equivalent).   Zinc content was quantified by ICP-MS.   No significant difference was seen with increasing LPS concentration when compared to LPS free zinc controls.   No significant trend towards increasing or decreasing cellular zinc content, with increasing LPS concentration, was observed for any zinc compound.   Data presented as mean±SEM, n=4 for all except '∧' where n=3 and '"' where n=2.
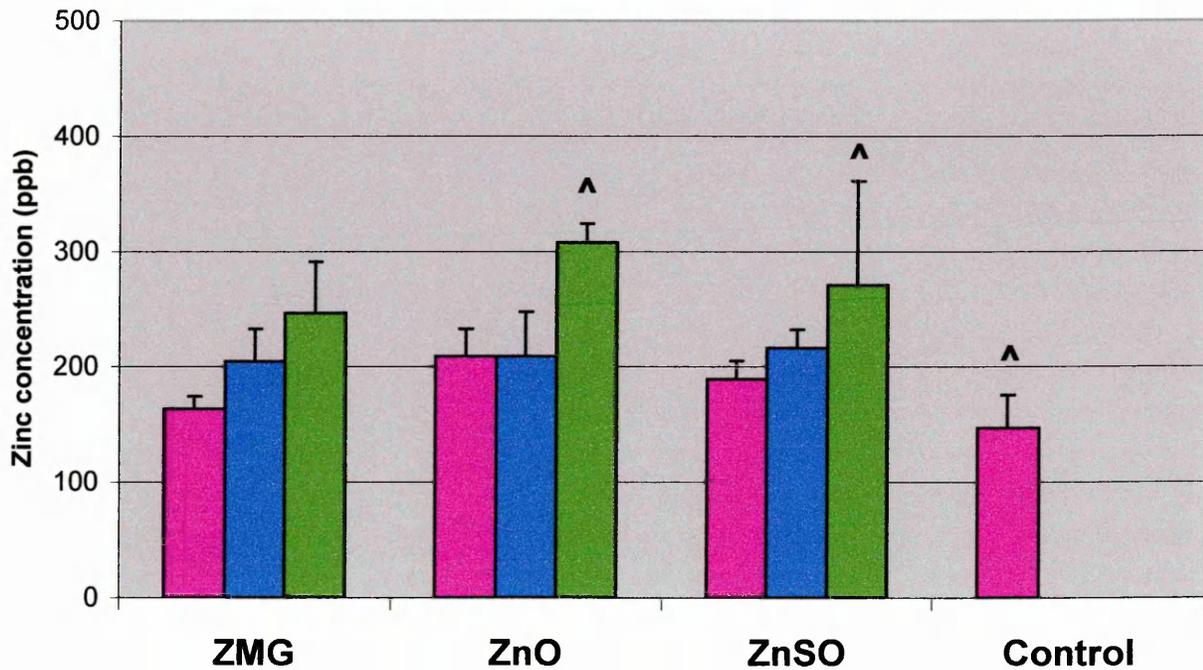
**Figure 3.23   Zinc analysis of Jurkat T-cells incubated with or without LPS and zinc compounds:** Cells were incubated 24 hours with 0, 1 and 10ng/ml LPS ( ■ , ■ and ■ respectively) and then incubated a further 24 hours with or without zinc compounds (25μM molar equivalent).   Zinc content was quantified by ICP-MS.   No significant trend was seen in any sample.   Data presented as mean±SEM, n=4 for all except data marked '˄' where n=3.

HGT-1 cells were incubated with a variety of substances to try and induce any level of zinc uptake beyond control levels (Figure 3.24). Initially it was thought that, being a gastric derived epithelial cell line, HGT-1 cells might have been secreting mucus that could interfere with zinc uptake. This was supported by zinquin (zinc specific fluorophore) staining of HGT-1 cells that indicated highly fluorescent areas on the slides that seemed associated with but not part of areas of HGT-1 cell growth. It is known that mucus is able to bind zinc in vivo and in vitro (Coleman and Young, 1979, Seal and Heaton, 1987) so HGT-1 cells were incubated with a mucolytic compound to remove any mucus. HGT-1 cells were also incubated with cytokines IL-1$\beta$ (Figure 3.24), TNF-$\alpha$ and the mitogen LPS (not shown). These were used in an attempt to activate the HGT-1 cells in a manner that might result in zinc uptake; normally cells react to cytokines or mitogens by producing more soluble mediators. This sudden upregulation of protein production absolutely requires zinc and might be expected to result in an influx of available exogenous zinc.

The results indicated that no external stimulus caused healthy HGT-1 cells to take up exogenous zinc. It was not possible to carry out repeated experiments of the HGT-1 cell analyses due to a lack of availability of the ICP-MS instrument and n=2 for each point except ZMG where n=3. However, it is clear from figure 3.24 that cells incubated with ZMG contain less zinc than the control cells incubated in zinc free media, indicating that zinc levels are in fact at baseline level. Comparison with figure 3.19 indicates that there is no difference between the HGT-1 cells incubated with ZMG and either n-acetyl cysteine (a mucolytic agent) of varying concentration or IL-1$\beta$ at 20 units/ml (Figure 3.24) or in fact, 2 units/ml (Not shown). These results are similar to those for cells incubated with LPS or TNF-$\alpha$, none of which displayed increase in zinc uptake compared to "no zinc" controls (Not shown). In fact no chemical treatment resulted in an increase of cellular zinc content in HGT-1 cells. Significantly, zinquin studies showed that HGT-1 cells grown on commercial cell culture materials did not display any zinc-related fluorescence but cells grown on glass cover slips displayed high levels of zinc-related fluorescence and also high levels of cell death. This observation is discussed later in the following section of photomicrographic results.
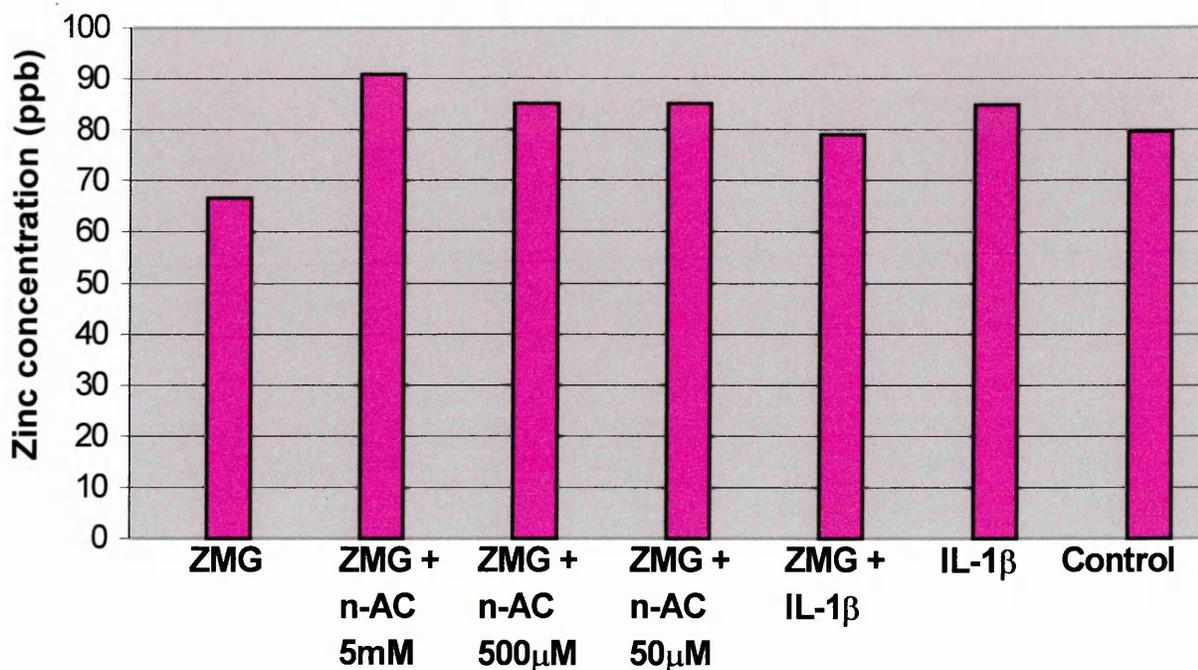
**Figure 3.24    Cellular zinc concentration of HGT-1 gastric carcinoma cell line incubated under various conditions:** HGT-1 cells were incubated with or without zinc (25μM molar equivalent) after pre-incubation with mucolytic agent n-acetyl cysteine (n-AC).    Alternatively the cells were incubated with inflammatory cytokine IL-1β in the presence or absence of zinc.    Data presented as mean, standard deviations not shown as n=2 for all points except ZMG alone where n=3.

### 3.2.4 Analysis of cellular zinc uptake by fluorescence photomicrography

These results are comprised of photomicrographs taken either by still camera on an Olympus fluorescent microscope under which cells fluoresce green/yellow or by still camera and computer controlled video image capture where cells fluoresce blue/white. The difference in colour is attributable to the differing excitation filters used and is not a function of either the zinc specific fluorophore zinquin or of cell function.

Figure 3.25 indicates the localisation of zinquin fluorescence to the cytoplasm and its exclusion from the nucleus in two different cell types. In figure 3.25a Jurkat T-cells were incubated with ZMG suspension for 2 hours then stained with zinquin ester and recorded by video capture. In figure 3.25b THP-1 cells were incubated with zinc sulphate and sodium pyrithione (a zinc ionophore), then co-incubated with zinquin and ethidium bromide. The ionophore allows zinc to bypass the normal uptake mechanisms of zinc uptake and was used to show that zinquin does not pass into the nucleus. These results agreed with the only publication at the time that had used zinquin (Zalewski et al., 1993) and confirmed that the fluorescence seen in this set of results was extranuclear, either cytoplasmic or membrane bound.

Occasionally, in highly fluorescing cells, the unstained nucleus is not clearly visible. However, this appeared to be attributable to the very high fluorescence itself, in that the cytoplasm surrounding the nucleus and particularly cytoplasm directly above the nucleus fluoresces brightly enough to mask the unstained nucleus. This is clear in figure 3.25b, especially in cells marked with a yellow arrow, where the highly fluorescent granules of cytoplasmic or membrane bound zinc are able to mask even the intense red stain of the ethidium bromide in the nucleus. Thus, the dark nuclei of the very bright fluorescing cells in figure 3.25a are masked.

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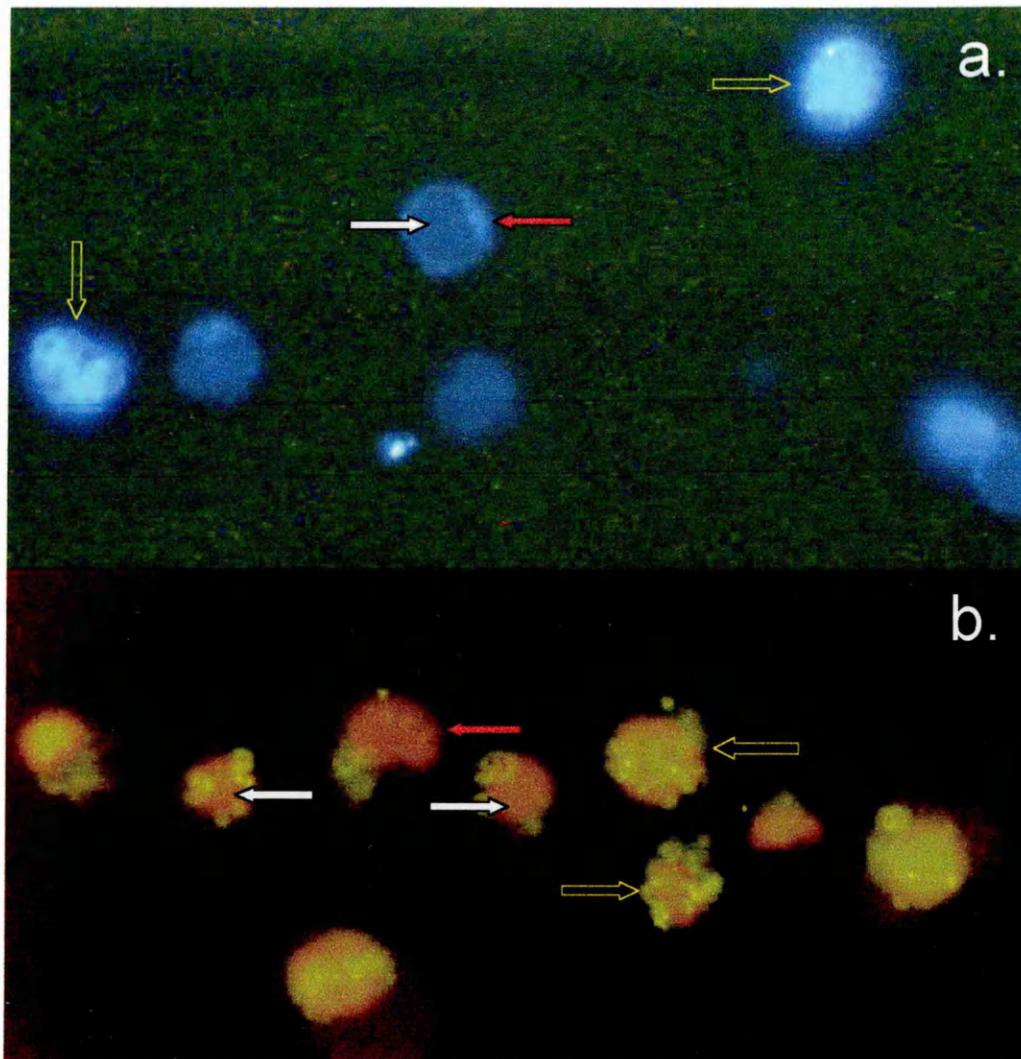
**Figure 3.25 Jurkat T-cells (Fig. 3.25a) and THP-1 monocytic cells (Fig. 3.25b) incubated with zinc then zinquin:** The Jurkat T-cells, incubated with ZMG suspension display a distinctive 'all-or-nothing' staining pattern of zinc uptake. THP-1 cells were incubated with zinc sulphate and sodium pyrithione and then ethidium bromide to stain cell nuclei. Yellow (open) arrows indicate 'positive for uptake', brightly fluorescing cells of both cell lines. Red arrows indicate 'negative for uptake' cells with little granular or diffuse zinc specific staining. White arrows indicate cell nuclei, unstained in the Jurkat cells and stained red with ethidium bromide in the THP-1 cells. Some cells clearly contain large amounts of granular fluorescence whilst the other cells remain relatively unstained. Original magnification x400.

Figure 3.25a is a typical example of the 'all or nothing' staining found in all cell types investigated. It is clear that some cells fluoresce brightly (marked by yellow arrow) whilst some display little more than background fluorescence (marked by red arrow). This pattern was displayed by all cell lines examined and appears to be an important function in cellular zinc control. The 'negative for uptake' cells that exclude zinc appear otherwise healthy with an intact nucleus and a visibly intact, relatively smooth cell membrane. These observations would argue that the cells were not dead from apoptosis, which is typified by nuclear blebbing, or necrosis, which is normally typified by outer membrane crenelation. The all-or-nothing uptake pattern is apparent even in the very low fluorescing HGT-1 cells (Figure 3.35, page 201).

Figures 3.26-3.29 respectively show zinc uptake of ZMG suspension, ZMG solution, zinc sulphate solution and no zinc vehicle control by THP-1 monocytic cells. The first point to note is that the all-or-nothing staining pattern was present to some degree in all figures except the zinc free vehicle control. However, compared to zinquin stained Jurkat T-cells in general, there was a far higher degree of cytoplasmic staining in all cells, not just the brighter stained ones. In the THP-1 cells, there appears little discernible difference in uptake patterns between cells incubated with different zinc preparations. Additionally, all zinc preparations give distinctly brighter fluorescence than the vehicle control.
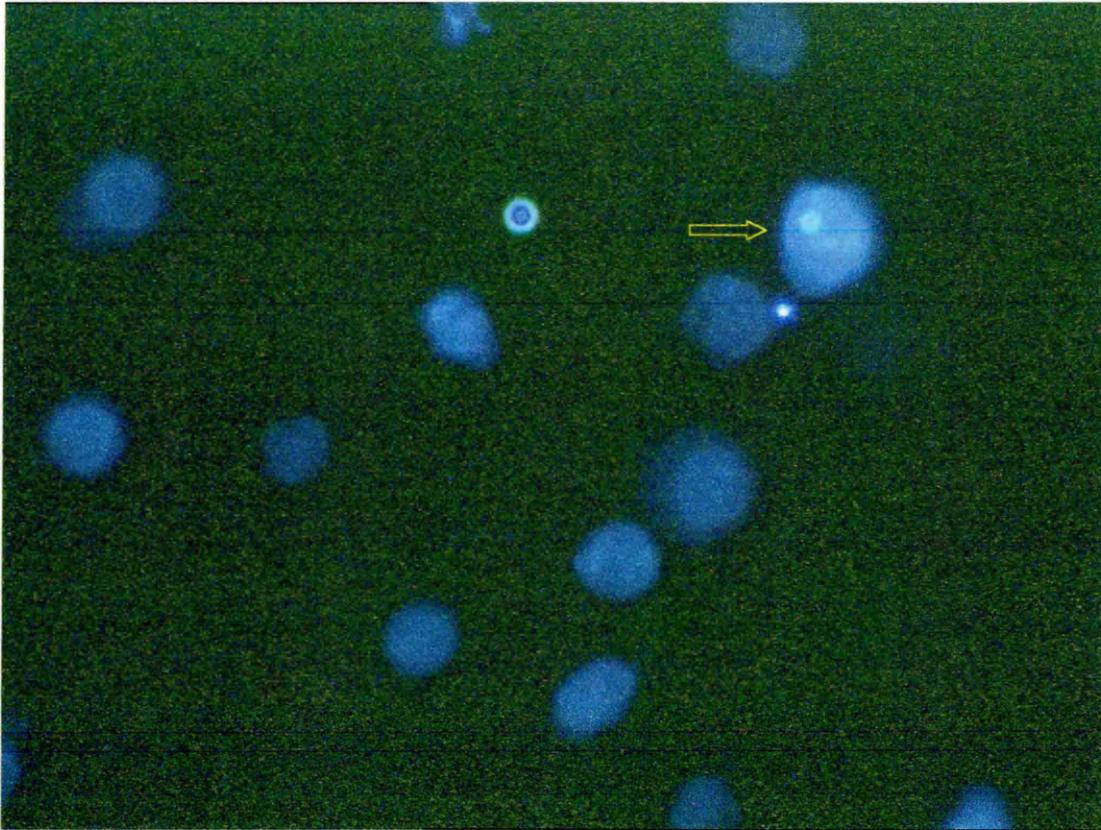
**Figure 3.26    Zinc content of THP-1 cells as shown by zinquin fluorescence:** THP-1 cells incubated 2 hours with ZMG dissolved in citric acid (25µM zinc) and then 30 minutes with zinquin (3µM), all appear to have some level of rather diffuse fluorescence. One strongly stained cell (yellow open arrow) that appears 'positive for uptake' is distinct from the less intensely stained cells. Original magnification   x 400
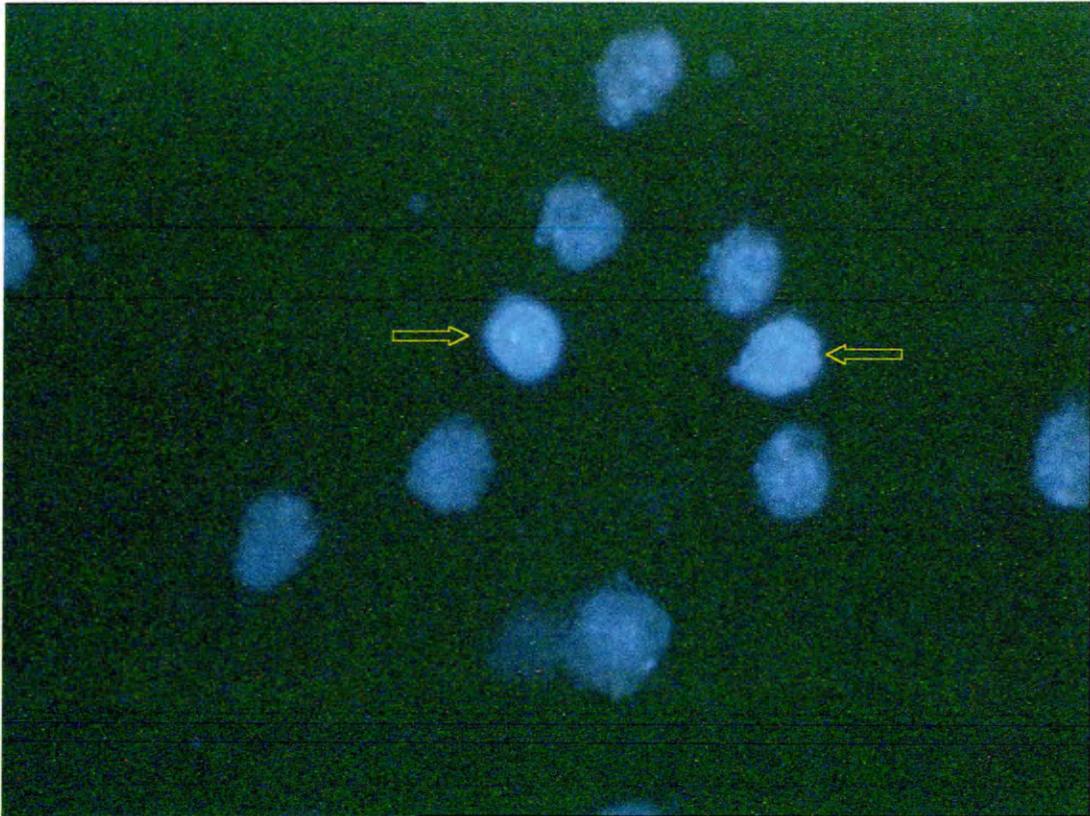
**Figure 3.27   Zinc content of THP-1 cells as shown by zinquin fluorescence:** THP-1 cells incubated 2 hours with ZMG suspension (25µM equivalent zinc) and then 30 minutes with zinquin (3µM).   Yellow (open) arrows indicate brightly stained positive cells. Although the diffuse stain of all cells appears greater than in figure 3.26 the distinction between the individual brightly stained cells is still apparent. Original magnification x400.
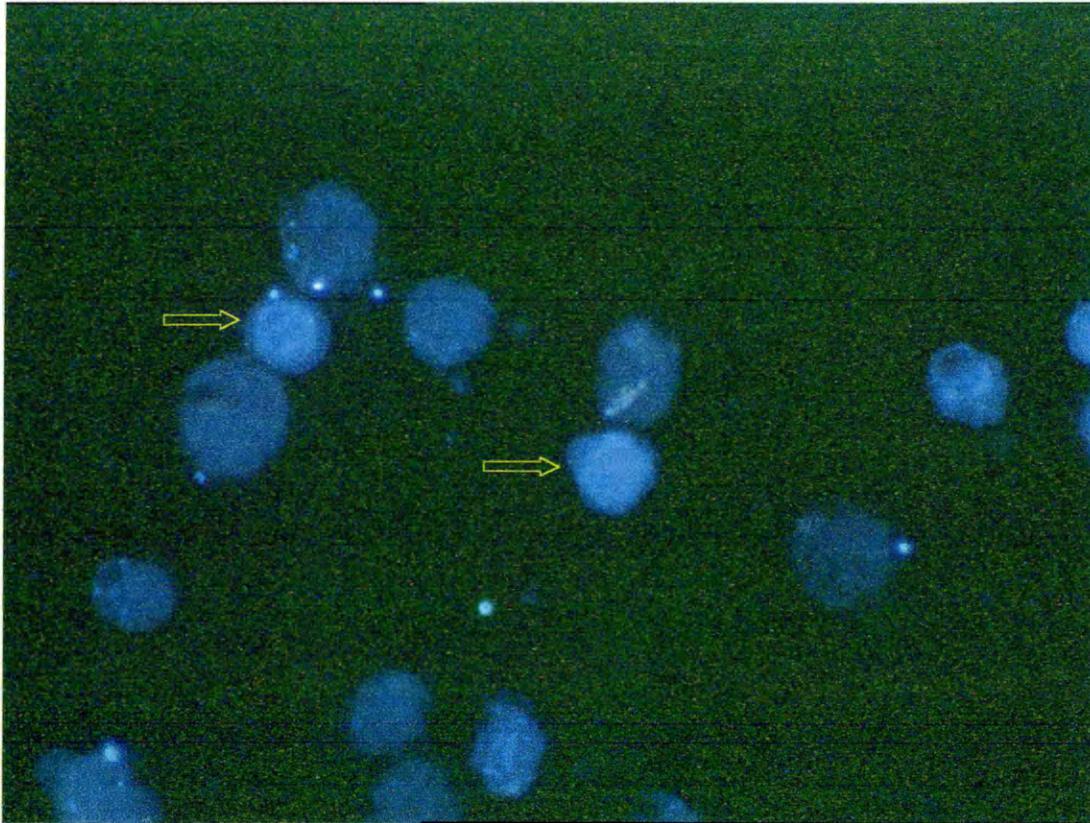
**Figure 3.28  Zinc content of THP-1 cells as shown by zinquin fluorescence:** THP-1 cells incubated 2 hours with zinc sulphate solution (25μM zinc) and then 30 minutes with zinquin (3μM).   Yellow (open) arrows indicate individual cells with positive zinc uptake, displaying more intense fluorescence than other cells. Original magnification x400.
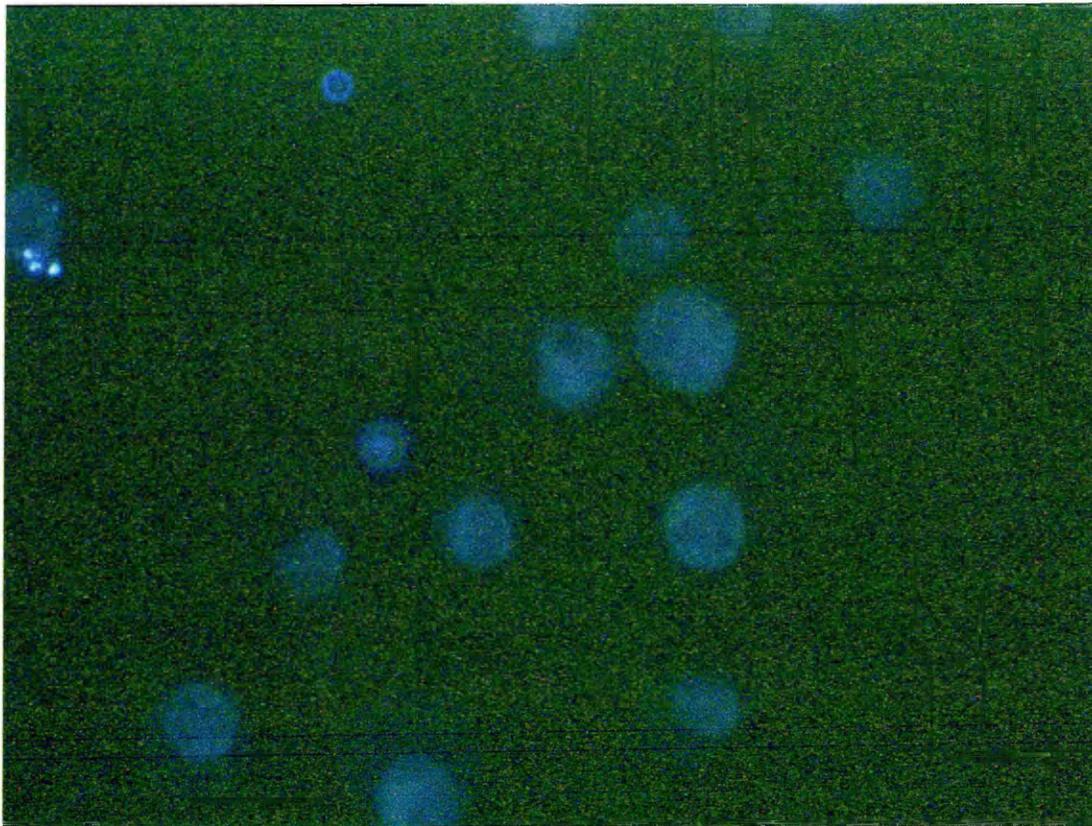
**Figure 3.29    Zinc content of THP-1 cells as shown by zinquin fluorescence:** THP-1 cells incubated 2 hours with zinc free vehicle and 30 minutes with zinquin (3μM). Endogenous zinc levels cause very low background fluorescence in cells incubated without zinc. *Original magnification x400.*

These photomicrographs do not make it immediately clear why ZMG suspension should provide a significantly greater cellular zinc concentration (Figure 3.15, page 173). However, it is possible that the ZMG suspension gave a larger number of the positively stained cells overall. It is also possible that the diffuse cytoplasmic staining was generally more intense in cells incubated with ZMG suspension compared to solutions of ZMG or zinc sulphate but without image analysis software this would be difficult to determine accurately. Figure 3.30 is a good example of this; THP-1 cells were incubated for 1 hour with either ZMG suspension or zinc sulphate (plus glycerol in this example) and photographed at a low magnification to acquire a larger field of cells than figures 3.26-3.29.

Figure 3.30a, cells plus ZMG, clearly shows a greater number of fluorescent cells than figure 3.30b, cells with zinc sulphate. However the positive cells in Figure 3.30b appear as intense as the positive cells in figure 3.30a. Thus supporting the argument that cells incubated with ZMG have a higher *general* diffuse fluorescence than cells incubated with zinc sulphate. The possibility that ZMG suspension might increase the number of highly fluorescent 'positive' cells cannot be discounted. However, without a densitometric analysis programme it would be difficult to say more than that the general fluorescence is apparently increased over fields of cells incubated with zinc sulphate. For reasons examined in the chapter summary both factors probably account for the significant difference in zinc uptake.
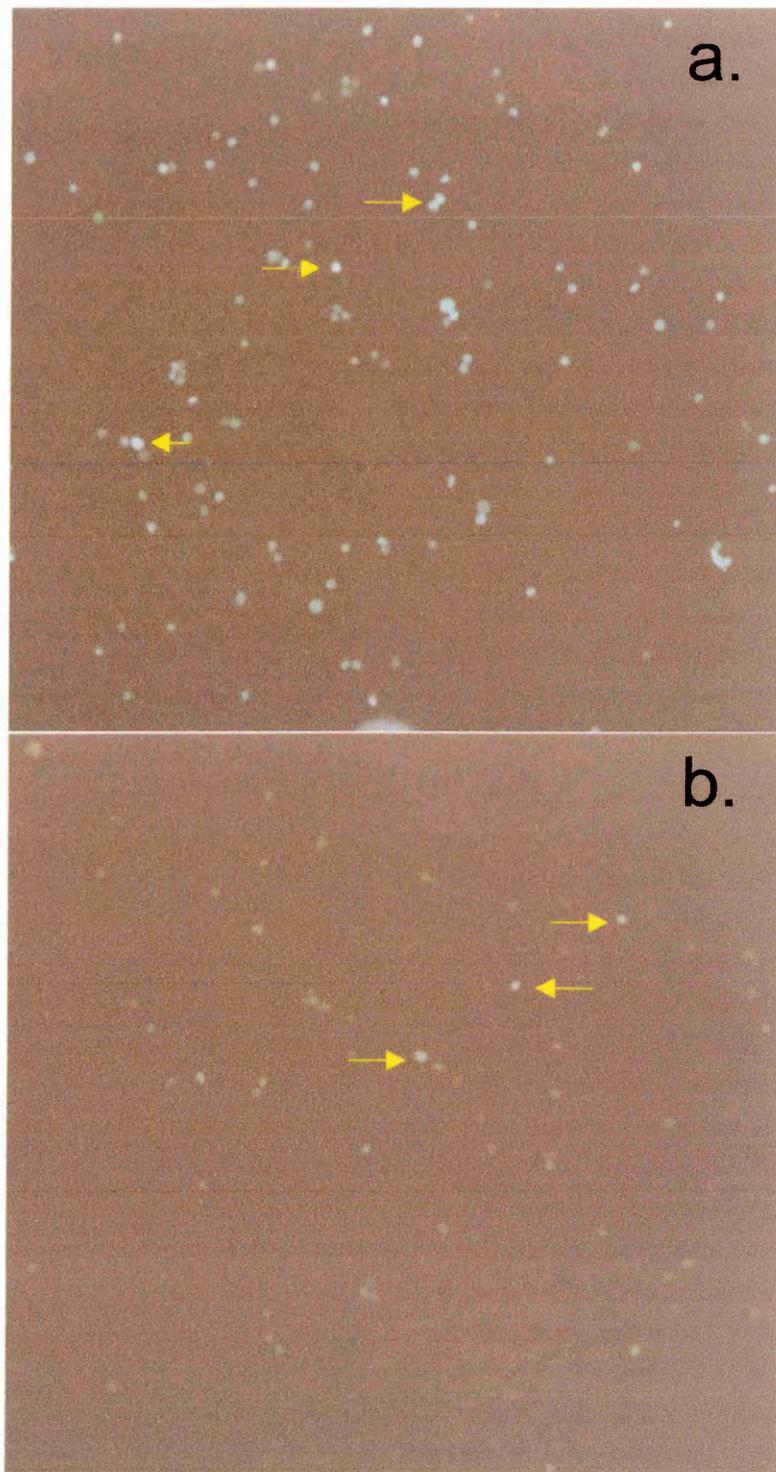
**Figure 3.30    Zinc content of THP-1 cells as shown by zinquin fluorescence:** THP-1 cells were incubated for 1 hour with ZMG in suspension or zinc sulphate + glycerol solution both providing an equivalent of 25$\mu$M zinc, then a further 30 minutes with zinquin (3$\mu$M).    Images were captured by still camera.    Cells with ZMG showed a high level of general fluorescence (Fig. 3.30a) as opposed to cells with zinc sulphate (Fig. 3.30b). Some high fluorescing cells are indicated in each image by yellow arrows. Original magnification X100.

Fluorescence in Jurkat T-cells incubated for 24 hours with ZMG solution and suspension, zinc sulphate solution or vehicle control is shown in Figures 3.31-3.34 respectively. It is not readily apparent that background staining is exceedingly low in the Jurkat T-cells as indicated in cells marked with a white arrow in the vehicle control cells (Figure 3.34). It meant that zinc negative cells were often too dim to register on the video capture system. Two such very faint cells, also indicated by white arrows, are shown in Figure 3.32 in cells incubated with ZMG suspension. Emphasised by this very low background fluorescence, the all-or-nothing staining pattern is quite marked with 'positively' stained cells far brighter than the low fluorescing cells.

Again, as with the THP-1 cells, the fluorescence profile appears similar for each zinc compound. However, unlike the THP-1 cells, there is an observable level of prominent punctate fluorescence (in the positive cells indicated with yellow arrows) especially in the ZMG suspension (Figure 3.32) but also in the ZMG solution (Figure 3.31) and the zinc sulphate solution (Figure 3.33). When filters were introduced to reduce fluorescence it was clear that these masses of fluorescence were distinct, highly fluorescent granules and, by correlation, granules of high zinc concentration. It should be noted that no filters, either on the microscope or by software manipulation, were used to clean up the photomicrographs presented here. The results for all cell lines are shown as taken under identical conditions and are shown as near to the original microscopic image as possible.
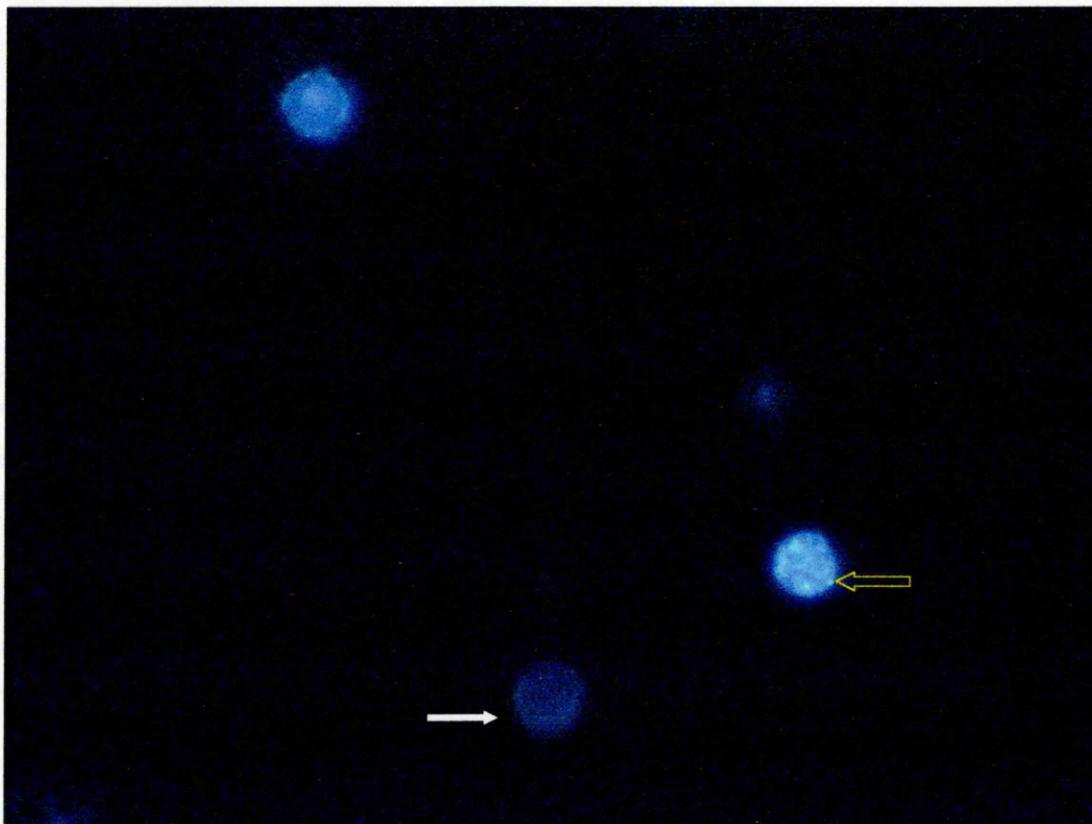
**Figure 3.31** **Zinc content of Jurkat T-cells as shown by zinquin fluorescence:** Jurkat T-cells were incubated 24 hours with ZMG (25μM equivalent zinc) dissolved in citric acid and then 30 minutes with zinquin (3μM). The large contrast between the Jurkat positive uptake and less intensely stained cells is shown here. Yellow (open) arrow indicates the strongly fluorescing cell, the low fluorescing cell being indicated by a white arrow. Original magnification x 400.
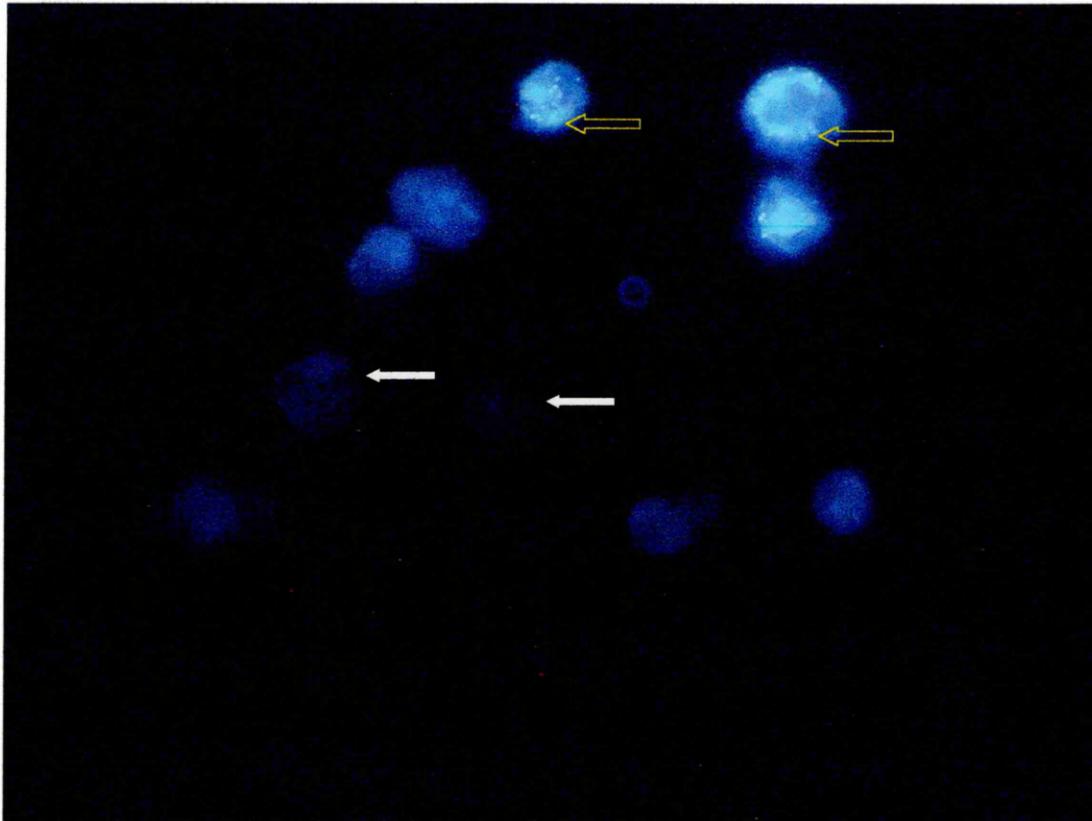
**Figure 3.32 Zinc content of Jurkat T-cells as shown by zinquin fluorescence:** Jurkat T-cells incubated 24 hours with ZMG suspension (25μM equivalent zinc) and then 30 minutes with zinquin (3μM). Yellow (open) arrows indicate the strongly fluorescing cells, the low fluorescing cells being indicated by a white arrow. Some punctate fluorescence is visible in the brightly fluorescing cells, indicating the presence of vesicular zinc. Original magnification x 400.
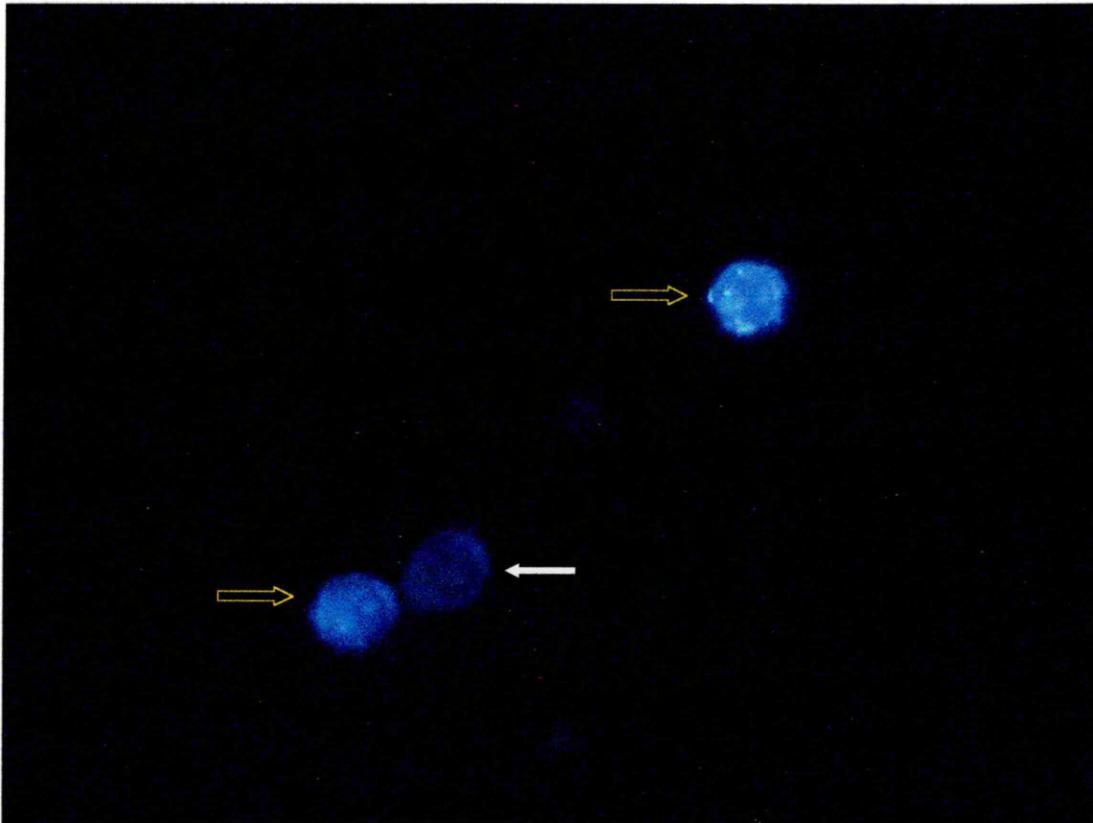
**Figure 3.33  Zinc content of Jurkat T-cells as shown by zinquin fluorescence:** Jurkat T-cells incubated 24 hours with zinc sulphate solution (25μM zinc) and then 30 minutes with zinquin (3μM). Yellow (open) arrows indicate the strongly fluorescing cells, the low fluorescing cell being indicated by a white arrow. *Original magnification x 400*
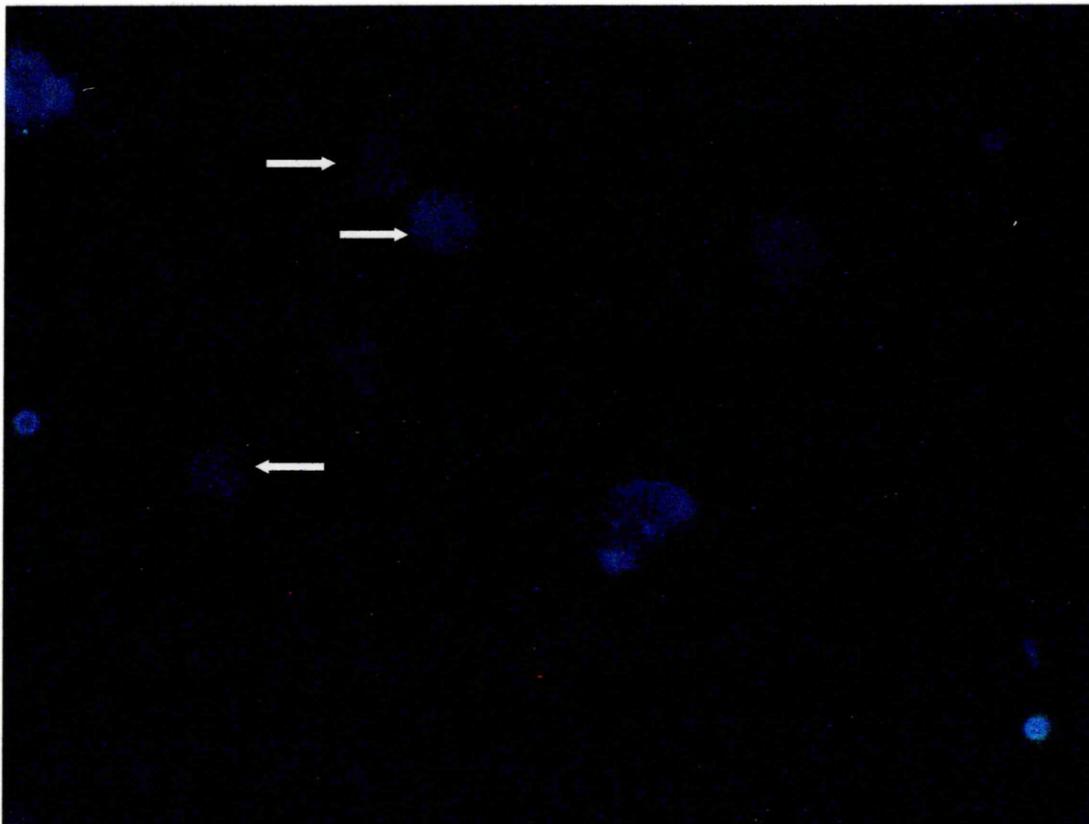
**Figure 3.34   Zinc content of Jurkat T-cells as shown by zinquin fluorescence:** Jurkat T-cells incubated 24 hours with zinc free vehicle and then 30 minutes with zinquin (3μM). A white arrow indicates the low fluorescing cells.   It is clear that the Jurkat T-cells have low levels of endogenous zinc as indicated by the very low background fluorescence in cells incubated without zinc. *Original magnification x400*

HGT-1 cells did not appear to appreciably take up zinc from any source; a representative photomicrograph of cells incubated 2 hours with ZMG solution is shown in Figure 3.35. The background fluorescence was higher than normal due to autofluorescence from the plastic of chamber slides used to culture the adherent HGT-1 cells. In experiments using coated glass slides or glass coverslips to culture HGT-1 cells cellular zinc/zinquin fluorescence was barely visible to the eye and practically invisible by photographic or video capture. This was also seen in the rat intestinal cell line IEC6 (Figure 3.36). It should be noted that the glass surfaces were not used to culture HGT-1 cells for general experimentation for reasons explained below and in Figure 3.37. However, as indicated by the yellow arrows, the all or nothing pattern is apparent even with this very low level of fluorescence, which would seem to indicate a level of zinc absorption. HGT-1 cells were also incubated with n-acetyl cysteine the mucolytic agent used in the ICP analyses of zinc uptake, to see if the gastric epithelial carcinoma cells were secreting mucus that might affect zinc uptake. It had no effect and cells still displayed fluorescence lower than the detectable limit for video capture.

Experiments using glass coverslips as a culture surface led to an observation that was not followed up due to lack of time, but it was noteworthy in itself. Cells grown on the coverslips grew normally and, when stained with zinquin, displayed exceedingly low fluorescence as observed previously (Figure 3.37a and 3.37b). However, when these cells were incubated with either zinc sulphate or ZMG to a final zinc concentration of 25$\mu$M (the standard concentration used for zinc uptake in all cell lines) the HGT-1 cells appeared to be killed without exception. The cells were highly crenelated and fragmented and no longer adhered to the coverslip. In addition to this they were highly fluorescent after staining with zinquin which indicated very high cellular zinc content (Figure 3.37c and 3.37d). Cell death was seen in both ZMG and zinc sulphate containing slides but not zinc-negative controls.
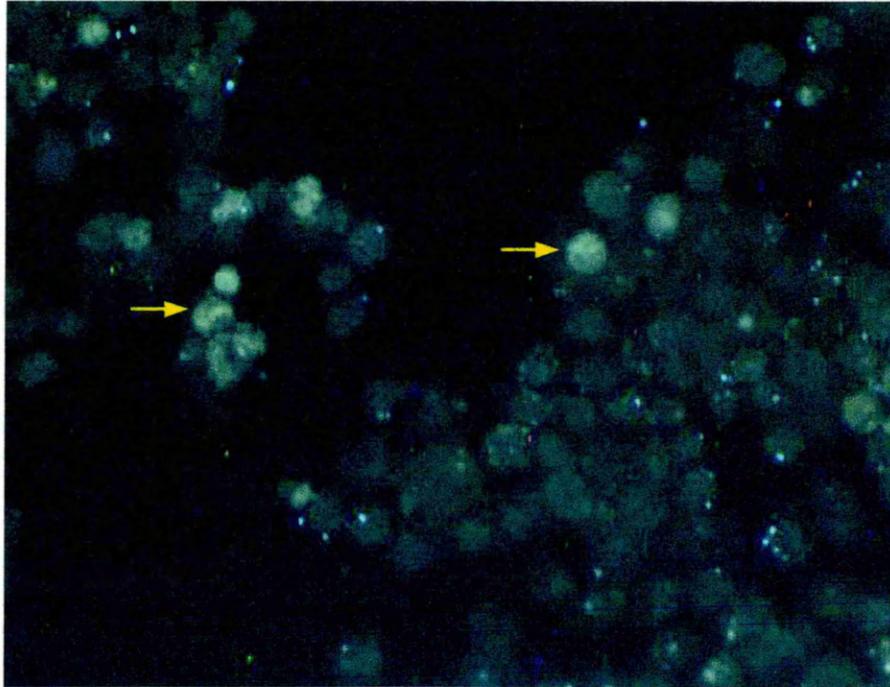
**Figure 3.35   Zinc content of HGT-1 cells as shown by zinquin fluorescence:** HGT-1 cells were incubated with ZMG dissolved in citric acid (25μM zinc) for 2 hours then washed and stained with zinquin (3μM).   Fluorescence was observed under UV light. This is a representative result for zinc uptake in HGT-1 cells.   It shows considerably more fluorescence than many HGT-1 incubations with zinc but it has been selected as it clearly shows that, despite very low overall fluorescence, the all-or-nothing uptake appears to be present in HGT-1 cells.   Positive cells (yellow arrow) are the only cells with any level of zinquin staining above background levels. *Original magnification x 200.*
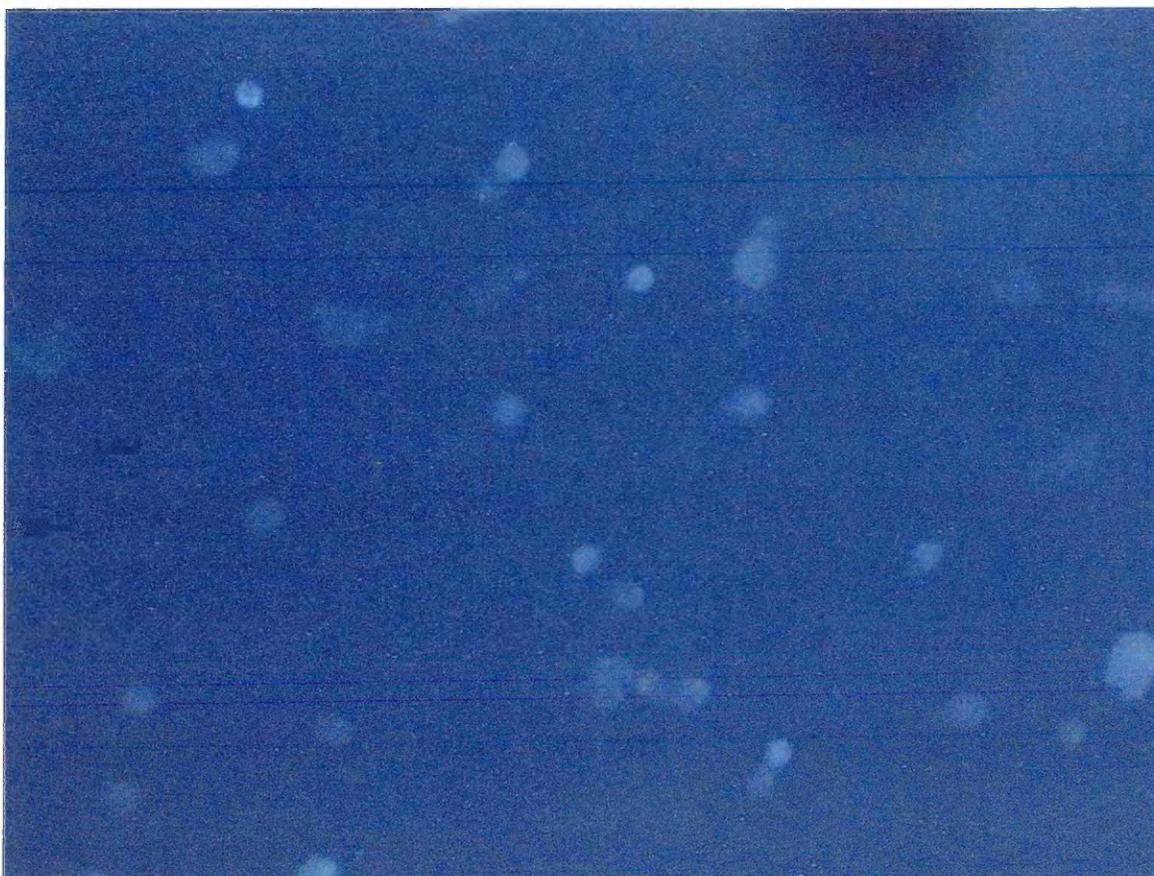
**Figure 3.36    Zinc content of IEC6 cells as shown by zinquin fluorescence:** Rat intestinal epithelial cells were incubated with ZMG dissolved in citric acid (25μM zinc) for 2 hours then washed and stained with zinquin (3μM).    Clearly, there are no brightly fluorescing cells, the cells being barely brighter than the background fluorescence of the plastic culture slide. *Original magnification x 200.*
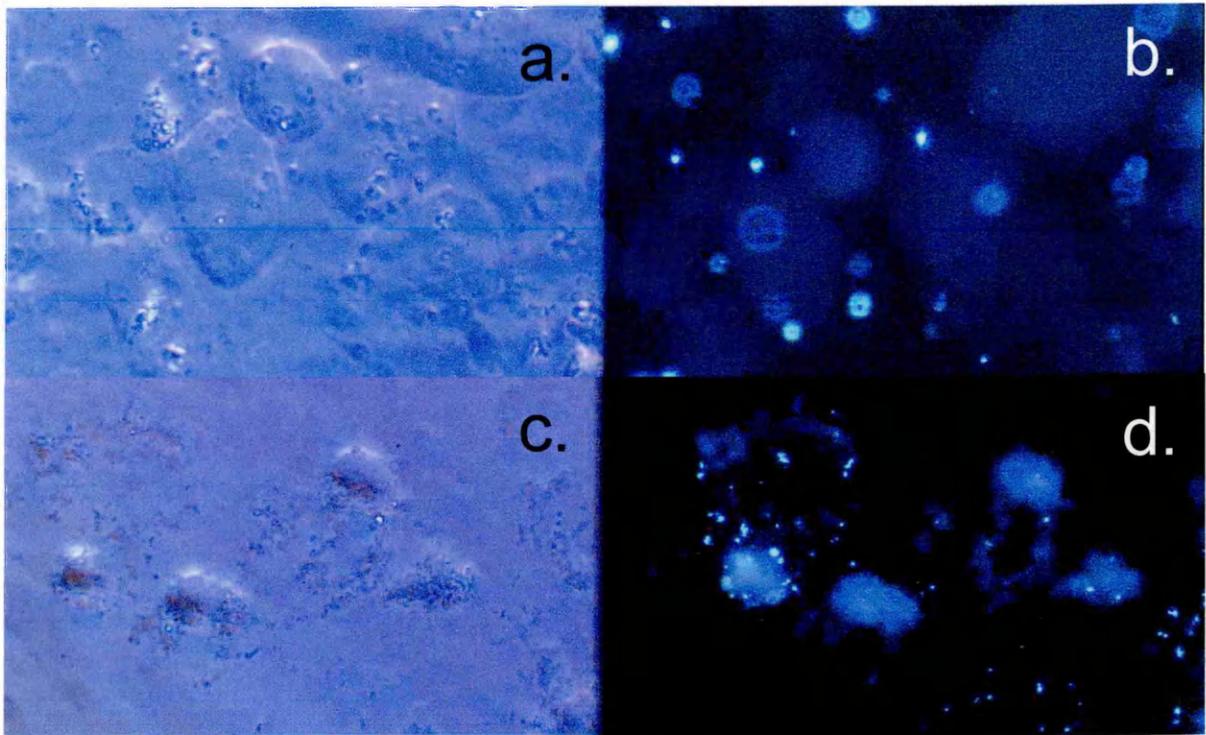
**Figure 3.37 HGT-1 cells grown on glass coverslips in the presence or absence of zinc:** HGT-1 cells were grown to confluence on glass coverslips and then incubated a further 24hr in the presence or absence of zinc (25μM zinc) and then stained with zinquin (3μM). Images were captured by video imaging. Figure shows two fields, a field of cells without zinc under white and UV light (a/b) and a field of cells with zinc under white and UV light (c/d). As may be seen control cells are flattened and adherent, healthy looking cells (Fig 3.37a) with low fluorescence (Fig 3.37b). However, those cells incubated with zinc were small and crenelated, were not adherent and were surrounded by fragments of cell membrane (Fig. 3.37c). All remaining intact cell bodies displayed high fluorescence compared to the cells without zinc and also compared with figure 3.35, HGT-1 cells grown in plastic chamber slides with zinc. Original magnification x 400

The rat intestinal epithelial cell line, IEC-6, became available and was briefly investigated in some experiments in order to compare the fluorescence in a gastric cell line other than HGT-1, the human gastric epithelial cell line. These cells also displayed fluorescence close to that of background when incubated with ZMG solution and zinquin (Figure 3.36).

As zinc was taken up in both punctate and diffuse manner, the vesicular uptake of zinc was investigated. THP-1 and Jurkat T-cells were incubated with zinc compounds and either co-incubated with FITC-dextran or stained with acridine orange. FITC-dextran was used to investigate vesicular transport as the long chain polymer is endocytosed in vesicles and the fluorescent FITC, which is most intense at neutral pH, allows the transport to be visually tracked under UV light. Acridine orange is a stain used to label a number of cellular compartments but has particular affinity for acidic compartments. It stained the cell cytoplasm orange (mainly staining mRNA) and the nuclear material and cytoplasmic vesicles green. It was thought that co-localisation of zinquin fluorescence with the FITC-dextran or acridine orange would indicate which compartment the zinc was occupying. The FITC-dextran was extensively tested but no FITC fluorescence was ever detected and certainly none that co-localised with zinquin fluorescence. It is conceivable that bright zinquin fluorescence may have masked the dimmer FITC fluorescence. Acridine orange staining was only tested twice and was very much a preliminary result. However, it did provide some indication that at least some of the punctate zinc staining is not localised to acidic vesicles. Figure 3.38 shows THP-1 cells incubated with ZMG solution and stained with zinquin alone for comparison (Figure 3.38a) or zinquin and acridine orange together (Figure 3.38b). Two granules appeared bright blue similar to the normal zinquin stain but three granules, apparently closer to the nucleus, are blue/green. The blue/green granules may represent a co-stain of the zinquin and acridine orange thus indicating the presence of zinc in an acidic vesicle. It further suggests that the two blue granules represent zinc in non acidic vesicles. Similar results were obtained with Jurkat T-cells (Figure 3.39), the quality of the image was not very good due to high levels of acridine orange fluorescence.
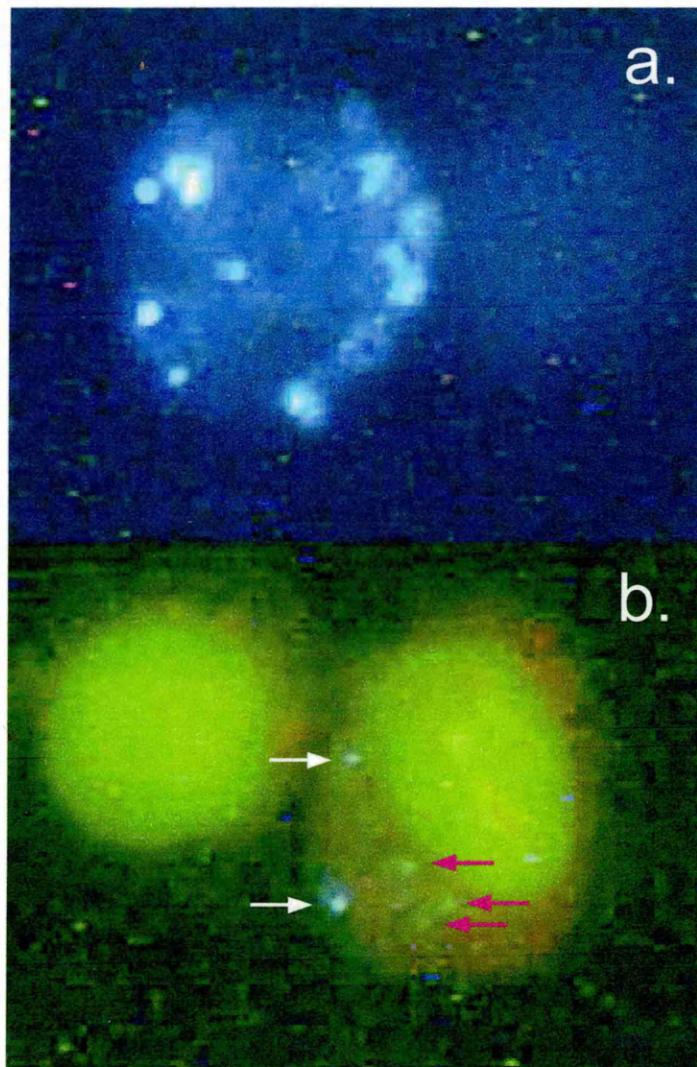
**Figure 3.38    Co-stain of THP-1 cells with zinquin and acridine orange:** THP-1 cells were incubated with ZMG solution and then stained with either zinquin (Fig 3.38a) alone or zinquin + acridine orange (Fig 3.38b).   The zinquin stain in figure 3.38a highlights the distinct granular punctate zinc uptake.   The acridine orange stain marks the nuclear material clearly green and the cytoplasm a cloudy orange colour.   Five definite granules are visible, two displaying the fluorescent blue of zinquin (white arrow) and three staining a blue green (purple arrow).   The blue vesicle appears nearer the outer membrane whilst the blue green vesicles appear to be predominantly nearer the nucleus. *Original magnification x 1000.*

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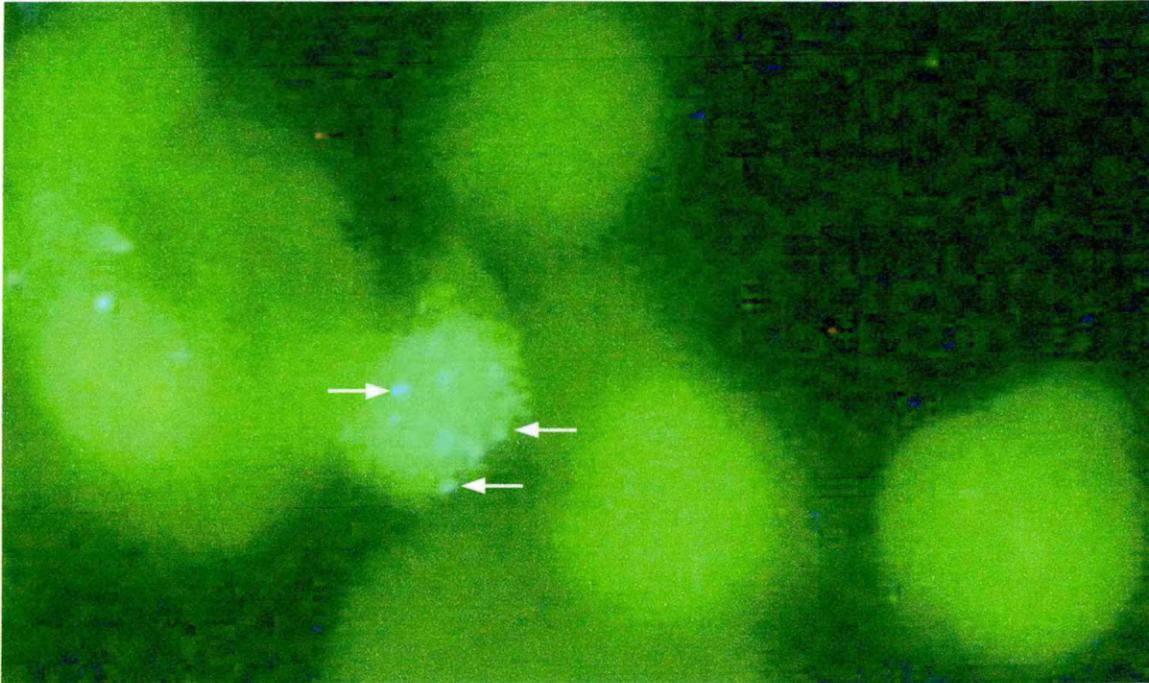
**Figure 3.39    Co-stain of Jurkat T- cells with zinquin and acridine orange:** Jurkat T-cells were incubated with ZMG solution and then stained with alone or zinquin + acridine orange.    The higher level of acridine orange fluorescence meant that cell structures were not as readily visible as they were in the THP-1 cells (Figure 3.38).    However, the blue punctate fluorescence in one 'positive for uptake' cell was clearly visible (white arrows).    The high level of acridine orange fluorescence combined with the large number of granules visible in this particular cell made it difficult to distinguish between vesicles with a blue fluorescence and those with a blue/green fluorescence. Original magnification X 1000.

**Summary**

The results from the viability experiments indicate that there is no differential effect on viability whether zinc salt or ZMG suspension or ZMG in acid solution is used as zinc source. They also provided information that indicated the viability of almost all cell lines was unaffected by increasing zinc concentration up to 120μM, zinc concentrations higher than 120μM resulting in increasing cell death in all cell lines. The exception to this was the HGT-1 cell line that displayed a decrease in viability after 48 hours in the absence of additional zinc; the zinc concentration in complete medium was 4nM as determined by ICP-MS.

There is a difference seen between cell lines in the reaction to increasing zinc concentration. The THP-1 cells appeared to be most resistant to high zinc concentration with Jurkat T-cells less resistant and HGT-1 cells appearing least resistant of all with no graduation of viability between no cell death, at 120μM zinc, and complete cell death at 600μM zinc.

Thus the viability experiments collectively suggest that the different cell lines may have different mechanisms for dealing with zinc. This was emphasised by the cellular zinc uptake data, which showed that the three cell lines dealt with different zinc compounds in distinctly different manners.

At all time points, 2, 4 and 24 hours, only incubation with ZMG suspension or zinc oxide suspension (with or without glycerol) resulted in cells with cellular zinc concentrations greater than control cells without exogenous zinc. In addition, only THP-1 cells and Jurkat T-cells appeared to take up zinc in amounts statistically significantly greater than controls. HGT-1 cells did not appear to take in exogenous zinc above control levels. THP-1 cells only appeared to display statistically significant levels of cellular zinc compared to control cells at early time points, 2 and 4 hours incubation with zinc compounds. Jurkat T-cells differed from the THP-1 cells in that they displayed statistically significant cellular zinc levels compared to controls at 4 and 24 hours but not 2 hours incubation with zinc compounds. So it appears that all three cells lines deal with exogenous zinc in distinctive fashions. As discussed in Chapter 1, this may be expected as zinc transport mechanisms are differentially displayed in the cells of various tissues.

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When uptake results were looked at cumulatively over time, THP-1 and Jurkat T-cells appeared to start with high cellular zinc concentrations, when incubated with ZMG or zinc oxide suspensions, which decreased over the 24 hour period. Overall the trends for cellular zinc content, when incubated with either ZMG suspension or either of the zinc oxide suspensions, were significantly different from that of the control cells, as determined by ANOVA. Interestingly both THP-1 and Jurkat cells incubated with zinc sulphate solution had cellular zinc contents that were not greater than control levels at 2, 4 or 24 hours individually but cumulatively the trend for cellular zinc levels over time was greater than that of control cells, as determined by ANOVA. This would seem to indicate that, whilst the zinc salt was not as good a source of bioavailable zinc as either ZMG or zinc oxide, it was nonetheless causing an increase in cellular zinc as distinct from control cells.

Zinc uptake was also examined under conditions that model some aspects of inflammation by stimulating the cells with the bacterial mitogen, LPS. None of the cell lines displayed a statistically significant increase or decrease in cellular zinc, at any single LPS concentration, when compared to cells incubated with zinc but without LPS. However, Jurkat T–cells appeared to display trends towards *increasing* cellular zinc with increasing LPS concentration when incubated with zinc compounds, whilst THP-1 cells appeared to tend towards *decreasing* cellular zinc with increasing LPS concentrations. The HGT-1 gastric cells did not appear to take in significant amounts of zinc under any conditions of stimulation. Even in the presence of cytokines, that might have been expected to stimulate secretion of other cytokines, failed to cause a noticeable increase in cellular zinc. As protein synthesis and secretion absolutely requires zinc and as many proteins use zinc as a structural component it seems strange that the HGT-1 cells did not appear to have measurable increases in zinc. It may be that the zinc levels in this cell line are very efficiently regulated and that they simply maintain sufficient zinc for homeostasis and no more.

The results from the zinquin stain of labile zinc in cells showed that, of the labile zinc that is available for zinquin binding, the patterns appear quite similar whether cells were incubated with ZMG suspension, ZMG dissolved in citric acid or zinc sulphate. However, there was a distinct difference between cell lines. THP-1

monocytic cells appeared to display a generally higher level of fluorescence than either Jurkat T-cells or the HGT-1 gastric cell line. This was manifested by a brighter diffuse fluorescence in the cells although the particularly bright 'positive for uptake' cells did not seem to be in any more abundance than in the Jurkat cells under the same conditions. The diffuse fluorescence was lower in the Jurkat cells which resulted in a very marked difference between 'positive for uptake' cells and all other cells. The HGT-1 cells were barely fluorescent after incubation with zinc compounds and then staining with zinquin. This observation of zinquin stained labile zinc was in agreement with the ICP-MS results that showed no increase in total zinc under similar conditions.

Interestingly, the comparison of large fields of cellular fluorescence after incubation with either ZMG suspension or zinc sulphate solution would seem to suggest, despite apparent similarity fluorescence patterns in THP-1 cells, in this case, that zinc sulphate was providing less labile zinc to the cells. The cells incubated with ZMG were generally much brighter than the cells incubated with zinc sulphate. This would seem to indicate that the higher level of total cellular zinc in THP-1 cells, incubated with ZMG as opposed to zinc sulphate, was probably due to an increase in the general diffuse fluorescence of the whole population rather than a larger number of 'positive for uptake' cells.

In light of the lack of zinquin fluorescence in HGT-1 cells under conditions that increased labile zinc fluorescence in THP-1 and Jurkat cells, the effect of growth surface on HGT-1 fluorescence was unexpected. HGT-1 cells grown on glass coverslips as opposed to plastic culture flasks or slides, in the presence of exogenous zinc appeared to display high levels of labile zinc as indicated by high zinquin fluorescence. The cells also appeared unhealthy and fragmented. As control cells were unaffected it would appear to indicate either that adhesion to the glass surface caused a change in the cells that allowed zinc influx and/or prevented excess zinc excretion.

The final set of results, the vesicular sequestration of zinc into zinquin stained or acridine orange stained granules hints at the route zinc may follow as it is taken up by cells. Although preliminary investigations, the results appear to indicate

that zinc may exist in both non acidic vesicles (fluorescing with zinquin alone) and acidic vesicles (fluorescing with zinquin *and* acridine orange).

As a whole, the results indicate that the three cell lines investigated treat exogenous zinc in quite distinctly different manner which would seem to indicate the presence of different mechanisms for zinc transport in each line. It may also be that they all share the same mechanisms but specific mechanisms predominate in different cell lines. This would fit in with the published observations of zinc transport mechanisms in freshly isolated cells, which are tissue and cell specific.

# Effects of zinc on cytokine production and signal transduction in inflammatory cells

## Background

These experiments were carried out in order to try and relate some cell functions with endogenously administered zinc. When this study was initiated very little was known about the activity of endogenous zinc in cells and even less was known specifically about the effect of endogenous zinc on cytokine function. As has been discussed in Chapter 1 the scientific literature contained under ten studies looking at the effects of endogenous zinc on cytokine activity in freshly isolated cells and none looking at the same effects on cultured cell lines such as Jurkat T-cells and THP-1 cells. This led to different lines of enquiry, specifically, which proteins endogenous zinc associated with, whether endogenous zinc had any effect on transcription factors controlling cytokine production and what effect endogenous zinc had on cytokine release by inflammatory cells.

## 4.1.1 Uptake of radiolabelled zinc – investigation into molecular-zinc associations

This was, in part, a continuation of the investigation of zinc uptake into cells. It was known that zinc absorbed by cells is rapidly associated with a number of proteins such as metallothionein and CRIP (See Chapter 1). However it was also known that zinc modulates cytokine levels *in vivo* and one of the major cytokine control points is the transcription factor NFκB, zinc finger protein. The labile zinc pool is increasingly being understood as a 'store' of zinc for metalloproteins. It was therefore decided to investigate the possibility that exogenous zinc, especially when administered as ZMG, may rapidly associate with proteins other than metallothionein and CRIP. These two zinc-binding proteins have low molecular weights, 6-7kDa for metallothionein and 8.5kDa for CRIP whilst NFκB has a molecular weight of between 50 and 130kDa depending on the subunit type and whether it forms a dimer. It was decided to use a non-denaturing polyacrylamide

gel to separate the proteins. This non-denaturing method does not affect the native protein characteristics and so any bound zinc should be retained in the molecules that bind it. Radiolabelled zinc could then be detected by exposure of the gel to autoradiographic film.

There is a complex procedure for determining molecular weight of an unknown protein in non-denaturing PAGE analysis. As distance run on the gel is not based solely on molecular size due to the intact secondary and tertiary protein structures, standard size markers must be recorded on a series of non-denaturing gels of different gel concentrations (Sigma Chemical Co. Technical bulletin no. MKR-137, 1986). The migration distance of markers and samples are measured on each percentage gel and plotted. The slopes of each graph can then be calculated and these slopes plotted against the log of the molecular weights of the marker and sample proteins to produce a Ferguson. In the experiments presented here, cell lysates were separated along with the markers but additionally, the bands displayed on the autoradiograph of the cells with labelled zinc were recorded separately and the figures incorporated into the final results.

### 4.1.2 Cytokine bioassay analyses

Bioassays were the mainstay of cytokine analysis until ELISA kits were commercially available (See section 4.1.3 for explanation of ELISA). Cells sensitive to specific cytokines provided a measure of the cytokine levels in their surrounding media. This meant that culture media could be recovered from cell lines of interest, after an intervention such as stimulation with a test compound. If the compound changed cytokine secretion by the test cells, this could be detected and quantified by incubation of the supernate from these cells with indicator cells. For example, T-cells incubated with LPS might produce increased levels of the cytokine IL-1 and IL-6. The supernatant medium would be harvested and added to indicator cells such as B9 cells that are sensitive to IL-6. The cell death or cell proliferation may be quantified in several ways but the most common methods use relatively colourless substrates, as MTT or XTT, which are metabolised within the cell mitochondrion to produce a dark insoluble product. These crystalline products must be solubilized by lysing the cells with detergent and dissolving the crystals with acidic solution in order to equalise the colour throughout the well.

The homogeneous solution can then be measured in a spectrophotometer at a specific wavelength. Another common method for measuring cell proliferation is to measure the uptake of a radiolabelled molecule. A molecule such as thymidine is readily taken up into cell nucleic acids and can be synthesised with tritium (an isotope of hydrogen). This uptake can be measured by scintillation counting, the levels of tritium being directly proportional to the cell proliferation. These techniques are normally carried out in 96 well microplates, suitable for cell culture, for which specific spectrophotometers have been designed for the colorimetric methods and specific cell harvesters have been designed for the tritium assay.

The bioassay provides information on the activity of the protein as well as its concentration in the test medium whilst ELISA simply quantifies protein. In the experiments presented here the cytokines TNF$\alpha$ and IL-2 are investigated using the L929 (Shahan et al., 1994, Levesque et al., 1995) and CTLL (Gillis et al., 1978, Heeg et al., 1985, Russell and Vindelov, 1998) assays respectively. In addition, MTT was replaced as an indicator by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate), an almost colourless mitochondrial substrate that provided a dark water-soluble product. As it was water-soluble it removed the need to disrupt the cells and solubilize the mitochondrial product as is required for MTT or XTT (Boehringer Mannheim technical sheet for product Cat. No. 1644807).

TNF$\alpha$ is a major inflammatory cytokine and is released both in an autocrine fashion and as a result of external stimulation. The L929 mouse fibroblast cell line is a very well characterised cell line that is sensitive to the cytokine TNF$\alpha$ specifically and for which an assay exists (Shahan et al., 1994). L929 cell death is directly proportional to the concentration of TNF$\alpha$ present within the surrounding medium. These experiments utilise the L929 cell line to determine changes in TNF$\alpha$ levels secreted into supernatant media taken from THP-1 monocytic cells.

IL-2 is the major control cytokine for Jurkat proliferation and function. It acts on and is released by T-cells and implements itself in an autocrine and paracrine manner. The most commonly used standard bioassay for IL-2 uses the CTLL murine lymphocyte cell line (Gillis et al., 1978). Supernatants are standardly harvested from the test cell line and are then added to the CTLL cells grown in 96 well microplates. Any increase in IL-2 level in the test culture media causes the

CTLL cells to proliferate proportionally. Cellular incorporation of tritiated thymidine is routinely used to determine cell proliferation, in the CTLL assay, instead of a colo rimetric method. In the experiments presented here the WST-1 col o rimetric detection system was used instead of thymidine uptake.

In both bioassay techniques a standard curve of cell killing or proliferation is constructed on each plate with known concentrations of TNF$\alpha$ or IL-2 respectively. When absorbance is measured on the microplate spectrophotometer, the unknown samples can be examined by comparison with the standard curve.

As a result of some of the studies described in previous chapters it was realised that if exogenous zinc can have such profound effects on THP-1 and Jurkat T-cells, then it is likely to have an effect on the bioassay cells. An extensive search of the literature revealed one paper which presented one figure indicating that zinc might inhibit TNF$\alpha$ mediated cell death in L929 cells (Fady et al., 1995). Only one concentration was examined, 75$\mu$M, and the thrust of the research was towards apoptosis rather than the bioassay specifically. This observation was backed up by subsequent studies by Wellinghausen et al., (1996) as described in Chapter 1, where the zinc content and zinc ligand concentration in culture media were shown to alter significantly cellular zinc content and activity. Although these workers did not directly examine bioassay function their results strongly suggested that the effects of exogenous zinc must be investigated if bioassays were to be used in this particular study.

## 4.1.3 Enzyme Linked Immunoassay (ELISA)

In this set of experiments the effect of zinc on cytokine release by THP-1 or Jurkat T-cells were analysed by ELISA. ELISA is a technique in which a protein is detected by a specific antibody, which is in turn bound by a secondary labelled antibody or biotinylated antibody. The detection systems are normally fluorescent or chromogenic reactions similar to those described above for visualisation of protein bands on Western blot membranes. The difference is that ELISA measures soluble protein in a fully quantitative manner. A 96-well microplate is coated with a capture antibody that can be the same as the primary detection antibody. When a culture medium containing the test protein is added to the well the protein will bind to the capture antibody and remain in the well after the well has been washed. The primary antibody is then added followed by the secondary antibody and the detection system, either chromogenic or fluorescent. A microplate spectrophotometer is used to measure the reaction, and test samples can be compared to a standard curve of the test protein at known concentrations, so allowing quantitation of the protein in the test samples.

The benefit of ELISA over bioassay is that it is rapid, usually around 3-5 hours in total and it is convenient as ELISA kits are readily available for most proteins. Bioassays can take up to 48 hours to complete and have a limited number of applications. ELISA kits are also very expensive whereas bioassays are relatively inexpensive. As they are usually commercial kits, ELISAs generally tend to be more consistent as all reagents are pre-tested and concentrations are guaranteed to be accurate.

## 4.1.4 Investigation into the effect of exogenous zinc on NFκB

As discussed previously, zinc can modulate cytokine production and NFκB is a central control point for many molecules including many cytokines. The possibility that endogenous zinc could influence the activity of the nuclear factor in cell lines was investigated by Western analysis. The technique starts by separation of proteins after boiling in the detergent SDS and running on a denaturing gel (SDS-PAGE), this unravels tertiary and secondary structure of proteins and allows sample separation by size. As distance migrated is inversely proportional to

protein size, a series of size markers are run on the same gel and the molecular weight of the protein can be determined by direct comparison or by correlation from a plot of marker migration distance versus marker protein size. The Western blot continues with the proteins being transferred from the gel onto a solid membrane by passing a weak current through the gel and membrane in the direction of transfer. The proteins are then fixed onto the membrane. A specific antibody, monoclonal or polyclonal can then be used to probe the membrane for the protein of interest (monoclonal recognising one site on the protein and polyclonal recognising many sites) (Roitt, 1991). The band on the membrane corresponding to the band on the original gel may be visualised by a number of methods utilising antibodies specific to the original probe antibody. These secondary antibodies can be conjugated to fluorescein for example, which would allow it to be observed under UV light or may be conjugated to biotin (Haugland, 1996). The protein streptavidin has a high affinity for biotin and may be conjugated to a large number of different detection reagents. For example, the streptavidin could be linked to a fluorescent dye such as phycoerythryn or may be linked to an enzyme such as horseradish peroxidase (Haugland, 1996). The fluorescent dye may be directly viewed under UV light but the enzyme requires a final step in which a chromogenic substrate is exposed to the membrane bound enzyme that converts it to an insoluble product. This product precipitates out of solution but remains closely associated with the site of the enzyme reaction on the membrane. The reason for so many layers of detection is that each successive layer is an amplification of the previous layer; for example, two secondary antibodies conjugated to several biotin molecules may each bind one primary antibody. Each biotin molecule may be bound by streptavidin with further amplification possible depending on the detection system used. The result is that very small amounts of protein can be detected in this manner.

In these experiments nuclear extracts were prepared from THP-1 and Jurkat T-cells. This is a preparation that isolates the cell nuclei from the cell cytoplasm and membrane (Andrews and Faller, 1991). The reason for doing this was because NFκB is normally bound to a control protein, IκB and is located in the cytoplasm. Upon activation, it dissociates from IκB and translocates to the nucleus. These sets of experiments were carried out in order to detect any increases of NFκB in the nuclei of THP-1 and Jurkat T-cells, incubated with zinc and stimulated with LPS.

Antibody detection can also be applied to the visualisation of proteins in cells. Cells are fixed to a slide and incubated with the antibody and secondary antibodies as described above. The fluorescent or chromogenic detection molecules are then seen localised to specific areas in the cell depending on the protein being detected.

## Experimental

### Reagents

Non-denaturing molecular markers, HEPES-KOH, $MgCl_2$, KCl, dithiothreitol, PMSF, EDTA, Tris-HCl, SDS, glycine, goat anti-mouse antibody, glycerol (SigmaUltra grade), $\beta$-mercaptoethanol, BCIP/NBT, Actinomycin-C and zinc sulphate (cell culture grade) were all molecular grade unless otherwise stated. $^{65}ZnCl_2$ was obtained from Amersham International. Mouse anti-human NFκB p50 antibody was obtained from Serotec. WST-1 cell proliferation indicator was obtained from Boeringer Mannheim. Human interleukin-2, human interferon-γ and human tumour necrosis factor-α were obtained from the National Institute of Biological Standards and Control. ELISA kits were obtained from Quantikine R&D.

### 4.2.1 Uptake of radiolabelled zinc – investigation into molecular-zinc associations

### Equipment

Electrophoresis equipment was obtained from Biorad.

### Method

*Preparation of gel*

Molecular markers were obtained in kit form and reconstituted in 1mM sodium phosphate buffer or distilled water as indicated. Polyacrylamide gels were prepared at different gel concentrations; the method described below is for preparation of a 7.5% gel. For gels of different concentrations the acrylamide and water volumes were altered as appropriate, all other concentrations were unchanged. Acrylamide (1.875 ml of a 30% stock solution), and distilled water (3.625ml) were inverted briefly to mix. Gel buffer (1.94ml of 181.5g/l Tris-HCl

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solution), was added and mixed by inversion. Immediately prior to pouring, ammonium persulphate (75μl), and TEMED (7.5μl), were added as gel polymerisation initiator and accelerant respectively. The gel was inverted rapidly and poured between clean glass plates, a gel well forming comb was inserted at the top of the plates and the gel was left to set for a minimum of an hour. The gel was either used immediately or wrapped in clingfilm for storage at 4°C overnight. Gels were not used if stored for longer than 24 hours.

Prior to use the gels were incubated in an electrophoresis tank containing electrophoresis buffer, 30g/l Tris-HCl, 140g/l glycine in aqueous solution.

## Preparation of samples and standards

For analysis on the PAGE system the cells were prepared as follows. THP-1, Jurkat or HGT-1 cells were incubated with radiolabelled or non-labelled zinc compounds as follows. For the radioisotope analysis $^{65}$Zn-ZMG (1mg) was dissolved in 2.5mM citric acid to a ZMG concentration of 978μM. The unlabelled or labelled ZMG solution was added to cells to a final concentration of 12.5μM. To other cells was added $ZnSO_4$ solution to a final concentration of 25μM. As a radioactive comparison for the radiolabelled ZMG in the autoradiographic analyses, 1μl of $^{65}$ZnCl$_2$ (10mM in 0.1M HCl solution), was added to 2ml zinc sulphate solution (2.5mM), and the resulting solution was added to the cells to a final concentration of 25μM. The small volume of radioactive zinc chloride was used as the isotope had high specific activity (2.28mCi/ml). All cells were then incubated for 24 hours at 37°C, 5% $CO_2$, before harvesting and washing twice in serum free media. Cells were pelleted and stored at −20°C until required.

## PAGE analysis of samples and standards

When ready for use the cells, radioactive or non-radioactive, were thawed and prepared as follows. Cell pellets were resuspended in 100μl Tris-HCl (10mM, pH8.8) and to each cell suspension was added a protease inhibitor cocktail, 6.4μl, to prevent breakdown of proteins. (Protease inhibitor cocktail: PMSF 1Mm, leustatin 10μg/ml, E64 20μg/ml, pepstatin A 10μg/ml).

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The protein content of each sample was then determined by BCA assay (described and reviewed Stoscheck, 1990) so that equal protein amounts could be loaded onto the acrylamide gel. The BCA assay uses a colorimetric method to determine protein concentration in samples and compares them to soluble bovine albumin at known concentrations. Samples or standards were dissolved in 1M HCl, 25$\mu$l in 225$\mu$l respectively. To each sample or standard was then added 250$\mu$l NaOH (1M). Samples or standards were added to a 96 well microplate, 20$\mu$l of each solution per well. BCA reagent (200$\mu$l) was added to each well and the plate was incubated at room temperature for 20 minutes or until colour reaction had occurred. Absorbances were then read on a microplate spectrophotometer at 570nm and the standard curve of albumin concentrations was plotted. Sample absorbances were correlated with standards and sample concentrations were determined from the standard curve.

With the sample protein concentrations known, aliquots of each sample were diluted to give equal concentrations. Samples (18$\mu$l) were then mixed with gel loading buffer (2$\mu$l) (Tris-HCl, 25% glycerol, 0.5% bromophenol blue) and applied to the gel. The gel was run at between 50-80V until the dye front reached 1cm-0.5cm from the base of the gel. At this point the method for the PAGE analysis of the radiolabelled cells diverges and is described below. The gel was then removed and placed in a protein stain and fixative solution for 12-24 hours or until the gel was stained darkly. (Stain solution: 45% methanol, 45% distilled water, 10% acetic acid, 0.25% Coomassie blue).

The gel was then destained with the stain solution minus Coomassie blue. The gel was left in the destain solution until all background stain was washed out of the gel leaving the protein bands stained and visible. The gel was then dried onto a backing of Whatman's filter paper under vacuum pressure and heat.

The dried gel was then ready for analysis of protein bands. Molecular weight is determined by plotting the migration distance (Rf) of a protein in PAGE gels of different concentrations. The slopes of these lines are determined for a number of standard proteins, in this case, $\alpha$-lactalbumin, carbonic anhydrase, ovalbumin, bovine serum albumin (monomer and dimer) and urease (trimer and hexamer). These slopes are then plotted against the log of the molecular weights of the standards to produce a Ferguson plot. The varying gel concentrations and plots

must be repeated for the unknown samples to obtain the slope values which may then be read off the Ferguson plot to determine the corresponding log of the molecular weight and which may then be converted to molecular weight by inversion. The proteins from the radiolabelled cell lysates were separated on gels of concentration 4%, 7.5% and 12.5% and relative mobilities obtained by marking the well positions and dye front position on the autoradiographic film lightly with a pencil. The bands detected by autoradiography could then be treated like the normal coomassie stained proteins and the molecular weights determined as described above.

## 4.2.2 Cytokine bioassay analyses – the effect of zinc on cytokine production and on bioassay activity

**Method**

*Bioassay sample preparation*

For examination of cytokine production, cells were incubated in 24 well plates normally for 24 hours, with or without zinc at varying concentrations, in the presence of phorbol myristic acetate (PMA) (50nM) or LPS (1-10μg/ml) depending on the experiment. For this cytokine stimulation Jurkat T-cells were incubated at $2.5 \times 10^6$ cells/ml and THP-1 cells were incubated at $1 \times 10^6$/ml. Supernatant media were removed, centrifuged to remove cells and then stored at −70°C until required.

*TNFα bioassay*

L929 murine fibroblast cells (100μl) were plated out into 96 well microplates at a density of $5 \times 10^4$ cells/ml. They were allowed to adhere to the plate overnight at 37°C, 10%$CO_2$. To the plate was then added a standard curve of TNFα (100μl), usually from 5000U/ml to 0.001U/ml, and also the sample supernatant media (100μl). U/ml is used for description of cytokine concentrations as the cytokine stocks are obtained from the National Institute for Biological Standards and Control as International Units per vial which are made up to a specific volume with solvent buffer. The concentration unit U/ml or IU/ml is routinely used in the

literature. Actinomycin-A (10μl) was then added to all wells to a final concentration of 1μg/ml. The cells were then incubated 24 hours at 37°C, 10%$CO_2$. To each well was then added 10μl WST-1 reagent and the cells were incubated until the colour developed. The absorbance of the plate was then read on a microplate spectrophotometer at 450nm. Cell death was inversely proportional to absorbance with no cell death displaying highest absorbance and complete cell death displaying the lowest absorbances.

In some experiments, to test specificity of the assay, an anti human TNFα antibody was added to the standard curve in concentrations indicated in the Results section.

*IL-2 bioassay*

This experiment was carried out in order to investigate the effect of zinc on the CTLL proliferation assay for IL-2. CTLL murine lymphocytic cells, 100μl, were plated out in RPMI, with or without foetal calf serum, at various cell densities as indicated on the figures in Results section. IL-2 diluted in RPMI was then added in concentrations from 2000U/ml-0.02U/ml. In some experiments zinc compounds, 1μM-100μM, were added to the IL-2 standard curves. Cells were then incubated 24 hours and treated with WST-1 as described for the L929 bioassay.

### 4.2.3 Enzyme Linked Immunoassay (ELISA) - Effect of zinc on IL-1β production

**Method**

THP-1 cells were incubated for 48 hours in serum-free medium in the presence or absence of zinc compounds (25μM free ion), LPS (1μg/ml), or LPS plus IFN-γ (100U/ml).   Cell supernatant media were then collected from the incubates and centrifuged to remove particulate material then stored at −70°C until required or used immediately.   Samples were then analysed using   commercial ELISA kits according to the instructions provided with the kits.   Samples (100μl) were added to wells in a microtitre plate pre-coated with anti IL1-β antibody and incubated before washing and addition of a second anti IL1-β antibody.   The samples were incubated and then washed a second time before addition of a detection antibody conjugated to the detection system incorporated by the kit.   The substrate for the detection system was finally added and the colour reaction allowed to develop before reading at 450nm on a microplate spectrophotometer.   Concentration of IL1-β in samples was calculated by comparison of sample absorbance with that of a standard curve of known IL1-β concentration.

### 4.2.4  Investigation into the effect of exogenous zinc on NFκB

**Materials and equipment**

Electrophoresis and western transfer equipment were obtained from Biorad or LKB.

**Method**

THP-1 cells and Jurkat T-cells were incubated with or without zinc as ZMG or zinc sulphate at 25μM zinc ion concentration, for 24hrs then with LPS for varying times. Cell nuclei were then isolated and DNA binding proteins isolated after the method of Andrews and Faller (1991).

*Nuclear extraction*

For nuclear extraction, cells were harvested, washed in serum free medium and pelleted in microfuge tubes. Cell pellets were resuspended, by flicking gently, in 400μl buffer A (10mM HEPES-KOH pH7.9 at 4°C, 1.5mM MgCl₂, 10mM KCl, 0.5mM dithiothreitol, 0.2mM PMSF). The cells were allowed to swell on ice for 10 minutes before being vortexed for 10 seconds. Samples were centrifuged for 10 seconds to pellet and the supernatant, containing cell membrane and cytoplasmic contents, was decanted for storage or discarded. The nuclear pellet was resuspended in 20-100μl of cold Buffer B (20mM HEPES-KOH pH7.9, 25% glycerol, 420mM NaCl, 1.5mM MgCl₂, 0.25mM EDTA, 0.5mM dithiothreitol. 0.2mM PMSF) and incubated 20 minutes on ice for the high salt extraction. Cell debris was removed by centrifugation for 2 minutes at 4°C. The resulting supernatant containing the DNA binding proteins was decanted and stored at −70°C until required.

*Analysis of nuclear extracts by Western blot of SDS-PAGE*

Polyacrylamide gels, normally 12%, were cast as described previously although the gel was not poured to the top of the gel cassette. Gels were prepared as previously described (p219-220) with the inclusion of 0.1% SDS in all solutions. Once set, a second gel, 4% acrylamide, was cast on top of the first gel taking up the remaining space and the wells created in this lower percentage gel. The second gel was a stacking gel and allowed all the proteins in the sample to travel rapidly through it so that when they reached the interface with the second gel they were compacted into a discrete band. The 12% gel was where the proteins were separated or resolved according to size, for this reason it was called the resolving gel. The tank buffer used for the denaturing gels contained SDS for denaturing conditions (in distilled water; Tris-HCl 30.3g/l, SDS 10g/l, glycine 144g/l).

Protein contents of samples were determined by BCA assay, as previously described, and equal protein amounts were aliquoted into microfuge tubes. To each tube was added 5x loading buffer (in distilled water; SDS 100mg/ml, Tris-HCl 75mg/ml, glycerol 250mg/ml, bromophenol blue 0.5mg/ml, 25% β-mercaptoethanol) and samples were then boiled for 1 minute to denature proteins.

The samples were then immediately loaded onto the gel, which was run at 80-130 volts until the dye front was near the bottom of the gel.

After electrophoresis the stacking gel was cut from the resolving gel and discarded. The resolving gel was then incubated for 15 minutes in transfer buffer (in distilled water; glycine 14.4g/l, Tris-base 3g/l, SDS 0.75g/l, methanol 20%). PVDF membrane was then cut to the size of the gel, pre-wet in methanol and then transfer buffer and placed onto the gel. The gel and membrane were placed in a Western blot cassette, which was in turn placed into a transfer tank containing transfer buffer. The proteins in the gel were then transferred onto the membrane at 60-90 volts for at least an hour. After this the membrane was dried and stored at 4°C or probed immediately.

### Detection of NFκB in nuclear extracts on Western blot

The membrane was re-wet briefly in methanol and then Tris buffered saline (TBS, in distilled water; Tris-base 1.21g/l, NaCl 8.8g/l) and then incubated for 1hr in blocking solution, commercial milk powder (50g/l) in TBST (0.05% Tween-20 in TBS). The membrane was next washed in TBST and incubated, with gentle agitation, for 2hrs with rabbit anti-human NFκB p50 antibody (Serotec) diluted 1:1000 in 2g/l milk powder TBS solution. The wash was repeated and the membrane incubated with the secondary antibody at 1:5000 dilution for 1.5-2hrs. The secondary antibody was goat anti-mouse antibody conjugated to alkaline phosphatase (Sigma/Aldrich). The membrane was washed again and incubated with an alkaline phosphatase substrate, BCIP/NBT, a chromogenic substrate, leaving a dark purple insoluble end product at the site of reaction with the alkaline phosphatase. The NFκB p50 bands visualised on the membrane by the antibody probe were photographed by still camera. Molecular weights of the bands were calculated by plotting the standard marker weights against relative mobility and correlating the unknown band relative mobilities with the standard graph to obtain unknown band molecular weights.

### 4.3.1 Uptake of radiolabelled zinc – Investigation into molecular-zinc associations

These results are divided up into two sections, the analysis of marker proteins and unlabelled cell lysates (Figure 4.1 and table 4.1) and the presentation of autoradiographic results from cell lysates labelled with $^{65}$Zn isotope, (Figures 4.2-4.3). The comparison of autoradiographic results with marker proteins and unlabelled cell proteins is then examined. Figure 4.1 shows the Ferguson plot derived from running standard marker proteins on gels of varying concentration. In essence it represents the trends of protein migration on the non-denaturing gels of different concentration compared to the molecular weight of the proteins. The molecular size of NFκB is between approximately 50kDa and 130kDa depending on subunit dimerization and so the gel concentrations selected are suitable for the PAGE separation of proteins of these sizes.

From the Ferguson plot the molecular weights of the major protein bands in THP-1 and Jurkat cells may be calculated and these are presented in Table 4.1. Treated like the standard proteins, the relative migration distances were calculated for bands in each lysate and plotted against gel concentration. The slope of each graph was read off the Ferguson plot (Figure 4.1) to obtain the molecular weight of the band (Table 4.1). It may be seen from table 4.1 that molecular weights of four or five bands have been calculated for each lysate. They were representative of the range of major protein bands found in the lysates although many more were present. It was not necessary to determine the sizes of all constituent proteins in the cell lysates as the reference ranges were required for approximate comparison of protein bands within the autoradiograph of the radiolabelled lysates.
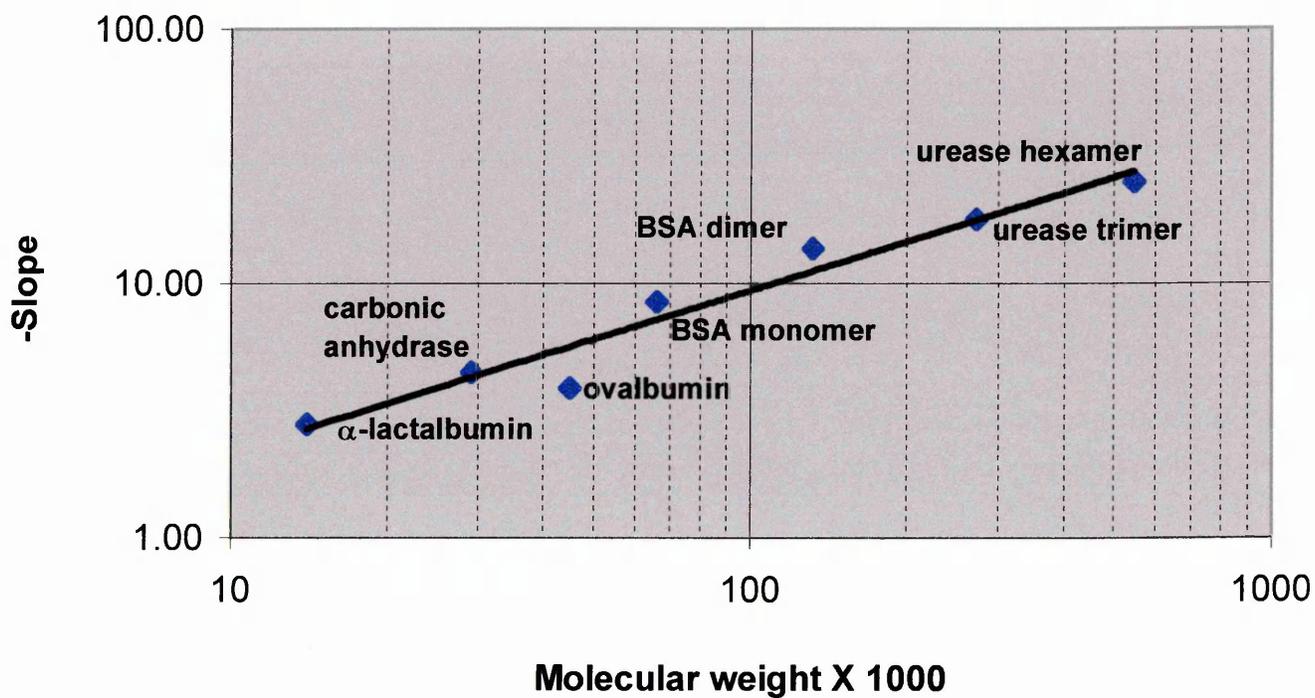
**Figure 4.1** **Ferguson plot of molecular weights against slope of graphs derived from relative mobilities of proteins in gels of varying concentration:** This calibration curve was constructed to determine the molecular weights of unknown proteins. Each point represents the graph slope of the relative mobilities of a standard protein on 6 different concentrations of acrylamide gel.

| Protein band number in order of mobility | Jurkat T-cell lysate protein band molecular weight (Da) | THP-1 cell lysate protein band molecular weight (Da) |
|---|---|---|
| 1 (lowest mobility) | 550000 | 485000 |
| 2 | 156000 | 123000 |
| 3 | 114000 | 73000 |
| 4 | 130000 | 57000 |
| 5 (highest mobility) | 27000 | |

Table 4.1    Molecular weights of selected major protein bands from PAGE analysis of cell lysates: Cells were incubated with zinc and analysed by PAGE.    Protein bands were visualised by Coomassie staining and molecular weights were determined for selected major protein bands spanning the molecular weight reference standard range as described in figure 4.1.   The slopes for graphs of relative mobilities for each protein were determined as described previously for standard proteins.    The slope values of the unknown proteins were correlated with molecular weights using the Ferguson plot to give the size of each protein band.    The bands selected were the prominent protein bands within the range of the molecules of interest (NFκB molecular family, 48-63kDa monomers, 100-115kDa dimers and larger multimeric complexes).

The reason for such elaborate calibration of molecular sizes is illustrated in table 4.1. The protein bands are numbered in order of mobility with 1 being least mobile and 5 being most mobile. It may be seen that protein band 4 from the Jurkat T-cell lysate is larger than protein band 3 but that it appears to have greater mobility than the smaller protein in band 3. This is due to the non-denaturing conditions of the PAGE analyses in which the secondary and tertiary protein structure is maintained. This means that proteins are not necessarily separated by size alone but also by molecular shape and charge.

Cell lysates labelled with radioactive zinc in the form of ZMG or free zinc salt ($ZnSO_4/^{56}ZnCl_2$) were analysed by PAGE and gels were then exposed to autoradiographic film shown in figures 4.2-4.3. These lysates were fractionated on 4.5%, 7.5% and 12.5% gels. Bands were present on the autoradiograph of the 4% gel but they were too faint to be detected without a light box and so the gel is not shown as a figure. The bands ran in two wells apparently at the same distance as the dye front. Figures 4.2 and 4.3 show autoradiographs of the 7.5% and 12.5% gels. Again the bands apparently correspond to proteins running with or immediately behind the dye front and certainly they would appear to be small by their relative mobilities. The molecular weight from the relative mobility was calculated from the Ferguson plot. However the molecular weights were well below the limits of the calibration graph standard proteins. The results indicate that the radiolabelled zinc from both $^{65}Zn$-ZMG and $ZnSO_4/^{65}ZnCl_2$ was not associated with proteins of molecular weights corresponding to any NFκB subunit or any dimer combination of the nuclear factor. The low molecular weights calculated for the $^{65}Zn$-labelled proteins were approximately 3kDa as determined from relative mobilities on the 7.5% and 12.5% gels. Whilst there may be low accuracy of the plot for low molecular weights these results suggest that exogenous labelled zinc was bound to metallothionein or CRIP, the two main zinc storage proteins in cells.
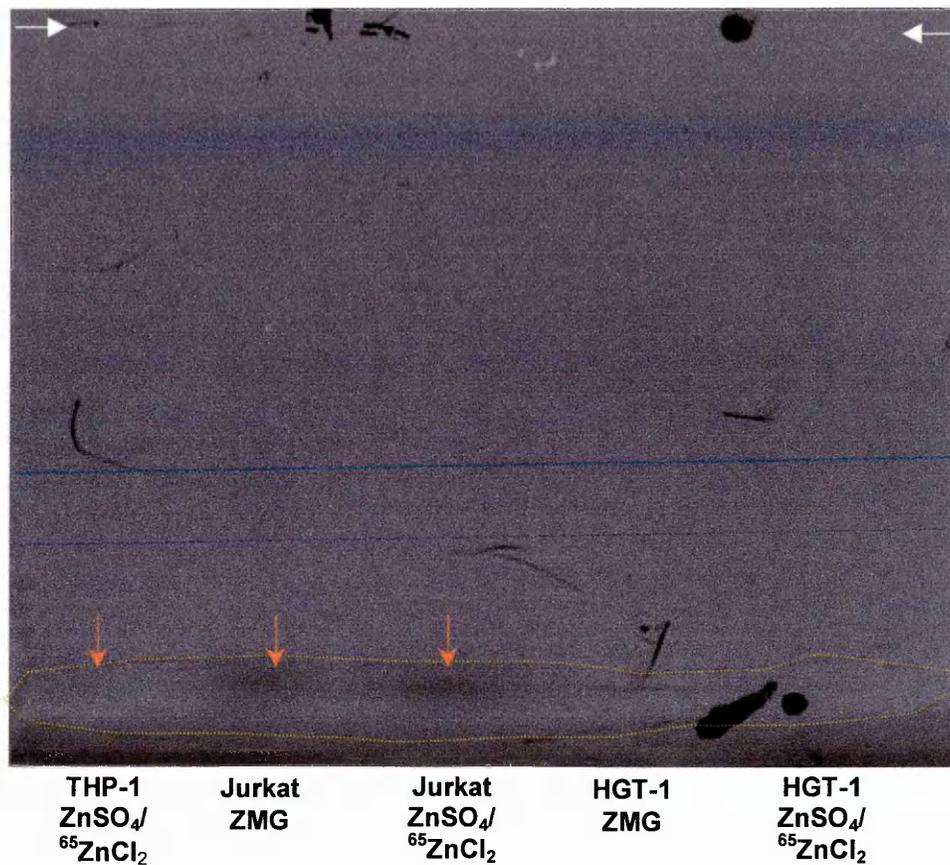
**Figure 4.2    Autoradiograph of cell lysates labelled with $^{65}$Zn and run on a 7.5% acrylamide gel:** Cells were incubated 24 hours with either $^{65}$Zn-ZMG or $ZnSO_4/^{65}ZnCl_2$. Lysates were analysed by PAGE followed by exposure to autoradiographic film for 39 days.   Bands are visible for Jurkat lysates within the dye front region (blue dotted line) and are more weakly visible in the THP-1 lysate (orange arrows).    Bands are not apparent in the HGT-1 lysates.   No bands appear above the dye front.   White arrows indicate start point for PAGE (well position) and blue dotted line indicates dye front position at end of PAGE run.   THP-1 cell lysate with ZMG is not shown due to severe marking on the autoradiographic film.   Close examination of this lane suggested the presence of an autoradiographic band but of lower intensity than that of THP-1+$ZnSO_4/$ $^{65}ZnCl_2$.
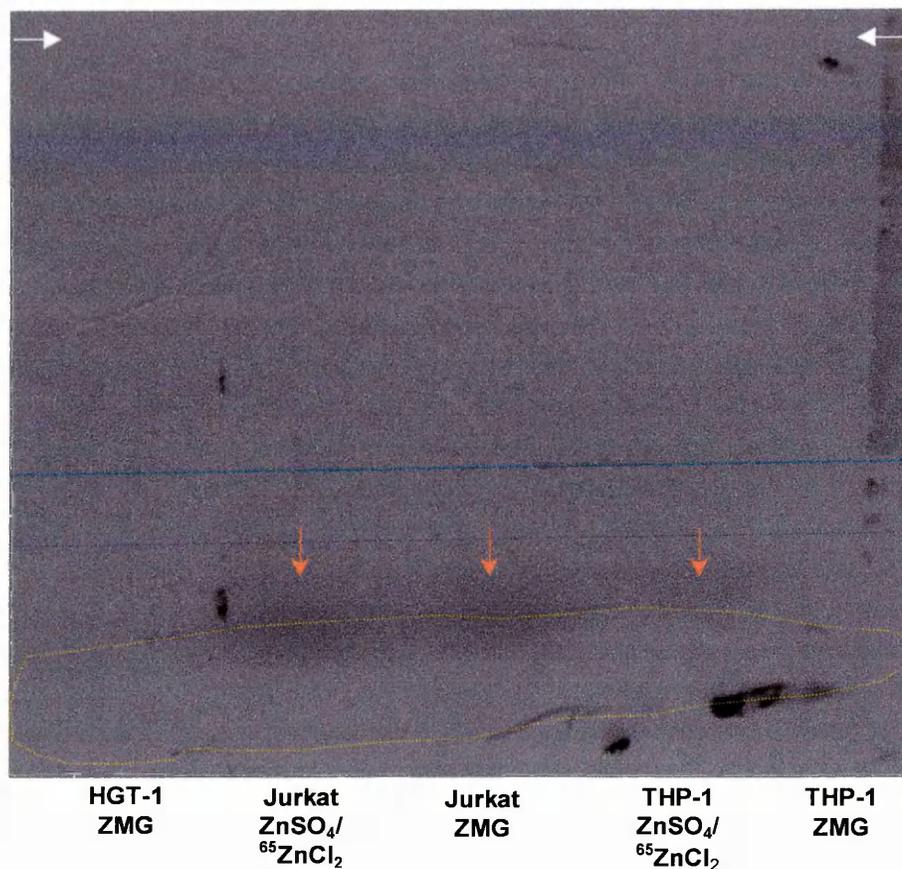
**Figure 4.3    Autoradiograph of cell lysates labelled with $^{65}$Zn and run on a 12.5% acrylamide gel:** Samples were run as described in figure 4.2, but sample lane order is reversed.    Again the bands are present in the Jurkat lysates with both zinc compounds and more weakly in the THP-1+ $ZnSO_4/^{65}ZnCl_2$ cell lysate (orange arrows).    In this concentration of gel the bands appear to be running slightly behind the body of the dye front.    No bands are apparent in the HGT-1 lysates and HGT-1+ $ZnSO_4/^{65}ZnCl_2$ lysate is clipped due to severe marking on the autoradiographic film.    White arrows indicate start point for PAGE (well position) and blue dotted line indicates dye front position at end of PAGE run.

L929 bioassay for TNFα

This assay is specific for TNFα and is a standard bioassay for the cytokine. However, there is normally an anti human TNFα antibody control on each assay plate to show that any cell death is TNFα specific. It was not possible to obtain a control antibody however an antibody was taken from a TNFα detection ELISA kit, normally used as the primary antibody which was specific for human TNFα. There was not enough antibody to use for each bioassay but a validation experiment was carried out using the small amount of antibody available to block a standard curve of TNFα induced cell death (Figure 4.4). This showed that the cell death caused by TNF could be blocked by anti-TNFα antibody in a dose dependent manner and provided a limited validation for the bioassay for the following set of experiments.

Figure 4.4 shows death of L929 cells incubated with a concentration of TNFα known to induce high levels of cytotoxicity (10U/ml) and which is blocked by increasing concentrations of anti human-TNFα antibody. This figure shows that a 1/400 dilution of antibody caused a significant decrease in TNFα induced cell death ($p < 0.01$). However higher concentrations of antibody did not prevent cell death. It is likely that the anti-bacterial agent (sodium azide) in the antibody preparation caused cell death independently, as there was cell death at high antibody concentrations where TNFα cytotoxic activity should have been inhibited. It then seems likely that as the antibody preparation was diluted down to 1/400 the sodium azide was also diluted and so the protective effects of the antibody were seen. As the antibody was diluted further, down to 1/2000, its protective ability was reduced and TNFα mediated cell death increased. This is another reason that the antibody could not be used as a control for the bioassay but these results do show that the 1/400 dilution of antibody can specifically significantly inhibit TNFα induced cytotoxicity of L929 cells.
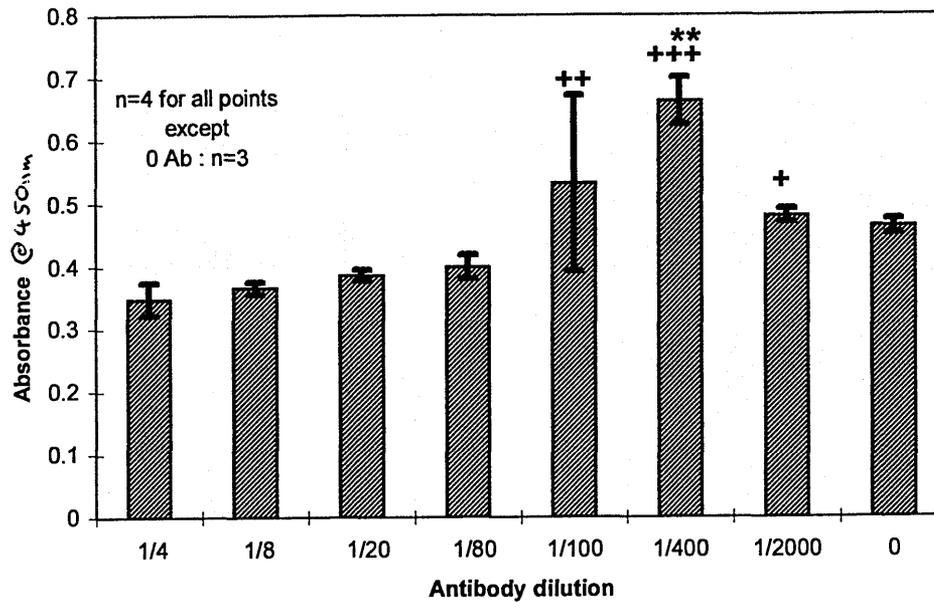
**Figure 4.4**  **L929 cell death by TNFα inhibited by anti-hTNF antibody:** L929 cells were incubated with TNFα (10U/ml), a high concentration causing a large quantity of cell death.    The antibody, at a dilution of 1/400, significantly blocked the cytotoxicity (**=p<0.01) and this was the only significant protection observed.    At higher concentrations the antibody itself appeared cytotoxic with a ¼ dilution of antibody displaying significantly higher cytotoxicity than 1/100, 1/400 or 1/2000 (+=p<0.05, ++=p<0.01, +++=p<0.001).    Increasing absorbance indicates decreasing cell death with results presented as mean ±SD.

Figure 4.5 shows the effect of increasing zinc concentration on the LD25 assay with values for cell death calculated as a percentage of absorbance of cells incubated in complete medium alone (taken as zero cytotoxicity). The zinc ion concentrations tested spanned a range of experimental concentrations of zinc used in the studies described here, and also within the general literature. Higher concentrations, $100\mu M$ and above, were found in publications where freshly isolated cells were used for examination of the effects of zinc. It is clear that the zinc concentration had a complex effect on the standard curves of $TNF\alpha$ induced cytotoxicity. Zinc sulphate at $25\mu M$ reduced cell death very significantly ($p<0.001$) in the mid-range of $TNF\alpha$ concentrations, but had no effect at either end of the standard curve. Zinc sulphate at $50\mu M$ appeared both to enhance the $TNF\alpha$ cytotoxicity at lower cytokine concentrations ($p<0.001$) and also to inhibit the cytotoxicity at higher cytokine concentrations ($p<0.001$-$p<0.05$). The effects of zinc ions up to $50\mu M$ were not significant at $TNF\alpha$ concentrations higher than 50U/ml (not shown) as cell death increased from 70%-100%.

Figure 4.6 shows the effect of increasing zinc concentration, as ZMG in citric acid solution, on the CTLL proliferation assay. The data are presented as percentage proliferation from the control of unstimulated cells, incubated without zinc or IL-2. It was clear that zinc had an effect on the bioassay standard curve of IL-2 induced proliferation, showing that it was significantly toxic to the CTLL cells at concentrations $60\mu M$ and above causing complete cell death. The lower zinc concentrations had no significant effect on the proliferation. There was no gradual concentration response of zinc action; $10$-$30\mu M$ zinc had no significant effect on the proliferation assay standard curve whilst $60\mu M$ zinc ion concentration, and above, caused complete cell death at every concentration of IL-2.
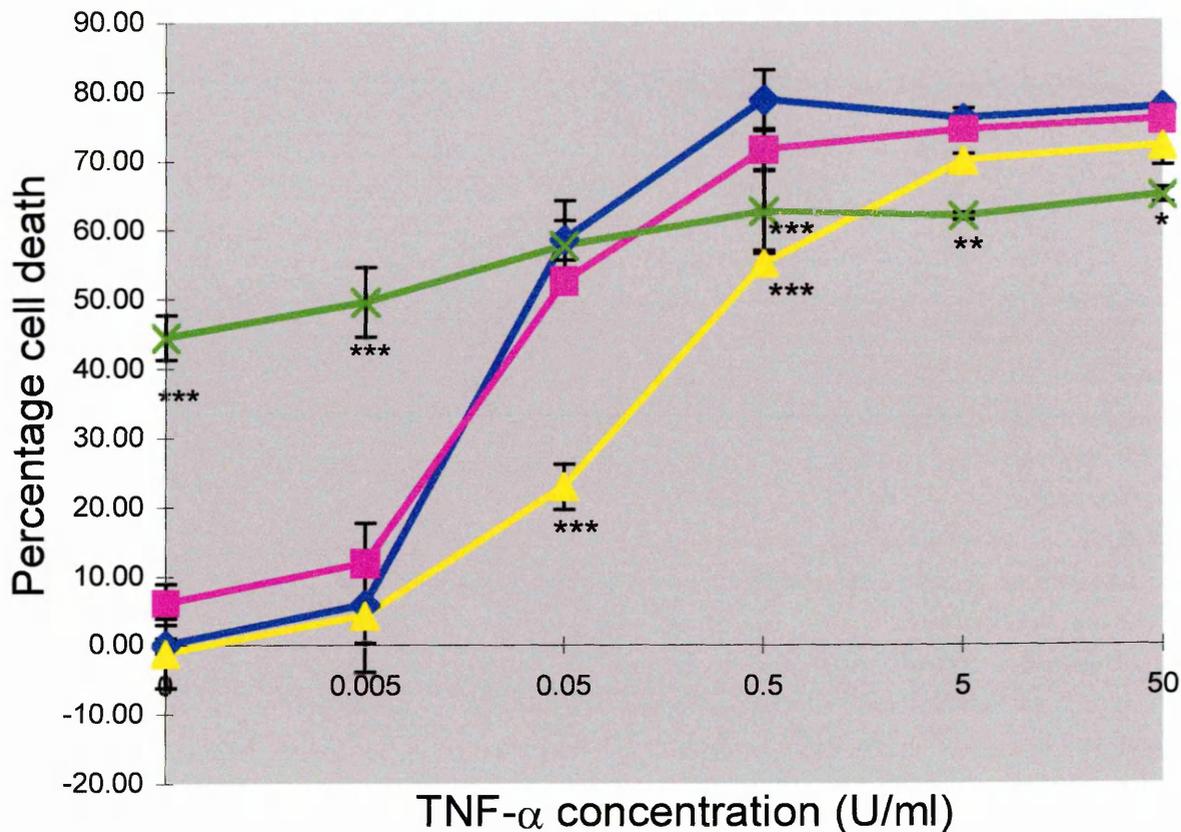
**Figure 4.5** **Effect of zinc on TNFα induced cytotoxicity in L929 cells:** L929 cells were incubated with increasing concentrations of TNFα in the presence or absence of different concentrations of zinc (ZnSO$_4$). It is clear that zinc significantly affects the TNFα induced cytotoxicity of L929 cells in this bioassay. Compared with those cells without zinc (diamond), cells with zinc at 12.5µM (square) did not alter the TNFα cytotoxicity however at 25.5µM (triangle) zinc ion appeared to significantly protect L929 cells from TNFα induced cell death at concentrations of 0.05-0.5 U/ml cytokine (p<0.001). Zinc ion 50µM (cross) caused a significant increase in cell death at lower cytokine concentrations (p<0.001) but significant protection from TNFα induced cytotoxicity at cytokine concentrations of 0.5, 5 and 50 U/ml (by ANOVA: p<0.001, p<0.01 and p<0.05 respectively). Data presented as mean ±SD, n=3 for all points.
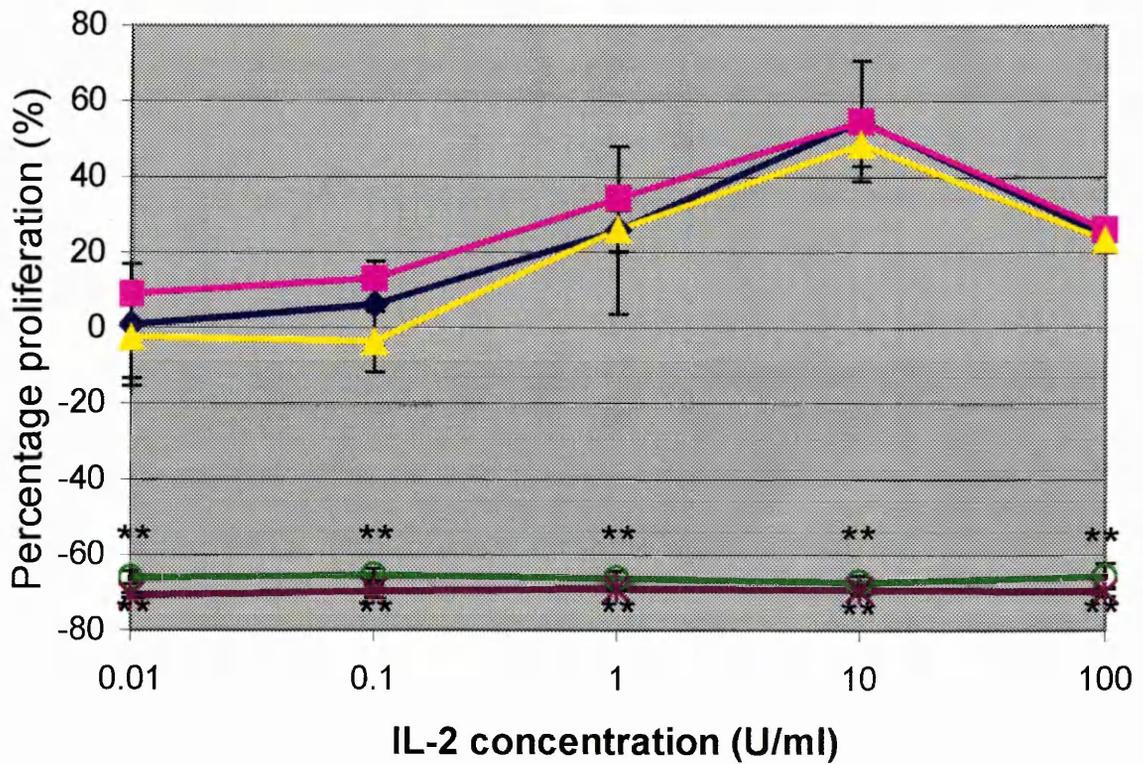
**Figure 4.6    Effect of zinc on IL-2 induced cell proliferation in CTLL cells:** CTLL cells were incubated with increasing concentrations of IL-2 in the presence or absence of different zinc concentrations (ZMG solution in citric acid).   Zinc at concentrations of $10\mu M$ (square) and $30\mu M$ (triangle) had no significant effect on the IL-2 induced CTLL proliferation when compared with control cells (diamond).   Zinc concentrations of $60\mu M$ (circle) and $90\mu M$ (cross) significantly inhibited the cell proliferation ($p<0.01$ for all points in both data sets).   Data presented as mean $\pm$SD, n=3.

The effect of zinc compounds on TNFα production by THP-1 monocytic cells is shown in figure 4.7. Values were calculated as percentage cell death in control cells without zinc or TNFα (taken as zero cell death). Data were analysed statistically by One way ANOVA and Dunnett Multiple Comparison to a control value, in this case cells with LPS alone as the effect of zinc on LPS stimulated cells was being investigated. It is clear that zinc had a significant effect on TNFα–mediated cell death in CTLL cells but no significant difference was observed *between* zinc compounds (analysed by Tukey-Kramer Multiple comparisons test). These experiments were carried out before the test of zinc concentrations on the bioassay itself as described in figure 4.5 and were, in part, the reason for those experiments.

Zinc appears to have a dual effect on TNFα release by THP-1 cells as shown in figure 4.7. At higher concentrations in the presence of LPS, 25μM and 50μM, it appears to enhance the cytotoxicity of the THP-1 supernatant media compared to media from THP-1 cells incubated with LPS alone. The levels of cell death corresponded to the concentrations of TNFα as indicated above each bar in figure 4.7. Zinc concentrations in the figure (6.25μM, 12.5μM, 25μM and 50μM) refer to zinc added to THP-1 cells to stimulate cytokine release. As the THP-1 supernatant media are diluted 1:2 on the L929 assay plate the effective zinc concentrations in the bioassay are half that of the THP-1 supernatant media, and are therefore 3.125μM, 6.25μM, 12.5μM and 25μM. The effect of these concentrations on the L929 assay were described previously in figure 4.5 and it is clear that zinc up to and including 12.5μM has no significant effect on the L929 assay. It may therefore be assumed that any change in levels of L929 cell death with zinc concentrations up to 12.5μM is due to the actions of zinc on the THP-1 cells rather than on the assay. The effect, on the bioassay results, of the samples with 25μM zinc is discussed later.

At lower zinc concentrations, 6.25μM and 12.5μM, the THP-1 supernatant media appeared to have significantly less cytotoxicity in L929 cells than media from THP-1 cells with LPS alone (p<0.01 in all cases). It was shown in figure 4.5 that a zinc concentration of up to 12.5μM had no effect on the L929 bioassay. Therefore the significant decrease in cell death seen at 6.25μM and 12.5μM in figure 4.7

appeared to correspond to a significant decrease in TNFα activity of THP-1 cell supernatants.

Shown in figure 4.7, the cell death of L929 cells incubated with supernatant media from THP-1 cells stimulated with LPS and incubated with 25μM and 50μM zinc was significantly greater than that from THP-1 cells incubated with LPS alone (p<0.01 in all cases). The media containing 25μM zinc from the THP-1 cell stimulation resulted in 12.5μM zinc in the L929 assay plate. This concentration of zinc was shown not to effect the bioassay (Figure 4.5). Thus changes in L929 cell death at this concentration were a result of zinc modulating of the THP-1 cell cytokine secretion and was not an effect of zinc directly on the bioassay. Therefore, stimulating THP-1 cells with 25μM zinc, produced media with significantly more cytotoxic activity than THP-1 cell media from cells incubated with LPS alone.

THP-1 media from cells incubated with LPS plus 50μM zinc also caused significantly more cell death in L929 cells than media from THP-1 cells incubated with LPS alone. It may be seen from figure 4.5 that 50μM zinc in the THP-1 media, corresponding to 25μM zinc in the L929 plate, significantly inhibited TNFα induced cell death. However, zinc only affected cytotoxicity of up to and including 0.5U/ml TNFα; from figure 4.7 it may be seen that 50μM zinc as ZMG solution or zinc sulphate solution produced 410U/ml and 580U/ml TNFα respectively from THP-1 cells. At these concentrations of TNFα, 25μM zinc had no effect on TNFα cytotoxicity and so the L929 assay results for THP-1 cells incubated with 50μM zinc can be assumed to be unaffected by zinc activity directly on the assay.
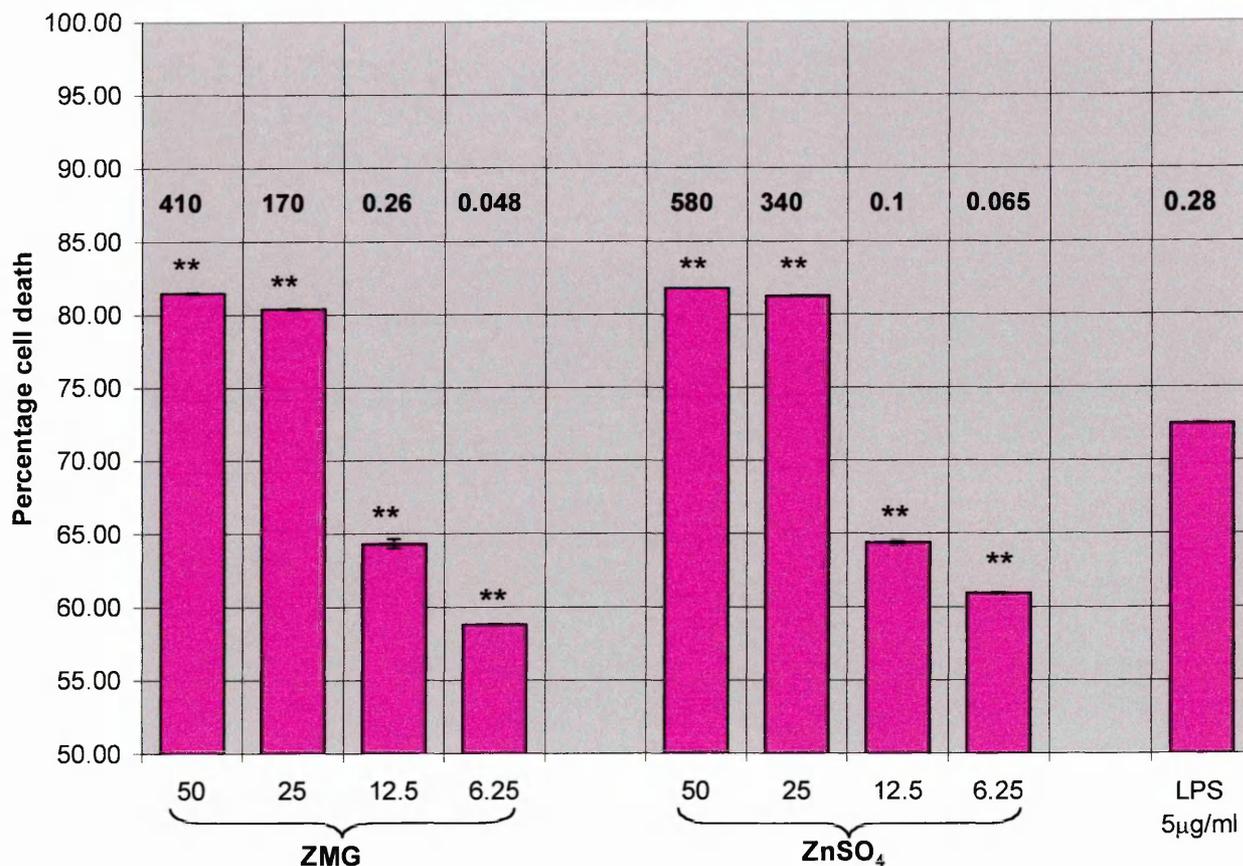
**Figure 4.7** **Cytotoxic activity of THP-1 cell supernatant media after stimulation with LPS with or without zinc compounds, as determined by L929 bioassay:** THP-1 cells were incubated 24 hours with 5µg/ml LPS and zinc compounds (ZMG citrate solution or $ZnSO_4$) from 6.25-50µM zinc ion. Supernatant media were recovered and assayed for TNFα activity by L929 bioassay. Results presented as cell death calculated as a percentage of unstimulated control cells (cells without LPS or zinc). Displayed above each bar is the TNFα concentration (U/ml), calculated from the standard curve, corresponding to percentage cell death as derived from a standard curve of TNFα cytotoxicity. Zinc concentrations of 25µM-50µM significantly increased the cytotoxic activity of the THP-1 supernatant media ($p<0.01$) indicating an increase in TNFα concentration. Zinc concentrations of 6.25µM-12.5µM significantly decreased the cytotoxic activity of THP-1 supernatant media ($p<0.01$) indicating a decrease in TNFα concentration. Values presented as mean ±SD (error bars present on all data columns) and n=3 for all points except 12.5µM ZMG where n=2; **=$p<0.01$.

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Jurkat T-cells were also stimulated with LPS and cytotoxicity of Jurkat culture media was determined by L929 assay (Figure 4.8). Almost no cytotoxicity was observed in the Jurkat supernatants and no significant difference in cytotoxicity was seen in cell supernatants from stimulated Jurkat T-cells incubated with or without zinc compounds at any concentration tested.

Both THP-1 cells and Jurkat T-cells were incubated with PMA with similar results to those in figure 4.8. Very little cytotoxicity was seen in the L929 assay for any of these cell supernatants. No significant difference was seen in supernatant samples from cells incubated with zinc compounds compared to the supernatant media of cells incubated with PMA alone.

The lack of stimulation of cytokine secretion in some of the experiments looking at LPS and PMA stimulation of cells was probably due to the batch of LPS or PMA used however time did not allow for further examination of this fact.
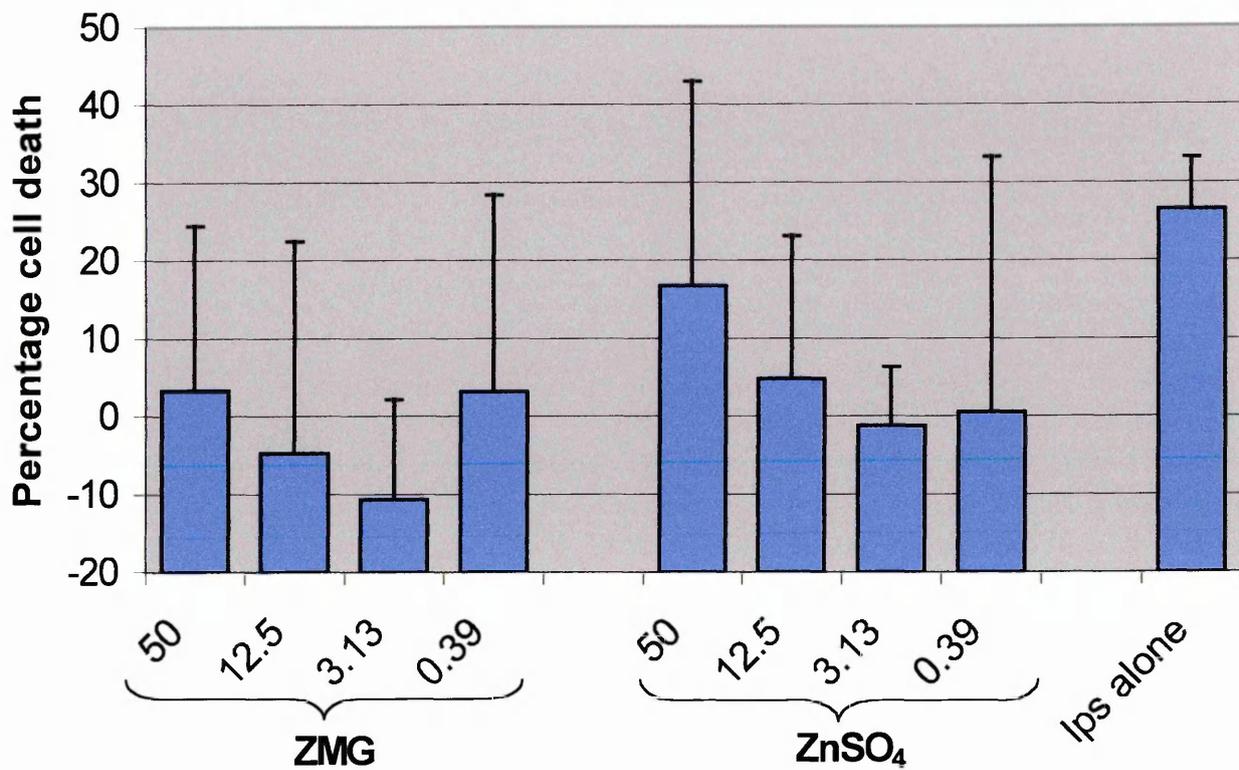
**Figure 4.8    Cytotoxic activity of Jurkat T-cell supernatant media after stimulation with LPS with or without zinc compounds, as determined by L929:** Jurkat T-cells were incubated 24 hours with 5μg/ml LPS and zinc compounds (ZMG solution or $ZnSO_4$ solution) from 0.39-50μM zinc ion. Supernatant media were recovered and assayed for $TNF\alpha$ activity by L929 bioassay.   Results are presented as cell death calculated as a percentage of unstimulated control cells (cells without LPS or zinc).   When calculated from the standard curve of $TNF\alpha$ cytotoxicity (0.05-5000U/ml) $TNF\alpha$ levels were very low and the highest concentration, the LPS control, induced a percentage cell death below the limits of the standard curve.   No significant cytotoxicity was observed in any media (ANOVA and Dunnet's multiple comparison to control).

## 4.3.4 Enzyme Linked Immunoassay (ELISA) - Effect of zinc on IL-1β production

Results for the IL-1β ELISA are shown in figure 4.9, the data were presented as means of two observations. Statistics have not been applied as samples were only run in duplicate as the results were intended as a preliminary examination of cytokine release.

It is clear from figure 4.9 that THP-1 cells appear to have a low level of IL-1β secretion in the unstimulated state. When cells were stimulated with LPS they secreted approximately ten times more IL-1β than without the mitogen and when stimulated with LPS plus PMA the THP-1 cells produced roughly 100 times more IL-1β than those stimulated with LPS alone. As samples were tested in duplicate it is only possible to say that none of the zinc compounds had a dramatic effect on the cytokine secretion of stimulated THP-1 cells. As a general observation, cells stimulated with LPS with or without PMA and incubated with ZMG suspension appeared to secrete less IL-β than stimulated cells incubated with vehicle control. As a preliminary study this may indicate an effect that should be examined further.
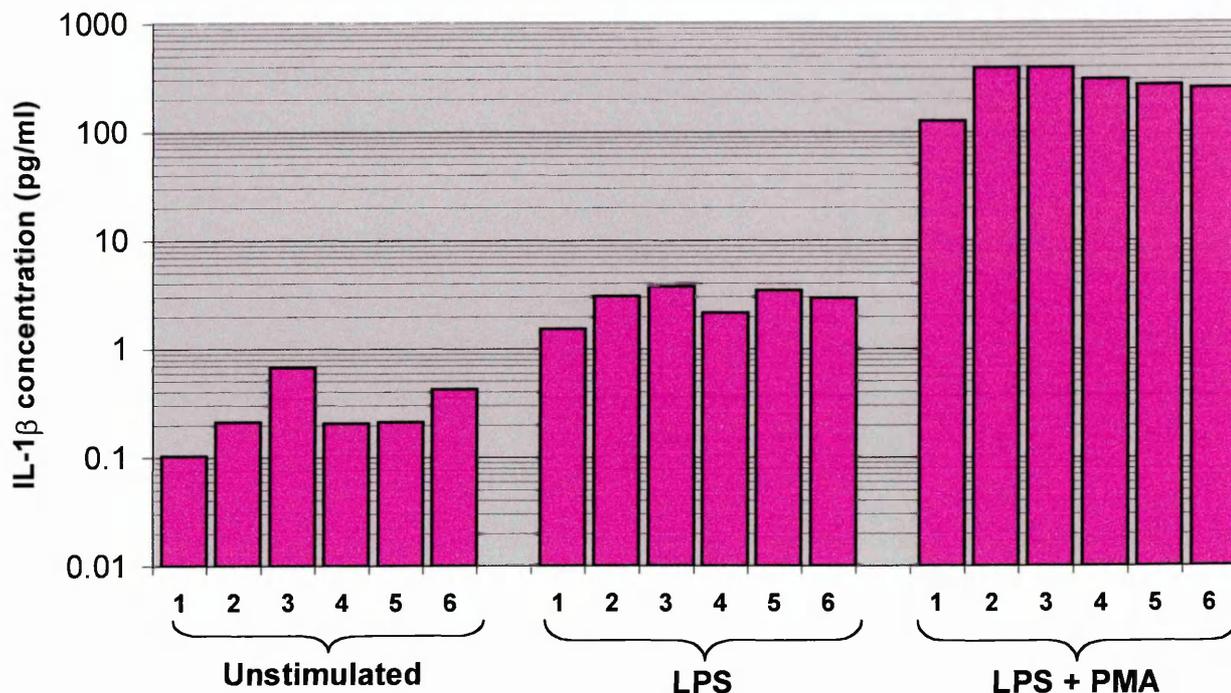
**Figure 4.9     Analysis of IL-1β secretion by THP-1 cells stimulated with LPS or PMA and incubated with zinc compounds:** THP-1 cells were incubated with either ZMG suspension (1), zinc sulphate solution (2), zinc sulphate solution plus glycerol (3), zinc oxide suspension (4), zinc oxide suspension plus glycerol (5) or vehicle control, distilled water (6).   All zinc compounds provided $25\mu M$ final zinc concentration.   Cells were concomitantly stimulated with LPS ($1\mu g/ml$) alone, LPS plus IFN-γ ($100U/ml$) or left unstimulated.   Cells stimulated with LPS secreted approximately 10 times more IL-1β than unstimulated cells.   Cells stimulated with LPS + PMA secreted approximately 1000 times more IL-1β than unstimulated cells and 100 times more of the cytokine than cells stimulated with LPS alone.   Values are presented as mean, n=2 for all points.

### 4.3.5 Investigation into the effect of exogenous zinc on NFκB

Although some preliminary work was carried out involving both THP-1 and Jurkat cells, time limitations meant that work had to be focussed on one cell line at a time. Figure 4.10 shows a comparison of lysates from THP-1 and Jurkat cells. The following two figures (Figures 4.11 and 4.12) describe tests carried out to determine conditions for the main assay, namely LPS concentration and incubation time. The final figure, figure 4.13, shows analyses of Jurkat cells incubated with zinc and LPS. The anti human NFκB antibody was indicated in the commercial literature as being specific to the p50 subunit and its p105 precursor protein. The Serotec data sheet, accompanying the antibody, further stated that in some gel assays it showed activity against p50:p50 and p50:p65 NFκB dimers. This should mean that only these bands were seen on membranes probed with this specific antibody, in fact in several cases extra bands were observed and so the molecular weights of all major bands were calculated. However, small differences in lane length due to gel shrinkage or slight skewing of the gel on the membrane during Western transfer could have affected the molecular weight calculations from relative mobilities. Thus in figures 4.10-4.13 the theoretical molecular weights, as expected from the information on the data sheet, were shown along with the calculated molecular weights where relevant.

Figure 4.10 shows unstimulated THP-1 and Jurkat whole cell lysates indicating that identical bands were detected in each case. It should also have given an indication of all the bands available for antibody binding and in fact five distinct bands are visible, the most intense corresponding to p50 (calculated mw = 56.4kDa). The top band corresponds to a calculated molecular weight of 110.96kDa, this may be the p50:p50 homodimer but is more likely to be the p50 precursor protein (105kDa). However, as can be seen from the THP-1 lane there is a suggestion that the 110.96kDa band may in fact be two poorly resolved bands. Given that samples were boiled with SDS it seems unlikely that these bands could represent either the p50:p50 homodimer or the p50:p65 heterodimer as these should not survive the SDS treatment.
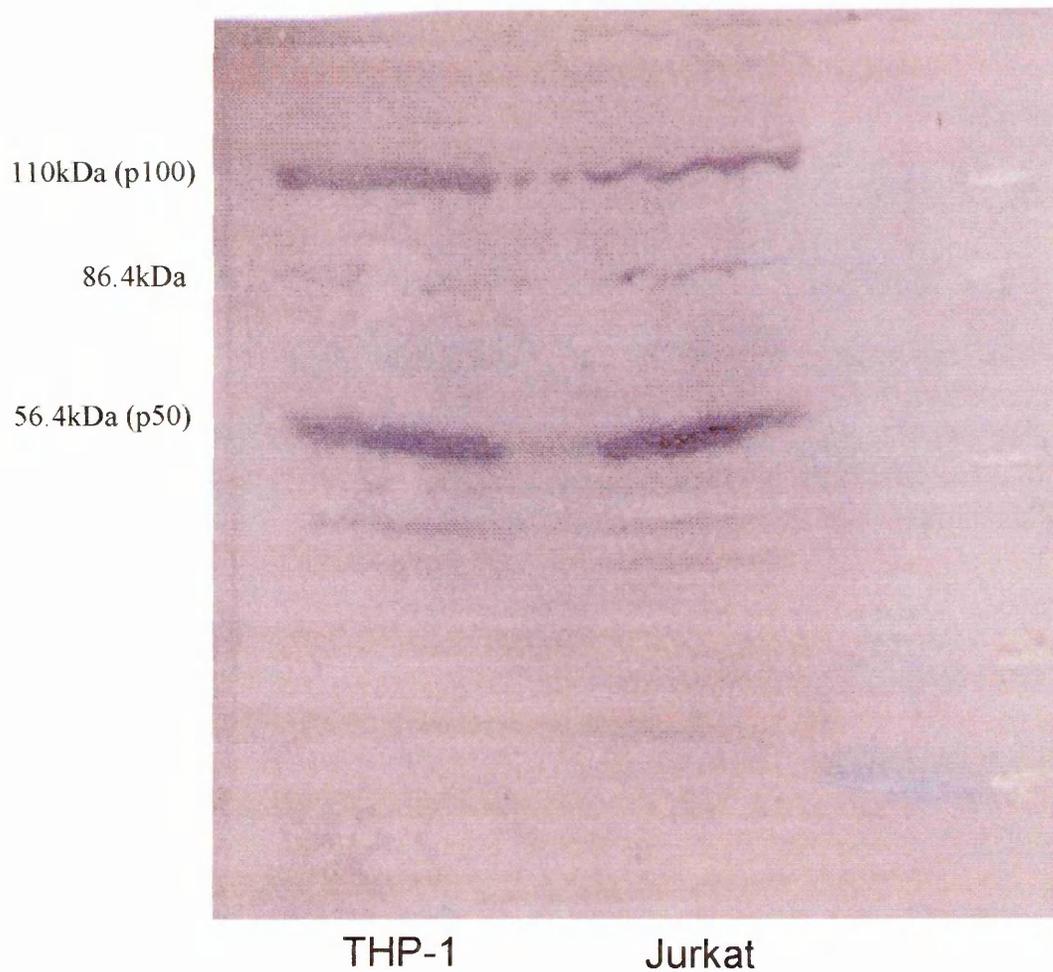
**Figure 4.10     Cell lysates analysed by Western blot and probe by anti NFκB p50 antibody:** Two major bands are apparent in each cell line sample, one of 56.4kDa and one of 110kDa as determined by comparison with relative mobilities of molecular marker proteins.    These bands are assumed to correspond to the p50 monomer and the p105 precursor allowing for some error and gel shrinkage during gel processing.

Results from later experiments looking at LPS and zinc stimulation of Jurkat cells would appear to agree with this observation (Figure 4.13) with two or three bands being observed in the high molecular weight range proteins of the nuclear extracts.

Figure 4.11 shows the effect of increasing time of incubation of Jurkat cell with LPS (1μg/ml). This was done to determine whether incubation time effected the levels of NFκB in nuclear extracts. The control lane is slightly occluded by incomplete transfer from gel to membrane during Western blot however the probe indicates apparently no difference between 30 minutes incubation and 120 minutes incubation. This incomplete transfer also affected the marker lane on the membrane so that relative mobilities of the molecular weight marker proteins were determined from both the membrane and the gel and the molecular weights of the unknown bands were determined from both the Coomassie stained gel and the membrane markers. There was no difference in molecular weights, the smallest protein detected corresponded to 55kDa. It must be assumed, unless antibody specificity is questioned, that this is the p50 subunit and that the corresponding 110kDa bands in figure 4.11 are likely to be the precursor protein, perhaps cytoplasmic contaminants from the nuclear extraction. Increasing incubation time of cells in LPS apparently makes no difference to the nuclear concentration of NFκB p50 monomer or dimer as detected by Western blot and probe. Image analysis would be required to confirm this quantitatively.

In addition to the p50 monomer and the 110kDa protein there appear two protein bands of 60.5kDa and 82.0kDa. These are not expected to be detected with the antibody according to the commercial literature. It seems unlikely that the antibody is detecting the p65 subunit of NFκB as the sizes are not correct for any combination of the molecule. If the p50 protein corresponds to a band of 55kDa then the same correction for the smaller unknown protein of 60.5kDa on the blot would make it in reality 55.5kDa.
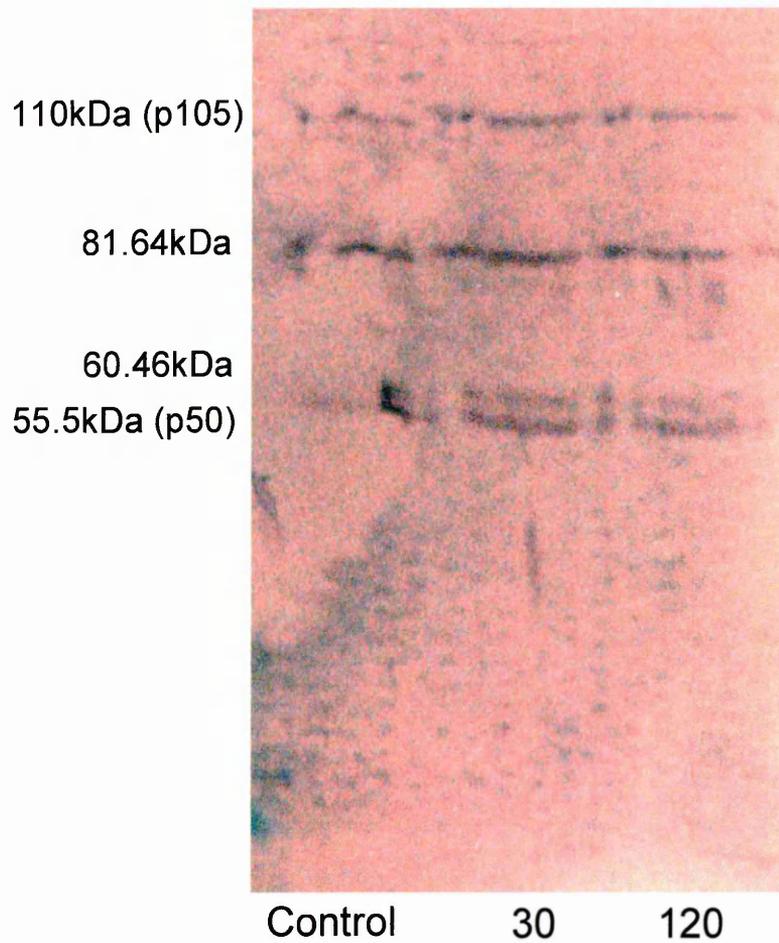
**Figure 4.11   Nuclear extract of Jurkat T-cells incubated for different times:** Jurkat T-cells were incubated for 30 and 120 minutes with LPS (1µg/ml) and nuclear extracts were prepared and analysed by Western blot and probe with anti NFκB p50 antibody.

Figure 4.12 shows Jurkat cell nuclear and cytoplasmic extracts from cells incubated with high and low concentrations of LPS (1 and 10 μg/ml respectively). The calculated molecular weight for the smaller major protein band was 60.1kDa. As with the previous figure, Figure 4.11, the specificity of the antibody indicates that this is p50. However, the broad nature of the band may conceal two bands larger and smaller than 60.1kDa that produce the average band of a size that corresponds to 60.1kDa. If this is the case antibody could possibly be detecting the p65 NFκB subunit. This would not matter in practice for interpretation of results as both the p50 and p65 monomers can constitute the dimer that translocates to the nucleus on stimulation with LPS or cytokines. There appears to be no great difference between intensity of bands of cytoplasmic 60.1kDa protein. There does appear to be slight increase in band intensity of the nuclear 60.1kDa protein with increasing LPS concentration, although this would require computer image analysis to confirm. Certainly this observation would be made clearer by repetition of this experiment with a larger range of LPS concentrations.

Two minor protein bands of 44.5kDa and 39.1kDa were also visible and appeared more intense in the high LPS than the control. Significantly, the NFκB inhibitory protein, IκB, has two isoforms IkB-α (37kDa) and IκB-β (45kDa) and these dissociate from NFκB when the nuclear factor is activated. An increase in NFκB activity should thus lead to an increase in IκB dissociation and to an increase in the concentration of IκB in the cytoplasm where it is mainly situated. Figure 4.12 shows 39.1 and 44.5kDa bands more intense than the control. Certainly this cannot be used as a measure of NFκB activity as it cannot be proved from these results that IκB was detected in addition to the NFκB specificity claimed by the supplier. It does however further bring into question the specificity of the antibody.
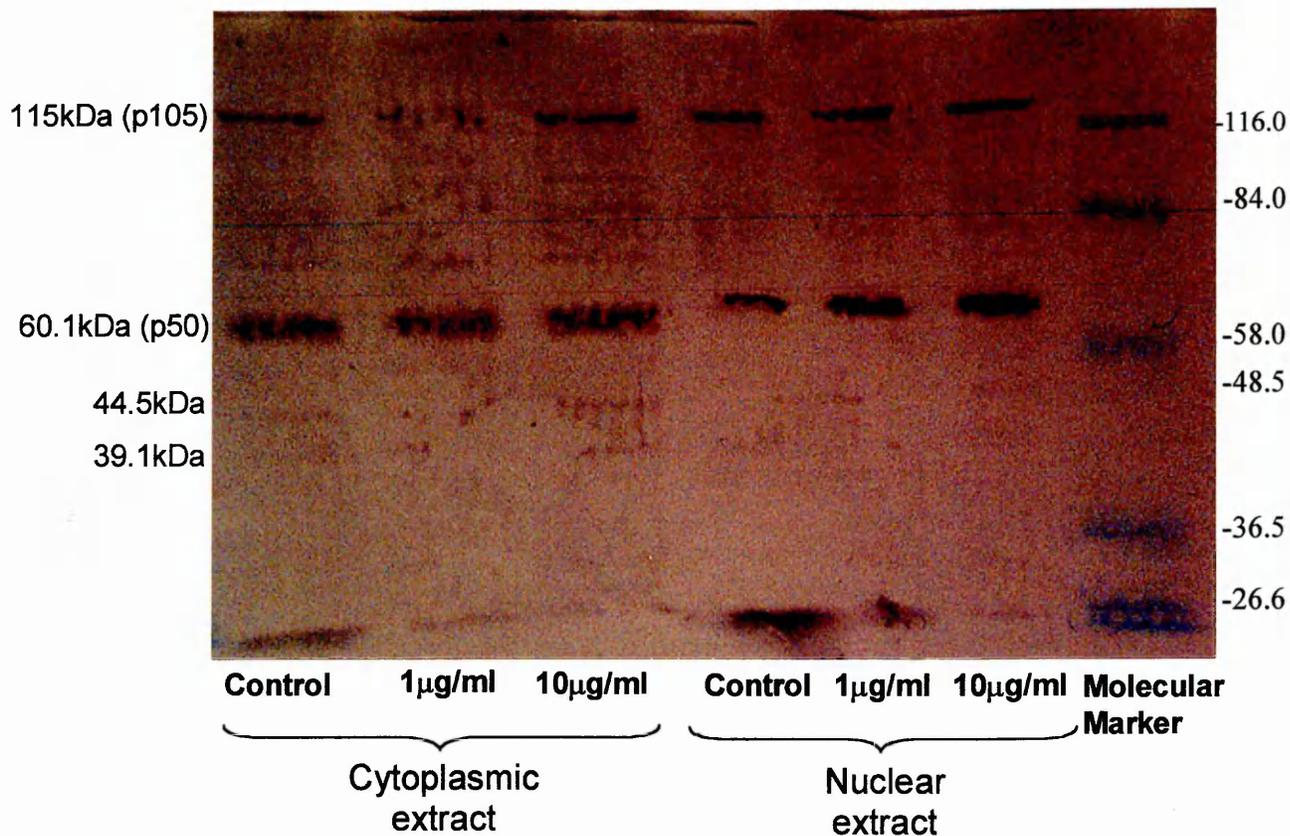
**Figure 4.12 Nuclear and cytoplasmic extracts of Jurkat T-cells incubated for 90 minutes with different concentrations of LPS:** The cells were incubated with LPS at 1 and 10µg/ml then nuclear and cytoplasmic extracts were analysed by Western blot and probed with an anti NFkB p50 antibody.

As there did seem to be a slight graduation of increasing NFκB band intensity in figure 4.12, the concentration of 1μg/ml was selected for examination of effect of zinc on the nuclear factor activation. This would allow zinc mediated increases or decreases in band intensity to be observed. Nuclear extracts from Jurkat cells incubated with zinc (ZMG and $ZnSO_4$ at either 12.5μM or 25μM) and LPS (1μg/ml) are shown in figure 4.13. There were two protein bands around the p50 area and they were calculated to be 54.7kDa and 58.5kDa in size. The larger 110kDa band is present as before and the bands in the nuclear extracts from cells incubated with zinc do appear to be less intense than the zinc free controls. As with figure 4.12, two bands at 39.2kDa and 44.8kDa are strongly present in all extracts.

There appeared to be a slight decrease in the intensity of the 54.7kDa and 58kDa bands in extracts from cells incubated with increasing ZMG solution. The control plus LPS 54.8kDa and 58kDa bands appear darker than the same protein bands from cells incubated with 12.5mM zinc (as ZMG solution) which in turn was darker than the same bands in the 25mM zinc (as ZMG solution) lane. This pattern of decrease in intensity was not seen in all the bands of these three lanes suggesting that it was not as a result of unequal sample load concentrations. The pattern was not visible at all in the lanes derived from cells incubated with equivalent concentrations of zinc sulphate instead of ZMG.

122.1kDa
118.0kDa
110.4kDa (p105)
66.9kDa
58.5kDa
54.7kDa (p50)
44.8kDa
39.2kDa

Control    Control    12.5μM    25μM        12.5μM    25μM
           + LPS

ZMG                    ZnSO₄
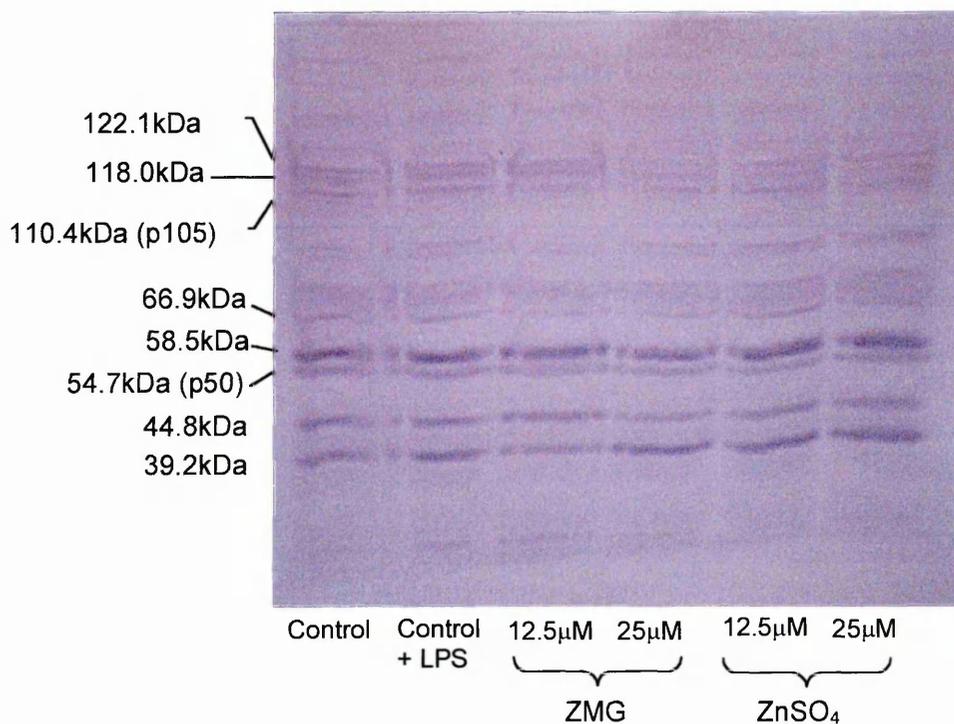
**Figure 4.13    Nuclear extracts of Jurkat cells incubated with zinc compounds and LPS:** Jurkat cells were incubated 24 hours with ZMG or zinc sulphate solution, 12.5μM and 25μM zinc ion concentration then for 90 minutes with LPS (1μg/ml). Nuclear extracts were prepared and analysed by Western blot and probe with anti NFκB p50 antibody.

### Summary

The inhibition and activation of cytokine secretion by zinc *in vivo* and *in vitro* has been discussed previously in Chapter 1 along with studies carried out on the control of cytokine levels by activation of the zinc finger nuclear factor protein NFκB. The experiments described in this chapter were conducted to investigate any possible links between zinc levels and NFκB control of cytokine activity. In addition, the studies examined, generally, the zinc control of cytokine secretion by cultured cell lines in vitro. In doing so questions arose and answers derived regarding the action of zinc on the cytokine assays themselves.

The results obtained from the PAGE analyses of radiolabelled zinc in Jurkat and THP-1 cells appeared to agree with the major part of the literature which indicated that zinc is absorbed by the cells and is rapidly bound by small storage molecules. Either metallothionein or CRIP appear to bind the rapidly exchangeable labile zinc component of cells. These may transport the ion back out of the cell or may act as a transfer point for zinc to other proteins. In the time period studied, 24 hours, it appears that none of the exogenous zinc is transferred to larger proteins such as NFκB in amounts detectable by autoradiography. It would also appear from these results that ZMG citrate solution is no different from zinc sulphate solution in this respect. It is possible that incubating cells with the radiolabelled zinc compounds for a longer period or co-stimulating the cells with the labelled zinc compounds and NFκB activators might result in incorporation of the radioactive zinc by NFκB in amounts detectable by the autoradiographic techniques used in this study. Time restrictions precluded further study of these possibilities, however increasing incubation time may not have been relevant to NFκB activation and subsequent cytokine stimulation, as discussed in the Discussion chapter.

As exogenous labelled zinc, examined by autoradiographs, did not seem to bind directly to the zinc-binding site of NFκB to activate it then it may have acted indirectly on NFκB resulting in its translocation to the nucleus. The SDS-PAGE analyses by Western blot were carried out to investigate this possibility.

The first figure in the Western analysis study of NFκB (Figure 4.10) indicated that NFκB p50 was expressed and detectable in both THP-1 and Jurkat T-cells. The following studies focussed on Jurkat T-cells and showed that the anti NFκB p50 antibody was not at all specific. The analyses did not show a distinct difference in nuclear or cytoplasmic NFκB p50 levels in LPS stimulated cells incubated with or without zinc. It may be that the method is not sensitive enough to detect these changes but it is highly likely that the lack of specificity affected the sensitivity of the detection system. The study indicated that the antibody detected major protein bands of sizes that corresponded to, variously, NFκB p65 monomer, c-Rel (an 85kDa member of the Rel family to which NFκB belongs) but also all combinations of NFκB homo- and heterodimers. As there are proteins in this family at 49kDa, 50kDa (NFκB p50), 52kDa, 55kDa and even the 65kDa NFκB subunit (p65) the lack of specificity of the antibody must be assumed to affect the observed results. If strongly detected by the antibody or if in high enough concentration proteins of similar size are unlikely to be resolved into discrete bands by SDS-PAGE or equally by immunoblot of proteins, transferred by Western blot, from the acrylamide gel. For this reason the results obtained cannot be conclusively explained. However as the antibody was primarily raised against NFκB p50 subunit it is reasonable to assume that it would be detected more than the other proteins in that family. Taking this into consideration there still does not seem to be any visible variation in the intensity of stain between nuclear extracts from cells incubated with or without zinc compounds thus indicating that p50 does not seem to have been activated and translocated to the nucleus. An antibody probe of Western blot membrane is not the best way to examine nuclear factor levels but it was chosen as best for the study in view of cost and speed of assay. Ideally electrophoretic mobility shift assay (EMSA) would be the method of choice for analysing NFκB activity.

As NFκB is a major control point for cytokine activity, the action of zinc on cytokine production was investigated. It was clear from the literature, as discussed in chapter 1, that zinc can modulate cytokine secretion but a search of the literature revealed no studies carried out on the effect of zinc on cultured

cell lines. As many such lines, including THP-1 cells and Jurkat T-cells, are very commonly used as models for cells of the same lineage *in vivo* it was of particular interest to examine the effect of zinc on cytokine production by these cell lines.

From the results obtained it is clear that zinc modulates cytokine secretion in the model cell lines but that it additionally has a significant effect on the bioassays used to measure the cytokine levels. This is an important observation and perhaps a logical one, but one that had apparently not been approached in the literature in any depth. The L929 bioassay for TNF$\alpha$ (Shahan et al., 1994) and the CTLL bioassay for IL-2 (Gillis et al., 1978) are two very common bioassays used for measurement of cytokine activity. However the results presented here indicate that levels of zinc used in experimental zinc studies could very easily affect the sensitivity of the assay. Concentrations of zinc of up to 50$\mu$M in the test samples caused a significant enhancement of L929 cell death in the assay. As the actions of zinc in peripheral blood mononuclear cells have been examined using zinc concentrations in excess of 200$\mu$M and often routinely at 100$\mu$M, the results presented here are highly relevant. Examination of cytokine production by bioassay would, under these conditions, be significantly affected by the presence of the high zinc concentrations. More generally, these results indicate the problems associated with studying a substance as ubiquitous and as active as zinc. The effect of the ion on all assay types should be taken into consideration.

Having tested the assay it seemed that most of the results obtained for the zinc modulation of cytokine secretion were carried out at zinc concentrations that had no effect on the assay. Thus it appears that zinc modulation of TNF$\alpha$ is bimodal in LPS stimulated THP-1 cells, increasing its secretion at higher ion concentrations, 25$\mu$M and 50$\mu$M whilst reducing it at lower concentrations, 12.5$\mu$M and 6.25$\mu$M. Why this might occur is not clear but the highly complex nature of cytokine secretion, the autocrine activity and paracrine activity of cytokines and the control of these mechanisms by zinc is only very recently being understood. The potentiation of LPS stimulated TNF$\alpha$ release by higher exogenous zinc concentrations and the inhibition of LPS stimulated release at

lower exogenous zinc concentrations (Figure 4.7) agrees with the available literature. These are discussed in greater detail in Chapter 5.

The IL-1$\beta$ ELISA was carried out as a preliminary investigation and all that was being looked for was a result that would warrant further study. Although there was no definite effect of zinc on cytokine secretion by the stimulated cells, those that were incubated with ZMG suspension appeared to produce lower levels of IL-1$\beta$ than control cells under both stimulatory conditions tested. This would appear to be a result worthy of further investigation.

Knowledge of how zinc affects cytokine production by most of the commonly used cell lines is scant. However, as the ion is found associated with many compounds, sample zinc concentration may have a significant effect on any cell investigation especially in the study of cytokines. This is reinforced by the results from the L929 study (Figure 4.7) that showed not only that zinc concentration significantly altered the assay parameters but also that well within normal experimental concentrations of zinc there was a bimodal effect of zinc on the TNF$\alpha$ secretion by THP-1 cells. These results suggest that it is highly important to continue the examination of zinc on cytokine release in cultured cell lines and extend it to encompass a greater number of cytokines.

## Discussion

The results presented in this thesis have extended the knowledge of ZMG in particular and have continued and extended the general understanding of the actions of zinc in biological systems. This discussion aims to present and comment on the findings of the thesis in the context of the known literature.

The chemical analyses of ZMG were carried out in order to understand better the action on cultured cells of either the ZMG suspension or ZMG dissolved in acid solution. It became clear from the results that the acid preparation of ZMG resulted in degradation of ZMG to give a solution of free zinc ions and glycerol. Thus no true dissolution of ZMG molecules occurred at acid pH. Where the term 'ZMG solution' or 'dissolved ZMG' has been used in relation to acid preparations both in this thesis and in the available literature (Fairlie et al., 1992, Tiekink et al., 1996), these results show the solutions contained mainly free zinc ion and glycerol. Whilst it was not explicitly concluded by Fairlie et al. (1992) that free ionic zinc was released, they carried out solubility experiments that produced a solubility profile for ZMG consistent with degradation of ZMG to release zinc and glycerol in direct proportion to the hydrogen ion concentration. The results presented here agree with this observation in that zinc from ZMG dissolved in citric acid solution had zinc elution profile identical to that of zinc sulphate solution. In addition to the HPLC analyses, the MALDI-ToF analysis of ZMG dissolved in dilute hydrochloric or citric acid showed that there was no peak of mass equivalent to the ZMG polymer of any substitution number. Together with the HPLC data, for both zinc and glycerol release, this indicated that in acid solution, the higher structure of ZMG was probably entirely degraded to give zinc and glycerol. This has important implications for ZMG administered orally for therapeutic purposes, in that initially it might be thought that ZMG solid, suddenly suspended in gastric juice would react very rapidly with stomach acid and degrade to release zinc and glycerol. ZMG would then be expected to react like any other soluble zinc salt, binding to ligands in digesta or secreted ligands from the mucosa. There can be little doubt that this does happen to some degree but the present study suggests that this reaction is only one of several

possible fates for ZMG in the stomach, and is probably the least important of them from a biological and therapeutic standpoint. To understand why this is true, the results relating to the physical nature of ZMG are examined below in greater detail.

The results presented in this thesis agree in part with a study by Tiekink et al. (1996), who used spectrophotometric techniques to measure dissolution of ZMG in aqueous suspension by its decrease in turbidity under varying conditions. Before the two sets of results may be compared, the method employed by Tiekink et al. (1996) should be looked at. Tiekink et al. (1996) carried out this analysis by adding the ZMG to the vehicle and shaking the cuvette containing the suspension before placing it in the spectrophotometer. It was observed during the course of the experiments in this thesis, that ZMG, when suspended in water or in fact most non-viscous media, settled very rapidly even when particle size was very fine. If this sedimentation factor of ZMG had been taken into account by Tiekink et al. (1996) is not made clear in the published study and presumably must affect the results obtained using this method of analysis. What they showed was that ZMG 'dissolution' in a variety of buffered aqueous media was dependent on the concentration of the solid particle dispersion and also probably to the square root of the hydrogen ion concentration. The results presented in this thesis indicate that in very mildly acidic water (>pH6.6), ZMG is not entirely degraded to release zinc and glycerol but releases something into solution that appears to be a soluble component of the ZMG molecule. This does not conflict with Tiekink et al. (1996), as release of the soluble fraction of ZMG from ZMG particles in suspension would simply appear as a decrease in turbidity in their results, which do not specify the fate of ZMG once dissolved. It should also be noted that Tiekink et al. (1996) use a lower concentration of ZMG than used in the current study, 1mg/ml maximum concentration as opposed to 100mg/ml used in the ZMG suspension studies presented here. It would be interesting to observe, using the HPLC analysis of glycerol, the effect of lower ZMG concentration on the soluble fraction of ZMG. This is discussed further in the Future Work section.

Where the results presented here do not entirely agree with those of Tiekink et al. (1996) is on the subject of polymer size. They observe, using fast atom bombardment analysis, a spectrum that showed the major fragment of ZMG to be $[(C_3H_6O_3)Zn]_4$ followed in decreasing abundance by $[(C_3H_6O_3)Zn]_3$, $[(C_3H_6O_3)Zn]_2$ and $[(C_3H_6O_3)Zn]$ (Tiekink et al., 1996). This is presented as a statement without data and seems odd, since fast atom bombardment normally requires that the target compound be dissolved in a solvent suitable to that compound (Cotter, 1997). Tiekink et al. (1996) used 3-nitrobenzyl alcohol as matrix without any indication as to the solubility of ZMG in that solvent and without description of the method employed, so it is difficult to determine what is being analysed in their preparation. Assuming that no dissolution or degradation occurred, only simple suspension, then the experiment appears similar to the MALDI-ToF analyses presented here, in which ZMG was prepared in 2,5-DHB matrix solution. The present results indicate that ZMG exists in polymeric form with a wide range of sizes approximately extending from $[(C_3H_6O_3)Zn]_4$ to $[(C_3H_6O_3)Zn]_{24}$ subunits. These results are not in complete disagreement with those of Tiekink et al., (1996) but suggest that the MALDI-ToF method was more sensitive than fast atom bombardment.

MALDI-ToF has not been used quantitatively in this thesis but it seems possible, by comparison of relative peak intensities, that $[(C_3H_6O_3)Zn]_4$ (620Da) and a larger fragment (840Da) may have been the most abundant fragments. Smaller fragments may be present in limited quantities but the MALDI-ToF matrix appeared to cause interference at lower masses, which made it difficult to distinguish lower mass peaks. The MALDI-ToF data add to the known structural information relating to ZMG as they provide a basis to determine molecular structure. Chapter 2 showed that ZMG polymers of different lengths have different end moieties as determined by examining mass differences (See Figures 2.14-2.15). These were derived by addition to and subtraction from the structures determined by X-ray crystallography of ZMG (Hambley and Snow, 1983). As the MALDI-ToF has an error margin between approximately 4-5 mass units it is possible that these are not the correct or only structures but the structures presented here are those that seemed to best fit the available data within that error margin.

It should be understood that the MALDI-ToF traces did not provide a picture of ZMG degradation, instead they are a picture of the range ZMG polymers in that one commercial preparation as supplied. As the results from HPLC analysis of glycerol release and zinc release both indicate, virtually no ZMG is degraded in aqueous suspension (>pH6.6) up to 15 minutes after suspension. In the MALDI-ToF analyses the ZMG was suspended in the matrix methanol solution for a very brief time, several seconds, before it evaporated to leave the dry ZMG/matrix residue. It is thus reasonable to assume that little, if any, ZMG degradation was likely to occur. The polymeric trace obtained for ZMG in the MALDI-ToF results is therefore likely to be very close to the true polymeric size range for the compound before any degradation and zinc release occurs. Thus, in aqueous suspension, different length polymers could be degraded at different rates as different end moieties might be more or less susceptible to attack. This could result in different release rates of zinc from polymers of varying lengths. The variation in polymer terminal structures could also affect the way that the ZMG polymer interacts with cell membranes. A chain with an exposed terminal zinc atom may have greater affinity for membrane-bound peptide zinc ligands than would a chain with a terminal hydroxyl or methyl group. In both cases, the different terminal structures could account for the slow release of zinc from ZMG preparations observed in the current study (Chapters 2 and 3) and in previous investigations (Whitehouse et al., 1990, Rainsford and Whitehouse, 1992, Fairlie et al., 1992). Use of the MALDI-ToF could determine the change in each polymer over time if ZMG were to be incubated in suspension prior to testing by this method. In this manner traces of the polymer mass range could be obtained at various points after degradation had started and could give an indication of the cleavage points on the ZMG chains.

That ZMG in suspension was shown in this present study to release zinc and glycerol over time is in agreement with the published data (Fairlie et al., 1992, Tiekink et al., 1996). However, the methods employed here have not been employed previously for this purpose. The analyses, with the Poros HPLC column, of zinc in solution provide a novel method for further study of ZMG

dissolution and degradation, but may also provide the basis for the analysis or purification of other zinc compounds. In addition, this is the first presentation of a simple quantitative HPLC method for directly analysing glycerol release from ZMG in suspension where previously an indirect quantitative iodometric method had been used (Fairlie et al., 1992). Together they may comprise a relatively simple HPLC based tool for further analysis of ZMG preparations, although it would probably require further refinement for use with the various media that may be used to suspend or dissolve ZMG.

The observation mentioned above, that ZMG may release both ionic zinc and a possible ZMG molecular component into solution, is a novel one. It has relevance for the action of ZMG *in vivo,* as a *molecule* of ZMG would be treated differently from released ionic zinc by most cells that have specific zinc uptake channels and transporters. Even if the dissolved ZMG molecule consisted simply of one zinc atom and one glycerol molecule i.e. one ZMG subunit, it is unlikely that it would have access to the normal zinc ion transporter mechanisms due to size and steric hindrance.

From the chemical studies of ZMG it was possible to relate properties of the compound to its action in cell culture systems as displayed in the various biological assays carried out in the present study. ZMG has been tested *in vivo* in rodent models of both arthritis and ulcer (Whitehouse et al., 1990, Rainsford and Whitehouse, 1992, Rainsford, 1992), its solubility had also been examined in various tissue homogenates (Fairlie et al., 1992). It was clear from these studies that various tissues including skin and probably gastric mucosa readily absorbed ZMG. ZMG was also variously shown to be less irritant than zinc sulphate in the gastric mucosa (Rainsford, 1992) and when injected subcutaneously (Whitehouse et al., 1990). This latter study also noted that in rats the skin readily absorbed $^{65}$Zn-ZMG powder (radiolabelled) more efficiently than zinc oxide when rubbed on and that the label ($^{65}$Zn) from the ZMG was excreted in the faeces. This indicated that dermal application of ZMG resulted in the ready absorption, presumably by the epidermal cells, and ready processing to excretion of the exogenous zinc. In addition, dermally applied or subcutaneously injected ZMG suspensions (in glycerol or water)

were far more effective than other zinc preparations of equivalent zinc concentration, including zinc sulphate and zinc oxide, at reducing inflammation in rat models of arthritis (Whitehouse et al., 1990). Together these results suggested that ZMG reacted with cells *in vivo*, particularly epidermal cells and cells mediating inflammation, in a manner different to that of other zinc compounds. The results presented currently are in agreement with the published literature and describe the uptake profile of ZMG in cell lines of different lineage.

The ICP-MS analyses of zinc uptake by THP-1, Jurkat or HGT-1 cells were designed to compare the bioavailability of zinc as ZMG suspension with that of an insoluble and soluble zinc control, zinc oxide and zinc sulphate respectively. In the cell lines where uptake occurred, THP-1 and Jurkat, the results were clear that ZMG and zinc oxide provided approximately 5 to 10 times more bioavailable zinc than zinc sulphate over short, 2-4hr time periods. However, over a 24hr period the difference diminished such that cells incubated with ZMG or zinc oxide contained almost the same amounts of zinc as those incubated with zinc sulphate. This would appear to indicate that the physical nature of ZMG caused it to be taken up by cells in a manner similar to zinc oxide and quite distinct from that of soluble ionic zinc.

As the uptake experiments were carried out in serum free media it is unlikely that ZMG or zinc oxide interactions with soluble proteins could account for the high levels of cellular zinc provided by these compounds. It is possible that proteins and peptides with zinc binding regions, secreted by the cells, could acquire the exogenous zinc and increase cellular uptake (Rowe and Bobilya, 2000, Prasad and Oberleas, 1970). In fact, this should positively favour the soluble, unbound zinc provided by zinc sulphate solution as it provided a more ready source of the ion than either ZMG or zinc oxide suspensions. The free ion would be more likely to be acquired by the ligand-binding route and so this cannot account for the higher levels of cellular zinc provided by ZMG or zinc oxide suspension. It would seem therefore to be a function of the particulate nature of both ZMG and zinc oxide suspensions that caused this rapid uptake after 2 and 4 hours, with resultant high cellular zinc concentrations. It may be

that whole particles are simply ingested by cells via an endocytic mechanism or that the particles, in close physical proximity to the cell membrane, release zinc in a concentrated 'bolus', stimulating uptake in a localised area of membrane via an endocytic mechanism such as potocytosis. It is not certain which of these two possibilities is most likely. For instance, exogenous zinc has been shown to stimulate phagocytosis in some cell types (Peretz et al., 1994). Certainly this is a possibility with the THP-1 cells, which are derived from the monocyte macrophage lineage, but it may also occur in a more limited fashion with the Jurkat T-cells. This process has been shown to occur in neutrophils where phagocytosed particles of zinc hydroxide were observed by electronmicroscopy (Ogino et al., 1994). This study further suggested that the zinc hydroxide itself stimulated the phagocytic uptake of the particles by these cells (Ogino et al., 1994). Also there is a commonly used technique known as 'glycerol shock' used to aid the uptake of DNA into cells during transfection, the mechanism of which does not appear to be recorded in the current literature (See Røttingen et al., 1995 for example of the method). However, the method involves DNA being precipitated onto cell surfaces often by addition of calcium phosphate. Glycerol is then added at as high a concentration as the cells will bear (this is ascertained empirically) for a brief time before being washed off. It may be that the sudden osmotic potential created by the high glycerol concentration causes a transient increase in cell membrane permeability. If so, a granule of ZMG could act in similar fashion at a highly localised point on the cell membrane. It would release glycerol at a single locus as it degrades, thus causing a transient increase in permeability at that point on the membrane resulting in increased uptake of the granule or of released ionic zinc.

The possibility, at the time intervals over which ZMG uptake was determined, that vesicular uptake of zinc from ZMG may have occurred, does not exclude the possibility of other mechanisms acting concurrently. In fact the degradation analyses of ZMG suggest that it is almost certain that, whilst particulate ZMG may be endocytosed by cells, ZMG is also releasing free ionic zinc over the 2-4hr time period which would be taken up by the same mechanisms as zinc from zinc sulphate. These would almost certainly include cation channels, zinc specific channels (McMahon and Cousins, 1998) and passive diffusion but

could also include protein ligand binding by cellular secreted proteins (Rowe and Bobilya, 2000) or uptake via anion channels if zinc was associated with an anion (Chang et al., 1998). The contribution of these other mechanisms to zinc uptake has not been quantified here, but as it would be equal to or less than the cellular concentrations provided by zinc sulphate solution, it may be assumed to be negligible compared to the zinc taken up by vesicular transport. However, the alternative mechanisms *may* account for the fact that incubation with ZMG suspension resulted in a generally higher mean cellular zinc content after 2hr and 4hr, than incubation with zinc oxide with or without glycerol. As zinc oxide is virtually insoluble in neutral aqueous suspension, it should not release free zinc to participate in the secondary uptake mechanisms in the same amounts as ZMG. The statistical significance of the apparently higher cellular levels of zinc from ZMG may or may not become clearer with more repetitions of the uptake experiment.

By 24 hours the normal mechanisms for processing zinc seem to take effect, as the zinc content of cells incubated with ZMG, zinc oxide or zinc sulphate gave approximately the same levels of total cellular zinc. The question must then be asked, why use ZMG or even zinc oxide when after 24 hours the cells *in vitro* have generally the same zinc content? The zinc uptake must be examined in the *in vivo* application of a tablet or suspension of ZMG.

The mechanisms for cellular zinc homeostasis appeared to remove a large amount of the zinc present in the cells, after 2 and 4 hour incubation with ZMG or zinc oxide, by the 24 hour time point. In cell culture the excess zinc could only be excreted back into the culture medium by zinc transport mechanisms such as the ion channels ZNT-1 and ZNT-2 or DCT1. However *in vivo*, the excess zinc can be transferred to adjoining cells or transferred into the circulation and also excreted back into the gastrointestinal tract or the surface of the skin, depending on where it was applied. Thus applied, ZMG would be well absorbed by a larger area of tissue than an equivalent concentration of free ionic zinc as the high zinc levels would distribute out between cells adjoining those that actually absorbed the initial dose of ZMG. So in addition to the lower gastric irritancy and better absorption through skin when compared

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to zinc sulphate or zinc oxide respectively, the cellular uptake characteristics of ZMG could favour whole tissue uptake of zinc from ZMG over zinc oxide or sulphate.

It should be noted that even prior to contact with the mucosal cells, the chemical and physical properties of ZMG might provide greater bioavailability of zinc by enhanced binding to the gastric mucus. Free ionic zinc from zinc salts may well bind to ligands in the gastric milieu, such as phytate that inhibits zinc uptake (Lonnerdal et al., 1988), and thus be poorly absorbed across the gastric mucus to the mucosa. It is known that gastrointestinal mucus binds zinc (Coleman and Young, 1979, Davies, 1980) and that this binding by the mucus layer is the rapid initial phase of zinc uptake into the mucosal cells (Davies, 1980). A bolus of ZMG would physically lie on the mucus and release zinc onto the mucosa in a concentrated area, which a solution of zinc salt could not do. As the initial binding of ionic zinc to the mucus was only 1% of the total zinc in the study by Davies (1980), it seems reasonable to expect that between physical settling onto the mucus, and possible affinity for the mucus the ZMG particles could provide greater than 1% zinc in that initial uptake. In the case of an ulcerated lesion the zinc particles might come into direct contact with mucosal cells but also T-cells and macrophages in the mucosa that could infiltrate the wound (Shigeta et al., 1998). The ZMG could then be expected to be taken up by those cells in a similar fashion to the uptake seen in the Jurkat T-cells and THP-1 monocytic cells of the present study.

The zinquin fluorescence and cell viability studies complemented the total zinc uptake experiments. The distinction between the ICP-MS analyses and the fluorescence analyses was that ICP measured total cell content of zinc whilst zinquin bound only to freely available labile zinc. This labile pool of zinc was shown to be important in prevention of apoptosis in cultured thymocytes, where it represented a freely available source of the ion for cellular processes that require a rapidly available source of zinc for molecular function (Zalewski et al., 1993). Zinquin allowed this zinc pool to be directly observed by microscopy but in addition to this, the fluorophore allowed observation of the effects of exogenously applied zinc on this labile pool. As an extension of this method

zinquin was used in the present study to follow the progress of exogenous zinc uptake into cells from various different zinc sources. The results presented here are supported by the observations obtained from the ICP-MS experiments particularly in the manner of cellular zinc uptake from ZMG suspensions. In particular the fluorescence studies indicated specific differences in treatment of exogenous zinc, between the THP-1 cell line and the Jurkat T-cell line, which were not apparent from the ICP-MS studies. In addition the fluorescence photomicrographs suggested reasons for the apparent lack of zinc uptake by that HGT-1 gastric cell line and acute sensitivity to high concentrations of zinc. These reasons are discussed later.

As mentioned above there could be a number of possible mechanisms for zinc uptake either from zinc solution or from particles of zinc compounds. These mechanisms could differ between cell lines. Before looking at differences between cell lines, a point of interest is that there was an observation common to all three cell lines. Some cells in each population appeared to be distinctly more able to take up exogenous zinc than others, regardless of zinc source. This 'all or nothing' uptake pattern was detectable in the Jurkat cell line and to a lesser degree in the HGT-1, due to generally low cellular fluorescence and THP-1 cell line due to generally high cellular fluorescence. The 'uptake-positive' cells did not generally appear to be physically different from other cells, which tended to rule out zinc entering after loss of membrane integrity due to apoptosis or necrosis. The viability experiments supported this observation as no significant cell death was shown for the zinc concentrations used in the uptake studies. It seems then that there may be a zinc uptake mechanism that can be switched on in one particular cell and that allows tolerance of higher than normal zinc concentrations. Whether or not this mechanism is active in some cells all the time or in all cells at specific times cannot be determined from these results, but it is an observation from which further investigation might provide an insight into cellular zinc regulation. Certainly, measurement of the zinc concentration by image analysis of zinquin fluorescence levels in such cells could determine the contribution made by 'uptake-positive ' cells to the total zinc in any given population of cells. The photomicrographs presented here indicate that this contribution might be

important in the Jurkat T-cell line with its distinctly high and low fluorescing cells and perhaps less important in the THP-1 cell line where all cells had a relatively high level of fluorescence after incubation with zinc compounds.

The treatment of exogenous zinc, and ZMG suspension in particular, was distinctly different in all three cell lines. The HGT-1 cell line, which did not take up measurable amounts of zinc in the uptake experiments, will be discussed later as it had a particularly unusual response to exogenous zinc. Considering then the two remaining cell lines, it may be reasonable to assume that it was the physical properties of ZMG or shared zinc uptake mechanisms that caused the ion to be taken up in similar amounts by THP-1 cells and Jurkat cells. However, the manner in which zinc was treated after the first initial uptake differed between the monocytic and T-cell lines. This might be expected as, for example, cells from different lineages may display differential expression of zinc specific transport molecules such as the ion channels ZNT-1, -2, -3 and –4 as discussed in chapter 1.

In light of the various known mechanisms for zinc transport and processing, it is possible to suggest reasons for the differences observed between the THP-1 and Jurkat cell lines. All THP-1 cells appeared to have relatively uniform diffuse zinquin fluorescence, some with a number of brightly fluorescing vesicles, after incubation with zinc compounds over 2-4 hours. Jurkat T-cells appeared to have a larger number of cells containing brightly fluorescing vesicles and some diffuse fluorescence although this was not uniform. There were a number of Jurkat cells that had little or no diffuse fluorescence. With the microscopy techniques employed to examine the zinquin fluorescence it was not possible to determine definitely whether the diffuse fluorescence, that was so marked in the THP-1 cells, was in the cytoplasm or on the outer cell membrane. It would be very important for an understanding of the response of the cells to high levels of zinc, to know whether the zinc levels were increased in the cytoplasm or whether the excess zinc had been rapidly localised to the outer membrane ready for excretion. If the excess zinc were rapidly removed from the cytoplasmic compartment to the membrane, then that would suggest a cell line with a rapid and efficient zinc removal mechanism. If, however, the

zinc remained in the cytoplasm then this would seem to suggest a cell line with a higher tolerance for zinc which was able to protect itself from zinc toxicity by binding the ion into storage molecules such as metallothionein. For several reasons discussed below it seems likely, but not certain, that the zinc was localised to the membrane.

The ICP-MS data revealed that in THP-1 total cellular zinc fell rapidly between 2 and 4 hours incubation with zinc compounds and levelled off over the subsequent 24 hours. The early rapid fall in cellular zinc levels would tend to indicate that the cells had started to remove the excess zinc almost as soon as it was taken into the cells. It would seem logical then, that by that time the zinc was less likely to be still situated within the cytoplasm and more likely to be situated towards the outside of the cells. This could occur in a number of ways but given the nature of vesicular processing by cells of the monocyte macrophage lineage and the fact that zinc has been shown to be required for phagocytosis in macrophages (Wirth et al., 1989), the excretion of zinc could well involve the endocytic/exocytic mechanism. It is also possible that the THP-1 cells could have the additional facility of sequestration of any free cytoplasmic zinc into vesicles as was shown in BHK cells with the ZNT-2 zinc transporter (Palmiter et al., 1996). This would mean that both zinc in endocytic vesicles and free cytoplasmic zinc subsequently were sequestrated into vesicles and could be removed from the cells by the relatively rapid method of exocytosis. Vesicular exocytosis would account for several of the features seen in the photomicrographs of the THP-1 cells. First there were few punctate, highly fluorescent vesicles, second there was a generally high level of diffuse fluorescence that could be situated at membrane level and third, total cellular zinc rapidly fell after only 2 to 4 hours incubation with exogenous zinc. Added to this proposed mechanism could be zinc excretion by membrane bound zinc transporters such as ZNT-1 and ZNT-4. These molecules are thought to remove zinc from the cytoplasm through the basolateral membrane of enterocytes and into the microcirculation or into adjoining cells (Palmiter and Findley, 1995, Murgia et al., 1999). These would also tend to cause zinc to be concentrated at the outer cellular membrane as it was pumped out of the cell.

The results obtained from the Jurkat T-cells could also fit the zinc control mechanism proposed above although they may be more similar to the BHK cells examined by Palmiter and co-workers (1996). They showed that the ZNT-2 transporter molecule could be expressed in vesicles and lead to vesicular sequestration of zinc from the cytoplasm. Once in the vesicles, the cytoplasmic zinc was no longer at toxic or near toxic concentration. What Palmiter et al., also showed was that the BHK cells did not appear to remove the zinc filled vesicles from the cell. In fact, the cells reduced the total zinc load by distributing half the zinc filled vesicles into either daughter cell during cell division (Palmiter et al., 1996). Thus the zinc filled vesicles remained in the cells but the total zinc in the cell population was reduced relative to the number of cells. They showed that there was some leakage of zinc from the vesicles and proposed that this acted as a pool of zinc should the cells require it (Palmiter et al., 1996). It may be similar with Jurkat T-cells, in that excess cytoplasmic zinc, obtained from the exogenous zinc sources, could be either pumped into vesicles where it would remain, or directly pumped out of the cell as discussed for THP-1 cells. This would account for the lower levels of generalised diffuse fluorescence and higher levels of vesicular fluorescence than the THP-1 cells. The ICP-MS data would tend to suggest that the Jurkat cells did not act exactly like BHK cells in that over 24 hours the total cellular zinc levels did fall dramatically. This would suggest that although zinc was highly localised to the vesicles, the Jurkat cells did indeed have a mechanism for removing the vesicular zinc, albeit far more gradually than the THP-1 cells.

Unlike the THP-1 cells or the Jurkat T-cells, the HGT-1 cells did not seem able to take up zinc in measurable amounts as determined by ICP-MS or by zinquin fluorescence. Obviously HGT-1 cells have some level of cellular zinc flux, as they would not survive without it, however these cells provide evidence that mechanisms for zinc processing can vary, sometimes dramatically, between cell lines. The lack of zinc uptake may or may not have been an aspect of the original gastric carcinoma cell from which this particular clone was derived, it is not currently possible to determine this. However, when grown on glass coverslips and subsequently incubated with a normally non-toxic concentration of zinc, the HGT-1 cells appeared both zinc rich and dead. This suggested

that contact with the glass surface had triggered a zinc uptake mechanism in the cells as cells grown in plastic culture flasks, with the same concentration of zinc, were healthy and contained no zinc. At first, it does seem unlikely that a gastric cell should be resistant to zinc uptake and so sensitive to zinc toxicity given that *in vivo* the cells of gastrointestinal tract must act as a highly selective barrier for zinc uptake. The protective barrier action of the gastric cells is thought to act at least in part, by capture of the zinc probably by metallothionein, and subsequent release back into the digestive tract (Davis et al., 1998, Albergoni and Piccinni, 1998), either by excretion though pathways as discussed above, or by cell death and sloughing. If the effect of excess zinc is to cause the cells to die and slough off into the gastrointestinal tract then the responses of the HGT-1 cell line to zinc can be explained as a defensive mechanism against high levels of zinc in the digesta.

As discussed previously, it has been observed in almost all *in vivo* studies, that orally administered zinc has little effect on disease state where circulating zinc levels are adequate (Remarque et al., 1993). Additionally, dietary supplementation with large doses of zinc does not seem to result in plasma zinc levels being elevated greater than normal (Coppen and Davies, 1987). Given the normally rapid turnover of gastric epithelial cells, the reaction of HGT-1 cells to zinc may indeed be part of the normal function of this cell type. That external stimuli, in this case the growth surface, are able to apparently to switch on zinc uptake, provides an interesting area for future research. It is not inconceivable that cell surface receptors in contact with glass instead of plastic, could be activated to send a signal resulting in upregulation of a zinc uptake mechanism. The different responses obtained by changing the growth surface could be a starting point to determine the pathways involved in HGT-1 zinc uptake and subsequent cell death.

The aspect of zinc processing that appeared common to both THP-1 and Jurkat T-cells, the zinc rich vesicles, was examined in a little more detail by co-incubation with either acridine orange or FITC-Dextran. The cells take up the large dextran molecules into vesicles where, under neutral conditions, the fluorescein label fluoresces under ultraviolet light. The experiments carried

out in this area gave very poor FITC-dextran labelling of the cells and little or no vesicular association with the FITC fluorescence. The acridine orange associates preferentially with acidic compartments of cells, not necessarily with vesicles alone (Palmiter et al. 1996). Some vesicles, especially lysosomes, do have a lower pH and it would be interesting to know whether zinc from ZMG suspension was associated with these low pH vesicles. In terms of zinc release, if a particle of ZMG was taken up into a vesicle with a high $H^+$ content then, according to the chemical analyses, it would be more likely to rapidly degrade and release the zinc. If the vesicle containing a putative ZMG particle were neutral pH, then the zinc release would be expected to occur more slowly. In these preliminary co-incubation experiments, THP-1 cells and Jurkat T-cells were examined for vesicles that appeared to fluoresce either blue from the zinquin, red from the acridine orange or a mixture of both. The results again were not conclusive but did seem to indicate that some of the zinc from ZMG suspensions was associated with non-acridine orange stained vesicles as indicated by normal blue zinquin vesicular fluorescence. There were other vesicles however that appeared blue/green in colour, which may have indicated vesicles fluorescing both blue and red. From this it seemed reasonable to conclude that zinc taken up from a ZMG suspension by THP1 cells and Jurkat cells was found definitely in non-acidic vesicles but also possibly in acidic vesicles. Using the techniques described in this thesis it was not possible to determine whether these vesicles contained ionic zinc or particulate ZMG but cytoplasmic ionic zinc has been shown to accumulate in acidic vesicles (Palmiter et al., 1996).

Subsequent experiments were carried out in order to determine the eventual destination of zinc taken up by cells incubated with radiolabelled zinc. This was done specifically to see if the zinc associated with any of the cell signalling molecules or nuclear factors. Zinc of course is required for cell function as discussed in detail in this chapter and Chapter 1, and exogenous zinc is acquired by molecules that require it for function, NFκB for example. It was also known that much of the loosely bound zinc in cells is to be found associated with the metallothionein molecule or the CRIP molecule, depending on cell type. The aim of the current study was, in part, to examine the effect of

zinc on cellular processes involved in the inflammatory response. Zinc can mediate relatively rapid changes in secreted cytokine levels (Scuderi, 1990) in many cases promoting increased levels of these inflammatory mediators. This suggested that the ion might control cytokine expression pathways either by direct association with a transcription factor or a metal responsive element on the cytokine gene or indirectly by controlling the activation of a nuclear factor.

NFκB is a zinc finger bearing transcription factor that is involved in the activation of a large number of cytokines including the inflammatory cytokines such as IL-1β and TNFα (Chen et al., 1999). NFκB was shown in the literature to have transcriptional enhancement activity independent of its own protein synthesis (Sen and Baltimore, 1986) indicating post-translational mobilisation of the protein as the control point for its activity. Later it was shown that NFκB is found inactivated in the cytoplasm and bound to IκB, a control protein (Reviewed Grimm and Baeuerle, 1993). From these initial studies and a large number of later publications it was clear that the control of cytokine activity by NFκB, being rapid, was most likely to occur through this post-translational activation of the nuclear factor. This was shown specifically in T-cells (Sen and Baltimore, 1986) and monocytes (Hass et al., 1992). If exogenous zinc was to have a direct effect on the nuclear factor activity under inflammatory conditions it would have to act on the post-translational NFκB molecule and not by stimulation of NFκB synthesis. As NFκB requires the zinc atom to co-ordinate DNA binding it seemed reasonable to determine whether zinc could exert its cytokine modulating effect by direct association with the active or inactive transcription factor. This possible mechanism was supported in the literature by the *in vitro* inhibition of NFκB DNA binding by zinc chelators and reconstitution of NFκB DNA binding ability by addition of zinc (Otsuka et al., 1995). The results obtained in the present study of cellular zinc uptake indicated that exogenous zinc was not directly associated with the NFκB molecule or with any other molecule of similar size over the time period required for cytokine activation. It is possible that the detection methods were not sensitive enough to detect low levels of NFκB or similar proteins that may have associated with the exogenous labelled zinc. Instead, the results implied that, as expected for zinc recently entering into a cell, the radiolabelled zinc

was associated with low molecular weight proteins. The assay was optimised for detection of proteins between approximately 20kDa and 150kDa so it was not possible to accurately determine the size of the bands detected which were smaller than 10kDa. However, this size range would encompass metallothionein and CRIP, the two important intracellular zinc storage and transport molecules.

As the analysis of cellular uptake of radiolabelled zinc indicated that exogenous zinc did not appear to associate and activate NFκB directly, indirect stimulation by zinc, of the nuclear factor, was examined. These experiments were carried out to investigate whether application of exogenous zinc could increase levels of NFκB in the nucleus. The technique applied to determine NFκB levels was the probe of Western blots of nuclear extracts by an antibody specific to the p50 NFκB molecule. Ideally, the antibody should have detected one major protein band however, although the antibody was sold as monoclonal with little or no cross reactivity with other related proteins, in this study the antibody appeared to recognise proteins from a wide range of sizes. Proteins that are much larger or smaller than the p50 protein of interest were unlikely to interfere with analysis of the p50 NFκB molecule levels but there are components of the NFκB system that were of similar size, 49-65kDa. If these molecules ran close to the p50 molecule in the Western blot and were also recognised by the antibody, then they could interfere with observations of changes in band intensity. Even if this occurred, it may not have altered the eventual result as these proteins may have been unaffected by the zinc or LPS used to stimulate NFκB. In addition, p50 can act as a homogeneous but also a heterogeneous dimer, p50 binding to p65 or possibly to other sized monomers (Grimm and Baeuerle, 1993). Therefore these other subunits would also act as indicators of NFκB translocation to the nucleus.

The cells were activated with LPS, which is known to cause the translocation of the nuclear factor (Sen and Baltimore, 1986) and also with zinc to determine whether endogenous zinc had any effect on this translocation. Therefore, LPS should have increased the levels of NFκB in the nuclear extracts and hence the intensity of the detected protein band. The effect of zinc on the intensity of

that band was observed. The data obtained did appear to indicate a slight increase in nuclear NF$\kappa$B p50, however definite conclusions could not be made without quantitation requiring image analysis. Whilst not conclusive, the immunoblot results do agree with the published literature which has recently shown a level of control over NF$\kappa$B activation by zinc. This topic is discussed below in relation to cytokine activity as the cytokine experiments carried out in this study indicate that *in vitro*, zinc is able to modulate cytokine secretion. It seems likely from the recent literature and the indication obtained from the current immunoblot experiments that this ability of zinc to modulate cytokine production and secretion is based on its ability to control the activity of NF$\kappa$B.

The cytokine experiments led in two directions, the first examined the effect of zinc on the cell based bioassays used to measure cytokine production and the second direction investigated the effect of zinc on cytokine production by the THP-1 and Jurkat T-cells cells. In only one publication found in a search of the relevant literature was there a mention of the effect of zinc on the commonly used bioassays for cytokine secretion. This seemed strange, in that zinc is such a ubiquitous ion and is required by cells for so many functions, that zinc levels could vary significantly depending on the indicator cell line and the test cell line. Cell bioassays are generally similar in methodology. Briefly, a test cell line is stimulated in order to secrete a specific substance into the culture medium, for example THP-1 cells stimulated by LPS to secrete TNF$\alpha$. That medium is then sampled and either centrifuged or filtered to remove particulate matter. The sample of medium containing the secreted substance is then place into a well containing indicator cells which are sensitive to the effects of this substance, for example murine L929 fibroblast cells which are sensitive to the cytotoxic effects of TNF$\alpha$. The indicator cells are either killed, as in the above example, or proliferate in the presence of the secreted substance, for example murine CTLL T-cells in the presence of IL-2. These two examples, the L929 assay and CTLL assay, were used in the present study and it seemed very strange that they had apparently not previously been tested for the effects of zinc. Zinc is known to protect against cell death, which could possibly affect TNF$\alpha$ cytotoxicity, and zinc is well known to modulate many aspects of IL-2

activity in T-cells, which could affect the CTLL assay.   In fact zinc was shown in this study to have significant effects on both assays.

When the standard concentration-effect curve of TNF$\alpha$-induced L929 cell death was carried out in the presence of either 25$\mu$M or 50$\mu$M zinc, there was found to be a change in the levels of cell death.   This change was present most importantly along the linear part of the curve for both concentrations, but in the case of the higher concentration, along the whole curve.   The higher concentration of zinc increased cell death at lower TNF$\alpha$ concentrations and protected against cell death at higher concentrations.   Therefore, if the zinc concentration of a sample were to be around 25$\mu$M then these results indicate that the L929 assay could show the TNF$\alpha$ concentration in the sample to be lower than it truly was.   If the sample zinc concentration was above 50$\mu$M, then the cytotoxicity of low TNF$\alpha$ levels in the sample may be masked in the L929 assay by the cytotoxic effect of the high sample zinc concentration.   Both concentrations are well within the ranges of zinc concentration used to study the effect of zinc on cytokine production *in vitro* (for examples see Fady et al., 1995, Dreissen et al., 1995).   For the TNF$\alpha$ bioassay it appears that zinc levels can have quite a wide range of effects, the CTLL assay was also affected by zinc concentrations similar to those used in many experiments.   No alteration in IL-2-induced CTLL cell proliferation was seen up to 30$\mu$M zinc, but at 60$\mu$M and 90$\mu$M zinc no cell proliferation was seen.   In fact, on visual examination the majority of cells appeared dead.   As samples are diluted 1:1 when added to the wells in the bioassay, 60$\mu$M and 90$\mu$M would correspond to 120$\mu$M and 180$\mu$M zinc in the original samples.   Although the latter concentration is probably near the top range of zinc concentrations used for *in vitro* study, the former is well within commonly used test levels for zinc stimulation of cells.   Whilst the study of the effects of zinc on cytokine stimulation *in vitro* is still relatively new with few publications, especially relating to the common cell culture models such as THP-1 or Jurkat T-cells, bioassays will be used in conjunction with ELISA analyses.   The results presented here indicate that not only must the zinc concentration of samples be taken into account but also they strongly suggest that the effects of zinc on the indicator cell lines, L929 and CTLL, should be studied more closely.   In addition, these

results strongly indicate that other bioassays should be examined for the effects of zinc concentration. These would include, for example, the WEHI assay for TNFα (Espevik and Nissen-Meyer, 1986), the B9 assay for IL-6 and D10 assay for IL-1 (Helle et al., 1988) and the HT-2 assay for IL-2 (Gieni et al., 1995).

After sample content of zinc was taken into account, the L929 assay results obtained from the zinc and LPS stimulation of the THP-1 monocyte cell line were particularly interesting. A bimodal effect was observed for increasing zinc concentration. The L929 assay indicated that supernatants of LPS-stimulated THP-1 cells incubated with zinc (6.25-12.5μM) contained significantly less TNFα than supernatants from cells incubated with LPS alone. In apparent contradiction, supernatants from LPS-stimulated cells incubated with higher concentrations of zinc (25-50μM) contained significantly more TNFα than cells incubated with LPS alone. The recent literature examining the effects of zinc on NFκB activation and function appear to support the observation of bimodal effects by zinc on cytokine secretion.

Wellinghausen et al. (1996a) showed that higher zinc levels, 50μM and 100μM, significantly increased TNFα and IL-1β production in LPS stimulated freshly isolated PBM cells. They showed that this increase in cytokine secretion might be due to alteration of the LPS molecular structure by zinc. This could account for the increase in TNFα production seen in the current study of the effects of zinc on TNFα production by the THP-1 monocytic cell line. However, they only showed this effect to be directional i.e. zinc cannot make LPS *less* effective, only *more* effective at inducing cytokine secretion therefore the bimodal effect of zinc must be through an unrelated mechanism.

It is possible that the decrease in TNFα secretion observed in the results from the present work was due to a membrane stabilising effect of zinc (Bettger and O'Dell, 1993) or possibly through alteration of some cytoplasmic signalling cascade involving NFκB (Shumilla et al., 1998, Kim et al., 1999). While both mechanisms are possible, as mentioned previously zinc has recently been

shown to mediate NFκB function as suggested by the results of the immunoprobe for NFκB presented in Chapter 4 .

NFκB exists as the inactive IκB/NFκB complex in the cell cytoplasm until it is degraded by phosphorylation of IκB by the IκB kinase (IKK). Exogenous zinc influx has recently been shown to inhibit NFκB activation (Connel et al., 1997). More specifically, zinc has recently been shown to inhibit IKK in LPS-stimulated monocytes, and so prevent the degradation of the IκB/NFκB complex (Jeon et al., 2000). This would in turn inhibit NFκB translocation to the nucleus and any subsequent promotion of cytokine synthesis. Zinc has also been shown by electrophoretic mobility shift assay (EMSA), to inhibit NFκB binding to DNA in nuclear extracts, albeit at high concentrations, >100µM (Shumilla et al., 1998). As the samples in this study were nuclear extracts, the NFκB in the EMSA was not associated with the cytoplasmic inhibitory molecule IκB (found in the cytoplasm not the nucleus), thus indicating a secondary point for zinc inhibition of NFκB activation.

In light of these publications (Wellinghausen et al. 1996a, Connel et al., 1997, Jeon et al., 2000, Shumilla et al., 1998) the TNFα bioassay results presented in Chapter 4 would seem to indicate that the synergistic effect of zinc on LPS activity is able to overcome the inhibitory effects of zinc on NFκB activity and that this effect is dose dependent. The effect of this interaction is seen in the inhibition or stimulation of TNFα production detected by the cytokine study. It is becoming increasingly clear that the role of free ionic zinc or zinc in the labile cellular pool is as important to cell function as enzymatic and structural zinc. Whether the zinc is free ionic zinc or whether it acts in a readily exchangeable state bound to metallothionein or CRIP remains to be determined. In either case the effects of supplementing this labile pool with exogenous zinc, in the form of ZMG or soluble zinc salts, are only beginning to be understood. New zinc-specific fluorescent indicators such as zinquin (Zalewski et al, 1993) or *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide, TSQ (Fredrickson et al., 1987) will be useful tools in investigating this pool.

As the active role of zinc in cell function is studied more widely the effects of ionic zinc and zinc metalloproteins, other than nuclear factors, on signal transduction and nuclear transcription will become clearer. An example of this role can be found when comparing the actions of zinc and metallothionein on control of apoptosis, programmed cell death. Zinc can inhibit apoptosis as shown by addition of exogenous zinc (Mathieu et al., 1996) or chelation of endogenous zinc (Ho et al., 2000) and as discussed above it can modulate NFκB activity (Otsuka et al., 1995). Similarly it has been reported that increasing cellular metallothionein levels will inhibit apoptosis (Kondo et al., 1997, Deng et al., 1999). In addition to this, metallothionein interacts with the p50 NFκB subunit and in doing so activates NFκB and possibly mediates DNA binding of the nuclear factor (Abdel-Mageed and Agrawal, 1998). Metallothionein and zinc have been shown to translocate to the nucleus at the same time during differentiation (Schmidt and Beyersmann, 1999, Apostolova et al., 1999) with cell lines containing higher levels of metallothionein displaying lower levels of apoptosis (Apostolova et al., 1999). It has also been shown that NFκB activation can, depending on stimuli, suppress apoptosis (Natoli et al., 1998, Mustapha et al., 2000). In light of these studies the question remains as to whether zinc can, for example, translocate to the nucleus and directly modulate NFκB to inhibit apoptosis, or whether it acts as part of the metallothionein molecule to the same end. This distinction is of importance to the action of novel zinc compounds. If the novel zinc compound directed zinc to a compartment where it is less likely to bind to metallothionein for instance, then the normal processing of exogenous zinc may be altered. This could result in the control by zinc of NFκB activation and subsequent cellular effects, being different depending on whether ZMG or free ionic zinc salts were used as the exogenous zinc source.

Use of the new zinc indicators for labile zinc will increasingly elucidate the cellular processing of labile zinc not only from common zinc salts but especially from novel zinc compounds such as ZMG or polaprezinc. This rapidly exchangeable and transportable fraction of zinc will almost certainly prove to be of utmost interest in relation to the novel zinc compounds. To continue the apoptosis and metallothionein example, metallothionein itself is inducible by

zinc through a metal responsive element on the metallothionein gene (Pulido et al., 1966). Cultured cells in the studies of Pulido et al., (1966) show an upregulation of metallothionein synthesis when incubated with zinc salts. As shown in this thesis soluble zinc salts provide a low level of cellular zinc whilst ZMG can provide a far higher level of zinc. This alone should be expected to cause a difference in levels of metallothionein gene induction with more zinc likely to reach the nucleus from ZMG. However the cellular activity should not be seen as unchanging. In fact ZMG provides a higher level of zinc that gradually falls over at least the 24 hour period examined in this study while soluble zinc salts maintain the low zinc concentrations over the same period. Thus ZMG could be expected to maintain metallothionein gene induction for longer and possibly at a higher level than simple zinc salts. This might result in greater protection by ZMG against apoptosis through the mechanisms described previously, than would be afforded by simple zinc salts. The importance of this labile pool is really only now beginning to be understood with the apoptotic control mechanisms discussed above representing just one facet of labile zinc activity. If ZMG provides different levels of labile zinc, then it is reasonable to assume that not only might the apoptotic response be different but, due to the ubiquitous roles of zinc (especially those relating to nuclear factor activation), the inflammatory and immune responses would be different. It then becomes particularly interesting to examine the relative effects of ZMG compared to zinc salts on the inflammatory and immune responses of cells *in vivo*.

Despite the generalised functions of zinc in the body as a whole, it is the surprising capacity for zinc to target and modulate specific cell activation pathways that makes the construction and function of novel zinc compounds particularly interesting. Whilst the unique properties of ZMG, that provide a higher bioavailability of zinc to cells, may be somewhat masked by dissolution in the extreme conditions of the stomach, there is perhaps a good argument for enteric coating of ZMG in a tablet form to act further down the digestive tract. The stomach is an important part of the overall digestive system although less involved in nutrient absorption and it may be that the truly interesting actions of ZMG may occur in the less acidic portions of the intestine where ZMG may be

better able to maintain its unique polymeric structure. Although ZMG almost certainly maintains enough structural integrity to bind to the mucus and mucosal cells in the stomach as discussed previously, a bolus of the compound might be expected to last far longer without the hydrogen ion levels found in the gastric juice. This would leave any ZMG particles free for longer to interact with the intestinal mucosa. This is interesting in general because the mucosa is a rapidly changing barrier for the highly dynamic and responsive intestinal tissue and zinc participates in rapid changes of cell function.

Of particular relevance to the present study would be the effect of intact ZMG particles on the immune cells of the gut-associated lymphoid tissue (GALT). The GALT is a discrete immune 'organ' in different species and acts as the interface with, and main line of defence against, infection from pathogens in the digestive tract (Hall, 1979, Ermak et al., 1990). It is organised into a network of lymph ducts linking external nodes such as the tonsils, adenoids, appendix and Peyer's patches to the lamina propria (Parham, 2000, King et al., 1997). Briefly, the nodes, such as the Peyer's patches, contain discrete T-cell and B-cell areas, and a germinal centre. Pathogens are transported across the mucosa and are processed by lymphocytes that enter the Peyer's patches from the general circulation (Parham, 2000). A search of the literature for publications on zinc in relation to GALT or Peyer's patches revealed nothing, however the evidence for zinc modulation of lymphocyte activity and T-cell function in particular is strong and has been presented above and in Chapter 1. It has also been shown that there is a subset of T-cells, intestinal intra-epithelial lymphocytes (IEL), which develop and mature outside of the thymus (Poussier and Julius, 1994). The site of lymphopoeisis for these cells is, as their name suggests, in the intestinal GALT. It was found that adult mice, irradiated or thymectomized and then irradiated, still recovered IEL T-cell numbers despite the lack of a working thymus (Yoshikai et al., 1991, Poussier and Julius, 1994).

Zinc is intimately linked with T-cell maturation and differentiation in the thymus and changes in body zinc levels can result in changes in T-cell subset production (Prasad et al., 1997, Oksel and Tanell, 1996, Mocchegiani et al.,

1999, Coto et al., 1992, Savino et al., 1984). For this reason alone it would be of interest to observe the effect of general zinc compounds on the maturation and differentiation of IEL. However, the results obtained for cellular zinc uptake from ZMG suspension in the present study, and the possibility that this uptake could lead to a localised increase in tissular zinc concentration, indicate that the compound might act as a particularly efficient zinc delivery system to immune cells beyond the mucosa. A search of the current literature for any relation of zinc levels to IEL revealed only one publication, in Portuguese (de Amorim et al., 1984). The abstract stated that the IEL population was decreased after a four week zinc deficient diet when compared to rats fed a normal diet with zinc supplements (de Amorim et al., 1984). So it seems that zinc levels *may* modulate IEL in the GALT, which seems a reasonable assumption given the body of evidence relating to zinc and thymic T-cell activity. The evidence that ZMG may bypass some of the normal cellular zinc uptake mechanisms suggests that it might be more effective than common zinc salts in upregulating IEL activity during zinc deficiency.

Although the IEL in GALT have been described here as a possible target for exogenous zinc control they are only one of several areas that have yet to be investigated but which would be worthy of examination in relation to ZMG and other zinc compounds. Recent evidence has indicated that the innate immune response of intestinal epithelial cells to bacterial infection *in vitro* is centrally mediated by NFκB which controls the expression of pro-inflammatory genes in these cells (Elewaut et al., 1999). The complex control of NFκB activation by both exogenous zinc and metallothionein suggests that this may be another specific target for exogenous zinc compounds to exert control over the gastric immune and inflammatory response. Again, a study of the relative effects of ZMG and other zinc compounds on prophylactic and acute zinc treatment of such infected cells would seem to be a worthwhile investigation.

The study of zinc and its involvement in the development and function of the immune system response to inflammatory insult and host defence is still in its relative infancy. Even more generally, cellular zinc specific transport mechanisms have been described, and the importance of the labile zinc pool

has been understood, really only in the last ten years. During this time it has become clear that zinc in structural and catalytic roles, but especially as the rapidly translocated and exchangeable labile zinc pool, is intimately involved in the response of immune cells to inflammatory insult. Zinc compounds such as ZMG, polaprezinc and zinc acexamate, which are able to bypass normal zinc transport and control mechanisms, might be expected to affect immune cells in ways that ionic zinc salts could not, due to the strict control of cellular ionic zinc flux. It may well be that the difference between the effects of ZMG administration compared to zinc salt administration *in vivo* is, as this thesis supports, the delivery by ZMG of zinc in higher concentration to cells than is possible with zinc sulphate for example. This does not exclude the possibility, as suggested, that in bypassing normal zinc uptake mechanisms ZMG may specifically direct ionic zinc to cytoplasmic compartments that simple zinc salts could not. The presence of a putative soluble fraction of ZMG as indicated in this thesis would increase this likelihood. The most important aspect of ZMG may well be seen in whole tissue studies of zinc uptake where the highly efficient cellular zinc delivery by the polymer could lead to far higher tissue zinc levels beyond the mucosa than would be achievable with zinc salts. This would certainly have an impact on the local cellular environment of the gastrointestinal tract whether or not the increased zinc levels were transferred into the general circulation.

The results presented in this thesis provide new information about the chemical structure of ZMG and the release of ionic zinc or a possible soluble ZMG component from the ZMG polymer that is in support and continuance of published data. The biological studies described in this thesis have shown that ZMG provides a greater level of bioavailable zinc without increased toxicity to the THP-1 monocyte or Jurkat T-cell lines when compared to common zinc salt. Aside from the actions of ZMG the data presented currently provides information on the action of ionic zinc in the THP-1 monocyte, Jurkat T-cell and HGT-1 gastric epithelial cell lines and also on the function of cell bioassays. In particular, the observation that the HGT-1 cell line is resistant to zinc uptake and is particularly sensitive to increased zinc levels may be of great relevance to the use of this line as a gastric cell model. The results from the cytokine

studies showed in detail for the first time that sample zinc levels may alter the sensitivity of the commonly used L929 bioassay for TNFα and the CTLL bioassay for IL-2. The cytokine studies also revealed a possible bimodal action of exogenous zinc on TNFα secretion in THP-1 cells. This observation is supported by the data published on the differential control of NFκB activation by zinc in LPS stimulated cells and the resulting modulation of cytokine production. As a whole, the data presented in this thesis expand on and agree with the current knowledge of zinc in general and ZMG in particular.

- **HPLC analyses of ZMG in suspension.** Release of both zinc and glycerol by ZMG in suspension should be examined over longer periods until, in both cases, maximum release is determined. In addition, more time points between 0min and 240mins should be taken to more accurately show the linear phase of both zinc and glycerol release.

- **Analysis of unknown soluble component of ZMG.** The unknown peak observed in the analysis of glycerol release by ZMG should be identified. The fractions containing the unknown element could be collected and dried. The sample could then be analysed by MALDI-ToF and ICP-MS to determine mass and composition. The release of this component by ZMG in aqueous suspension could then be quantified.

- **Analysis of ZMG degradation by MALDI-ToF.** MALDI-ToF could be used to determine the changes in ZMG polymer length over time. ZMG would be incubated in aqueous suspension and filtered, as for the HPLC analyses, and the residue analysed. The mass peaks in the polymeric traces presented in this study would be expected to shift to the left as they released zinc and glycerol.

- **Cellular uptake of zinc – total zinc analyses.** It would be desirable to confirm cellular zinc uptake results obtained by ICP-MS by increasing experiment repetition number. This may show differences between zinc compounds that may have been masked by low 'n' values, especially the difference between ZMG, zinc oxide and zinc oxide plus glycerol.

- **Cellular uptake of zinc – labile zinc analyses.** A closer analysis of ZMG, zinc oxide and zinc salt by cell lines between 0min-120mins, the initial zinc uptake period, would provide useful information. Of particular interest would be observation of zinc uptake into the vesicles, whether this develops at the cell membrane or within the cytoplasm. The former would support endocytosis as a probable uptake mechanism, the latter would indicate vesicular zinc sequestration prior to excretion of the excess zinc.

- **Cellular uptake of ZMG – zinquin analysis.** The localisation of putative ZMG particles into vesicles was preliminarily examined in the present study using ethidium bromide and FITC-dextran. The continuation of this would

expand on the fate of zinc from ZMG once inside the cell. As discussed previously, an acidic vesicular environment might encourage zinc release from a ZMG particle, whilst in a neutral vesicle the zinc release may be slower. An uptake marker such as FITC-dextran would also indicate whether the vesicular zinc was derived from external endocytosed zinc or cytoplasmic zinc. Zinc and dextran rapidly co-localised to vesicles would suggest that they were entering the cell at the same time. The initial presence of FITC-dextran alone followed by increasing zinquin fluorescence in vesicles would indicate that zinc was not being taken up at the same time but instead that it were entering from the cytoplasm.

- **Cellular uptake of ZMG – radiolabel analysis.** As a direct continuation of the radiolabelled ZMG analysis, it had been intended to label ZMG with $^{14}$C-glycerol to examine the fate of glycerol released from ZMG during cellular uptake. Using scintillation counting of cells it could be possible to quantify the labelled glycerol content of cells and compare with cells incubated with the $^{65}$Zn labelled ZMG. It may then be possible to determine whether glycerol uptake equalled zinc uptake from ZMG, which would indicate cellular uptake of ZMG particles as opposed to free zinc and free glycerol.

- **Tissue uptake of ZMG.** It is important that the cell uptake profile of ZMG be studied with tissue and *in vivo* experiments. Whilst the *in vitro* cell studies can suggest mechanisms for ZMG uptake, the variable conditions of cells in whole tissue cannot be easily recreated in culture. The difference between gastric absorption of ZMG compared to intestinal absorption of the compound is likely to be quite different given the effect of hydrogen ion concentration on ZMG zinc release. Given the increased bioavailability of zinc from ZMG it would be interesting to compare the uptake rates of zinc from the compound or from zinc salts using *in vivo* models of zinc deficiency. The present cellular zinc uptake results suggest that ZMG would be more efficient than zinc salts at providing zinc as a dietary supplement however correlation between cell culture and *in vivo* treatment may not be exact. This would support the need for analysis of ZMG uptake in gastric tissue preparations or *in vivo* uptake experiments.

- **Cellular treatment of exogenous zinc.** Both THP-1 cells and Jurkat T-cells reduced the high levels of zinc from ZMG over a 24hr period. Recently it has been suggested that metallothionein may act as a barrier to zinc

uptake in gastric cells (Davis et al., 1998). CRIP, expressed in immune cells may interact with metallothionein and has been suggested to participate in modulation of immune and inflammatory response (Fernandez et al., 1997, Hempe and Cousins, 1992). If the excess exogenous cytoplasmic zinc is bound or actively transported from immune cells in particular then it is probable that one of these two proteins is involved. Antibody labelling of metallothionein or CRIP could determine whether there is directed translocation of either molecule during the reduction of zinc levels.

- **Effect of exogenous zinc on NFkB activation.** The antibody probe of Western blots in the current study indicated that NFkB activation and translocation to the nucleus after stimulation by LPS, could be modulated by exogenous zinc. The results were inconclusive due in part to the possibility of cross reactivity. The same experiments should be carried out using EMSA as indicated in the Discussion section. This relies on the binding of specific labelled nucleotide sequences to the activated nuclear factor and is highly specific.

- **Analysis of HGT1 cells.** The lack of quantifiable zinc uptake by this cell line suggests that it is not the best model cell line for analysis of all gastric cell functions. As gastric epithelial cells, however, this line may provide an interesting model for the analysis of zinc control mechanisms. Determining the metallothionein, CRIP and zinc transporter expression in these cells may elucidate novel zinc control mechanisms for this zinc sensitive cell line.

- **Zinc modulation of cytokine stimulation.** Zinc was shown to modulate bimodally the secretion of TNFα in the THP-1 cell line in agreement with what is know about the effect of zinc on NFkB activation by LPS. These results support further study of this differential effect.

- **Zinc modulation of cell bioassays.** Zinc was shown in the present study to affect bioassay results at concentrations used for the *in vitro* study of zinc. It likewise has the potential to interfere in the study of other zinc bearing molecules such as insulin. If zinc or zinc bearing molecules are tested on a cell line for effect on secreted cytokine, and the medium is then tested on a bioassay specific for that cytokine then the zinc content remaining in the media sample could affect the result obtained. This suggests that the effect

of zinc ion should be tested on every cell bioassay commonly used, especially for the study of the action of zinc on cytokine secretion.

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