The effect of hyperthermia on the phosphoinositide signalling system of tumour cells

Kwong, Wing Yee

The effect of hyperthermia on the phosphoinositide signalling system of tumour cells

by

Wing Yee Kwong
(B.Sc. London)

A thesis submitted in candidature for the degree
of Doctor of Philosophy

Department of Biological Sciences
The University of Durham
(Graduate Society)
1994

給父母親

Abstract

The effect of heat on the phosphoinositide signalling pathway was investigated in CHO-K1 cells and WRK-1 cells. Heat caused a decrease in 1,2-diacylglycerol (1,2-DAG) levels but did not have any effect on monoacylglycerol (MAG) levels in both cell types. On the other hand, an increase in triacylglycerol (TAG) level was observed in both cell lines. This heat-induced decrease in 1,2-DAG level in WRK-1 cells was not due to an increase in turnover rate of 1,2-DAG to phosphatidic acid (PA) since the decrease in 1,2-DAG was not affected when cells were heated in the presence of the DAG kinase inhibitor, dioctanoylethylene glycol (diC_8EG). The increase in TAG level may be due to a rapid, heat-induced increase in TAG synthesis from 1,2-DAG, thus leading to decreased levels of 1,2-DAG. Heat also led to an increase in inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) but not inositol monophosphate ($InsP_1$) or higher inositol phosphate ($InsP_{4/5/6}$) levels in WRK-1 cells. The increase in $InsP_2$ and $InsP_3$ was both temperature and heating time-dependent. A transient increase in $InsP_3$ was observed at 11 min, and did not require extracellular calcium nor did it depend on the heat-induced increase in cytosolic free calcium ($[Ca^{2+}]_i$). The magnitude of the heat-induced increase in $InsP_3$ was comparable to that obtained upon incubation in AlF_4^-. Stimulation of WRK-1 cells with vasopressin at 45°C distorted the pattern of inositol phosphate metabolism. However, the vasopressin-sensitive phosphoinositide signalling pathway remained intact after a severe heat shock, sufficient to lead to the death of greater than 95% of the cells. Heat also led to an increase in $[Ca^{2+}]_i$ in WRK-1 cells which came primarily (solely?) from calcium influx from the extracellular medium. This influx was unlikely to occur through voltage-gated calcium channels because calcium channel blockers, such as La^{3+} and nifedipine, did not inhibit the heat-induced elevation in $[Ca^{2+}]_i$. This heat-induced increase in $[Ca^{2+}]_i$ may have a protective role in hyperthermic cell death.

Acknowledgement

I would like to thank the North of England Cancer Research Campaign for financial support. I would like to express my thanks to Dr. R. Manning and Professor K. Bowler their helpful advice and help in all respect of research and critical reading of the manuscript.

I would also like to thank Dr. C.J. Barker for providing the WRK-1 cells, Dr. D. Hyde for advice in calcium experiments and Dr. J. Anstee for allowing me to use Fig-P graphic package on his computer to generate HPLC chromatogram. I am also grateful to Dr. P. Humle for advice in statistics.

I would also like to express my thanks for Miss J. Chambers for her technical assistance and Mr. J. Gilroy for his help in HPLC.

Furthermore, I would also like to thank J. Cunningham for doing the pH measurement of the calcium calibration buffers, E. Jones for assistance in one of the critical experiment. A. Hong and J. Kwong for their help in producing some of the diagrams, D. Peries and A. Baloch for introducing me to Cricket Graphic and Microsoft Word package and M. Cuculescu for advising me SPSS statistical package and Quattro Pro spreadsheet package. Furthermore, I would also like to thank the staffs of the Computer Centre.

Finally I have to thank all my friends for their encouragement during the writing up this thesis.

Contents

List of Figures

List of Tables

Glossary

BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Cytosolic free calcium concentration
cAMP	adenosine 3'5'-cyclic phosphate
1,2-DAG	sn-1,2-diacylglycerol
DAG	Diacylglycerol (unspecified isomer)
DiC_8EG	Dioctanoylethylene glycol
DMSO	dimethylsulphoxide
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
FBS	Foetal bovine serum
Gpp(NH)p	5'-guanylylimidodiphosphate
GroPIns	Glycerophosphoinositol
GroPIns4P	Glycerophosphoinositol 4-phosphate
GroPIns 4,5-P_2	Glycerophosphoinositol 4,5-bisphosphate
GTPγS	Guanosine 5'-o-(3-thiotrisphosphate)
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	High performance liquid chromatography
Ins(1)P_1	Inositol 1-phosphate
Ins(4)P_1	Inositol 1-phosphate
Ins(1:2cyc)P	Inositol 1:2-cyclic phosphate
Ins(1,3)P_2	Inositol 1,3-bisphosphate
Ins(1,4)P_2	Inositol 1,4-bisphosphate
Ins(1:2cyc,4)P_2	Inositol (1:2-cyclic,4) bisphosphate
Ins(1,3,4)P_3	Inositol 1,3,4-trisphosphate
Ins(1,4,5)P_3	Inositol 1,4,5-trisphosphate
Ins(1:2cyc,4,5)P_3	Inositol (1:2-cyclic,4,5) trisphosphate
$InsP_3$	unresolved isomers of inositol trisphosphate
Ins(1,3,4,5)P_4	Inositol 1,3,4,5-tetrakisphosphate
Ins(1,3,4,6)P_4	Inositol 1,3,4,6-tetrakisphosphate
Ins(3,4,5,6)P_4	Inositol 3,4,5,6-tetrakisphosphate
Ins(1,3,4,5,6)P_5	Inositol 1,3,4,5,6-pentakisphosphate
Ins(2,3,4,5,6)P_5	Inositol 2,3,4,5,6-pentakisphosphate
$InsP_6$	Inositol hexakisphosphate

MAG	Monoacylglycerol
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
PA	Phosphatidic acid
PCA	Perchloric acid
PI-PLC	Phosphoinositide-specific PLC
PKC	Protein kinase C
PtdCho	Phosphatidylcholine
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdSer	Phosphatidylserine
PTX	Pertussis toxin
r_{av}	Average radius of rotation
TAG	Triacylglycerol
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TMB-8	8-(diethylamino)-octyl 3,4,5-trimethoxybenzoate

Materials

All regents were of analytical grade unless stated otherwise.

Amersham International plc. Amersham, Buckinghamshire.
myo[2-^{3}H]inositol

BDH Ltd., Aycliff Industrial Estate, Durham.
Acetic acid
Ammonium formate
ammonium phosphate (HiPerSol grade)
Chloroform
Methanol
diethyl ether
Ethyl acetate
Perchloric acid
Trichloroacetic acid
Orthophosphoric acid (HiPerSol)

BioRad, Mayland Ave., Herts.
AG1x8 formate form, 200-400 mesh resin

B.O.C. Ltd., Vigo Lane, Birtley Co., Durham.
Oxygen-free nitrogen
Carbon dioxide

Cambridge Bioscience, Cambeidge.
Fura-2/acetoxy methylester (Fura-2/AM)
Fura-2 free acid
Pluronic acid

Canberra Packard, Pangbourne, Berks.
Ultima Flo AF
Pico-hang in vials

Coulter Electronics Ltd., Luton, Bedfordshire.
Isoton II

Flow Laboratories Ltd., Richmansworth, Hertfordshire.
Ca^{2+}, Mg^{2+}-free PBS
Eagles Minimum Essential Medium (EMEM)
Ham's F12
L-glutamine
Non-essential amino acids
Penicillin/streptomycin solution
Sodium bicarbonate (7.5% solution)
Trypsin 2.5% (w/v)

Gibco, Paisley, Renfrewshire.
Inositol-free EMEM medium
Glucose
i-inositol
L-arginine
L-leucine
L-methionine
Di-sodium cysteine

Medicell Internal Ltd., London
Spectra/por 6 molecular porous dialysis membrane with molecular cut off at 3000

National diagnostics, Hessle, Hull.
Ecoscint A
Uniscint BD

NEN, Stevenage, Herts.
[2-^{3}H]glycerol
[5,6,8,11,12,14,15-^{3}H]arachidonic acid
[^{3}H]inositol(1,3,4)P_3
[^{3}H]inositol(1,3,4,5)P_4
[^{3}H]inositol hexakisphosphate

Sera Laboratory, Crawlley Down, Sussex.
Foetal bovine serum

Tocris Neuramin Ltd., Bristol.
[^{3}H]inositol(1)P
[^{3}H]inositol(1,4)P_2
[^{3}H]inositol(1,4,5)P_3

Whatman, Maidstone, Kent.
Partisphere SAX column
Partisphere WAX column
Pellicular guard column

All other regents were obtained from Sigma Chemical Co., Poole, Dorset.

Chapter 1

General Introduction

Hyperthermic treatment of mammalian cells is defined as an increase in the environmental temperature greater than 37°C, often in the range 40-45°C, by external means. The use of elevated temperature to treat cancer dates back at least 5000 years — the Edwin Smith papyrus showed a fire-drill used by surgeons to cauterise carcinoma of the breast. Later, it was discovered that prolonged fevers due to a variety of infections may lead to regression of tumours. However, the use of hyperthermia in cancer therapy was not widely employed until recently as a result of the development of modern technology such as microcomputer-controlled, microwave generators and new forms of thermometry. With the present technique, hyperthermia used alone can cause complete clinical regression in 10-15% of tumours, but relapse occurs in the majority of cases. The greatest potential for use of hyperthermia in tumour therapy at the present time appears to be in combination with chemotherapy or radiation in order to give an enhanced effect. It has been shown that heat enhances the effectiveness of many chemotherapeutic drugs such as nitrosoureas, cisplatin and bleomycin (Hahn, 1979). The increased effect seen by combining cytotoxic agents with hyperthermia is complex, but it has been suggested to be due to altered drug pharmacokinetics such as increased solubility, altered plasma membrane protein binding and activation of enzymatic processes (Vernon, 1992). Cells at different stages of the cell cycle respond differently to hyperthermia, with the most sensitive part being in the late S phase, which is generally the most radioresistant, again suggesting that hyperthermia might be both additive and complementary to radiation therapy (Westra and Dewey, 1971).

Some workers have suggested that tumour cells show differential thermal

sensitivity compared to their normal counterparts, but this is controversial. Early studies suggested that tumour cells are more sensitive to elevated temperature than normal tissue cells (Cavaliere *et al.*, 1967; Levine and Robbins, 1970; Giovanella *et al.*, 1976). In contrast, comparison of the effect of heat on normal liver cells and hepatoma cells did not show any difference in response to high temperature (Harisiadis *et al.*, 1975). Similarly, the heat sensitivity of 7 x-ray transformed C3H10T1/2 cells was comparable to normal C3H10T1/2 cells (Raaphorst *et al.*, 1985). Nowadays, it is believed that the differential thermal sensitivity of tumour and normal tissue *in vivo* is due to the difference in vascular supplies between tumour and normal tissue. Blood supply in tumour tissue is more disorganised and more sluggish than in the normal tissues, which gives rise to poorer cooling mechanisms (Field, 1987). The deprivation of blood supply also results in an inadequate supply of oxygen and nutrients as well as the failure to remove the by-products of metabolism, leading to accumulation of lactic acid (low pH) which also sensitises the cells to heat (Field, 1987; Vernon, 1992).

Studies with mammalian cells cultured *in vitro*, such as CHL V79 cells (Raaphorst *et al.*, 1979), EMT6 cells (Leith *et al.*, 1977), CHO cells (Gerweck, 1977; Westra and Dewey, 1971) and HeLa cells (Gerner *et al.*, 1976; Bhuyan *et al.*, 1977), appear to show different heat sensitivities. For example, LD_{90} values of CHO cells heated at 43°C were reported to be 94 min (Bhuyan *et al.*, 1977) and 83 min (Gerweck, 1977). The LD_{90} values of HeLa cells upon 43°C treatment were 150 min (Gerner *et al.*, 1976) and 86 min (Bhuyan *et al.*, 1977). However, even the same cell lines studied in different laboratories seem to have different heat responses. In order to investigate if the difference in thermal sensitivity is due to different inherent cellular properties or different culture conditions used in different laboratories, the thermal sensitivities of 7 established cell lines that were derived either from different species or from the same type of tissue within the same species, were grown and heated under the same conditions (Raaphorst *et al.*, 1979). A variation in thermal sensitivity was observed between

different cell lines, and it was concluded that it was impossible to predict the heat sensitivity of a specific cell line based on its origin. Furthermore, the thermal sensitivity of cells is also affected by the nutrient conditions such as the amount of glucose and availability of oxygen, the pH of the medium and stage of the cell cycle (Hahn, 1982).

It was shown that cells heated under hypoxic conditions were more sensitive to hyperthermia than cells heated under normal conditions (Hahn, 1982). Enhancement in thermal sensitivity was also observed in CHO cells chronically deprived of serum (Hahn, 1974). The effect of extracellular pH on thermal sensitivity has been investigated in at least 10 different cell lines, including rat (Dickson and Oswald, 1976), mouse (Overgaard, 1976b), Chinese hamster (Gerweck, 1977) and human cells (Gerweck and Richards, 1981). All of these cells showed an increase in thermal sensitivity when the pH of the medium was reduced. For example, when CHO cells were exposed to 42°C for 4 h, it was found that thermal sensitivity was not significantly affected when cells were heated in medium at pH values of 7.1-7.6, but thermal sensitivity increased as the pH of the medium decreased below 7.1 (Gerweck, 1977). However, the relationship between extracellular pH and thermal sensitivity was not fully understood at that time. Later it was reported by Chu and Dewey (1988) that the relationship between thermal sensitivity and pH upon heat treatment of CHO cells at 43.5°C for 55 min (a heat dose that killed 90% of cells) was most strongly correlated with intracellular pH (pH_i) rather than extracellular pH, especially when extracellular pH (pH_e) was below 7.1. In contrast, no correlation between pH_i and cell death was observed in human epidermoid A-431 cells (Kiang *et al.*, 1990). Exposure of A-431 cells to 45°C for 10 min, the pH_i decreased from 7.42 to 7.22 but no cell death was observed as assayed by trypan blue exclusion. The discrepancy between these 2 observations could be due to the difference in heat dose as well as the method chosen for pH_i measurement. In CHO cells, pH_i was determined by the distribution of [2-^{14}C]5,5-dimethyl-2,4-oxazolidinedione within the cells whereas the pH_i of A-431 cells were monitored in 2',7'-bis(carboxyethyl) carboxyfluorescein-

loaded cells by spectrofluorimetry (Kiang *et al.*, 1990).

One of the principal motives that rekindled interest in hyperthermia as an adjuvant cancer treatment modality was the observation that the stage of the cell cycle responding to maximum thermal sensitivity was complementary to that of radiation sensitivity (Westra and Dewey, 1971; Read *et al.*, 1984). The thermal sensitivity at different stages of the cell cycle was demonstrated in CHO cells (Westra and Dewey, 1971). Taking the advantage of the reduced ability of the rounded mitotic cells to adhere to their growth surface, the mitotic cells were dislodged by vigorous shaking. The collected mitotic cells were then cooled at 4°C to arrest the forward passage in the cell cycle. Once the cells were returned to 37°C, the progression of the cell cycle initiated again. At specified times after returning to 37°C, cells were exposed to 45.5°C for various periods. It was found that the most heat-resistant cells appeared to be those in early G_1 and the most sensitive were mitotic and late S phase cells. Since S phase is generally the most radioresistant phase, this suggests that hyperthermia might be both additive and complementary to radiotherapy in tumour treatment. The dependency of the thermal sensitivity on cell cycle stage was also observed in synchronised CHO cells (Bhuyan *et al.*, 1977), L1210 cells (Bhuyan *et al.*, 1977) and EMT6 cells (Leith *et al.*, 1977).

The most commonly accepted end point of hyperthermic cell death is the loss of reproductive ability of the cells. Hence, the clonogenic assay is the most commonly used method for the determination of cell death (Puck *et al.*, 1955), in which a known, constant number of cells (e.g. 600 cells) is seeded into a number of flasks and the cells are then heated. About 7-10 days after heating, the number of colonies is counted and the percentage of cell death can be calculated. Each colony is believed to arise from replication of a single surviving cell. When the percentage of survival is plotted against duration of heating at a fixed temperature, using a log-linear scale, a survival curve is obtained (Figure 1.1). The curve is generally characterised by a shoulder region followed

by a log-linear region for longer exposure time. This type of survival curve has been observed in almost all cell lines except for HeLa cells in which a shoulder was absent (Gerner *et al.*, 1976). The existence of the shoulder region has been suggested as a measure of the ability of the cells to sustain sublethal damage (Hahn, 1982).

When CHO cells were heated at temperatures below 43°C, a slightly different pattern of the survival curve was observed (Hahn, 1982). The initial portion of the survival curve was similar to the one described above, i.e. a shoulder region followed by a log-linear region. However, at longer heating times, the survival curve becomes concave upward and forms a third phase which has a much shallower slope. This phenomenon was suggested to be due to either a difference in thermal sensitivity within a mixture of populations (e.g. cells at different stage of the cell cycle) or, more likely, some cells were able to induce thermal resistance during longer exposure to heat. This phenomenon is termed 'thermotolerance', and is the development of a transient thermal resistance in cells or tissues. Thermotolerance can be induced by 2 ways, depending upon temperature and heating duration. When cells are heated at temperatures below 43°C, thermotolerance is developed during continuous heating whereas, when cells are heated above 43°C, thermotolerance usually develops after the first heat exposure and cells or tissues become resistant to subsequent heat treatment (Bauer and Henle, 1979; Spiro *et al.*, 1982). Although extensive studies have been performed on cultured cells in order to find out the mechanism(s) underlying the induction of thermotolerance, this mechanism is still unclear. It has been found that appearance of thermotolerance is usually associated with the induction of a set of proteins, known as heat shock proteins (HSP), but the importance of HSPs in thermotolerance is controversial. It has been shown that microinjection of purified human HSP70 into CHO cells led to an increase in thermal resistance (Li *et al.*, 1991). By using heat-resistant mutants, it was demonstrated that these mutants expressed an elevated level of HSPs (Laszlo and Li, 1985). However, Smith and Yaffe (1991) showed that HSP induction is not required for thermotolerance

acquisition in yeast. It is worth mentioning that the role of HSPs is not only confined to protecting cells from heat shock but they are also involved in many other cellular functions. For example, the HSP70 family act in the cytosol to keep proteins destined for mitochondrial import in an unfolded, translocationally-competent state and act inside the mitochondria to accept the unfolded proteins as they are being transported (Dice *et al.*, 1991). The HSP70 family also helps to target specific proteins to lysosomes for degradation (Dice *et al.*, 1991). HSP60 facilitates the proper folding and assembly of the newly-transported proteins in mitochondria (Horwich *et al.*, 1991). HSP47 appears to be involved in collagen assembly (Hightower, 1991). HSP82 is required for steroid hormone receptors to achieve an activation-competent state and may help to tether some receptors such as the glucocorticoid receptor to the cytoskeleton (Lindquist and Craig, 1988).

Although extensive searching for the exact target(s) for hyperthermic cell death has been carried out at single cell level by using tissue culture, the critical target(s) (primary lesion site) and the molecular mechanism(s) of heat-induced cell killing remain unclear. This is due to the all-pervasive effect of heat. The identification of the primary lesion site of cell death is further complicated by the fact that the observed damage may not result from a direct effect of heat, but rather it may result from secondary and perhaps tertiary damage resulting from the 'knock-on' effects of primary damage (Bowler, 1987). In fact, at one time or another, it has been suggested that all the major cellular structures have been implicated as having a significant role in heat injury (Roti Roti and Laszlo, 1988).

Models for cellular heat injury have been proposed by Jung (1986) and Bowler (1987). Jung's model proposes that cell killing is a 2 step processes. Non-lethal damage occurs initially, which is then converted into lethal damage during continuous heating and results in cell death. The conversion of non-lethal to lethal lesions are proposed to be

random events and depend only on temperature. The model proposed by Bowler (1987) resembles the model proposed by Jung (1986), but it differs in that the primary lesion, which may or may not be irreversible, causes a cascade of secondary, and perhaps tertiary, damage with time (Figure 1.2).

Alterations in organelles such as lysosomes and mitochondria during heating have been reported by several groups and these organelles have been suggested as possible targets in heat-induced cell death (Hahn, 1982). Lysosomes are organelles which contain many different types of hydrolytic enzymes including proteases, nucleases, phospholipases, phosphatases and sulfatases and are responsible for intracellular digestion of macromolecules. Exposure of HeLa cells to 43°C for 2 h resulted in a decrease in the number of lysosomes (Heine *et al.*, 1971). An increase in lysosomal enzyme activity was found in heated mouse mammary tumours (Overgaard and Overgaard, 1972) and the destruction of lysosomes was observed in solid mouse mammary tumours heated *in vivo* at 42.5°C for 30 min (Overgaard, 1976a), indicating that heat-induced structural alterations may lead to functional alteration. However agents such as trypan blue, which is known to enhance the susceptibility of lysosomal membrane to damage, did not affect thermal sensitivity (Hofer *et al.*, 1979). Thus the involvement of lysosomes in heat-induced cell death is unclear. Since lysosomes are involved in the destruction of dead cells, the changes in lysosomal activity may well be a consequence rather than a cause of heat damage.

A number of structural changes occurring within the mitochondria were observed when rat fibroblasts were exposed to 42°C for 3 hours. They appeared swollen, the cristae were more prominent and the intracisternal spaces appeared enlarged (Welch and Suhan, 1985). Similar changes were observed when exposing monolayer CHO cells to 41.5°C (Coss *et al.*, 1979). It has been suggested that the morphological changes of mitochondria may be related to the inhibition of glycolysis and respiration in heated

cells. In a detailed study on mitochondria from mouse brain, liver and Ehrlich ascites tumour cells, Christiansen and Kvamme (1969) found that heat treatment at 45°C for 10 min resulted in the loss of respiratory control and uncoupling of phosphorylation from electron transport, as a consequence of damage to mitochondrial membranes. Glycolysis and respiration are rapidly inhibited in severely heated cells (Dickson and Calderwood, 1983), and it has been suggested that a lethal lack of energy may be the primary event in hyperthermic cell killing (Haveman and Hahn, 1981)

A strong correlation between cell thermal sensitivity and cellular ATP levels was obtained in CHO cells by Laval and Michel (1982), who showed that a decrease in ATP level by treatment with inhibitors resulted in an increase in heat sensitivity. Similar results were obtained by Gerweck *et al.* (1984) who showed that reducing the cellular ATP level of CHO cells (by varying glucose concentration of the medium) resulted in an increase in thermal sensitivity. However, when the relationship between heat killing and energy status was investigated in CHO cells, the role of energy status in the cellular response to heat seemed unlikely (Calderwood *et al.*, 1985; Calderwood, 1987). Determination of the adenylate energy charge (an indicator of the degree of phosphorylation of the ATP-ADP-AMP system) and phosphorylation potential (the mass action constant for ADP phosphorylation) showed that more than 99% of thermal cell death occurred before the fall in these parameters of energy status occurred (Calderwood *et al.*, 1985). However, it is clear that further studies are necessary to clarify the correlation between energy status of cells and cell killing in other cell types and/or *in vivo*.

The structure and function of cellular proteins are known to be severely affected by heat and this may be an important component of heat cell death. For example, the activation energy for cell killing is similar to that observed for denaturation of protein (Johnson, 1974; Bauer and Henle, 1979). A variety of experimental data are consistent

with the view that one or more cellular proteins are critical elements at elevated temperatures. For example, when Chinese hamster V 79 cells were heated in the presence of sulfhydryl-rich compounds, which destabilise proteins, cells became very heat sensitive (Kapp and Hahn, 1979). CHO HA-1 cells were protected against heat cell death by the substitution, during heating, of D_2O for H_2O in the medium (Hahn *et al.*, 1978; Fisher *et al.*, 1982). This protective role of D_2O against heat-induced cell killing has been ascribed to the strengthening of hydrogen bonding, thus stabilising macromolecules whose higher order structure depends primarily upon weak bonding (Fisher *et al.*, 1982). As with D_2O, glycerol has a protective role against thermal cell killing (Back *et al.*, 1979). When CHO cells or HeLa cells were exposed to 45°C in the presence of 1 M glycerol, hyperthermic cell death was decreased (Henle and Warters, 1982). Thermal protection by glycerol was also observed in HeLa S3 cells (Kampinga *et al.*, 1989). The thermal protection was suggested to be due to the stabilisation of either protein or membranes by glycerol (Back *et al.*, 1979; Lin *et al.*, 1984). By employing a differential scanning calorimeter, it was clearly shown that glycerol protects cellular protein from denaturation in CHL V79 cells (Lepock *et al.*, 1990). Furthermore, changes in one or two amino acids by mutations can alter the temperature-dependent stability of proteins by as much as 10°C (Brock, 1985). Other examples of disruption of protein structures by heat include breakdown of the mitotic spindle (Coss *et al.*, 1982), depolymerisation of microtubules (Coss *et al.*, 1982) and damage to the cytoskeleton (Glass *et al.*, 1985). However, as far as inhibition of protein synthesis and thermal cell killing is concerned, it was found that protein synthesis was completely inhibited when cells were heated at 45°C for 10 min, but this process resumes over the period 4-8 h after heating, suggesting that inhibition of protein synthesis is temporary, and is unlikely to be a cause of cell killing (Henle and Leeper, 1979).

The nucleus contains the major portion of the cell's genetic information encoded in DNA. However, it seems unlikely that DNA is implicated in hyperthermic cell death

since the melting temperature of DNA *in vitro* is 87°C. It has been shown that the synthesis of DNA in CHO cells was depressed immediately upon 45°C heat treatment, but the synthesis reverts to normal once cells were returned to 37°C (Henle and Leeper, 1979). On the other hand, heat has been reported to inhibit the repair of x-ray induced DNA damage due to the denaturation of the replicative enzymes by heat, suggesting that heat and radiotherapy can give an enhanced effect. On exposure to various heat treatments, it was found that the DNA isolated from heated CHO cells (Tomasovic *et al.*, 1978) or from Hela cells (Roti Roti and Winward, 1978) was associated with nonhistone protein and the amount of bound protein was a function of heat dose. In other words, an increase in temperature resulted in an increase in the protein/DNA ratio of the nuclei isolated from cells. The increase in protein/DNA ratio is time and temperature-dependent and there is a good correlation between its increase and heat-induced cell killing (Roti Roti and Laszlo, 1988). This heat-induced increase in the protein/DNA ratio is probably due to an increase in protein mass, since no significant loss of DNA has been found in CHO cells and HeLa cells exposed to hyperthermia (Warters and Henle, 1982). The presence of excess nuclear proteins may be involved in the inhibition of DNA replication (Laszlo, 1992). Furthermore, it has been suggested that chromosome aberrations are involved in heat cell death because there was a log-linear relationship between cell survival and the number of chromosome aberrations when S-phase CHO cells were heated at 45°C. It was found that the heat dose that reduced survival by 1/e (i.e 37% of the initial value) produced one chromosome aberration per cell (Dewey *et al.*, 1971).

The cytoskeleton of eukaryotic cells is a complex network of protein filaments and tubules ramifying throughout the cytoplasm. Cytoskeletal organisation is involved in maintaining cell shape, cell movement, the movement of chromosomes during mitosis and meiosis and intracellular transport of vesicles and organelles. The main structural elements of the cytoskeleton are microtubules, microfilaments and intermediate filaments. All these elements have been reported to be altered by heat and the severity

and type of alterations depend on the temperature, heating duration and on the cell type examined.

The microtubules play a role in the orderly segregation of the genetic material at cell division by forming the mitotic spindle and the disruption of the mitotic apparatus could lead to cell death of the heat sensitive mitotic cells. Exposure of mitotic CHO cells to 45.5°C for about 15 minutes led to complete disassembly of the mitotic spindle and no reformation of this spindle was observed (Coss *et al.*, 1982). This implies that the ability of centrosomes to nucleate microtubule assembly was impaired, since centrosomes are associated with the *in vivo* control of assembly of microtubules.

Formation of stress fibres is necessary for the attachment of cultured cells to the substrate and the formation of stress fibres appears to require transmembrane linkages. An increase in the number of stress filaments after heat shock was observed in HeLa cells and gerbil fibroma cells (Thomas *et al.*, 1982). On the other hand, treatment of Reuber H35 hepatoma cells and neuroblastoma N2 cells at 43°C for 30 min resulted in the destruction of stress fibres (Van Bergen en Henegouwen *et al.*, 1985). Furthermore, the effect of heat on stress fibres is reversible. When interphase CHO cells were exposed to 45°C, disruption of stress fibres was observed within 5 min, but intact stress fibres were observed by 24 h after treatment (Glass *et al.*, 1985). Since hyperthemia has a pleiotropic effect on the plasma membrane, the loss of stress fibres might be a consequence of the effect of heat on this membrane.

Intermediate filaments are tough and durable protein fibres found in the cytoplasm of most eukaryotic cells. They form a basket around the nucleus and extend out in gently curving arrays to the cell periphery. They can be categorised into 4 groups: keratin, vimentin, neurofilaments and nuclear lamins (Goldman *et al.*, 1986). In some cell lines, such as HeLa cells (Van Bergen en Henegouwen *et al.*, 1985), rat fibroblasts (Welch and

Suhan, 1985) and mouse neuroblastoma cells (Van Bergen en Henegouwen and Linnemans, 1987) heat-induced collapse of vimentin-containing intermediate filaments have been observed.

A system linked with the organisation of the cytoskeleton is the calcium-calmodulin complex. Many of the components involved in cytoskeleton organisation are controlled by this complex (Means *et al.*, 1982). It was found that the presence of calmodulin antagonists such as W7, trifluoperazine or calmidazolium during heating resulted in the potentiation of hyperthermic cell killing of neuroblastoma N_2A cells (Wiegant *et al.*, 1985), hepatoma H_{35} cells (Wiegant *et al.*, 1985) and mouse tumour clone C cells (Evans and Tomasovic, 1989). An inverse relationship between cell killing and cytoskeletal alteration was observed (Wiegant *et al.*, 1985). The potentiation of heat cell death by calmodulin antagonists has been proposed by Weigant *et al.* (1985) to involve: (a) A heat-induced increase in cytosolic free calcium ($[Ca^{2+}]_i$) which binds to calmodulin to form a calcium-calmodulin complex, resulting in the activation of calmodulin. (b) This complex then binds to microtubule-associated protein and causes depolymerisation of microtubules. The presence of calmodulin antagonists prevents the alteration of cytoskeletal organisation upon heating. However, the role of cytoskeletal alteration in causing cell death remains to be clarified. Since the cytoskeleton is assumed to be an important structural linkage between the nucleus and the plasma membrane, it is tempting to postulate that hyperthermic-induced disruption of the cytoskeleton will result in its collapse towards the nucleus and this may possibly be linked to the heat-induced increase in nuclear protein content.

The plasma membrane is the cell boundary which is in immediate contact with the extracellular environment, and it has been suggested that this membrane plays a major, if not primary role in hyperthermic cell killing. In fact, the plasma membrane has received extensive consideration as a target for hyperthermic cell killing (Hahn, 1982; Bowler,

1987; Konings, 1988). Direct evidence for the effect of heat on the plasma membrane comes from morphological studies of CHO cells. Upon heating at 43°C or higher, blebbing was observed. The percentage of cells covered with blebs was dependent on the temperature and duration of heating (Kapiszewska and Hopwood, 1986). In addition, a correlation between hyperthermia-induced blebbing and survival in synchronous G_1 CHO cells (in suspension) was found (Borrelli *et al.*, 1986). However, this correlation between membrane blebbing and cell survival holds only for cells heated in the G_1 phase of the cell cycle.

Before the effect of heat on the plasma membrane function is described, it is necessary to consider the physical properties of biological membranes, which is best described by the fluid mosaic model (Singer and Nicholson, 1972). The phospholipid molecules are arranged in such a way that the hydrophilic polar head group are oriented to the outer surface whereas the hydrophobic tails point towards the interior, forming a hydrophobic core. Hence the plasma membrane acts as a barrier to prevent the free diffusion of ions and solutes across the membrane. Membrane proteins play an extremely important role in facilitating the flow of information between cells and their environment and regulating the molecular and ionic composition of the intracellular medium.

Two types of membrane proteins have been identified, which are known as integral and peripheral proteins, depending on their locations (Singer and Nicholson, 1972). Integral proteins penetrate the hydrophobic interior of the membrane to a greater or lesser extent, whereas peripheral proteins are believed to be associated with the polar head groups in the outer faces of the lipid matrix. Investigation of the properties of membranes employing pure phospholipids suggest that a membrane exists in two states — gel and liquid-crystalline states. At low temperature, the movement of lipid molecules is very slow and this state is known as the gel state of the membrane. As temperature increases, the rotational and lateral movement of lipids in the plane of the membrane, as

well as flexing of the hydrocarbon chains, increase. As temperature continues to rise, a transition temperature is achieved at which the movement of hydrocarbon chains increases abruptly, giving rise to the liquid-crystalline state (Lee and Chapman, 1987). In other words, low temperature increases the order of the lipid matrix (decreases fluidity), while higher temperature causes decreased order (increased fluidity) of the lipid matrix. The situation in natural membranes is far more complicated since the order of the lipid matrix is not only affected by temperature, but also by levels of cholesterol, proteins and the degree of unsaturation of the fatty acyl chains of membrane phospholipids. Therefore, a sharp transition temperature is not observed, and the order of the membrane lipid matrix decreases gradually as ambient temperature increases.

In concert with the changes in membrane fluidity, the functions of membrane proteins are also affected. As suggested by Cossins *et al.* (1981), the hydrophobic core of a biological membrane, in which the proteins are floating, is ordered and is anisotropic. The tertiary structure of these proteins must be relatively loose so as to allow the molecular flexibility necessary to fulfil their roles, such as catalysis. As temperature increases, the order of lipid matrix decreases. Under these conditions, membrane protein may adopt conformations that are inactivating (Cossins *et al.*, 1981; Stubbs, 1983). To compensate for the effect of temperature on membrane fluidity and protein functions, microorganisms (Sinensky, 1974) and poikilothermic animals (Cossins and Raynard, 1987) have the ability to modify the degree of saturation of their membrane lipids. This adaptive response is known as 'homeoviscous adaptation' (Sinensky, 1974).

To investigate the correlation between hyperthermia and membrane fluidity, membrane fluidising agents such as aliphatic alcohols and local anaesthetics have been used. Exposure of CHO cells to hyperthermia in the presence of alcohol increased cytotoxicity (Li and Hahn, 1978; Henle, 1981). Similarly, an increase in cytotoxicity was observed when *E. coli.* cells were heated in the presence of procaine, a local anaesthetic

(Yatvin, 1977). Potentiation of heat cell death was observed when AKR leukaemia cells were heated in the presence of lidocaine (Robins *et al.*, 1984) and when CHO cells were heated in the presence of procaine (Dynlacht and Fox, 1992a). Alcohols and local anaesthetics are thought to interact with the plasma membrane, resulting in an increase in membrane fluidity, bringing about the additive cytotoxic effect of heat and these agents (Hahn and Li, 1982). Further evidence to support the role of the plasma membrane as a target of heat was obtained by using amphotericin B, an antibiotic that binds specifically to cholesterol. When Chinese hamster cells and EMT6 mammary sarcoma cells were exposed to different concentrations of amphotericin B, substantial cell killing was observed upon exposure to 43°C but no significant effect was observed when cells were incubated at 37°C (Hahn and Li, 1982). The increase in effectiveness at 43°C may be due to an increase in the availability of cholesterol binding sites or an increase in lateral mobility of cholesterol-amphotericin B complexes to form the necessary aggregates for transmembrane channel formation. The cells die when too many 'holes' are formed in the membrane (Hahn, 1982).

Another way to manipulate the membrane lipid fluidity of cells is by altering the amount of polyunsaturated fatty acid in the extracellular medium during cell culture or by feeding an animal with a diet high in polyunsaturated fatty acid. This results in incorporation of polyunsaturated fatty acids into membrane phospholipid, thus altering the membrane composition experimentally in culture or *in vivo*. When *E. coli.* cells were supplemented either with oleic acid (18:1) or linolenic acid (18:3), it was found that the 18:3 supplemented cells were more heat sensitive than 18:1 supplemented cells (Yatvin,1977). Similarly, an increase in thermal sensitivity was obtained in L1210 murine leukaemia cells grown in 22:6-supplemented media compared with cells grown in 18:1-supplemented media (Guffy *et al.*, 1982), and in mouse fibroblast LM cells grown in 20:4-supplemented media compared with cells grown in medium supplemented with saturated fatty acid (Konings and Ruifrok, 1985). When murine P388 cells were

grown in animals fed a diet high in polyunsaturated fatty acids, such as safflower oil which is enriched in 18:2, the cells were more thermosensitive than those cells grown in animals fed a diet high in saturated fatty acid, such as beef tallow (Mulcahy *et al.*, 1981)

Using Hepatoma Tissue Culture (HTC) cells, Ladha *et al.* (1993) demonstrated that when these cells were grown in medium supplemented with arachidonic acid (20:4), an increase in thermal sensitivity was observed. This was associated with incorporation of the arachidonic acid into membrane phospholipid and a consequent increase in membrane fluidity, determined by fluorescence polarisation. Furthermore, alteration in membrane fluidity by arachidonic acid resulted in an increase in heat sensitivity of alkaline phosphodiesterase I, a membrane bound protein.

Cholesterol is another determinant of membrane fluidity and acts as a buffer to decrease the membrane fluidity at temperatures above the phase transition and increase membrane fluidity below that temperature (Stubbs, 1983). The closer packing of lipid molecules in the presence of cholesterol reduces membrane permeability (Demel *et al.*, 1972) and reduces the activity of Ca^{2+}-ATPase of sarcoplasmic reticulum in a manner dependent upon the cholesterol content (Madden *et al.*, 1974). Sabine (1983) has proposed that cells have an optimal level of membrane cholesterol for function, and levels above or below that value will result in impaired membrane function. In general, the higher the cholesterol level, the lower the fluidity of the membrane. In an investigation of cholesterol content and thermal sensitivity in 5 different cell types, an inverse correlation was observed between the cholesterol/protein ratio of the cell membrane and heat sensitivity, consistent with the stabilizing role for cholesterol in membranes (Cress and Gerner, 1980). Using 7 cell lines, a positive correlation was found between thermal sensitivity and weight ratios of cholesterol : protein and phospholipid : protein content (Cress *et al.*, 1982). However, other workers failed to

confirm this observation (Konings and Ruifrok, 1985; Raaphorst *et al.*, 1985)

Effects of heat on membrane fluidity have also been determined by fluorescence polarisation spectroscopy. Using this technique, Dynlacht and Fox (1992b) showed that hyperthermia caused persistent changes in the membrane fluidity of CHO cells but these changes were not directly correlated with cell survival. By using different cell lines, including AG1522 human foreskin fibroblasts, CHO cells, radiation-induced mouse fibrosarcoma and Crandall feline kidney cells, Dynlacht and Fox (1992a) found that the higher the ability of cells to resist changes in plasma membrane fluidity, the more resistant those cells were to heat cell death. Based on these observations, it has been suggested that the initial level of membrane fluidity may be less important, compared with the extent of changes in plasma membrane fluidity during hyperthermia.

The lipid fluidity per se is probably of little importance for the cell, but it becomes important for the stabilisation of membrane protein conformation. Changes in membrane fluidity may lead to changes in protein-lipid binding and thus alter membrane transport and cell communication events. A large number of observations imply that an alteration in the structure and/or function of membrane proteins occur as a consequence of hyperthermia. For example, the effect of heat on membrane proteins has been investigated in Chinese hamster V79 cells (Lepock *et al.*, 1983). Measurement of intrinsic protein fluorescence and of the energy transfer from protein fluorophore to trans-paranaric acid demonstrated the existence of an irreversible transition in membrane protein structure above 40°C, both in mitochondria and in the plasma membrane (Lepock *et al.*, 1983). These authors further proposed that the alteration in the structure of membrane protein above 40°C could cause many of the observed changes in the plasma membrane and may be involved in hyperthermic cell killing.

The plasma membrane acts to establish gradients of low molecular weight solutes

and ions between the cell and its environment. This is achieved by passive diffusion as well as the action of ionic pumps and specific transport systems (Wilson, 1978). Many reports have suggested that those transport functions were impaired after hyperthermia. Exposure of rat thymocytes to temperatures ranging from 39-43°C resulted in a striking inhibition of Na^+-dependent amino acid transport (Lin *et al.*, 1978). Conversely, glucose transport into CHO cells upon 45°C treatment was stimulated by heat (Vidair and Dewey, 1993). The uptake of thymidine (Slusser *et al.*, 1982) and the DNA specific dye Hoechst 33342 (Rice *et al.*, 1985) were inhibited in CHO cells exposed to 45°C. Hyperthermia also alters the permeability of the plasma membrane to several other compounds such as adriamycin (Hahn and Strande, 1976) and polyamines (Gerner *et al.*, 1980).

The effects of hyperthermia on ion fluxes (both influx and efflux) are equivocal. For example, an increase in total cellular K^+ concentration ($[K^+]$) was observed within 15 min when CHO HA-1 cells were heated at 42°C. This increase in $[K^+]$ was reversible when cells were returned to 37°C (Stevenson *et al.*, 1983). On the other hand, a decrease in $[K^+]$ was observed when mouse LM fibroblasts were heated at 44°C (Ruifrok *et al.*, 1985). In constrast, when plateau phase CHO cells were heated at 45°C for up to 30 min, no significant effect on total Na^+, K^+, and Mg^{2+} levels was observed in the following 22 h post-treatment (Vidair and Dewey, 1986).

Several observations suggest that hyperthermia causes changes in the affinity of agonist-receptor interaction and/or receptor number during signal tranduction at the plasma membrane. For example, the affinity of epidermal growth factor (EGF) membrane-bound receptor for its agonist in Rat-1 fibroblasts decreased as temperature increased, and the binding was fully inhibited when the cells were exposed to 45°C for 30 minutes (Magun and Fennie, 1981). It was also found that, on exposure of CHO HA-1 cells to 43-45°C for some time, the binding of insulin to its membrane receptor was

inhibited due to a reduction in receptor number, rather than a decrease in receptor affinity (Calderwood and Hahn, 1983). Similarly, the binding of monoclonal antibodies to the histocompatibility antigens on the surface of murine lymphoma cells in suspension culture was inhibited after hyperthermia (43-45°C), as a result of reduction of receptor number (Mehdi *et al.*, 1984).

Ca^{2+} is an important regulator for a variety of cellular processes, either through its own action or by forming a calcium-calmodulin complex. Disruption of Ca^{2+} homeostasis has been shown to be a cause of several kinds of cell death (Trump *et al.*, 1980; Orrenius *et al.*, 1989). It was found that, following hyperthermia, the total calcium content increased in EAT cells (Anghileri *et al.*, 1985a,b) and CHO cells (Vidair and Dewey, 1986). Heating Reuber H35 hepatoma cells in elevated extracellular calcium concentration (7.5 mM) resulted in an increase in thermal cell killing (Wiegant *et al.*, 1984). Alternatively, when Morris hepatoma cells were incubated in low extracellular calcium concentration (achieved by addition of EGTA), cells were protected against heat-induced cell killing (Lamarche *et al.*, 1985). It is known that the intracellular concentration of free calcium ($[Ca^{2+}]_i$) is responsible for modulation of several cellular functions, so the determination of $[Ca^{2+}]_i$ upon heating is more relevant in investigating the role of $[Ca^{2+}]_i$ in hyperthermic cell death. Analysis of $[Ca^{2+}]_i$ by flow cytometry, spectrofluorimetry and digitised fluorescent microscopy in heated cells has shown conflicting results. A heat-induced increase in $[Ca^{2+}]_i$ was observed in CHO HA-1 cells (Calderwood *et al.*, 1988), Drosophila salivary gland (Drummond *et al.*, 1988), human colon HT-29 cells (Mikkelsen *et al.*, 1991a), mouse mammary FM3A cells (Kondo *et al.*, 1993), human epidermoid A-431 cells (Kiang *et al.*, 1992) and NIH3T3 fibroblasts (Stege *et al.*, 1993b). On the other hand, no change in mean $[Ca^{2+}]_i$ was observed in mouse mammary MMT060562 cells analysed from 100 cells by fluorescent microscopic technique (Furukawa *et al.*, 1992), in $[Ca^{2+}]_i$ analysed in HeLaS3 cells in suspension (Stege *et al.*, 1993a), L5178Y-S (Stege *et al.*, 1993b) and L5178Y-R cells (Stege *et al.*,

1993b) during heat treatment. This differential effect of heat on $[Ca^{2+}]_i$ could be due to a difference in experimental conditions, including heat dose. To investigate the relationship of the heat-induced increase in $[Ca^{2+}]_i$ to hyperthermic cell death, 6 different cell lines were employed (Wierenga *et al.*, 1994). It was found that only mouse fibroblast 3T3 cells showed an increase in $[Ca^{2+}]_i$ upon 44°C treatment for up to 60 min, a heat dose that killed more than 90% in all 6 cell types. These results imply that there is no correlation between hyperthermic cell death and heat-induced increase in $[Ca^{2+}]_i$, and that the heat-induced increase in $[Ca^{2+}]_i$ is solely cell type dependent. A similar result was observed in C3H10T1/2 cells and NIH3T3 cells when changes in $[Ca^{2+}]_i$ upon heating were measured in indo-1 loaded cells by flow cytometry (Vidair *et al.*, 1990). By varying the extracellular calcium concentration, $[Ca^{2+}]_i$ was altered. When NIH3T3 cells were heated at 45°C for 30 min in medium containing 0.03 mM extracellular calcium concentration (achieved by addition of EGTA), $[Ca^{2+}]_i$ achieved 200 nM, which was comparable to those cells incubated at 37°C in medium containing 2 mM extracellular calcium and is lower than those incubated in 5 mM extracellular calcium medium at 37°C, which gave rise to 300 nM $[Ca^{2+}]_i$. On the other hand, when cells were heated in the presence of 15 mM extracellular calcium at 45°C for 30 min, $[Ca^{2+}]_i$ increased up to 1000 nM. No alteration of cell killing under these conditions was found. However, it must be noted that, in the method employed in this study, the measurement of $[Ca^{2+}]_i$ in single cells was performed at room temperature after cells were heated, so that $[Ca^{2+}]_i$ was not measured during the heating period. Similarly, no correlation between $[Ca^{2+}]_i$ and cell killing was found in fura-2 loaded HT-29 cells determined by digitised fluorescent microscopy during heating at 44°C for 1 h, because more than 80% of the cells showed a $[Ca^{2+}]_i$ greater than 200 nM but greater than 40% remained viable following heating, as determined by clonogenic assay (Mikkelsen *et al.*, 1991a). However, a linear relationship was found between cells having $[Ca^{2+}]_i > 200$ nM at 4-6 h post-heating and cell killing (Mikkelsen *et al.*, 1991a). The heat-induced increase in $[Ca^{2+}]_i$ can trigger a variety of cellular responses such as activation of Ca^{2+}-dependent

enzymes or alteration of the cytoskeleton, which would be expected to be permissive to heat killing.

The source of heat-induced $[Ca^{2+}]_i$ elevation has been suggested to be either Ca^{2+} influx from the extracellular medium (Kiang and McClain, 1993), Ca^{2+} release from internal Ca^{2+} stores mediated by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (Stevenson *et al.*, 1986; Drummond *et al.*, 1988) or both influx and Ca^{2+} release. $Ins(1,4,5)P_3$ is generated upon hydrolysis of a small class of membrane phospholipid, phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$), and is responsible for releasing Ca^{2+} from internal stores (Berridge, 1984; Berridge, 1993). The relationship of changes in inositol trisphosphate ($InsP_3$) and $[Ca^{2+}]_i$ during heating has been investigated in CHO HA-1 cells, but the identity of the $InsP_3$ isomer that accumulated upon heating was not resolved (Stevenson *et al.*, 1986). In CHO HA-1 cells it was found that heating at 45°C caused a 70% increase in $InsP_3$ level, which was observed as early as 1 min heating and remained at the same level for up to 5 min heating. This heat-induced increase in $InsP_3$ preceded the increase in $[Ca^{2+}]_i$, implying that $[Ca^{2+}]_i$ elevation may be mediated by the heat-induced changes in $InsP_3$, though the possibility that heat perturbed the intracellular calcium store by a mechanism that is unrelated to $InsP_3$ was not ruled out by these authors (Stevenson *et al.*, 1986). On the other hand, Kiang and McClain (1993) suggested that the increase in $[Ca^{2+}]_i$ in A-431 cells resulted from Ca^{2+} influx through a reverse mode of the Na^+/Ca^{2+} exchanger induced by heat. The increase in $[Ca^{2+}]_i$ was proposed to activate a calcium-dependent phosphoinositide-specific phospholipase C (PI-PLC) which hydrolysed $PtdIns(4,5)P_2$ and formed $InsP_3$, leading to a further increase in $[Ca^{2+}]_i$.

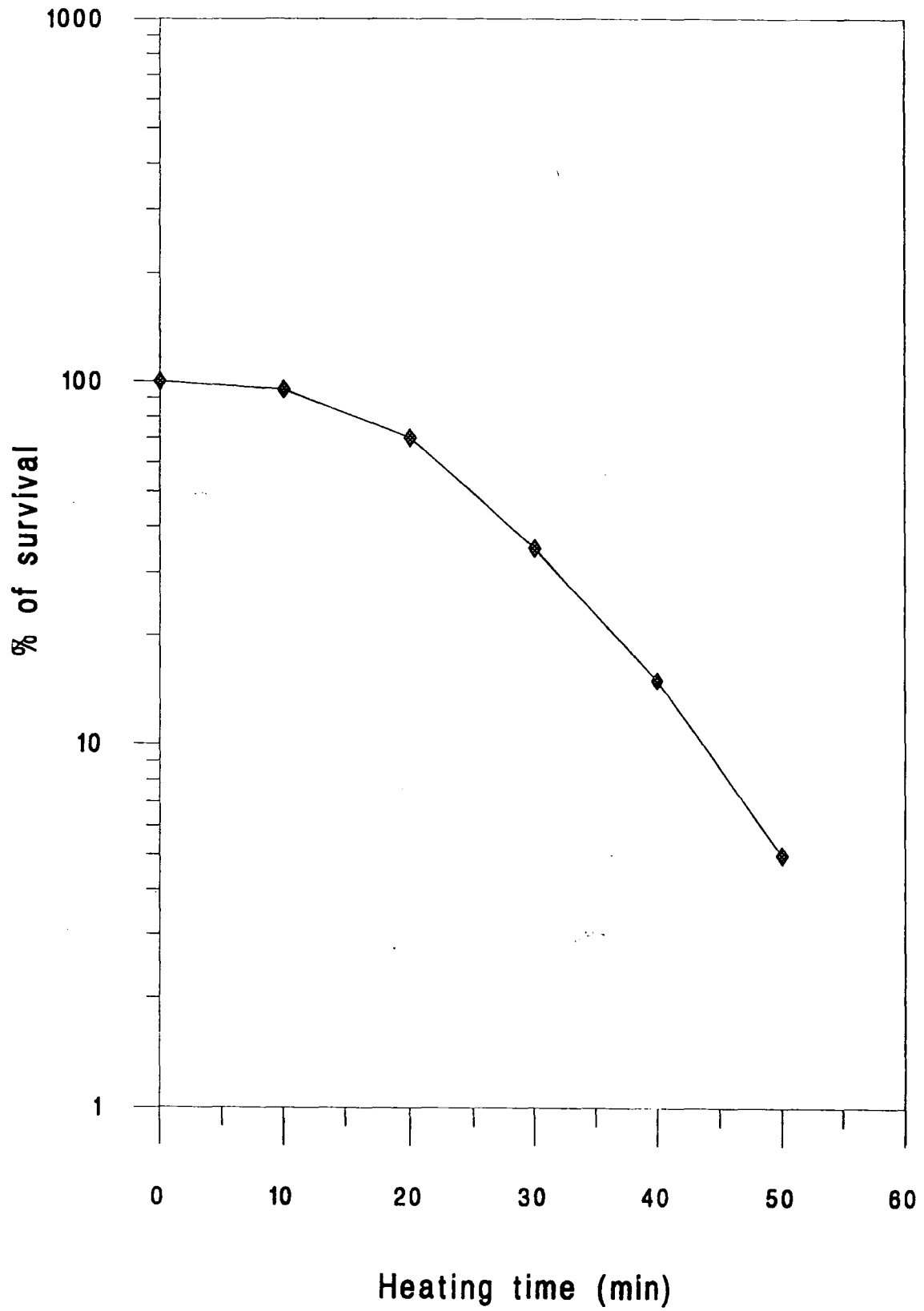
In a recent study, Calderwood and Stevenson (1993) found that heat led to an increase in $InsP_3$ formation, probably via the activation of PI-PLC. A heat-induced increase in $InsP_3$ was observed in many different cell lines including CHO HA-1 cells,

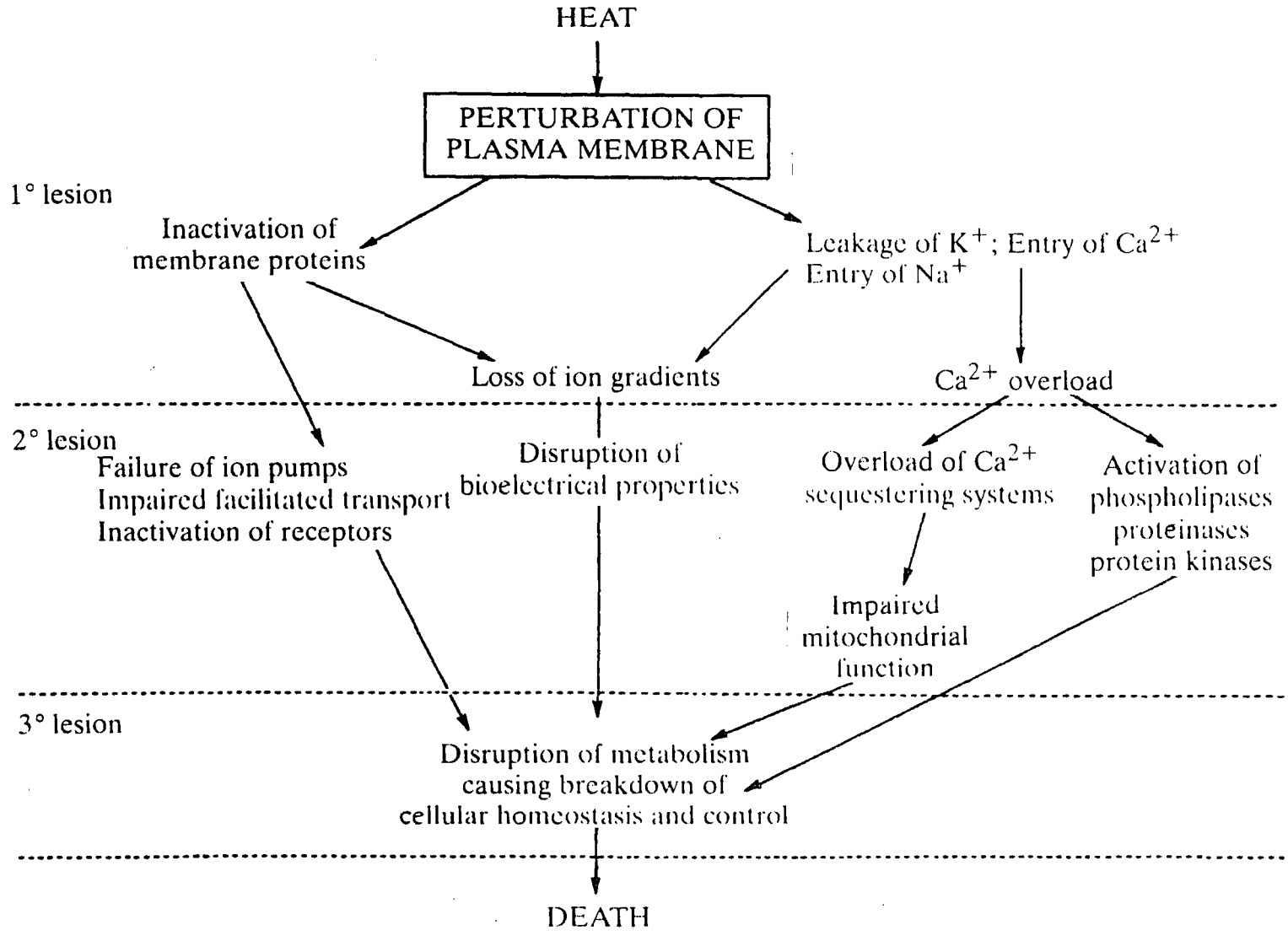
NIH3T3 cells, Balb C 3T3 cells and PC 12 cells upon 45°C heat treatment. For example, when HeLa cells were heated at 45°C for 30 min, the $InsP_3$ level increased to 70% of unheated level. Exposure of CHO HA-1 cells to 45°C for 15 min resulted in a 400% increase of $InsP_3$ level compared with unheated cells. A rapid, heat-induced increase in diacylglycerol level was also observed (about 2-3 min) upon 45°C treatment of CHO HA-1 cells, though the elevation was transient and declined rapidly to the unheated level by 4 min (Calderwood *et al.*, 1987). Using permeabilised CHO HA-1 cells, it was found that the heat-induced $InsP_3$ formation (presumably resulting from PLC activation) is guanyl nucleotide dependent, thus suggesting that a G protein may be affected by heat (Calderwood *et al.*, 1993). The participation of a G protein in the activation of PLC is a common pathway associated with receptor-mediated PI-PLC activation (Berridge, 1984; Exton, 1994). The implication of this finding is that the action of hyperthermia may resemble that of an agonist, suggesting that physical stress such as hyperthermia may be converted to a chemical message in the cell.

Given the importance of the phosphoinositide signalling pathway in controlling a variety cell functions through the action of either the $InsP_3/Ca^{2+}$ pathway or the 1,2-diacylglycerol/protein kinase C pathway, disruption of this signalling pathway might in turn lead to the loss of cellular homeostasis and produce chaotic changes in cell function.

As mentioned earlier, the mechanism and the primary lesion site that leads to hyperthermic cell death is obscure, but the identification of possible factors involved in hyperthermic cell death could be useful in optimising use of hyperthermia in cancer therapy. For example, if heat-induced cell death is due to the disruption of calcium homeostasis, agents such as local anaethetics that have been shown to perturb the calcium homestasis and increase in calcium influx could be used in collaboration with hyperthermia to potentiate the effect. The main aim of this study is to clarify further the effect of heat on the phosphoinositide signalling pathway in tumour cells using rat

mammary tumour WRK-1 cells and Chinese hamster ovary CHO-K1 cells as the model systems. Levels of 1,2-diacylglycerol (and other neutral lipids), inositol phosphates and $[Ca^{2+}]_i$ were measured during and after hyperthermic treatment of the cells.


1000
100
10
1
% of survival
0
10
20
30
40
50
60
Heating time (min)


HEAT
PERTURBATION OF PLASMA MEMBRANE
1° lesion
Inactivation of membrane proteins
Leakage of K^+; Entry of Ca^{2+} Entry of Na^+
Loss of ion gradients
Ca^{2+} overload
2° lesion
Failure of ion pumps
Impaired facilitated transport
Inactivation of receptors
Disruption of bioelectrical properties
Overload of Ca^{2+} sequestering systems
Activation of phospholipases proteinases protein kinases
Impaired mitochondrial function
3° lesion
Disruption of metabolism causing breakdown of cellular homeostasis and control
DEATH

Chapter 2

Characteristics of the Culture System for CHO-K1 and WRK-1 Cells

2.1 Introduction

The effect of hyperthermia on biological systems can be studied using both cell cultures and experimental animals. Cell culture may be used in the first instance to study the response of a single cell type to heating *in vitro*, followed by investigation of the cellular and functional responses of tissues in experimental animals to hyperthermia.

The first successful experiment in maintaining tissue *in vitro* was done by Roux and his co-workers in 1885 using chick embryo. They found that it was possible to keep this fragment alive for several days when it was incubated in warm saline. In 1910, Burrow introduced the plasma clot culture method that allowed the tissue to be grown *in vitro* and laid the foundation for subsequent development of the technique. Later, Eagle (1955) made the first systematic investigation of the nutritional requirements of cells in culture and found that animal cells could propagate in a defined mixture of small molecules supplemented with a small proportion of serum proteins. Since then several well-defined media have been manufactured and, at present, the commonly used media are easily available from the manufacturers. The choice of culture medium is usually determined by the cell line being employed.

Serum-free media with the composition mimicking that of blood serum have been used successfully for the growth of HeLa cells (Blaker *et al.*, 1971), human fibroblasts (Hammond *et al.*, 1984) and lymphoblasts (Iscove and Melchers, 1978). However, it seems that serum is still required for maximal cell growth since it contains many

biomolecules with different growth-promoting and growth-inhibiting activities. Apart from providing growth factors (Gospodarowicz and Moran, 1974) and hormones (Fredin *et al.*, 1979; McLean *et al.*, 1986), it also provides attachment and spreading factors for cells (Fisher *et al.*, 1958), binding proteins (Guilbert and Iscove, 1976; Iscove and Melchers, 1978; Barnes and Sato, 1980) and lipids and minerals (Ham and McKeehan, 1978). Serum proteins also contribute viscosity, which is important in protecting cells from mechanical damage during resuspension of trypsinized cells, and anti-protease which is necessary for arresting proteolysis after trypsinization .

Depending on the cell types, cells can be grown as monolayer or suspension cultures. In both cases, the cell growth is largely affected by nutrients, pH, temperature and growth area. In order to make sure that there is no deprivation of growth factors in the medium, it is a good practice to change the medium every two or three days.

Animal cell growth is optimal in the pH range 7.2-7.4. In order to operate in an effective buffering system, sodium bicarbonate is usually included in the growth medium in conjunction with a gas phase of air/CO_2 (95:5, v/v) to give rise to an effective CO_2-buffering system (Eagle, 1973).

The optimal temperature for mammalian cell growth is about 37°C. Although cells can tolerate a rapid and considerable drop in temperature (during cryopreservation), they will die if the temperature is maintained slightly higher than the normal growth temperature. Hence the temperature at different parts of the incubator must be kept very constant. This is achieved by circulating the air by a fan to give an even temperature. Apart from having an effect on cell growth, temperature also influences the pH of the medium due to the increased solubility of CO_2 at lower temperatures.

The growth area is another factor that affects the cell growth. Depending on the

cell number required, flasks and plates with different surface area are used. In this study, the most commonly used plasticware were 25 cm^2 flasks and 6-well plates (9.6 cm^2 per well). Nowadays, flasks with a folded surface are available which provides a greater surface area for cell growth but keeps the occupying space to a minimum.

Although aseptic techniques are used, contamination is still a problem in cell-culture. Bacterial and fungal contamination can be suppressed by adding antibiotics such as penicillin and streptomycin (Von Hoff *et al.*, 1986) and anti-fungal agents such as nystatin and amphotericin B (Pearlman, 1979) to the medium. Mycoplasma infection is a very serious problem in cell culture and can alter the metabolism of cultured cells but allows the cells to grow satisfactorily and appear normal under the light microscope (Stanbridge *et al.*, 1975; States *et al.*, 1978). Hence either tylosin (Friend *et al.*, 1966) or ciprofloxacin (Schmitt *et al.*, 1988) is often included in the medium as an anti-mycoplasma agent.

To prevent cell loss from contamination, incubator failure and to avoid genetic instability as the cell population ages, it is a common practice to freeze healthy cells at an early passage as "seed stock". The problems concerned with cell freezing include the formation of intracellular ice crystals (Meryman, 1974) and osmotic effects (Lovelock, 1953; Meryman, 1974). These problems can be overcome by adding a cryoprotective agent such as DMSO or glycerol. Although DMSO can penetrate cells more rapidly and give a greater protective effect, several reports suggested that DMSO leads to gene activation and differentiation in a variety of cellular systems and it is advisable to use glycerol as cryoprotectant (Rudland *et al.*, 1982; Higgins *et al.*, 1983).

Once the cells reach confluence (in monolayer), a subcultivation involving harvesting the cells then reseeding them at lower density in fresh medium is necessary. Whether harvesting is done by a mechanical method (using rubber policeman) or

enzymatically (trypsin-EDTA), some degree of trauma to the cell is expected. The most effective way to reduce cell loss during harvesting is by washing the cell monolayer first with warm Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, followed by treatment with EDTA-trypsin solution. The cells detach from the culture vessel and are then pelleted by centrifugation, resuspended in pre-warmed growth medium, plated out at a lower cell density and allowed to grow for several days before reaching confluence again.

Following subcultivation and re-seeding mammalian cells, 3 stages of cell growth can be observed. An initial lag period of about 24 h is detected which is the recovery period for cells after trypsinization. During this period the cells attach to the substrate and spread, and enzymes such as DNA polymerases increase in activity, followed by the synthesis of new DNA and proteins. This lag period is followed by an exponential growth period, "the log phase", during which the cell numbers increase rapidly until they approach confluence. At the end of the log phase the growth rate is reduced, and growth ceases in most cell types as soon as cells reach confluence, whereas in other cases the cells tend to grow on top of the first layer and form a multilayer culture. The length of the log phase depends on the seeding density, the growth rate of the cells and the nutritional conditions. If the cell growth is followed for several days, the doubling time and the maximum cell density for a particular surface area can be determined.

Several factors need to be considered before commencing research with a particular cell line. These factors include the suitability of the cell line for the proposed work, type of culture required (monolayer or suspension culture), cell number required as well as the facilities that are available.

The effect of hyperthermia on signal transduction pathways has been studied in CHO HA-1 cells (Stevenson *et al.*, 1986), Balb C 3T3 fibroblasts (Calderwood *et al.*, 1987), HeLa cells, PC 12 rat pheochromocytoma cells (Calderwood *et al.*, 1988) and

human epidermoid A-431 cells (Kiang and McClain, 1993). In the present study, 2 cell lines were used : the Chinese hamster ovary (CHO-K1) cells and rat mammary tumour (WRK-1) cells.

CHO-K1 cells are fast growing cells with a doubling time about 15 h and a high split ratio (1:20); hence a large number of cells can be produced in a rather short period. Different clones of this cell line have been employed by other workers in the study of hyperthermic effects such as the correlation of cell blebbing and cell death following heat shock (Borrelli *et al.*, 1986; Kapiszewska and Hopwood, 1988), alteration in membrane fluidity (Gonzalez-Mendez *et al.*, 1982; Dynlacht and Fox, 1992a,b), DNA damage (Warters *et al.*, 1985), thermotolerance and heat shock protein induction (Li *et al.*, 1982; Li and Hahn, 1987; Lee *et al.*, 1992), the effect of heat on calcium homeostasis (Stevenson *et al.*, 1987) and the phosphoinositide signalling system (Calderwood and Stevenson, 1993) and thermal sensitivity of growth factor receptors (Calderwood and Hahn, 1983). All these factors stimulated the use of this cell line as one of our models for hyperthermic study.

WRK-1 cells were chosen as another suitable model for this study. Although the effect of hyperthermia on this cell line has not been investigated before, the well-characterised phosphoinositide signalling system in WRK-1 cells (Monaco and Lippman, 1982; Guillon *et al.*, 1986a,b; Monaco, 1987a; Monaco, 1987b; Barker *et al.*, 1992; Wong *et al.*, 1992) provides a valuable tool for investigating the hyperthermic effect on this second messenger system. The doubling time of this cell line is about 20 h, hence making this system a suitable cell line to use. However, it has one drawback as rat serum is an essential component for cell growth.

The purpose of this chapter is to characterise the growth behaviour of both cell

lines, in order to establish fundamental properties of the cells (doubling time, plating efficiency etc) which were vital in planning further experiments.

2.2 Materials and Methods

2.2.1 Cell Types

(i) CHO-K1 cells

CHO-K1 is a clone of Chinese hamster ovary cells that was first isolated by Puck and others (1958). Later a defined medium was developed by Ham (1962) to support the growth of different clones of CHO cells. CHO-K1 has a modal chromosome number of 21 and it has been demonstrated that proline is indispensable for its growth (Kao and Puck, 1967).

(ii) WRK-1 cells

WRK-1 is a cloned cell line from long-term tissue culture originally derived from 7,12-dimethylbenz(a)anthracene-induced rat mammary tumour of a 50-day-old Sprague-Dawley rat (Kidwell *et al.*, 1978). The cells grow in monolayer culture and appear to possess of many of the ultrastructural characteristics of mammary secretory epithelium. They do not have receptors for oestrogens, androgens, progesterone and prolactin. Although they do not form tumours when injected into nude mice, they appear to be transformed because they have a modal chromosome number of 80 and "pile up" in culture (Kidwell *et al.*, 1978).

2.2.2 Culture Techniques

(i) Growth media and conditions

In this study, CHO-K1 was purchased from Flow Laboratories (Rickmansworth, Hertfordshire) at passage 24 and WRK-1 cells were a gift from Dr. C.J. Barker (University of Birmingham) at passage number between 15 to 24. Both cell types were grown as monolayers either in flasks or in plates depending on the nature of the experimental work. WRK-1 cells were also grown on coverslips when they were used for calcium measurements.

CHO-K1 cells were grown in Ham's F12 medium (purchased from Flow Laboratories) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 i.u./ml), streptomycin (100 μg/ml) and tylosin (10 μg/ml). Cells were maintained at 37°C in an air/CO_2 (19:1, v/v) atmosphere.

WRK-1 cells were grown in Eagles Mimimum Essential Medium with Earle's salts (purchased from Flow Lab as 10X medium w/o L-glutamine and sodium bicarbonate) supplemented with FBS (5%, v/v), heat inactivated rat serum (2%, v/v), 2 mM L-glutamine, penicillin (100 i.u/ml), streptomycin (100 μg/ml), tylosin (10 μg/ml) and non-essential amino acids (1%, v/v) and the medium was buffered to pH 7.4 with 24 mM sodium bicarbonate. Cells were maintained at 37°C in an air/CO_2 (19:1, v/v) atmosphere.

All the cell culture supplements were purchased from Flow Laboratories apart from FBS. FBS had been batch-tested to give the best cell growth, and was purchased from Sera Labs. The rat serum was prepared from mature, non-pregnant female rats,

with most preparations consisting of sera pooled from 10-50 rats. The serum was heat inactivated by treatment at 56°C for 30 min, filter-sterilised and stored frozen at -20°C.

(ii) Cell harvesting and subcultivation

Both CHO-K1 and WRK-1 cells were grown in 25 cm^2 flasks. As cells reached confluence, a subcultivation was found necessary. Cells were subcultivated no more than 10 times from the passage number of receipt to avoid alteration of growth characteristics as cells aged.

The growth medium in the flask was decanted and the monolayer was washed twice with 5 ml Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), added to the side of the flask opposite the cells. The PBS was discarded and the monolayer was detached by addition of 1 ml trypsin-EDTA solution which contained 0.25% (w/v) trypsin and 0.2% (w/v) EDTA in PBS followed by an incubation at 37°C for 3-5 min until the cells rounded up and detached from the flask surface. Then 10 ml of growth medium was added to terminate the action of trypsin. The cell suspension was then transferred to a sterile plastic universal vial and centrifuged at 80 g (r_{av} = 11 cm) at 20°C for 3 min, to sediment WRK-1 cells or at 120 g for 5 min to sediment CHO-K1 cells. The supernatant was discarded and the pellet was resuspended in a known volume of growth medium (usually 1-3 ml). The cells were disaggregated by sucking them up and down a syringe, fitted with a no. 25 gauge hypodermic needle, two to three times. Cells were counted using either a haemocytometer or Coulter Counter and then seeded into fresh growth medium. The medium was changed the following day and every 2 to 3 days thereafter.

(iii) Cryopreservation of cells

Subconfluent cells from a 175 cm^2 flask were harvested as described. The pellet

was then resuspended in 10 ml of freezing medium consisting of FBS/glycerol (92:8, v/v), and disaggregated. A 1 ml aliquot of the suspension (approximately 3 x 10^6 cells) was transferred into a plastic bio-freeze vial and the vials were then placed in a specially designed container (Nalgene cryo 1°C freezing container) to provide a better control on the freezing rate (-1°C per minute) when placed in a freezer set at -80°C. After 4-15 h, the vials were transferred to a liquid nitrogen container where they could be stored for several years.

(iv) Recovery of frozen stock

Frozen cells were thawed rapidly by placing the vial in a 37°C water bath. The cells were then transferred to a 25 cm^2 culture flask containing 9 ml of pre-warmed growth medium and the flask was then incubated in an incubator at 37°C in an air/CO_2 (19:1, v/v) atmosphere. The medium was changed after 24 h and every 2 to 3 days thereafter. The cells were not used for experiments until they had been subcultured once.

2.2.3 Determination of cell number, cell viability and cell size

(i) Estimation of cell number and viability via haemocytometer

The most commonly used method for determining cell viability or total cell number in a cell suspension is the dye exclusion method.

After harvesting and resuspending the cell pellet in a known volume of growth medium and disaggregating the cells, a small aliquot of cell suspension (100 µl) was mixed with 100 µl trypan blue solution (0.6% w/v trypan blue in PBS) such that approximately 50-100 cells were observed over each of the 9 large squares of the haemocytometer chamber. The number of viable (unstained) cells and total cells (stained

and unstained cells) in the large centre square and the four large corner squares was counted. Then the total cell number and the percentage viability of a particular cell line could be determined.

The large squares in the haemocytometer have an area of 1 mm^2. When the coverslip is passed over the grid, the depth of the chamber is 0.1 mm

Thus the total volume over each large square = 1 x 1 x 0.1

$= 0.1\ mm^3$

$= 1x10^{-4}$ ml

Cells were counted from 5 large squares and the mean cell number (n) was calculated. Since the cell suspension was mixed with same volume of trypan blue, i.e. diluted by 2-fold

$\therefore$ the no. of cells per ml in the suspension = $n \times 10^4 \times 2$

From the cell number obtained from the haemocytometer the cell viability can be calculated:

$$\% \text{ viability} = \frac{\text{viable cell number}}{\text{total cell number}} \times 100$$

(ii) Determination of cell number by Model D Coulter Counter

Although the haemocytometer can provide information such as viability and cell number, it is a time-consuming method and limited the number of samples that could be handled in a day. Nowadays, automatic cell counting equipment is available, and the Coulter Counter is one of the most commonly used machines. The determination of cell number is based on the generation of voltage pulses as the particles (cells) are forced to pass through a small aperture having an immersed electrode on either side. As a particle

passes through the aperture, it changes the resistance between the electrodes. This produces a voltage pulse of short duration having a magnitude proportional to the particle size. The series of pulses were then electronically scaled and counted.

The volume of the particle size (V) = t.I.A

Where t = threshold, I = Aperture current, A = Attenuation

and the particle diameter in μm = $k\sqrt[3]{V}$

Where k = calibration constant for the machine

After harvesting and resuspending in growth medium, a small aliquot of cell suspension was suitably diluted with Isoton II solution (Coulter Electronics, Luton). The blank was also counted using Isoton II solution. Optimum settings for each cell type were established and they were A = 2, I = 0.017 and t = 20 for CHO-K1 cells and A = 4, I = 0.017 and t = 20 for WRK-1 cells.

(iii) Determination of cell size distribution using micrometer

Cells from a 25 cm^2 flask were harvested and the cell pellet was respended in medium. A small aliquot was diluted with medium (5000 cell per ml) and the cell diameters were measured using a Watson-Barnet micrometer with a calibrated eye piece on a Zeiss light microsope.

(iv) Determination of cell size distribution by Coulter Counter

Cell sizing was done by harvesting exponentially growing cells using trypsin-EDTA and resuspending the cells in 10 ml growth medium. Then an aliquot of cell

suspension was added to 200 ml Isoton II such that the cell count was less than 10,000 when the lowest settings (A=1, I=0.0033, t=10) were used. The cell size distribution was obtained by determining the cell counts at a number of different aperture (A), threshold (t), and current settings (I) as recommended in the Coulter manual. A background count was performed using Isoton II and counted at different settings. In order to obtain statistical accuracy for each size level, the following rule as recommended in the Coulter manual was followed:

6 counts for number less than 100

4 counts for number between 100 to 1000

2 counts for number more than 1000

2.2.4 Determination of growth characteristics of CHO-K1 and WRK-1 cells

(i) Growth curve

Exponentially growing cells were harvested from a 75 cm^2 flask and the cell number was determined by Coulter Counter as described. Different numbers of cells ($5x10^4$-$5x10^5$ of CHO-K1 cells or $2.1x10^4$-$5x10^5$ WRK-1 cells)were then seeded into a number of 6-well plates with each well containing 3 ml growth medium. The medium was changed every day. Every 24 h, a plate of different seeding density was harvested and the total number of cells per well was determined by Coulter Counter.

The doubling time during exponential growth can be calculated as follows:

$$\text{Doubling time} = \frac{t_2 - t_1}{3.32(\log N_{t2} - \log N_{t1})}$$

Where $t_2 > t_1$, t_2 = time at point 2, t_1 = time at point 1

N_{t2} = cell number at t_2 N_{t1} = cell number at t_1

(ii) Determination of the time taken for the cells to attach to substratum

Exponentially growing cells were harvested from a 25 cm^2 flask and the cell number was determined. The cell suspension was then suitably diluted to give a density of 200,000 cells/ml. An aliquot of the diluted cell suspension (1 ml) was added to several 25 cm^2 flasks with each contained 9 ml of growth medium and the cells were incubated at 37°C in an incubator. Every half an hour, the medium was discarded from 2 flasks and cells attached to these flasks were harvested and counted. Hence the number of cells that attached to the bottom could be determined.

(iii) Plating efficiency

Different numbers (100-600 cells) of CHO-K1 cells were seeded in several 25 cm^2 flasks. The medium was changed the following day and every 2-3 days thereafter. On day 9, cells were fixed and stained with trypan blue and the number of colonies were counted. Each colony is assumed to derive by clonal growth from a single cell, so the number of cells that attached after seeding is equivalent to the total colony number.

$$\text{Then plating efficiency (\%)} = \frac{\text{no. of colonies formed}}{\text{no. of seeded cells}} \text{x100}$$

The method for WRK-1 cells was slightly different because these cells tend to migrate together, hence the clonogenic assay was not suitable for determining plating efficiency.

Instead, different numbers of WRK-1 cells were seeded into a number of 25 cm^2 flasks with a grid at the bottom. After 24 h, the medium was discarded, and the cells were fixed and stained as described in section 2.2.4 (iv). The number of cells remaining attached were counted under an inverted microscope. Since the cell number does not

change in the first 24 h, the cell number remaining was directly related to the plating efficiency.

(iv) Cell fixing and staining

Growth medium was discarded and cells were fixed with 10 ml PBS/methanol (92:8, v/v) for 15 min. The fixative was then discarded and the cells were stained with 0.6% (w/v) trypan blue in PBS for 10-15 min. Stain was then discarded and the cells were washed once with PBS to remove the trypan blue residue. Cells or colonies were counted using an inverted microscope.

2.3 Results

Initially, the average cell size was determined using a light microscope fitted with a micrometer gauge, and by using the Coulter Counter.

The cell size distribution curve of CHO-K1 cells obtained from micrometer readings gave a mean cell diameter of 12.50 μm and a standard deviation of 1.56 μm (Figure 2.1a). The mean cell diameter obtained via Coulter Counter was 13.40 μm and a standard deviation of 3.99 μm (Figure 2.2a) which was comparable to that obtained from the micrometer. It was important to establish that the Coulter Counter readings gave a true reflection of the cell number in the suspension. Hence a direct comparison between cell number obtained from the haemocytometer and Coulter Counter was performed (Table 2.1a). The result suggested that the settings for counting particles of diameter of 8.60 μm or above correlated with the result obtained from the haemocytometer. These settings corresponded to A = 2, I = 0.017 and t = 20. Thus by using these settings, the Coulter Counter could be used confidently to determine cell number in future experiments.

The cell size distribution curve for WRK-1 cells obtained from micrometer readings gave a mean cell diameter of 18.80 μm and a standard deviation of 2.83 μm (Figure 2.1b). The result obtained from the Coulter Counter was comparable to that obtained from the micrometer, with a mean diameter of 20.10 μm and standard deviation of 5.52 μm (Figure 2.2b). A direct comparison of cell counts from the haemocytometer and Coulter Counter suggested that settings of A = 4, I = 0.017 and t = 20, which counted particle diameters of 10.90 μm or above (Table 2.1b), were the best settings to use in future experiments.

The growth curve for a particular cell line is important in order to predict the cell

number and the growth period required to achieve a particular cell density. Since most of the future experiments were to be performed in 6-well plates, these plates were used for the determination of the growth characteristics of both cell lines.

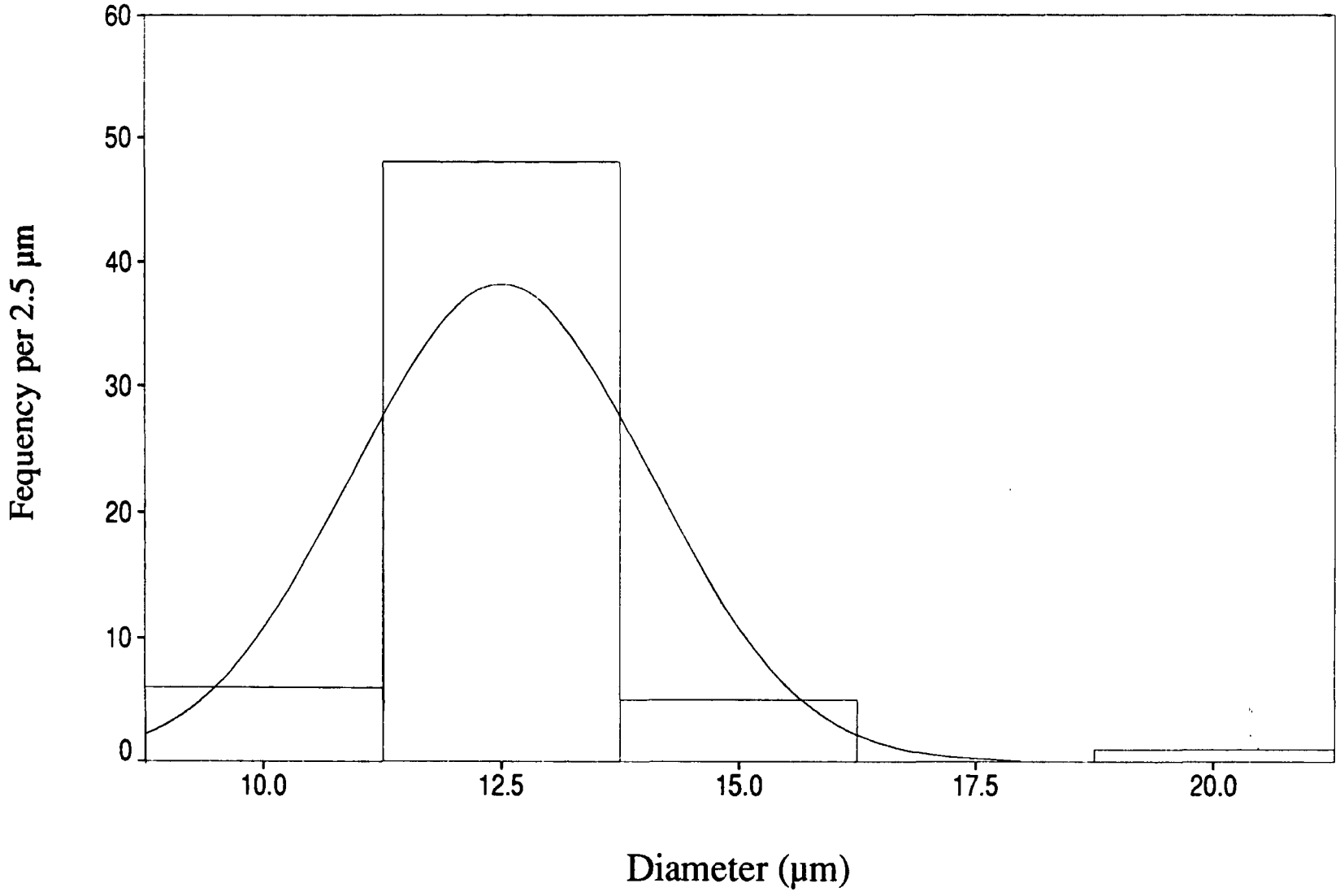
The growth curve of CHO-K1 cells showed that all three different seeding densities had a lag period of 24 h (Figure 2.3a). When cells were seeded at low densities i.e. 5 x 10^4, the cell number was reduced by 60% after 24 h, whereas if seeded at high densities this was not observed. This suggested that the seeding efficiency was affected by the seeding density. The cells then entered the log phase within 48 h. The doubling time of different densities at log phase were very similar with an average doubling time of 15.40 h. When the cell number in each well reached about 2 million, the growth rate slowed down and finally reached plateau with a density of 7 million per well.

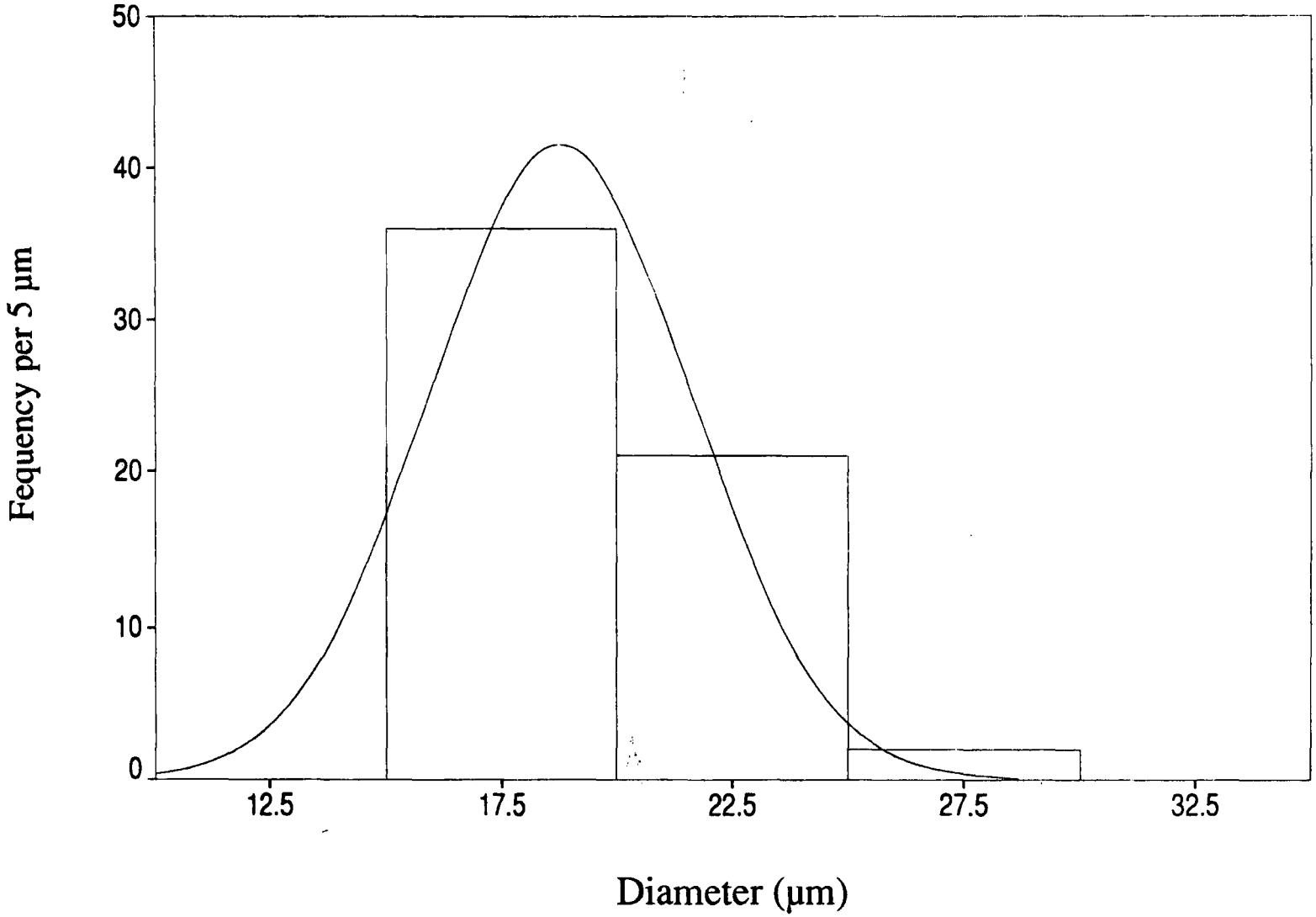
The growth curve of WRK-1 cells suggested that when cells were seeded at low densities 21 x 10^3, 43 x 10^3, the cell number decreased by 15.9% and 35.8%, respectively during a lag period of 24 h (Figure 2.3b). If the cells were seeded at higher densities i.e. 87 x 10^3, 175 x 10^3 and 500 x 10^3, the cell number remained similar to the seeded number, again suggesting that the seeding efficiency was affected by the seeding density. The cells entered the log phase within 48 h irrespective of the seeding density. During the log phase, the average doubling time was 21.60 h, and different seeding densities gave a similar result. When the cell density reached 1 x 10^6 per well, the growth rate slowed down and finally reached confluence with a maximal density not exceeding 2 million cells in a well.

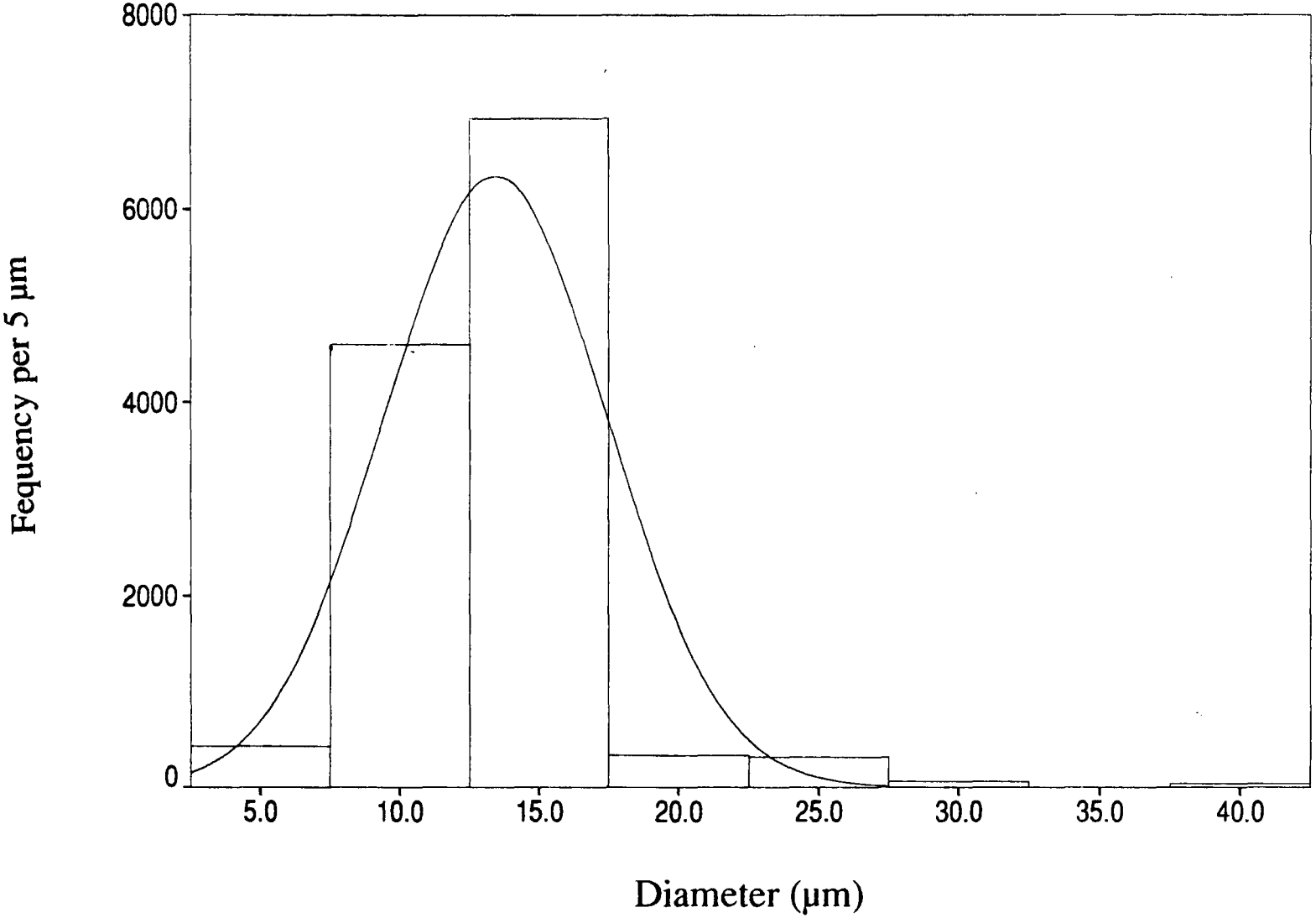
Prior to assessing the effect of temperature on hyperthermic cell death, plating efficiency was determined. For CHO-K1 cells, it was determined by counting the number of colonies formed from a low inoculum of cells (100-600 cells/flask) after 9 days. For WRK-1 cells, it was determined by inoculating low number of cells (100-500

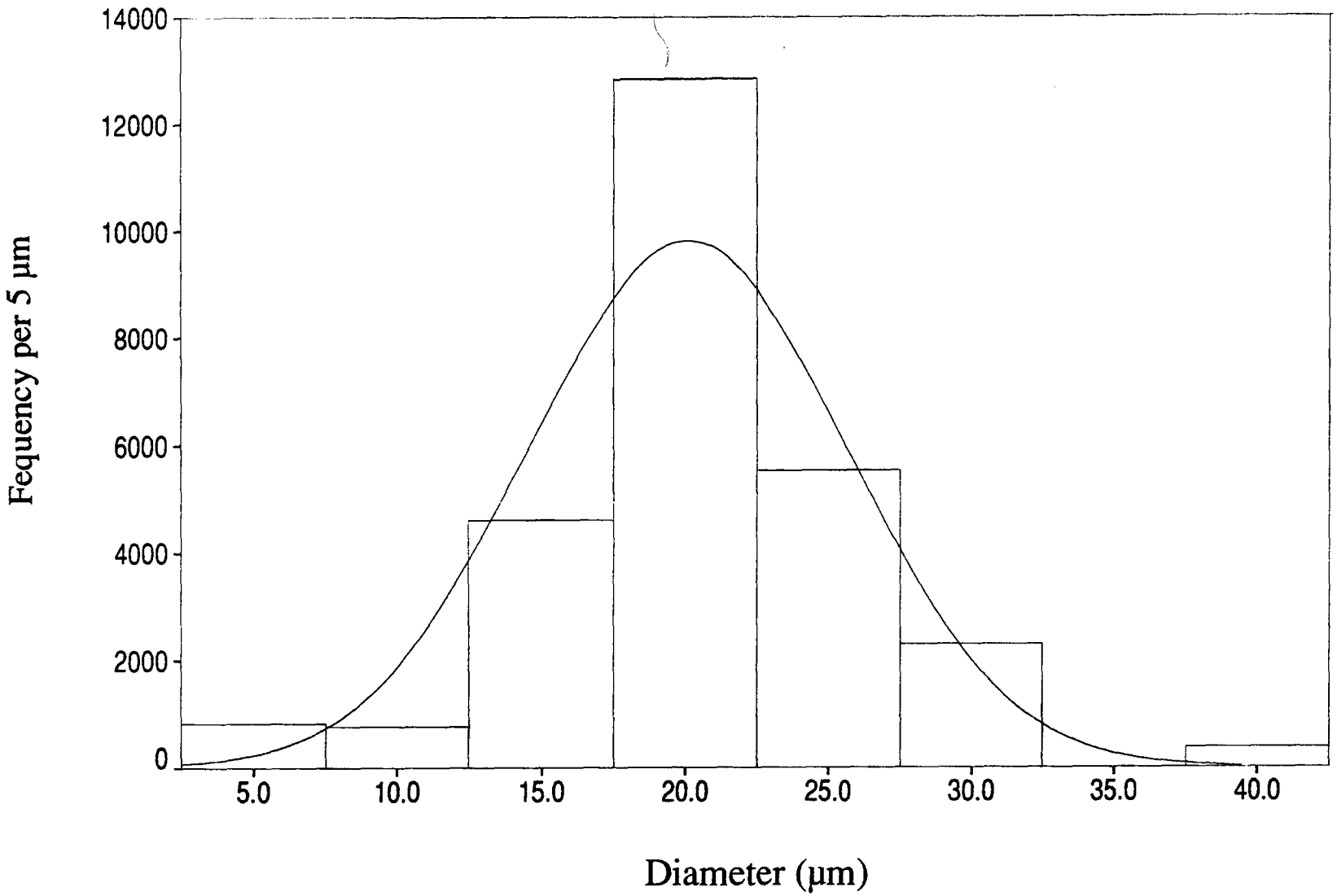
cells/flask) and the number of cells remaining attached was counted on the following day. The plating efficiency of CHO-K1 cells at different seeding density gave similar results with an average plating efficiency of 94.2% (Table 2.2a). In the case of WRK-1 cells, the average plating efficiency was 63.8% (Table 2.2b).

During heating, cells tend to detach from substrate and it is important to allow sufficient time for the viable cells to re-attach before the medium is changed. Hence the time taken for cells to re-attach to the substrate was determined (Figures 2.4a and 2.4b). From the graphs, it could be concluded that at least 3 h was required for most of the cells to re-attach. Longer times only improved the percentage of adhered cells slightly.


60
50
40
30
20
10
0
Fequency per 2.5 µm
10.0
12.5
15.0
17.5
20.0
Diameter (µm)


50
40
30
20
10
0
Fequency per 5 µm
12.5
17.5
22.5
27.5
32.5
Diameter (µm)


8000
6000
4000
2000
0
Fequency per 5 µm
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Diameter (µm)


14000
12000
10000
8000
6000
4000
2000
0
Fequency per 5 µm
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Diameter (µm)

Tables 2.1a. Comparison of CHO-K1 cells count via Coulter Counter and haemocytometer

CHO-K1 cells were harvested from 25 cm^2 flask. The pellet was then resuspended in 3 ml of medium. The number of cells in the suspension was then counted via Coulter Counter or haemocytometer.

Cell count from haemocytometer = 4.48 x 10^6 cells/ml

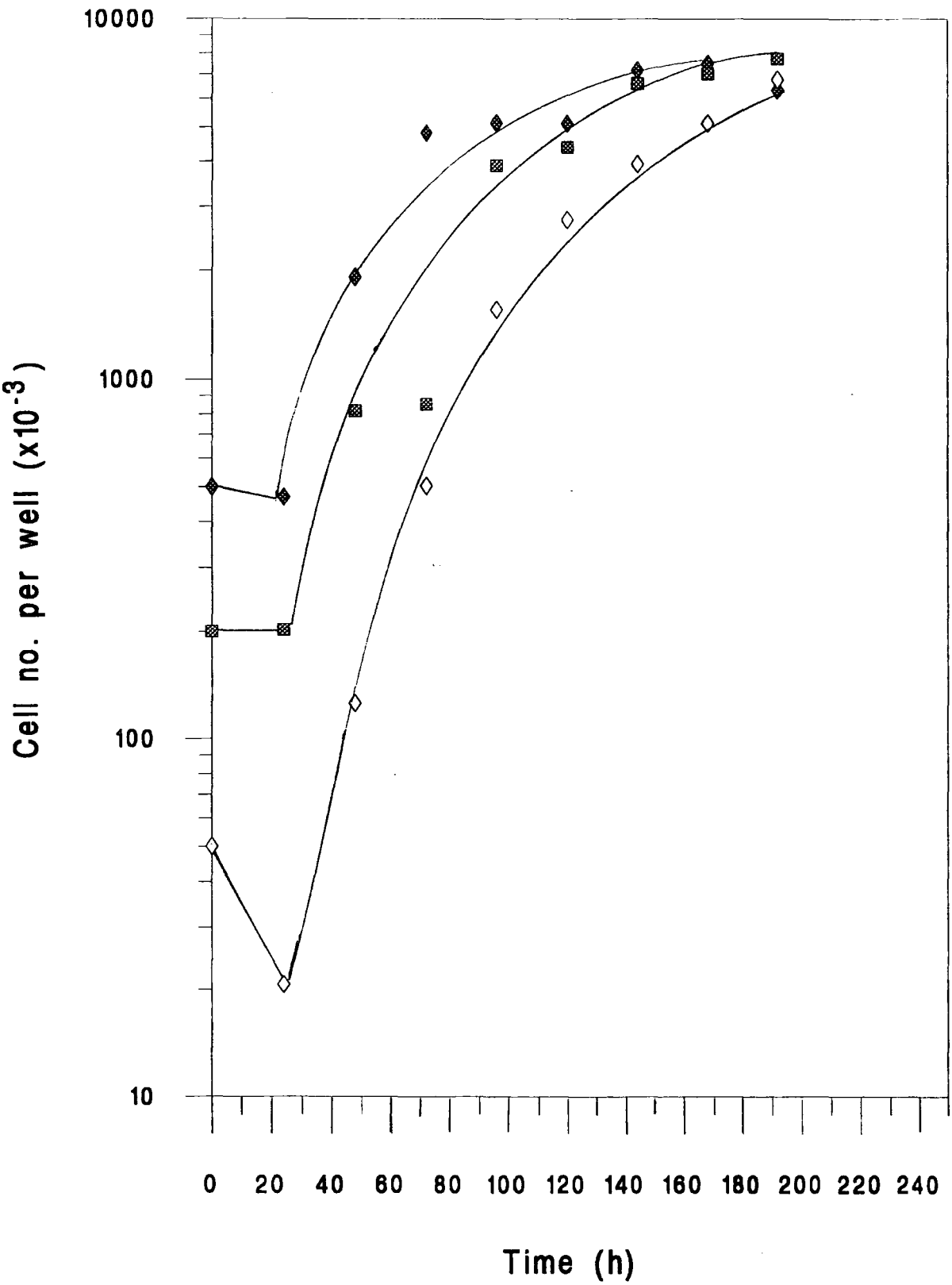
Minimum diameter (μm)	Count from Coulter Counter (x10^6 cells/ml)
5.6	4.93
8.6	4.48
10.9	3.60
12.4	1.77
13.7	0.73

Tables 2.1b. Comparison of WRK-1 cells count via Coulter Counter and haemocytometer

WRK-1 cells were harvested from 25 cm^2 flask. The pellet was then resuspended in 3 ml of medium. The number of cells in the suspension was then counted via Coulter Counter or haemocytometer.

Cell count from haemocytometer = 576 x 10^3 cells/ml

Minimum diameter (μm)	Count from Coulter Counter (x10^3 cells/ml)
8.6	631
10.9	575
13.7	548
17.1	273
19.5	86
21.5	42


10000
1000
100
10
Cell no. per well (x10-3)
0
20
40
60
80
100
120
140
160
180
200
220
240
Time (h)

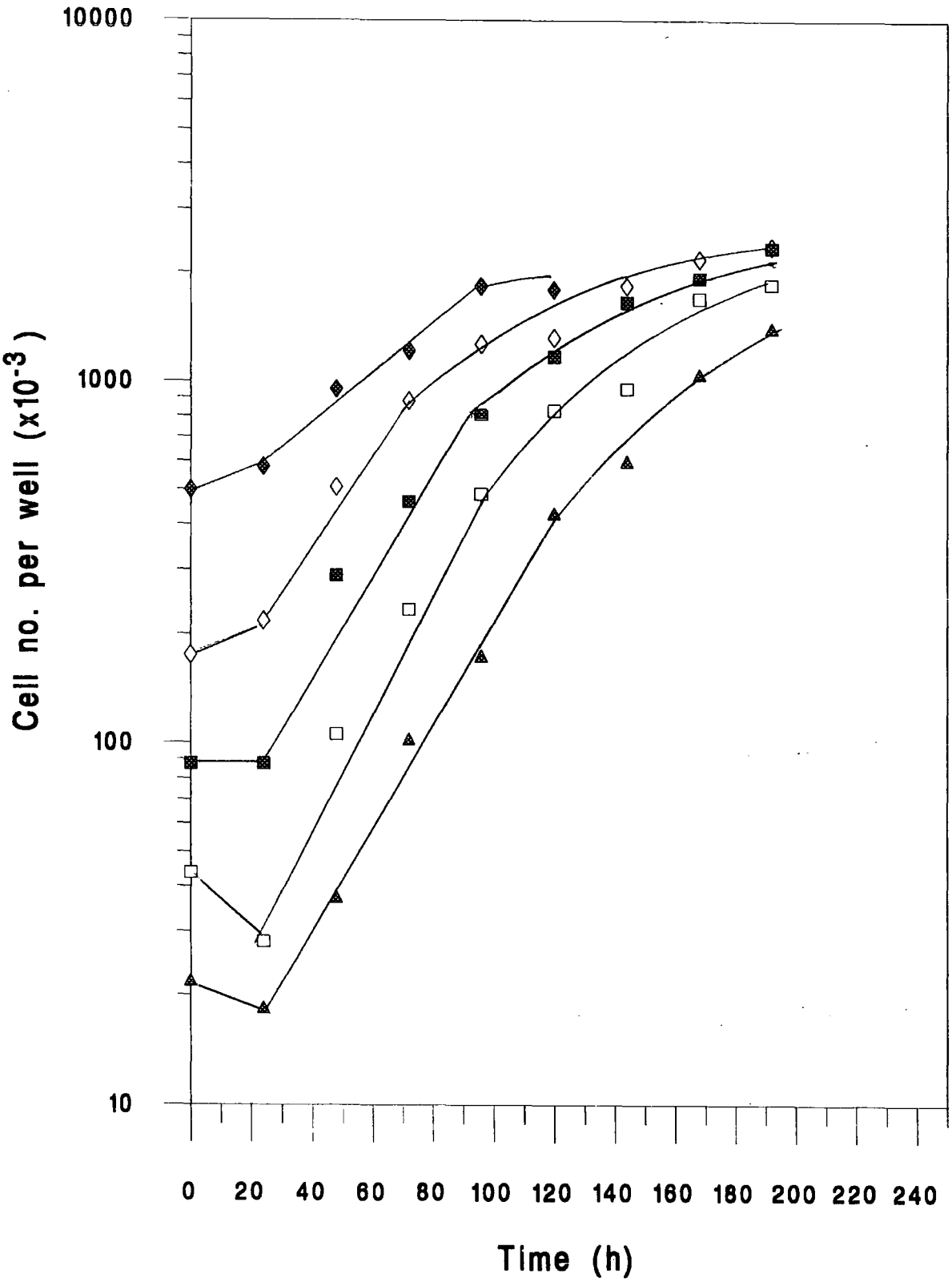

10000
1000
100
10
Cell no. per well (x10-3)
0
20
40
60
80
100
120
140
160
180
200
220
240
Time (h)

Table 2.2a. Plating efficiency of CHO-K1 cells

Cells were harvested and seeded as described. Medium was changed after 24 hours and on day 4 and 7. On day 9 the cells were fixed, stained and the number of colonies were counted.

No. of cells inoculated (cells/flask)	No. of colonies	Average plating efficiency (%)
100	104±7.75	103
200	186±13.4	93
300	294±8.8	98
400	370±16.7	92
500	443±13.9	89
600	540±9.09	90

Average plating efficiency = 94.2%

For CHO-K1 cells, results were obtained from 4 determinations per seeding density.

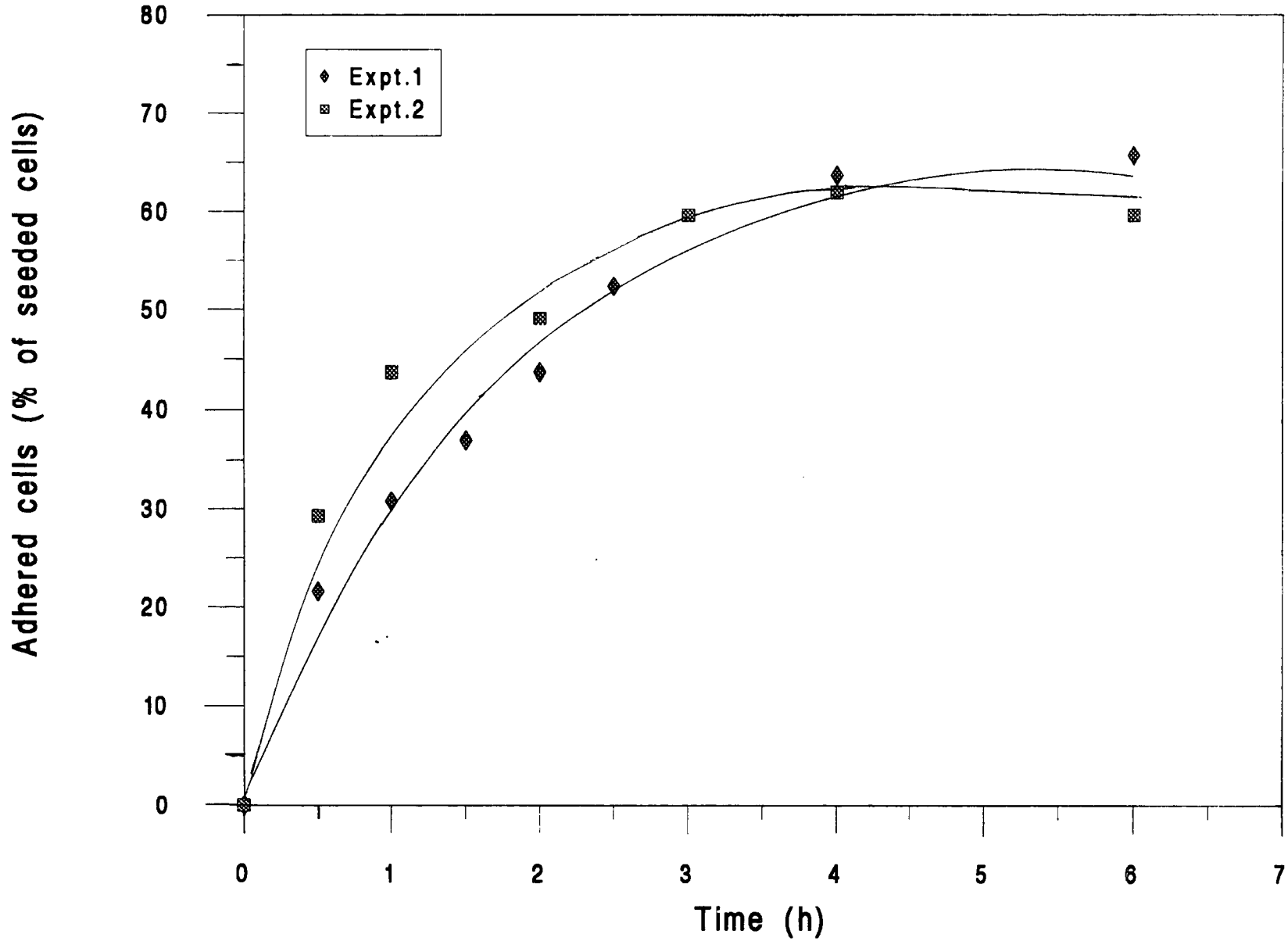
Table 2.2b. Plating efficiency of WRK-1 cells

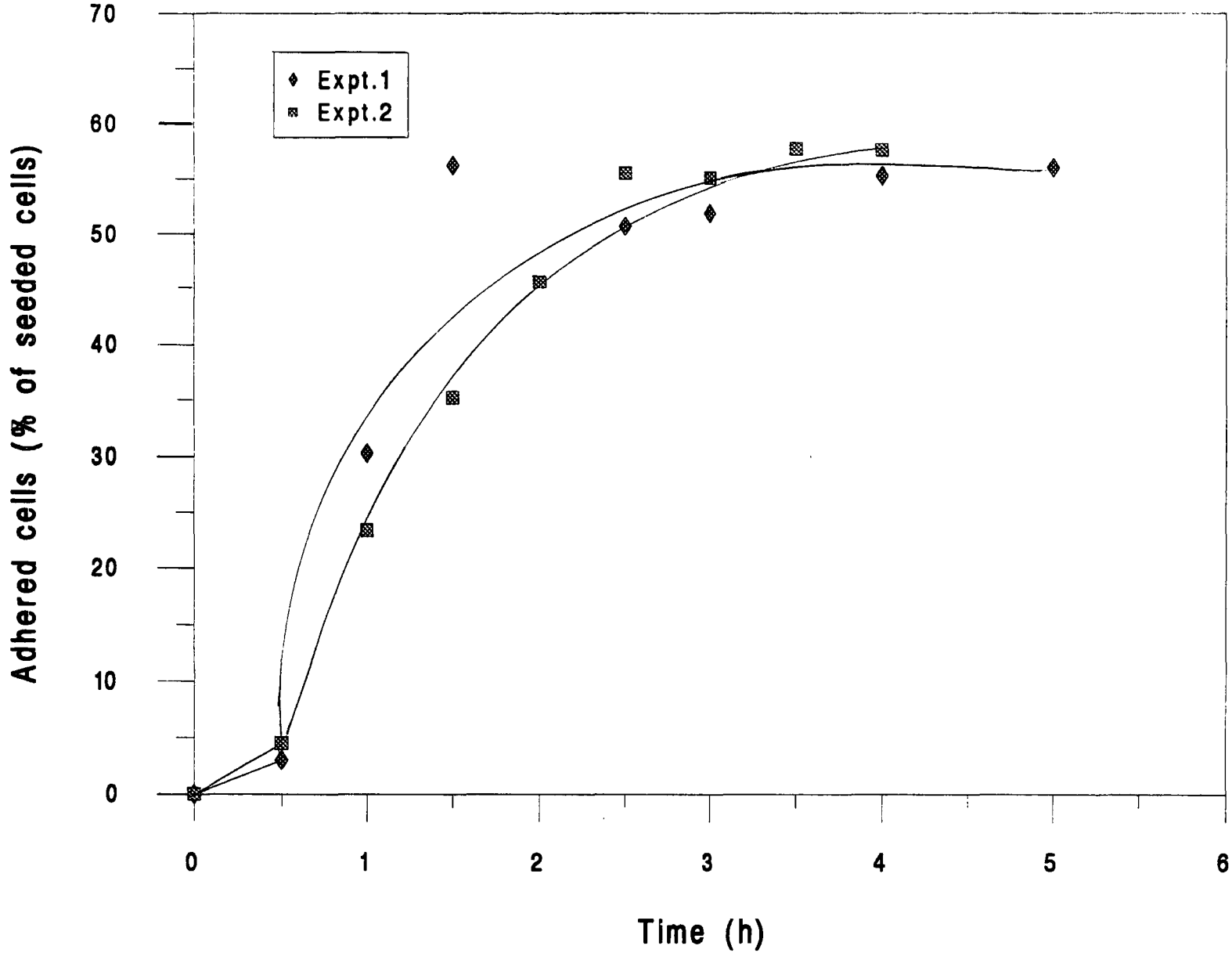
Cells were seeded at different densities into 10 ml growth medium in a number of 25 cm^2 flasks with grid at the bottom. After 24 h in culture, the medium was decanted and the cells were fixed with PBS/methanol (1:3, v/v) for 15 min. The fixative was then discarded and the cells were stained with trypan blue for 10 min. Cells were then washed with PBS once and the cells remaining were then counted on an inverted microscope.

No. of cells inoculated (cells/flask)	Average cell number	% of plating efficiency
100	73	73
200	115	58
300	186	62
400	257	64
500	309	62

Average plating efficiency = 63.8%

For WRK-1 cells, results were obtained from a single experiment with duplicate flasks per density


Adhered cells (% of seeded cells)
80
70
60
50
40
30
20
10
0
Expt.1
Expt.2
0
1
2
3
4
5
6
7
Time (h)


Expt.1
Expt.2
Adhered cells (% of seeded cells)
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (h)

2.4 Discussion

Before using a particular cell line for experimental work, it is important to optimise methods to quantify cell number, and the kinetics of cell growth must be determined. The first part of this chapter describes the method used to establish correct Coulter Counter settings. Then the growth characteristics of the cells, such as doubling time and plating efficiency, were determined.

The Coulter Counter provides a quick method to count cells. Since a large number of cells are counted at one time, it also provides a greater accuracy for determination of cell number. However there are some drawbacks in using this machine: 1) It cannot discriminate between live and dead cells. 2) It counts cells which exceed a certain diameter but cannot discriminate single cells from clumps of cells. 3) It may count cell debris as particles (cells) if the particle size is set too low. The first disadvantage was not a problem if the machine was only used for determining cell number in a cell suspension as cells remained viable after harvesting, but, it would be a problem if the machine was used for determination of cell viability after cytotoxic treatments e.g drug treatment. The second disadvantage was resolved by efficient disaggregation of cells before measurement. In view of these drawbacks, the correct setting of the Coulter Counter was crucial to give a true reflection of the cell number. In order to find out the best settings for both cell types, a comparison of counts from the haemocytometer and Coulter Counter was performed. This suggested that for CHO-K1 cells the best settings were A = 2, I = 0.017 and t = 20, whereas for WRK-1 cells, settings of A = 4, I =0.017 and t = 20 were optimum.

The growth curves for both cell types suggested that the seeding efficiencies were reduced at low seeding densities. This could be explained if cells required some cell-derived diffusible signals or conditioning factors which might be absent or too dilute at

low cell densities, thus affecting the seeding efficiency. It was observed that the growth of WRK-1 cells increased noticeably on the day following a medium change, but on the second day after a medium change the growth rate slowed down (result not shown). In order to obtain a uniform cell growth, the medium was changed every day when determining the cell growth for both cell types. The time taken for cells to reach stationary phase depended upon the seeding densities, the growth area and the nutritional conditions.

The doubling time for CHO-K1 cells in this study was 15.40 h which was rather longer than the 10 h reported by Kao and Puck (1967). For WRK-1 cells the doubling time had not been reported before, and in this study it was found that the doubling time was 21.60 h during log phase.

Although the doubling time of WRK-1 cells had not been reported before, it had been reported that rat serum was indispensable for its growth (Kidwell *et al.*, 1978). In the absence of rat serum the growth rate declined and cells finally died after 5-7 days. Fatty acid analysis of the rat serum suggested that it had 19-fold higher linoleic acid content than FBS. Addition of pure linoleic acid to FBS-containing medium improved the growth rate by four times compared with growth in FBS-containing medium. However linoleic acid was not totally capable of replacing rat serum suggesting that some component(s), such as hormone(s), in the rat serum was necessary for maximum cell growth (Kidwell *et al.*, 1978).

The plating efficiency of CHO-K1 cells had been reported to be largely affected by the concentration of proline in the medium (Kao and Puck, 1967). A constant plating efficiency (78%) was obtained when proline concentration was between 3×10^{-5} to 1×10^{-2} M. Proline concentrations above 0.1 M or below 1×10^{-5} M would give a 0% plating efficiency. The plating efficiency obtained in this study was 94.2% and different inoculated numbers gave similar results. The variation in the plating efficiency could be

due to the different concentration of serum being used (2% in the previous study and 10% in this study). Plating efficiency was also affected by the stage of cell growth and the method of harvesting.

As mentioned previously, WRK-1 cells migrated together, so the method for determining plating efficiency had to be modified. The average plating efficiency for this cell type was found to be 63.8%.

Different cell types have different affinities for the culture substrate and would attach to substrate at different rates. From the curves of cell attachment (Figures 4a and 4b), it was determined that at least 3 h was required for both cell types to re-attach to the substrate. Hence 4 h was chosen as the time to be allowed routinely for maximum attachment before proceeding with any further experimental manipulations.

In summary, this phase of the work established the culturing techniques, growth conditions and growth characteristics for both CHO-K1 and WRK-1 cells.

Chapter 3

Effect of heat on monoacylglycerol, 1,2-diacylglycerol and triacylglycerol levels

3.1 Introduction

The rationale for the use of hyperthermia in cancer treatment is the putative differential heat sensitivity of tumours as a consequence of poorly developed blood flow, relative nutrient deprivation and the accumulation of metabolic waste products (Field, 1987). Furthermore, the complementary or additive effect with radiotherapy and additive effect with chemotherapy all render hyperthermia an attractive modality for cancer treatment (Ross and Watmough, 1986).

Temperature has an all pervasive influence on cellular structures and this makes it difficult to identify the primary sites of lesion as well as the mechanistic relationship between events that lead from thermal damage to cell death. Indeed, at one time or another, all the major cellular structures have been implicated as having a significant role in heat injury (Roti Roti, 1982).

The plasma membrane forms the boundary of the cell and is in direct contact with the environment, and it has been suggested that this membrane plays an important, if not a primary role, in hyperthermic cell death (Yatvin, 1977; Hahn, 1982; Bowler, 1987; Laszlo, 1992). Evidence for this notion comes from the synergistic effect of heat with other membrane-active agents such as aliphatic alcohols and local anaesthetics (Yatvin, 1977; Hahn, 1982; Kim, 1988).

Many of the specific functions associated with the plasma membrane are carried out by proteins that are embedded in the membrane. Several reports suggest that heat affects the biological activities of a number of membrane proteins including membrane-bound ATPase enzymes, nutrient transporters and cell signalling molecules. For example it has been reported that the Ca^{2+} ion transport function of purifed Ca^{2+}-ATPase from sarcoplasmic reticulum reconstituted into an artificial membrane was inactivated at hyperthermic temperature (Cheng *et al.*, 1987). It has also been reported that glucose transport into CHO cells is impaired above 45°C (LeCavalier and Mackillop, 1985) and uridine uptake is inhibited at temperatures above 45°C in several mammalian cell types (Magun, 1981). Investigation of both mitochondrial and plasma membrane protein transition in Chinese hamster V79 cells (CHL V79 cells) by measuring both intrinsic protein fluorescence and energy transfer from membrane protein to lipid soluble fluorescent probe (trans-paranaric acid) suggested that irreversible protein transitions occur in membranes at 40°C to 41°C (Lepock *et al.*, 1983). This membrane protein transition correlated well with hyperthermic cell death of CHL V79 cells, so it was suggested that protein-associated functions of membranes would be impaired at elevated temperature, and hyperthermic cell killing may be associated with the effects of heat on the protein components of the plasma membrane.

A great deal of interest has been shown in the possibility of cell signalling mechanisms being modified by hyperthermia. The binding of insulin to its receptors in CHO HA-1 cells was inhibited when cells were exposed to 43°C to 45°C and the inhibition was found to be due to a reduction in receptor number and not receptor affinity (Calderwood and Hahn, 1983). On the other hand, EGF binding to its membrane-bound receptors in Rat-1 fibroblasts was inhibited after cells were exposed to 45°C for 30 min, and this inhibition was due to a decreased affinity of the receptors for the ligand (Magun and Fennie, 1981). The effect of heat on modifying the phosphoinositide signalling pathway has been suggested and it is further postulated that

prolonged heating (45°C for longer than 15 min) would result in the depletion of polyphosphoinositides, which might be associated with cell killing, either as a result of disruption of membrane integrity or calcium homeostasis which could result from the accumulation of inositol trisphosphate (Calderwood *et al.*, 1987). The aim of this study is to investigate the effect of heat on the phosphoinositide signalling pathway by measuring the changes in the levels of inositol phosphates, 1,2-diacylglycerol and cytosolic free calcium concentration. In order to understand the effect of heat on phosphoinositide signalling pathway, it is necessary to consider the general phenomenon of this pathway.

The first indication that inositol phospholipids may play a role in intracellular signal transduction was described by Hokin and Hokin (1953). They found that stimulation of pancreatic acinar cells with acetylcholine led to the incorporation of $^{32}P_i$ into the phospholipid fraction. It was then realised that this rapid, agonist-induced phosphorylation of lipid is essentially confined to phosphatidylinositol (PtdIns) and phosphatidic acid (PA). The increase in PA was the consequence of phosphorylation by DAG kinase of 1,2-diacylglycerol (1,2-DAG) that was generated from hydrolysis of inositol lipids. PA was then recycled for PtdIns synthesis. However, the linkage between PtdIns hydrolysis and the cellular events was unclear until 1975, when Michell proposed that PtdIns turnover may be one of the earliest event of the signal cascade, preceding and perhaps causing calcium mobilisation. This hypothesis was supported by the result obtained by Berridge and Fain (1979) who showed that the ability of 5-hydroxytryptamine (5-HT) to stimulate Ca^{2+} movement across the plasma membrane of the blowfly salivary gland was lost upon prolonged stimulation, and that this response can be reinstated if the desensitised glands are incubated in a medium containing inositol. This result suggested that an inositol-related substance is essential for receptor-controlled mobilisation of Ca^{2+}. Similar results were observed by other groups using different cell types (Abdel-Latif *et al.*, 1977; Michell *et al.*, 1981). The subsequent

acceleration of the pace of phosphoinostide signalling research was provoked by 3 discoveries, (1) the lipid hydrolysed in response to receptor activation is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Michell *et al.*, 1981); (2) 1,2-diacylglycerol (1,2-DAG) formed from hydrolysis of PtdIns(4,5)P_2 activates protein kinase C (PKC) (Takai *et al.*, 1979); (3) the released inositol 1,4,5-trisphosphate ((Ins(1,4,5)P_3) from the head group of PtdIns(4,5)P_2 causes release of Ca^{2+} from an intracellular store (Streb *et al.*, 1983) (Figure 3.1).

The mechanism of agonist-stimulated PtdIns(4,5)P_2 hydrolysis involves a receptor, a G protein (in case of growth factor receptors a G protein is not necessary), and an effector protein, phosphoinositide-specific PLC (PI-PLC). Binding of agonist to its receptor induces a conformational change of the receptor. This conformational change then transmits the signal to a heterotrimeric G-protein which is composed of α, β and γ subunits, allowing the tightly bound GDP on the α-subunit to exchange with GTP. Binding of GTP to the G protein leads to dissociation of the α-subunit from βγ-dimer. The dissociated α-subunit then activates PI-PLC, causing the breakdown of PtdIns(4,5)P_2. The bound GTP is then hydrolysed to GDP by the GTPase enzyme activity intrinsic to the α-subunit. Upon GTP hydrolysis, the α-subunit undergoes a conformational change, regains its high affinity for the βγ complex, and the α-subumit dissociates from the PI-PLC, inactivating this enzyme (Taylor, 1990; Cockcroft and Thomas, 1992).

Orginally, it was thought that the α-subunit of a G protein was solely responsible for the activation of an effector protein, whereas the βγ-subunit complex acted solely as a regulatory component for the α-subunit by stabilising the GDP-bound form of α, presenting the α-subunit to the receptor and serving as a membrane anchor for the oligomer. However, growing evidence supports the idea that the βγ complex can itself interact functionally with effector protein. For example, an undefined form of PLC in

HL-60 granulocytes is markedly activated by βγ (Camps *et al.*, 1992). The βγ complex also plays a role in regulating the action of adenylyl cyclase in peripheral tissues and PLA_2 in rod outer segments (Exton, 1994). Thus, the dissociation of a G protein can generate parallel and/or interactive signals via both α- and βγ-subunits.

To date, at least 21 distinct G protein α-subunits, 4 β-subunits and 6 γ-subunits have been identified (Hepler and Gilman, 1992). These G-proteins can be further divided into 2 groups depending on whether they are pertussis toxin-sensitive (PTX-sensitive) or not (Berridge, 1993). Members of the G_q family, comprising at least of G_q, G_{11}, G_{14}, G_{15} and G_{16}, are PTX-insensitive G proteins whereas members of G_o and G_i families are PTX-sensitive (Cockcroft and Thomas, 1992).

The phosphoinositide signalling pathway is further complicated by the identification of isoforms of PI-PLC. Three families of PI-PLC have been identified, known as β, γ and δ and the existence of at least 2 more families (α and ε) has been suggested. Each family has subtypes as well, and at least 9 isoforms of PI-PLC have been characterised so far (Rhee *et al.*, 1989; Rhee and Choi, 1992a). PI-PLCβ is coupled to a specific class of G protein, G_q, which is PTX-insensitive (Rhee *et al.*, 1989). PI-PLCγ1 is activated upon the phosphorylation of 3 tyrosine residues at 771, 783 and 1254 by receptor tyrosine kinases and phosphorylation of tyr 783 was shown to be essential for its activation (Rhee and Choi, 1992b). Phosphorylation of tyr 753 and 759 is responsible for the activation of PI-PLCγ2 (Rhee and Choi, 1992b). The mode of activation of PI-PLCδ is unknown. The identification of PI-PLCα was based on the use of an antibody against a purified PI-PLCα from uterus to screen the RBL I library (Bennett and Crooke, 1987). The lack of sequence homology of the putative PI-PLCα, compared with other PI-PLC isoforms, suggests that it is a distantly-related species and, in fact, it shows more similarity to a thiol-protein disulphide oxidoreductase (Cockcroft and Thomas, 1992). PI-PLCε has been found to be under the control of an unidentified

G protein (Martin *et al.*, 1991).

There is common agreement that the α-subunit of the PTX-insensitive G_q family stimulates PI-PLCβ isoenzymes, with PI-PLCβ2 being less responsive and PI-PLCβ1 and PI-PLCβ3 more responsive to αq. On the other hand, $G_{\alpha 16}$ stimulates PI-PLCβ2 only. Apart from regulation by PTX-insensitive G proteins, it is also known that the PI-PLCβ isoenzyme can be regulated by a PTX-sensitive G protein (Exton, 1994). However, efforts to identify the PTX-sensitive α-subunit that activates PI-PLC isoezymes have generally been unsuccessful, and it is now thought that activation of PI-PLC by PTX-sensitive G proteins involves the βγ complexes. Although the G proteins that release βγ subunits as a result of receptor activation are not absolutely identified, there is direct evidence that they are subtypes of G_i and G_o (Exton, 1994). The complexity and the numbers of G proteins, the diversity of the α and βγ interactions with effectors and the multiple isoforms of PI-PLC endows cells and organisms with extraordinary capacity for fine tuning both the magnitude and the nature of their responses to agonists. Furthermore, different PLC enzymes may be involved in different functions due to their substrate specificity and different mode of activation (Exton, 1994).

Hydrolysis of PtdIns(4,5)P_2 via PI-PLC upon agonist-stimulation will give rise to Ins(1,4,5,)P_3 and 1,2-DAG (Berridge, 1984; Berridge, 1987; Catt *et al.*, 1991; Exton, 1994). Ins(1,4,5)P_3 is released into the cytosol and causes calcium mobilisation from internal stores such as ER (Berridge and Irvine, 1984; Berridge, 1993) or calciosomes (Volpe *et al.*, 1988). The action of Ins(1,4,5)P_3 is terminated by metabolism to Ins(1,3,4,5)P_4 or Ins(1,4)P_2. These 2 compounds may be dephosphorylated sequentially to give rise to free inositol, which is then recycled to replenish the inositol lipid pool. The Ins(1,4,5)P_3 metabolism is very complex and some of the metabolites may have a functional role (see Chapter 4).

1,2-DAG remains in the membrane where it binds to and activates a serine, threonine-directed protein kinase (PKC) in the presence of phosphatidylserine (PtdSer) and Ca^{2+} (Nishizuka, 1984; see below). In unstimulated cells, 1,2-DAG is almost absent from membranes, but is transiently produced in response to extracellular signals. Unlike the other known messengers such as cAMP and Ins(1,4,5)P_3, which are H_2O-soluble and can be released into the cytosol to interact with their targets directly, the only way for 1,2-DAG to move from one region of the plasma membrane to another or to the cytoplasmic space is by forming a complex with a lipid transfer protein. Thus the movement of 1,2-DAG is far more restricted than cAMP and Ins(1,4,5)P_3. It has been suggested that 1,2-DAG is responsible for conveying information (by action of PKC) that is responsible for long-term responses such as proliferation and differentiation whereas Ins(1,4,5)P_3 is responsible for rapid cellular changes such as alteration in $[Ca^{2+}]_i$ as well as the activation of calcium-dependent enzymes (Liscovitch, 1992).

To date, 12 subspecies of PKC have been identified in mammalian tissues, which are α, βI, βII, γ, δ, ε, ζ, η, θ, ι, λ and μ (Dekker and Parker, 1994). These subspecies are categorised into 3 different groups according to the sequence homology (Stabel and Parker, 1991; Nishizuka, 1992). They are group A (also known as cPKC), group B (nPKC) and group C (aPKC). Group A PKC includes α, βI, βII and γ which are activated by Ca^{2+}, 1,2-DAG and PtdSer. The activation is also enhanced by cis unsaturated fatty acid and *lyso*phosphatidylcholine (*lyso*PC). Group B consists of δ, ε, η and θ subtypes. The lack of a Ca^{2+}-binding site on this group eliminates the requirement of Ca^{2+} for their activation. This group of PKCs has been found to be integrated into the signalling cascade that is initiated by growth factor receptors, eventually leading to the regulation of a nuclear event such as cell cycle control (Rossomando *et al.*, 1992). The ζ subtype is classified as group C, and the activation is dependent on PtdSer but is not affected by 1,2-DAG and Ca^{2+}. The signal to activate this group of PKC and the

mechanisms of activation of this PKC family remains unknown. No sequence data are available for ι, λ and μ subtypes. However it has been disclosed that PKC-μ is a member of the group B superfamily whilst PKC-ι and λ are related to PKC-ζ (group C).

A small amount of 1,2-DAG is sufficient to activate PKC at physiological calcium concentrations as long as it contains at least one unsaturated fatty acid (irrespective of the chain length of the other fatty acyl moiety) and has a 1,2-*sn* configuration. On the other hand, 1,3-diacylglycerol (1,3-DAG), triacylglycerol (TAG) and monoacylglycerol (MAG) do not have any effect on PKC (Ganong *et al.*, 1986). In the absence of agonist, most of the PKC is located in the cytoplasm and the pseudosubstrate site of PKC is bound to the substrate binding site (Figure 3.2). Upon agonist stimulation, an increase in $[Ca^{2+}]_i$ results from Ins(1,4,5)P_3 formation and subsequent release of Ca^{2+} from intracellular stores, causing PKC to interact with membranes (due to its Ca^{2+}-dependent phospholipid binding domain) where it remains in an inactive, but conformationally distinct membrane associated state. Association of PKC with membrane phospholipids is not sufficient to elicit activation of the enzyme (Huang, 1989) and activation occurs only when an activator such as 1,2-DAG becomes accessible to the membrane-bound enzyme at the intracellular membrane surface. These activator molecules appear to exert their effect by promoting the insertion of the inactive membrane-associated PKC into the membrane. During this insertion, PKC undergoes an additional conformational change such that the pseudosubstrate site is unmasked from the substrate site, thus rendering the enzyme active and capable of phosphorylating cellular substrate (Huang, 1989; Burns and Bell, 1992). Group B PKC lack the Ca^{2+}-binding domain, so the association with the membrane is not dependent upon $[Ca^{2+}]_i$. After membrane attachment, activation of these family members occurs in a manner identical to their Ca^{2+}-dependent counterparts (Burns and Bell, 1992). This model, however, does not adequately address the activation of PKC family members by effectors such as arachidonic acid and other fatty acid which appear to exert their effects in the absence of membranes. In 1982, Castagna and co-

workers reported that PKC can also be activated by 'tumour promoters' such as 12-o-tetradecanoyl-13-acetylphorbol by mimicking the effect of 1,2-DAG. This finding also suggests that some of the pleiotropic actions of tumour-promoting phorbol esters may be mediated through the action of PKC.

Since the members of the PKC family respond differently to various combinations of Ca^{2+}, PtdSer, 1,2-DAG and other phospholipid degradation products (cis unsaturated fatty acid and *lyso*PC), the pattern of activation of enzymes may vary in duration. Furthermore it has been suggested that the intracellular localisation of the PKC isoforms may vary as well, though the spatiotemporal distribution of these enzymes within cells is poorly understood.

PKC can control a wide range of cellular responses as a result of the phosphorylation of proteins. For example it has been postulated that PKC plays a role in maintaining calcium homeostasis. The greatest decrease in calcium following an agonist-stimulated $[Ca^{2+}]_i$ elevation was observed when cells were pre-treated with phorbol ester (Drummond, 1985). On the other hand, it has been proposed that PKC may play a role in enhancing calcium entry because microinjection of PKC or phorbol esters into cells enhanced the voltage-sensitive calcium current (DeRiemer *et al.*, 1985). A possible role of PKC in activating Na^+/K^+-ATPase in peripheral nerve has also been proposed (Greene and Lattimer, 1986).

PKC has also been shown to exert a negative feedback effect on the PtdIns(4,5)P_2 signalling system. The inhibition is due to the phosphorylation of PI-PLC by PKC since it has been found that treatment of intact cells with phorbol esters decreases the agonist-induced PtdIns(4,5)P_2 hydrolysis and Ca^{2+} mobilisation (Labarca *et al.*, 1984; Portilla *et al.*, 1988). Ryu and co-workers (1990) have shown that PI-PLCβ in PC 12 and NIH3T3 cells is phosphorylated by PKC at ser 887, thus modulating its interaction with

G_q. Park *et al.* (1992) also reported that phosphorylation of PLCγ1 at ser 1248 in Jurkat cells by PKC prevented receptor-induced tyr phosphorylation of PLCγ1, as well as PtdIns(4,5)P_2 hydrolysis. On the other hand, PKC seems to participate in a positive feedback loop responsible for the activation of PLD and PLA_2 (Liscovitch, 1992, Figure 3.3). Hence it has been hypothesised that PKC acts to switch activation of receptor coupling from PI-PLC at early stages of agonist stimulation to PLD and PLA_2 later in the response.

The 1,2-DAG formed from hydrolysis of phospholipids in the plasma membrane is removed by the actions of DAG kinase and DAG lipase. Monoacylglycerol (MAG), formed via DAG lipase action, is further hydrolysed to yield fatty acid (FA) and glycerol. On the other hand, 1,2-DAG can be phosphorylated to PA via the action of DAG kinase, and PA may then be used for the resynthesis of phosphatidylinositol (PtdIns).

PtdIns(4,5)P_2 is not the only source for 1,2-DAG production. It was observed that the agonist-stimulated formation of 1,2-DAG is biphasic and consists of an early peak, which is rapid and transient (parallels the increase in Ins(1,4,5)P_3 and Ca^{2+}), followed by a late phase which is sustained over many minutes (Exton, 1990). Analysis of the fatty acyl chain composition of 1,2-DAG from these 2 phases suggested that the 1,2-DAG from the early phase has a fatty acyl composition similar to that obtained from PtdIns(4,5)P_2 whereas the fatty acyl composition of 1,2-DAG from the second phase showed a similar composition to phosphatidylcholine (PtdCho) (Bocckino *et al.*, 1987). Thus, it is proposed that PtdIns(4,5)P_2 hydrolysis is rapidly desensitised and may play a minor role in certain physiological events such as mitogenesis whereas 1,2-DAG from hydrolysis of PtdCho is the likely candidate to be involved in controlling such events. PtdCho is a substrate not only for PLC but is also for PLD and PLA_2, so the action of different phospholipases on PtdCho will give rise to multiple lipid messenger molecules

such as arachidonic acid, *lyso*PC, PA and 1,2-DAG (Exton *et al.*, 1991).

The 1,2-DAG generation from PtdCho can result from (1) action of PtdCho-specific PLC (PC-PLC), (2) PLD action followed by action of phosphatidate phosphohydrolase. Two experimental approaches have been employed for investigating the involvement of these enzymes in the formation of 1,2-DAG upon agonist stimulation. The first approach is to prelabel the cells with *lyso*[^{32}P]phosphatidylcholine, which is then converted to PtdCho by acylation. The production of ^{32}P-phosphocholine would require a PLC activity whereas production of radioactive PA would require a PLD activity (Billah and Anthes, 1990). When hepatocytes were labelled with this radioisotope followed by vasopressin stimulation, formation of both radioactive phosphocholine and PA was observed (Augert *et al.*, 1989). The second approach is based on the properties of PLD which, in addition to lipid hydrolysis, also catalyzes transphosphatidylation. In the presence of a suitable acceptor alcohol, this enzyme transfers the phosphatidyl group from PtdCho to the alcohol. In most cases ethanol was used since it is non-toxic to cells. Formation of phosphatidylethanol in stimulated cells in the presence of up to 1% ethanol is taken as evidence of PLD activation. It was found that in several cell types such as hepatocytes and HL 60 cells, phosphatidylethanol is formed upon agonist stimulation (Billah and Anthes, 1990). So far, no signalling role has been proposed for choline or phosphocholine released by PtdCho cleavage.

The effect of heat on the phosphoinositide signalling pathway has been investigated in several cell lines (Calderwood *et al.*, 1989; Calderwood and Stevenson, 1993; Kiang and McClain, 1993). The level of DAG (unspecified isomer) in heated cells has been determined in CHO HA-1 cells (Calderwood *et al.*, 1989). Following a 2 h labelling of CHO HA-1 cells with ^{3}H-arachidonic acid in growth medium, followed by washing to eliminate excess radioactive isotope, cells were subjected to 45°C treatment. Lipids were then extracted from both heated and unheated cells and separated by TLC.

The radioactivity in ^{3}H-DAG was then determined. It was found that heat induced a 60% increase in ^{3}H-DAG (unspecified isomer) in the first 2-3 min at 45°C and the level then returned to the control level by 4 min and was maintained at the same level for up to 15 min heating. On the other hand, a sustained increase in DAG was observed when cells were heated at 45°C for 10 min followed by a 2 h recovery at 37°C, with DAG levels achieving 160% of the unheated control by 2 h. It was not clear from the experiment performed by Calderwood and co-workers whether the DAG isomers were separated or not. Since 1,2-DAG, but not 1,3-DAG is an activator of PKC, it is necessary to determine the effect of heat on this particular isomer. In addition, alteration in 1,2-DAG levels may lead to alteration in activity of PKC.

Investigation of the effect of heat on PKC activity has been performed in P388 lymphoid leukaemia cells (Bagi and Hidvégi, 1990). After the cells were heated at 40-45°C for 1 h, the activity of the enzyme was assayed by its ability to phosphorylate H1 histone. A significant decrease in activity of this enzyme was observed following heating. Although the activity of PKC reduced after heating, it was found that the phosphorylation of proteins having molecular weights of 33, 25, and 14 kD increased. The significance of increased phosphorylation of these proteins is uncertain and the authors postulated that the changes in protein phosphorylation may alter the regulation of cell metabolism which might lead to cell death.

More direct evidence for the role of PKC in hyperthermic cell death was obtained by using tamoxifen and H7, both of which are PKC inhibitors (Mikkelsen *et al.*, 1991b). By employing human colon cancer HT-29 cells, Chinese hamster lung V79 fibroblasts and human mammary carcinoma MCF-7 cells, it was found that the presence of either inhibitor potentiated hyperthermic cell death at 44.5°C for up to 6 h. On the other hand, when using HA1004, a less potent inhibitor of PKC, hyperthermic cell death was similar to that in cells heated in the absence of this inhibitor. These results suggested an

important role of PKC activity in the response of cells to hyperthermic treatment.

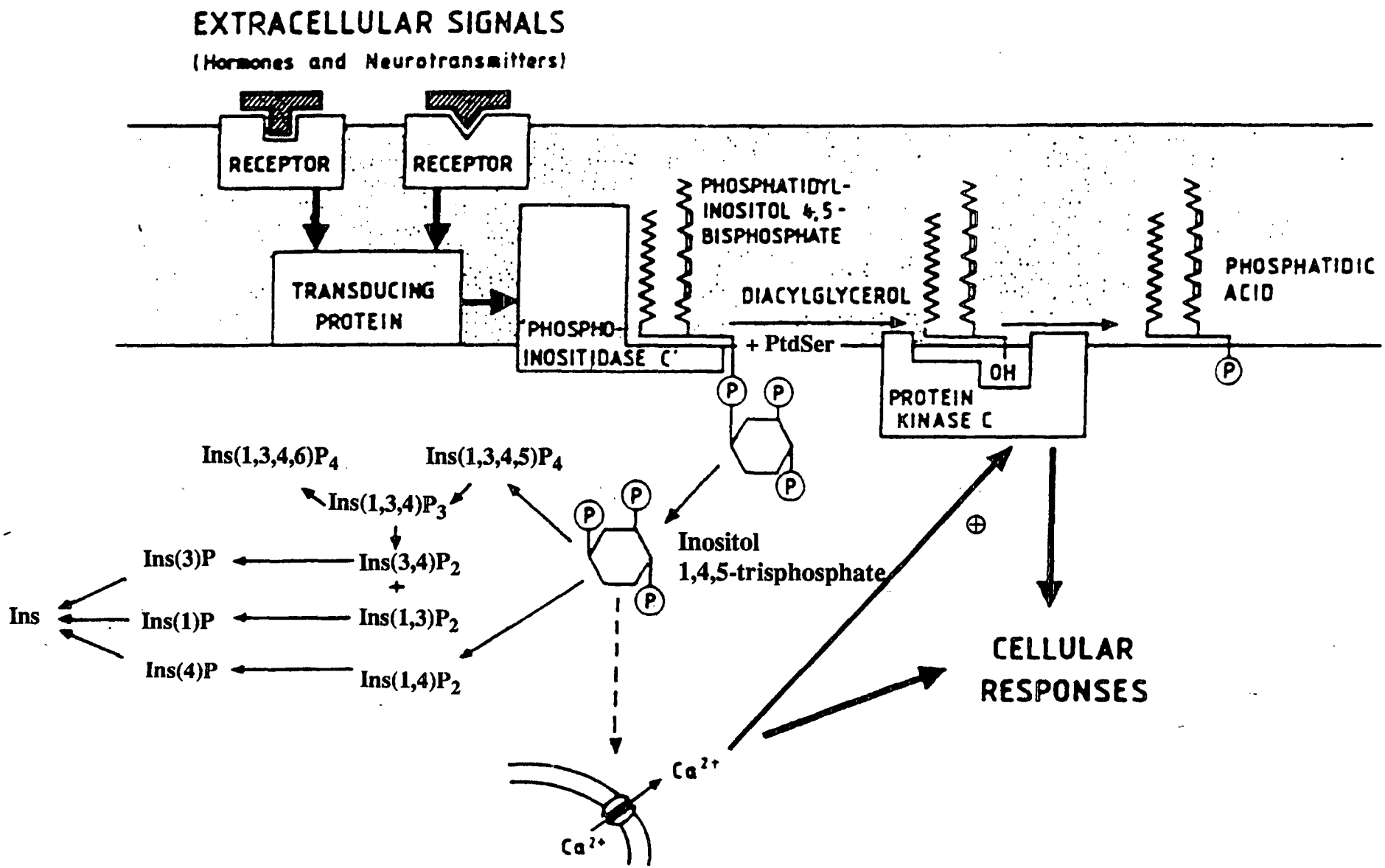
Using CHO HA-1 cells, Balb C 3T3 cells, HeLa cells and PC 12 cells prelabelled with myo[2-^{3}H]inositol, Calderwood and Stevenson (1993) found that heat treatment at 41°C or higher resulted in an increase in ^{3}H-inositol trisphosphate levels. This increase in inositol trisphosphate could be transient or sustained depending on cell type and heating duration. Since the separation of inositol phosphates was achieved by simple anion exchange chromatography, the isomers were not resolved. Increases in inositol monophosphate and inositol bisphosphate were also observed, which could be the metabolites of inositol trisphosphate. Increases in inositol monophosphate, bisphosphate and trisphosphate were also observed in A-431 cells during heating at 45°C (Kiang and McClain, 1993). The effect of heat on inositol phosphate levels of WRK-1 cells is reported in Chapter 4.

A heat-induced elevation in cytosolic free calcium ($[Ca^{2+}]_i$) has also been detected in several cell types such as CHO HA-1 cells (Stevenson *et al.*, 1986), human colon HT-29 cells (Mikkelsen *et al.*, 1991a) and NIH3T3 fibroblasts (Stege *et al.*, 1993a,b). Being an important cofactor for various key enzymes in regulating a variety of metabolic pathways and cell proliferation, the disruption of calcium homeostasis may result in the abnormal activation of intracellular proteases and lipases, which may ultimately lead to cell death (Schanne *et al.*, 1979; Trump *et al.*, 1980; Farber, 1981). The effect of heat on $[Ca^{2+}]_i$ in WRK-1 cells is reported in Chapter 5.

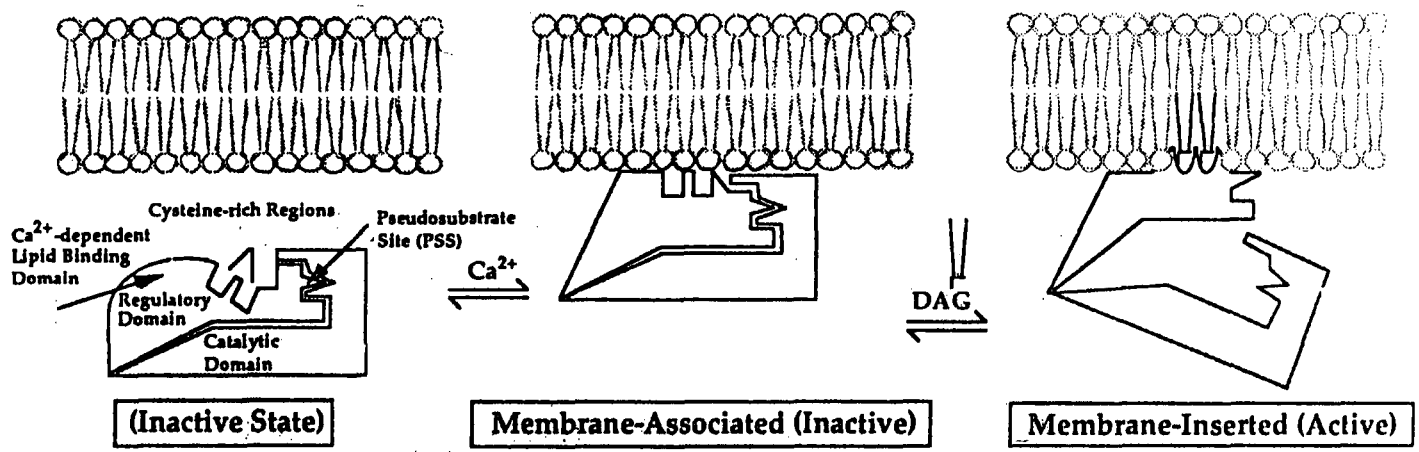
Given the importance of 1,2-DAG/PKC and Ins(1,4,5)P_3/Ca^{2+} pathways in the regulation of a variety of cellular responses, it seems reasonable to suggest that disruption of phosphoinositide signalling pathway may affect the level of the second messengers generated in this signalling pathway, resulting in alteration in activities of a variety of enzymes, including Ca^{2+}-dependent enzymes and PKC, which perhaps leading

to hyperthemic cell death.

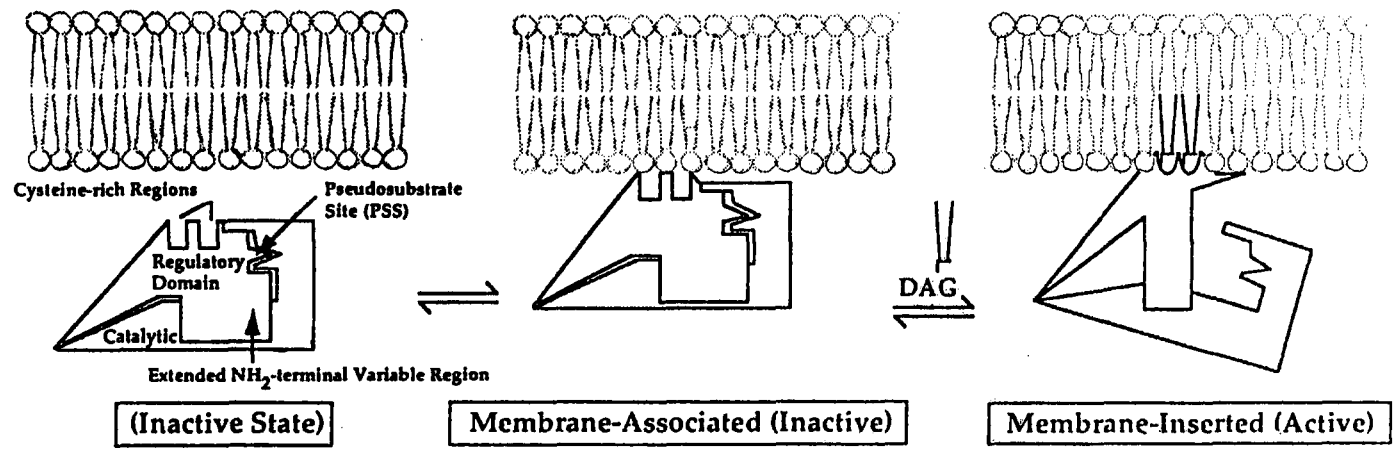
In this part of the study, the effect of heat on 1,2-DAG levels during and after heating was investigated in both CHO-K1 and WRK-1 cells. Furthermore, the effect of heat on other neutral lipid classes was also investigated in both cell types.

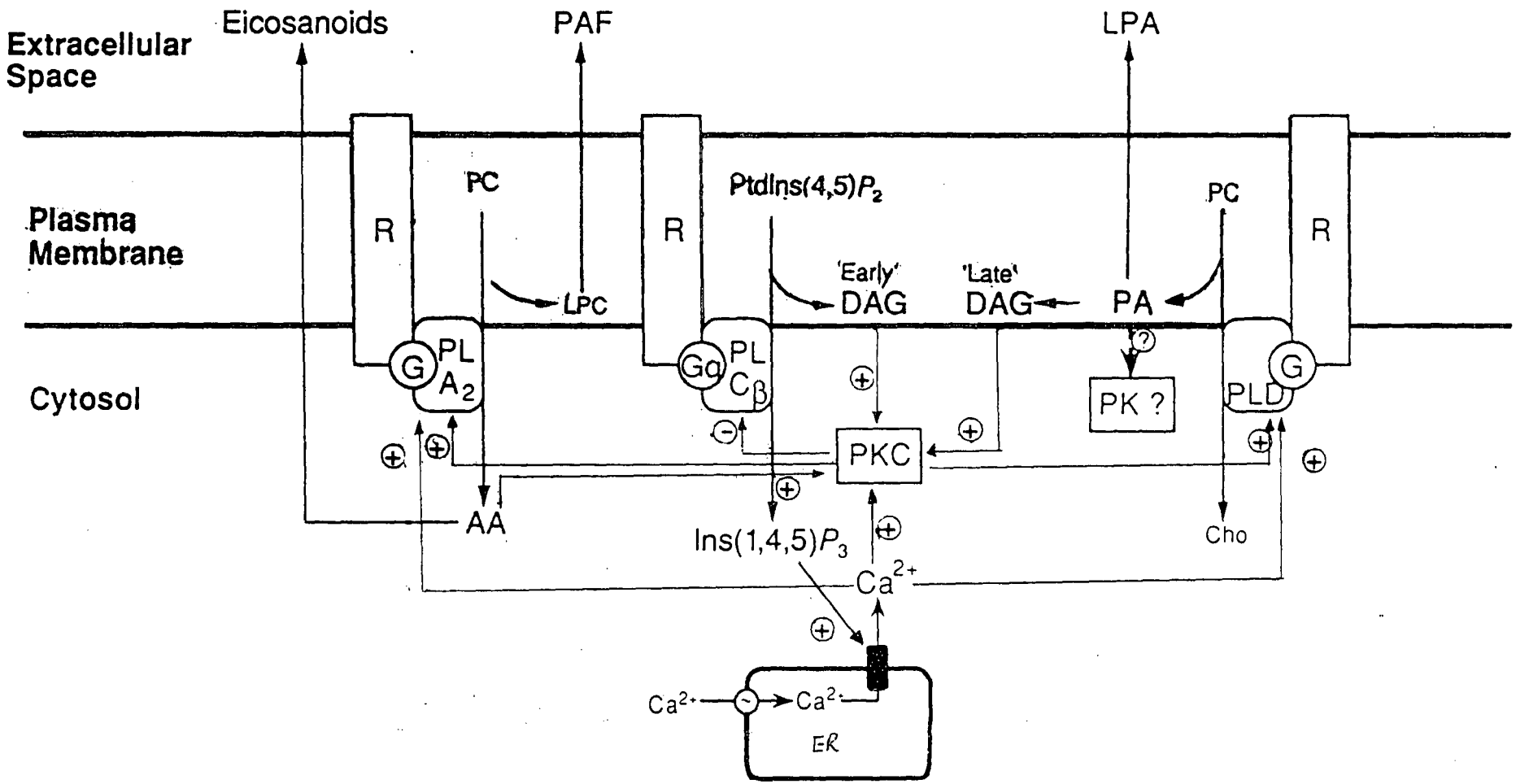

EXTRACELLULAR SIGNALS
(Hormones and Neurotransmitters)
RECEPTOR
RECEPTOR
TRANSDUCING PROTEIN
PHOSPHO-INOSITIDASE C
PHOSPHATIDYL-INOSITOL 4,5-BISPHOSPHATE
DIACYLGLYCEROL
+ PtdSer
PHOSPHATIDIC ACID
OH
PROTEIN KINASE C
Ins(1,3,4,6)P4
Ins(1,3,4,5)P4
Ins(1,3,4)P3
Ins(3)P
Ins(3,4)P2
Ins
Ins(1)P
Ins(1,3)P2
Ins(4)P
Ins(1,4)P2
Inositol 1,4,5-trisphosphate
CELLULAR RESPONSES
Ca2+
Ca2+

Group A Ca^{2+}-dependent PKC family members



Group B Ca^{2+}-independent PKC family members




Extracellular Space
Eicosanoids
PAF
LPA
Plasma Membrane
R
R
R
PC
PtdIns(4,5)P_2
PC
LPC
'Early' DAG
'Late' DAG
PA
Cytosol
G
PL A_2
Gq
PL C_β
PK ?
G
PLD
PKC
AA
Ins(1,4,5)P_3
Cho
Ca^{2+}
Ca^{2+}
Ca^{2+}
ER

3.2 Materials and Methods

3.2.1 Methods for determination of cell survival

Hyperthermic cell death may be determined by a clonogenic or a colorimetric assay depending upon the cell type (Kingston *et al.*, 1989). In the case of CHO-K1 cells, the clonogenic assay was used to determine thermal sensitivity. However, the tendency of WRK-1 cells to aggregate prevented the use of the clonogenic assay for determination of thermal sensitivity of this cell type. Instead, a method which depends on the the ability of metabolically viable cells to reduce yellow 3-[4,5-dimethyl thiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT) to blue formazan product was used (MTT assay) (Slater *et al.*, 1963). Before the MTT assay is used for cell viability measurement, optimal conditions such as the length of incubation with MTT solution, the concentration of the MTT solution to be used and the relationship between absorbance value and cell number have to be determined (Mosmann, 1983; Denizot and Lang, 1986).

(i) Determination of the optimal incubation time

WRK-1 cells were harvested and centrifuged at 80 g (r_{av} = 11 cm) at 20°C for 3 min to sediment the cells. The pellet was suitably diluted with growth medium to give 100,000 cells/ml. Then 1 ml of the diluted cell suspension was added to a number of 24-well plates and plates were returned to culture at 37°C. After 24 h, the medium was removed and plates were blot dried. Then 200 µl of 1 mg/ml MTT solution (see section 3.2.1(iv)) was added to each well and incubated for 0-5 h at 37°C in an air/CO_2 (19:1, v/v) atmosphere. Then the formazan crystals were dissolved by 1 ml of DMSO added directly to the well at various times. Duplicate samples (2 x100 µl) from each well were transferred into 96-well flat-bottomed microtiter plates by mean of a multichannel pipettor. The absorbance was determined on a Titertek Multiscan MCC/340 plate reader

using a test wavelength of 540 nm and a reference wavelength of 690 nm.

(ii) Determination of optimal MTT concentration

WRK-1 cells were harvested from a 175 cm^2 flask and counted. The pellet was then resuspended in an appropriate amount of growth medium to give 100,000 cells/ml. Then 1 ml of cell suspension was added to each well in a number of 24-well plates and plates were returned to culture at 37°C. After 24 h, the medium was removed and the wells were blot dried. Then 200 µl of MTT solution at different concentrations (0-4 mg/ml) (see section 3.2.1 (iv)) was added to each well and plates were returned to culture at 37°C. After 4 h, 1 ml of DMSO was added directly to the well to dissolve the formazan product. Duplicate samples (2 x 100 µl) from each well were transferred into 96-well flat-bottomed microtiter plates by mean of a multichannel pipettor. The absorbance was determined on a Titertek Multiscan MCC/340 plate reader using a test wavelength of 540 nm and a reference wavelength of 690 nm.

(iii) Relationship between absorbance and cell number

WRK-1 cells were harvested and counted. Cells were then suitably diluted to give 1,000,000 cells/ml. Different numbers (4×10^3 - 600×10^3) of cells were seeded into a number of 24-well plates which were returned to culture at 37°C. After 12 h, the medium was removed and the wells were blot dried, then 200 µl of 1 mg/ml MTT solution (see section 3.2.1 (iv)) was added to each well and the plates were returned to culture at 37°C for 4 h. Following this incubation DMSO (1 ml) was added directly to the well to dissolve the formazan product, and duplicate samples (2 x 100 µl) from each well were transferred into 96-well flat-bottomed microtiter plates by mean of a multichannel pipettor. The absorbance was then determined on a Titertek Multiscan

MCC/340 plate reader using a test wavelength of 540 nm and a reference wavelength of 690 nm.

(iv) Preparation of MTT solution

Stock MTT solution was prepared at 2 mg/ml in 20 mM Hepes, pH 7.4. The stock solution was then filter-sterilised through a 0.22 µm filter (Sartorius) and was stored in the dark at 4°C until used. By storing at 4°C and in the dark, the MTT stock solution was stable for 2 weeks. Prior to use, stock solution was diluted by mixing with an equal volume of double strength phenol-red free, serum-free EMEM medium containing 48 mM sodium bicarbonate, pH 7.4. The solution was filter-sterilised and 200 µl of the MTT solution was then added to each well. When different MTT concentrations were needed, 10 mg/ml MTT stock was prepared and the stock was suitably diluted with phenol-red free, serum-free EMEM medium to give the required concentration.

3.2.2 Determination of hyperthermic cell death at elevated temperatures

(i) The clonogenic assay of cell survival of CHO-K1 cells after hyperthermic treatment

CHO-K1 cells were harvested from a 25 cm^2 flask on day 0. Cells were then sedimented by centrifugation at 120 g (r_{av} = 11 cm) for 5 min at room temperature. The pellet was then resuspended in growth medium and counted via a haemocytometer. The cell suspension was further diluted to give 200 cells/ml. Then 1 ml of the cell suspension was added to a number of 25 cm^2 flasks, each containing 9 ml of growth medium and 20 mM Hepes, pH 7.4 (heating medium). The flasks were then returned to culture at 37°C to allow the cells to attach to the substrate. After 4 h, flasks were sealed with nescofilm and thermoequilibrated by submersion in a 37°C water bath for 15 min. Flasks were then

transferred rapidly to a 47°C (± 0.1°C) water bath for 2 min before submersion in a 45°C water bath. By doing so, the lag period for thermoequilibration was reduced to 3 min. The temperature of the growth medium was monitored continuously inside the flask by a thermocouple inserted into a 'dummy flask' containing heating medium only, which was heated under the same conditions as the experimental flasks. Flasks removed from the water bath at various times were cleaned with 70% (v/v) alcohol, unsealed and returned to culture at 37°C. The medium was replaced with fresh growth medium on days 1, 4 and 7. On day 9, the medium was removed, then cells were fixed and stained as described in section 2.2.4 (iv). Colonies having 50 cells or more were counted. The percentage of cell survival was calculated as follow:

$$\% \text{ of cell survival} = \frac{\text{no. of colonies formed}}{\text{no. of cells plated}} \times 100$$

(ii) The colorimetric assay of cell survival of WRK-1 cells after hyperthermic treatment

WRK-1 cells were harvested from a 25 cm^2 flask and sedimented by centrifugation at 80 g (r_{av}= 11 cm) for 3 min at room temperature. The cell pellet was resuspended in growth medium and counted via a haemocytometer. The cell suspension was suitably diluted with growth medium to give 70,000 cells/ml. Then 0.1 ml of the cell suspension was added to a number of 24-well plates with each well containing 0.9 ml of growth medium. Plates were then returned to culture at 37°C (day 0) and medium was changed on day 1. On day 3, the medium was replaced by 1 ml of growth medium containing 20 mM Hepes, pH 7.4 (heating medium). The plates were then returned to 37°C incubator in an air/CO_2 (19:1, v/v) atmosphere and gassed for 20 min. Plates were then sealed and thermoequilibrated by submersion in a 37°C water bath for 15 min. Plates were then transferred rapidly to a water bath set at 1.5°C higher than the desired temperature for 4.5 min before transfer and rapid submersion in the water bath at the

desired temperature, in order to reduce the lag period. Plates were removed from the water bath at various times, cleaned with 70% (v/v) alcohol and unsealed, then returned to culture at 37°C. The medium was changed on day 4. The MTT assay was performed on day 6, as described in section 3.2.1 (iii). The percentage of cell survival was calculated as follows:

$$\% \text{ of cell survival} = \frac{\text{absorbance from heated cells}}{\text{absorbance from control cells}} \times 100$$

3.2.3 Effect of heat on monoacylglycerol, 1,2-diacylglycerol and triacylglycerol levels

In this study, 2 radioisotopes were used. WRK-1 cells were labelled by growth in [2-^{3}H]glycerol and the abundant labelling of glycerolipids shows that these cells contain glycerokinase, allowing formation of *sn*[2-^{3}H]glycerol 3-phosphate which is a major precursor for lipid synthesis. In contrast, CHO-K1 cells did not produce labelled lipid when grown in [2-^{3}H]glycerol suggesting that these cells do not contain glycerokinase. Therefore, CHO-K1 cells were grown in [5,6,8,11,12,14,15-^{3}H]arachidonic acid, thus labelling the acyl moiety of glycerolipids.

(i) Long term labelling of the lipid pool of CHO-K1 cells

CHO-K1 cells were harvested from a 25 cm^2 flask and sedimented by centrifugation. The cell pellet was resuspended in growth medium and cell number was determined via a haemocytometer. The cell suspension was suitably diluted with growth medium to give 100,000 cells/ml. Then 1 ml of the diluted cell suspension was added to 2 ml of growth medium in each well of a number of 6-well plates (day 0). The medium was changed the following day and on day 3. On day 4, the medium was replaced with 1.5 ml of growth medium containing [^{3}H]arachidonic acid (0.75 μCi) and cells were

returned to culture at 37°C. After 24 h, the radioactive medium was removed and cells were washed 3 times with 3 ml of non-radioactive growth medium prewarmed at 37°C. Then 3 ml of growth medium containing 20 mM Hepes, pH 7.4 (heating medium) was added to each well and plates were sealed and thermoequilibrated by submersion in a 37°C water bath for 15 min before rapid transfer and submersion in a 47°C water bath. A plate with the same volume of heating medium in the well was taken through the whole procedure and was used for monitoring the temperature with a thermocouple. When the medium in the well reached 44°C, 1.2 l of water from the water bath was exchanged with 1.2 l of water at 9°C. By doing so, the lag period for equilibration to 45°C was reduced to 2.5 min. Reactions were terminated by addition of 3 ml of ice-cold absolute methanol. Lipids were then extracted and neutral lipid classes were separated by TLC as described in section 3.2.3 (v).

(ii) Long term labelling of WRK-1 cells

WRK-1 cells were harvested from two 25 cm^2 flasks and cells were sedimented by centrifugation. The cell pellet was resuspended in growth medium and cell number was determined via haemocytometer. The cell suspension was suitably diluted with growth medium to give 100,000 cells/ml, then 1 ml of the diluted cell suspension was added to 2 ml of growth medium in each well in a number of 6-well plates on day 0. On days 1 and 3 the medium was replaced with 3 ml of growth medium containing [2-^{3}H]glycerol (10 µCi). On day 5, the medium was replaced with 3 ml of non-radioactive medium and the plates were returned to culture for 2 h. The medium was then replaced with 3 ml of heating medium, plates were sealed and thermoequilibrated by submersion in a 37°C water bath for 15 min before heating as described in section 3.2.3 (i). Reactions were terminated by addition of 3 ml of ice-cold absolute methanol, then lipids were extracted and neutral lipid classes were separated by TLC as described in section 3.2.3 (v).

(iii) Short term labelling of CHO-K1 cells

Cells were harvested and seeded on day 0 as described in section 3.2.3 (i). The medium was changed on day 1 and on day 3. On day 5, the medium was replaced by 1 ml of radioactive medium containing [^{3}H]arachidonic acid (0.5 µCi). Cells were then returned to culture at 37°C for 2 h. After 2 h the cells were washed 3 times with non-radioactive heating medium prewarmed at 37°C. Then 3 ml of heating medium was added to the well, plates were sealed and thermoequilibrated by submersion in a 37°C water bath for 15 min before heating as described in section 3.2.3 (i). Reactions were terminated by addition of 3 ml of ice-cold absolute methanol. Lipids were then extracted and neutral lipid classes were separated by TLC as described in section 3.2.3 (v).

(iv) Short term labelling of WRK-1 cells

Cells were harvested and seeded on day 0 as described in section 3.2.3 (ii). The medium was changed on day 1 and on day 3. On day 5, the medium was replaced by 1 ml of radioactive medium containing [2-^{3}H]glycerol (5 µCi). Cells were then returned to culture at 37°C for 2 h. After 2 h the cells were washed 3 times with non-radioactive heating medium prewarmed at 37°C. Then 3 ml of heating medium was added to the well, plates were sealed and thermoequilibrated by submersion in a 37°C water bath for 15 min and heated as described in section 3.2.3 (i). Reactions were terminated by addition of 3 ml of ice-cold absolute methanol. Lipids were then extracted and neutral lipid classes were separated by TLC as described in section 3.2.3 (v).

(v) Lipid extraction

The method described by Bligh and Dyer (1959) was used for the extraction of lipids. After reactions were terminated by 3 ml of ice-cold absolute methanol, cells were

scraped off from the well and transferred to a pyrex glass tube. Then another 0.75 ml of ice-cold absolute methanol was added to the well and the remaining cells scraped from the well. The 2 extracts were combined together. To the extract, was added 7.5 ml of chloroform: methanol (1:1, v/v) containing 0.05% (w/v) butylated hydroxytoluene (BHT) and 30 µl of a mixture of the neutral lipid standards. This lipid standards mixture consisted of 600 µl of 10 mg/ml of MAG, 1,3-DAG and TAG mixture, 200 µl of 10 mg/ml 1,2-DAG and 200 µl of 10 mg/ml cholesterol. The extract was mixed for 30 s followed by addition of 3.75 ml of chloroform and the mixture was mixed on a vortex mixer for 30 s. Then 3.75 ml of water was added to each sample and it was mixed again on a vortex mixer for 30 s. The samples were then centrifuged at 1000 g (r_{av} = 22.3 cm) for 10 min at room temperature. The bottom phase was transferred to a test tube and was washed twice with 'synthetic top phase' (see below) with centrifugation at 1000 g (r_{av} = 22.3 cm) for 10 min at room temperature after each wash to facilitate phase separation. The bottom phase was dried under O_2-free N_2. The dried lipid was then redissolved in 1 ml of chloroform containing 0.05% (w/v) BHT, mixed for 30 s, and 2 x 50 µl aliquots of the lipid solution were transferred to glass scintillation vials by using a 50 µl glass syringe. The lipid solutions in the scintillation vials were dried under O_2-free N_2 and 5 ml of Ecoscint A scintillation fluid was added to each vial and mixed for 30 s. Radioactivity was measured by liquid scintillation counting in a Packard scintillation counter (model no. 300).

The remaining 0.9 ml lipid solution was dried under O_2-free N_2 and redissolved in 70 µl chloroform. The neutral lipid classes were separated by TLC using 20 cm x 20 cm silica gel 60 plates (Merck) in a solvent system consisting of toluene: diethyl ether: ethyl acetate: acetic acid (80:10:10:0.2, by volume). Lipids were located with iodine vapour and their identity was verified by comparison with authentic standards. After removal of iodine, spots were scraped off from the plates, 5 ml Ecoscint A scintillation fluid was

added and mixed for 30 s. Radioactivity was measured by liquid scintillation counting as described above.

(vi) Preparation of synthetic top and bottom phases

To a large separating funnel, appropriate amount of chloroform, methanol and water were mixed in a ratio of 2:2:1.8 (by volume). The mixture was left overnight to allow equilibration. The 2 phases were separated and 5 mM non-radioactive glycerol as carrier was added to the top phase which was stored in a brown bottle at room temperature.

3.3 Results

The thermal sensitivity of different cell lines vary, so it is necessary to determine the relationship between heat dose and the thermosensitivity of each cell type. The commonly accepted end point of hyperthermic cell death is the loss of reproductive ability of cells. In this study, clonogenic and colorimetric assays were employed for assessing the cell viability of CHO-K1 cells and WRK-1 cells, respectively. The clonogenic assay is the most commonly used assay for reproductive ability following heat damage in which a known number of cells (about 100-500 cells) were seeded into a 25 cm^2 flask and the number of colonies appearing after 7-10 days was assessed. The survival fraction is then calculated as the number of colonies formed divided by the number of cells plated. This method was used for the determination of cell viability of CHO-K1 cell at 9 days following heating in this study.

However, due to the tendency of WRK-1 cells to aggregate, the clonogenic assay could not be used for these cells. Instead, the colorimetric assay (MTT assay) was used which relies on the ability of metabolically viable cells to reduce yellow 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to blue formazan product by succinate dehydrogenase via coupling at 2 points along the cytochrome oxidase system (Slater *et al.*, 1963).

Before the colorimetric assay was used in the determination of cell survival, it was necessary to determine the optimal assay conditions such as the MTT concentration, the incubation time with MTT solution and the relationship between cell number and absorbance value.

It has been reported previously that the presence of phenol red or serum in the assay solution would give high background values in the MTT assay (Denizot and Lang,

1986). Thus, phenol red-free and serum-free EMEM medium was used in the MTT assay for WRK-1 cells. Furthermore, it has been reported that the pH of the solution affects the absorbance and it was found that as pH decreased below pH 6.5 the absorbance reduced dramatically, whereas the optimal absorbance value was obtained when the pH of the medium was around 7.2 (Jabbar *et al.*, 1989). Thus, 20 mM Hepes, pH 7.4, was included in the MTT stock solution and 24 mM sodium bicarbonate was also included in the phenol red-free and serum-free EMEM medium.

It has been pointed out that the the optimal MTT assay condition varies between cell type, thus the optimal assay condition for WRK-1 cells had to be elucidated before the assay was employed for cell survival measurement (Carmichael *et al.*, 1987; Plumb *et al.*, 1989).

In order to determine the optimal incubation time for the maximal conversion of MTT to formazan, 100,000 cells were exposed to MTT solution (1 mg/ml) for various times for up to 5 h and the absorbance was then determined. A gradual increase in formazan production was observed and the absorbance value reached a plateau after 3 h incubation (Figure 3.4). Although 5 h incubation showed a slightly higher level of formazan production, the increase was small compared with the absorbance obtained after 3-4 h incubation (Figure 3.4). Thus, a 4 h incubation time was chosen before formazan product was solubilized with DMSO.

The optimal concentration of MTT solution was then determined. When 1 x 10^5 cells were incubated with MTT solution with concentrations ranging from 0-4 mg/ml, it was found that the amount of formazan produced increased rapidly from 0-0.5 mg/ml and reached a plateau at concentrations between 0.5 mg/ml and 3 mg/ml (Figure 3.5). A decreased absorbance value was observed when 4 mg/ml was used. Taking into account the cost of the MTT agent and also the optimal absorbance achieved at concentrations

between 0.5-3 mg/ml, it was decided to use concentration of 1 mg/ml MTT solution in the subsequent study of cell viability of WRK-1 cells.

It was known that, in future experiments, when cell survival of WRK-1 cells would be assessed by the colorimetric assay, the cells would be seeded for 3 days before heating and then grown for another 3 days before carrying out the colorimetric assay. Consequently, it was necessary to ensure that the relationship between cell number and absorbance remained linear by the time of the colorimetric assay such that the cell survival would not be overestimated. In order to determine the optimal cell number for which a linear relationship was maintained, cells were seeded into a number of 24-well plates and the MTT assay was performed at 12-14 h after seeding such that sufficient time was allowed for cells to settle but not enough time for them to overcome the lag period and to divide. The data points were fitted with regression line and it was found that the best correlation was found for density up to 3 x 10^5 cells/well (seeded cell number) or 2.5 x 10^5 (actual cell number) in a 24-well plate (Figure 3.6). Hence in future experiments, 7 x 10^3 cells/well in a 24-well plate were seeded on day 0, such that by the time of measurement (day 6) the relationship of absorbance reading and cell number remained linear for control cells, and there were sufficient viable cells in the heat-treated plates to be detected by the MTT assay. Once the optimal condition of MTT assay had been established, the effect of heat on CHO-K1 cells and WRK-1 cells was determined by the clonogenic and the MTT colorimetric assay, respectively.

When log percentage of cell survival is plotted against time of heating, the thermal survival curve for many cell lines is curvilinear (Figure 3.7). The curve is characterised by a shoulder region at low heat dose, then the curve becomes linear at longer exposure time to elevated temperature. The curve can be described by survival parameters, D_q, D_0 and the LD_{90} value (Figure 3.7). D_q measures the width of the shoulder region and is obtained from back-extrapolation of the log-linear part of the curve to the 100% survival

point. The reason(s) for the existence of the shoulder region is unknown but it has been pointed out by Hahn (1982) and Jung (1986) that the shoulder region may determine the ability of the cells to sustain sub-lethal damage or to repair the thermal damage. Therefore, the higher the D_q value, the higher the ability of the cells to repair the damage. D_0 is the reciprocal of the slope of the linear portion of the curve, and is the time required to reduce the cell survival on the exponential part of the curve to 1/e times its initial value. Hence, the higher the D_0 value, the higher the thermal resistance of a particular cell type. In contrast to D_q and D_0 the time taken to kill 90% of the cells (LD_{90}) at a certain temperature reflects the effect of both the shoulder region (D_q) and the slope of the curve (D_0) (Bhuyan, 1979)

Thermal survival curves for CHO-K1 cells and WRK-1 cells are shown in Figure 3.8 and Figure 3.9, respectively, and the D_q and D_0 values were calculated as described in Figure 3.7. The D_q, D_0 and LD_{90} values for CHO-K1 cells heated at 45°C were 5.52 min, 14.5 min and 20.1 min, respectively. When WRK-1 cells were heated at 43.5°C to 45°C, it was found that both the D_q and D_0 values decreased as temperature increased. The D_q values were 68.3 min, 53.8 min and 21.4 min at 43.5°C, 44°C and 45°C, respectively. The D_0 values were 42.4 min, 38.4 min and 14.5 min at 43.5°C, 44°C and 45°C, respectively. LD_{90} values were 110.6 min, 89.2 min and 35.9 min at 43.5°C, 44°C and 45°C, respectively.

Having characterised the hyperthermic cell death of CHO-K1 cells and WRK-1 cells, the effect of heat on 1,2-DAG, MAG and TAG levels in both cell types was investigated. Several methods are available for determination of the changes in DAG level. Initally, 1,2-DAG may be separated from other lipids by TLC and/or HPLC, then the mass of 1,2-DAG can then be analysed by elution and charring (Kabara and Chen, 1976), charring and densitometry directly on TLC plates (Takuwa *et al.*, 1986), absorbance of the HPLC effluent (Boccking *et al.*, 1985) or acetylation of the free 3-

hydroxyl group of 1,2-DAG with labelled acetic anhydride and purification followed by radioactivity counting for the resulting labelled derivative (Banochback *et al.*, 1974). In the densitometric and acetic anhydride methods, relatively high blanks are encountered, and there is poor sensitivity and precision of these assays, particularly in biological samples which may have hydroxylated substances that are not fully separated from 1,2-DAG by chromatographic methods. Better purification of 1,2-DAG results after HPLC and 2-dimensional TLC but these are expensive and time-consuming methods. Alternatively, the use of DAG kinase to phosphorylate 1,2-DAG to PA provides a quick and precise method since it only measures *sn*-1,2-DAG but not the 1,3 isomer which is incapable of activating PKC (Preiss *et al.*, 1986). In this method, purified DAG kinase from bacterial sources (available commercially) is incubated with [γ-^{32}P]ATP together with detergents and extracted lipids from samples of interest. Incorporation of ^{32}P into 1,2-DAG results in the formation of ^{32}P-PA, which can be extracted into organic solvents and readily purified by one-dimensional TLC. The level of radioactivity in PA serves as an accurate index of 1,2-DAG content of the sample, and by comparison to ^{32}P-PA formed from known amounts of 1,2-DAG standard (e.g. diolein), the mass of 1,2-DAG of the sample can be determined. However, although the DAG kinase method allows the direct measurement of 1,2-DAG mass, this method cannot distinguish ether-linked analogues from 1,2-DAG.

Another method for measuring changes in 1,2-DAG is the use of radioactive isotopes in which the lipid fraction is labelled by a precursor and the changes of radioactivity in 1,2-DAG upon treatment are followed. The commonly used isotopes for labelling 1,2-DAG include [^{3}H]glycerol and [^{3}H]fatty acid. Although [^{3}H]glycerol labelling is not specific, as it labels all the glycerolipids, if [^{3}H]glycerol is labelled in the 2-position, it will reflect 1,2-DAG levels rather than ether lipid analogues which are synthesized exclusively from dihydroxyacetone phosphate (Farese and Cooper, 1990).

The degree of incorporation of [2-^{3}H]glycerol into lipid depends upon the intracellular activity of glycerokinase which phosphorylates ^{3}H-glycerol, thus labelling the *sn*-glycerol-3-phosphate precursor pool. ^{3}H-glycerol-3-phosphate is then converted via the action of acyltransferase to phosphatidic acid, which is the precursor of phospholipids. Therefore, if cells lack glycerokinase, it is not possible to use [2-^{3}H]glycerol to label the lipid pool. Alternatively, lipids can be labelled using [^{3}H]fatty acid. In particular, [^{3}H]arachidonic acid is commonly used since this fatty acid is very rapidly incorporated into phospholipids such as PtdCho and PtdIns by transacylation processes, rather than by *de novo* phosphatidate synthesis, and since hydrolysis of these phospholipids may be responsible for the increase in arachidonate-rich diacylglycerol and phosphatidic acid observed upon agonist stimulation.

In this study, [^{3}H]arachidonic acid was used for labelling the lipid fraction of CHO-K1 cells due to the lack of glycerokinase in this cell type, whereas [2-^{3}H]glycerol was used for labelling the lipid fraction of WRK-1 cells.

In order to determine the effect of heat on 1,2-DAG as well as other neutral lipids such as triacylglycerol (TAG) and monoacylglycerol (MAG), a method that is able to separate different classes of neutral lipids, but 1,2-DAG from 1,3-DAG, is necessary since only the former isomer is the activator of protein kinase C. It was found that neutral lipid classes, including 1,2-DAG and 1,3-DAG, were well separated on 20x20 cm plates coated with silica gel 60 (0.25 mm thick, Merck) following 1-dimensional development in a solvent system of toluene: diethyl ether: ethyl acetate: acetic acid (80:10:10:0.2, by vol.) (**Figure** 3.10). Thus this solvent system was used in this study.

Once the method for separation of 1,2-DAG and other neutral lipid classes had been established, the effect of heat on 1,2-DAG was investigated in CHO-K1 cells and WRK-1 cells. In this study, CHO-K1 cells were labelled with [^{3}H]arachidonic acid for

either 2 h or 24 h, while WRK-1 cells were labelled with [2-^{3}H]glycerol for either 2 h or 4 days (Farese and Cooper, 1990). The long term labelling protocol allows sufficient time for the precursor pools and relevant lipids to attain constant specific radioactivity. On the other hand, short term labelling enables the turnover of small, metabolically active lipid pools to be detected (Farese and Cooper, 1990).

When CHO-K1 cells were labelled with [^{3}H]arachidonic acid for 24 h then heated at 45°C for up to 10 min (a heat dose that killed 50% cells), the levels of ^{3}H-MAG in heated and unheated cells were similar throughout the heating time employed (Figure 3.11a). A decrease in mean ^{3}H-1,2-DAG level was observed in heated cells, especially at 5 min and 8 min heating time (Figure 3.11b). A small decrease in mean ^{3}H-TAG level was found in heated cells at 1-3 min (Figure 3.11c).

When WRK-1 cells prelabelled with [2-^{3}H]glycerol for 4 days were heated at 45°C for up to 12.5 min (a heat dose that would kill 20% of WRK-1 cells), no difference in the mean level of ^{3}H-MAG was found between heated and control cells (Figure 3.12a). Conversely, a consistent decrease in the mean level of ^{3}H-1,2-DAG in heated cells was observed (Figure 3.12b). On the other hand, a consistent increase in ^{3}H-TAG level was observed for heating time longer than 3.5 min (Figure 3.12c).

The results in Figures 3.11 and 3.12 suggested that acute exposure of both cell types to 45°C for 10-12.5 min resulted in a decrease in ^{3}H-1,2-DAG whereas an increase in ^{3}H-TAG level was observed in WRK-1 cells only. In order to investigate the effect of prolonged heating on ^{3}H-1,2-DAG levels, long term labelled cells were exposed to 45°C for up to 45 min, a heat dose that led to more than 95% cell death in both cell types.

When CHO-K1 cells, prelabelled for 24 h with [^{3}H]arachidonic acid, were heated at 45°C for 45 min, no difference in ^{3}H-MAG levels between unheated and heated cells

was observed (Figure 3.13a) whereas a decrease in ^{3}H-1,2-DAG level was observed throughout 45 min heating except at 30 min (Figure 3.13b). There was an increase in ^{3}H-TAG level in heated cells especially at heating time longer than 20 min (Figure 3.13c). In heated WRK-1 cells, prelabelled with [2-^{3}H]glycerol for 4 days, an increase in ^{3}H-MAG level was observed at 45 min heating (Figure 3.14a). On the other hand, a decrease in ^{3}H-1,2-DAG level was observed for up to 30 min at 45°C heating, whereas at 45 min the ^{3}H-1,2-DAG levels of heated and unheated cells were similar (Figure 3.14b). An increase in ^{3}H-TAG level was observed in heated cells and the level returned gradually to control level by 45 min (Figure 3.14c).

The results suggested that heat led to a decrease in ^{3}H-1,2-DAG level and an increase in ^{3}H-TAG level in long-term labelled CHO-K1 cells and WRK-1 cells. As mentioned earlier, long term labelling of lipids with radioisotopes enables glycerolipids to achieve equilibrium labelling. However, it may be possible that a small, metabolically active pool with a high turnover rate is involved in heat-induced changes in 1,2-DAG and the existence of such metabolically active lipid pools has been reported in many cells types including C3H fibroblasts (Holmsen *et al.*, 1989), GH_3 cells (Cubitt *et al.*, 1990) and WRK-1 cells (Monaco and Woods, 1983; Koréh and Monaco, 1986; Monaco and Gershengorn, 1992). In such case, experiments using cellular lipids labelled to near to isotopic equilibrium may not detect small changes in metabolically active pools (Farese and Cooper, 1990). To address this problem, both CHO-K1 cells and WRK-1 cells were labelled for 2 h with [^{3}H]arachidonic acid and [2-^{3}H]glycerol, respectively, then washed with non-radioactive growth media and subjected to hyperthermia.

In CHO-K1 cells, prelabelled with [^{3}H]arachidonic acid for 2 h, it was found that the ^{3}H-MAG levels obtained from heated and unheated cells were similar (Figure 3.15a). A decrease in ^{3}H-1,2-DAG level was observed when CHO-K1 cells were heated at 45°C for longer than 10 min (Figure 3.15b). An increase in ^{3}H-TAG level was observed when

these cells were heated at 45°C apart from 20 min heating in which the level of ^{3}H-TAG in unheated cells was higher than heated cells (Figure 3.15c). The ^{3}H-TAG level returned to the control level by 45 min. Similar results were obtained from WRK-1 cells when these cells were heated at 45°C. In WRK-1 cells, no difference in ^{3}H-MAG level was observed between heated and unheated cells (Figure 3.16a) whereas a decrease in ^{3}H-1,2-DAG level was detected in heated cells except at 32.5 min (Figure 3.16b). An increase in ^{3}H-TAG level was observed for heating time longer than 12.5 min (Figure 3.16c).

In summary, results obtained from long term and short term labelled CHO-K1 cells and WRK-1 cells following acute or chronic exposure to 45°C suggested that heat led to a decrease in ^{3}H-1,2-DAG and an increase in ^{3}H-TAG levels in most cases.

The experiments described so far employed serum-containing medium. However, serum contains several hormones and growth factors, so the addition of fresh serum-containing medium prior to heat treatment could trigger the hydrolysis of some of the inositol lipids and may disturb the heat-induced changes in the phosphoinositide signalling system to be detected. To eliminate this possibility, long term labelled CHO-K1 cells and WRK-1 cells were heated in serum-free medium together with 20 mM Hepes, pH 7.4.

On heating of long-term labelled CHO-K1 cells in serum-free medium at 45°C for up to 45 min, no difference in ^{3}H-MAG level was found in heated cells (Figure 3.17a). On the other hand, a consistent decrease in ^{3}H-1,2-DAG level was observed throughout 45 min heating (Figure 3.17b). No difference in ^{3}H-TAG level was found between heated and control cells (Figure 3.17c). Similarly, when long term labelled WRK-1 cells were heated in serum-free medium for up to 47.5 min, no difference in ^{3}H-MAG level was found (Figure 3.18a). A small decrease in ^{3}H-1,2-DAG was observed in heated cells

(Figure 3.18b). An increase in ^{3}H-TAG level was found in heated cells at 12.5 min and 32.5 min (Figure 3.18c).

Being a second messenger, 1,2-DAG must undergo rapid turnover in order to terminate the cellular responses. Following introduction of different radioactive, naturally occurring 1,2-DAG analogues (bearing different fatty acyl compositions) into NIH3T3 fibroblasts using the liposome fusion technique, 3 routes for 1,2-DAG metabolism have been suggested (Florin-Christensen *et al.*, 1992; Florin-Christensen *et al.*, 1993). They are (1) transferase-catalyzed conversion to PtdCho and TAG, (2) lipolytic breakdown of 1,2-DAG to MAG and fatty acid, (3) phosphorylation of 1,2-DAG to PA by DAG kinase and PA is then recycled for replenishing the inositol lipid pool (assuming that DAG formation comes from inositol lipid pool). The fate of the 1,2-DAG is totally dependent upon the fatty acyl composition of the 1,2-DAG analogues (Florin-Christensen *et al.*, 1992; Florin-Christensen *et al.*, 1993).

It seems unlikely that the decrease in ^{3}H-1,2-DAG level in heated cells could be explained by the rapid degradation of 1,2-DAG to MAG through lipolysis since the MAG level in both heated and unheated cells was similar for acute or chronic exposure to 45°C. However, it is possible that the observed reduction in ^{3}H-1,2-DAG in heated cells may result from a rapid conversion of 1,2-DAG to PA in heated cells as a result of increased activity of DAG kinase at elevated temperature (as a result of increase in kinetic energy). Under this circumstance, any heat-induced accumulation of 1,2-DAG may not be detected. In order to address this possibility, cells were heated in serum-free medium containing the DAG kinase inhibitor, dioctanoylethylene glycol (diC_8EG). Following 4-day labelling of WRK-1 cells with [2-^{3}H]glycerol and washing to eliminate the radioactive medium, WRK-1 cells were then incubated at 37°C for 10 min in either (1) serum-free EMEM medium containing 0.01% (v/v) DMSO, or (2) serum-free EMEM medium containing 100 μM diC_8EG and 0.01% (v/v) DMSO. After plates were

sealed and thermoequilibrated for 15 min at 37°C, they were heated at 45°C for up to 12.5 min. It was found that the ^{3}H-MAG level in cells heated with or without diC$_8$EG was similar to the control level in cells at 37°C in either medium (Figure 3.19a). A decrease in ^{3}H-1,2-DAG level was found in heated cells after 12.5 min heat treatment, in the presence or absence of diC$_8$EG, but not at other heating times (Figure 3.19b). When cells were heated in the absence of diC$_8$EG, an increase in ^{3}H-TAG was observed at 4.5 min, 6.5 min and 12.5 min heating compared with cells at 37°C in the absence of diC$_8$EG (Figure 3.19c) whereas those heated in the presence of diC$_8$EG showed an increase in ^{3}H-TAG level at 6.5 min and 12.5 min compared with diC$_8$EG-treated cells (Figure 3.19c).

In order to investigate the effect of chronic heating of WRK-1 cells in the presence of 100 μM diC$_8$EG, the experiment was repeated again as described above but this time cells were heated up to 45 min. No difference in ^{3}H-MAG level was observed between heated and unheated cells either in the presence or absence of diC$_8$EG (Figure 3.20a). On the other hand, a decrease in ^{3}H-1,2-DAG level was observed in heated cells, in the presence or absence of diC$_8$EG, compared to unheated cells incubated in the presence or absence of diC$_8$EG, respectively (Figure 3.20b). The levels of ^{3}H-1,2-DAG obtained from heated cells in the presence or absence of diC$_8$EG was similar. On the other hand, when cells were heated in the presence of diC$_8$EG, an increase in ^{3}H-TAG was observed at 20 min and 45 min compared to cells heated in the absence of the inhibitor or cells maintained at 37°C in the presence or absence of inhibitor (Figure 3.20c). When cells were heated in the absence of diC$_8$EG, an increase in ^{3}H-TAG level was observed at 30 min and 45 min heating compared with levels of ^{3}H-TAG in diC$_8$EG-untreated, unheated cells (Figure 3.20c).

In summary, none of the heating conditions employed in this study showed an increase in ^{3}H-1,2-DAG levels in heated cells. On the other hand, a decrease in ^{3}H-1,2-

DAG level was observed in most cases. It has been reported previously that a transient increase in ^{3}H-1,2-DAG level was observed in CHO HA-1 cells after 2-3 min exposure to 45°C, and this level returned rapidly to the control level by 4 min (Calderwood *et al.*, 1987). Furthermore, a sustained elevation in ^{3}H-DAG level was observed in CHO HA-1 cells during the 2 h recovery period following a 10 min heating at 45°C (Stevenson *et al.*, 1986). In order to investigate the effect of heat on MAG, 1,2-DAG and TAG levels during the 2 h post-heating period, both cell types were heated at 45°C for 10 min and then cells were returned to the 37°C water bath.

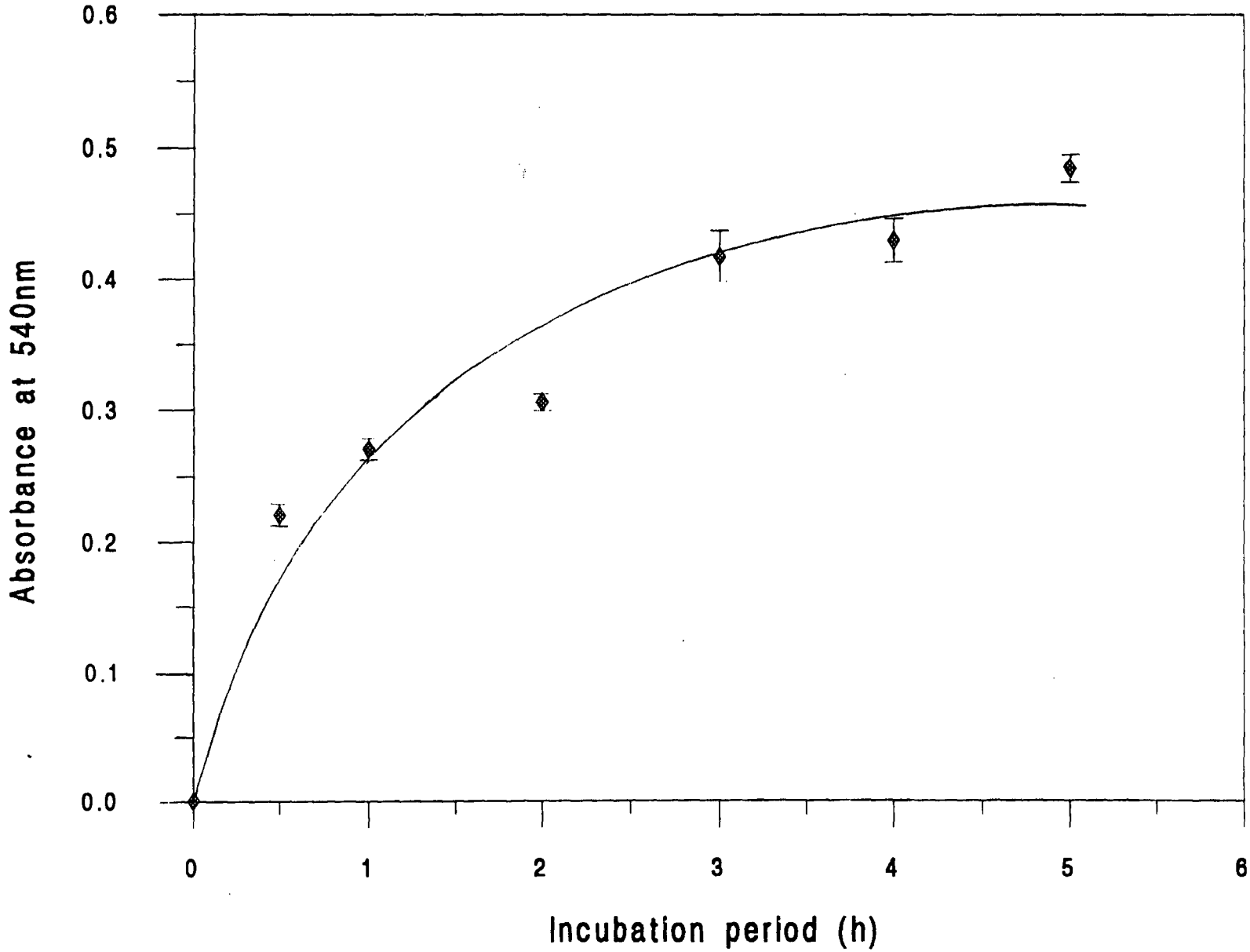
Monolayer CHO-K1 cells were labelled for 24 h with [^{3}H]arachidonic acid, heated in serum-containing medium at 45°C for 10 min and returned to 37°C for up to 2 h. The MAG, 1,2-DAG and TAG levels were measured during this 2 h period. No difference in ^{3}H-MAG level was observed between heated and unheated cells except at 2 h post-heating (Figure 3.21a). A decrease in ^{3}H-1,2-DAG level was detected immediately after 10 min heat treatment. As incubation at 37°C proceeded, the ^{3}H-1,2-DAG level in heated cells gradually returned to control levels, and by 2 h the ^{3}H-1,2-DAG levels in heated and unheated cells was similar (Figure 3.21b). An increase in ^{3}H-TAG level was observed in heated cells when measured immediately after 10 min heating. During the recovery period, there was an increase in ^{3}H-TAG levels in heated cells at 40 min and 130 min (i.e. 30 min and 120 min post-heating) whereas ^{3}H-TAG levels in both heated and unheated cells were similar at other time points (Figure 3.21c).

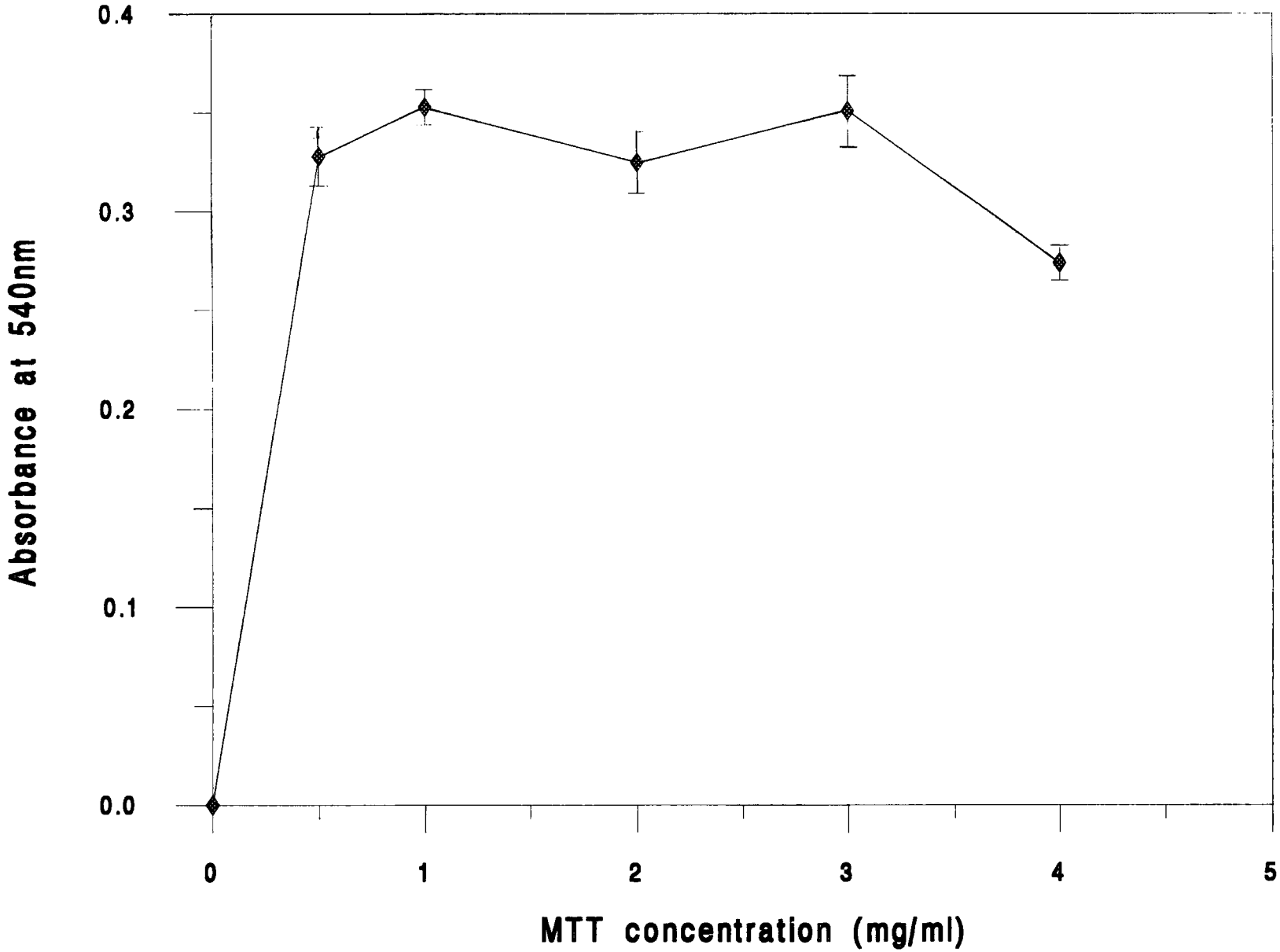
WRK-1 cells were labelled for 4 days with [2-^{3}H]glycerol, heated at 45°C for 10 min in serum-containing medium and returned to 37°C. The levels of ^{3}H-MAG, ^{3}H-1,2-DAG and ^{3}H-TAG was monitored during the 2 h recovery period. An increase in ^{3}H-MAG level were observed between heated and unheated cells at 30 to 60 min post-heating (Figure 3.22a). On the other hand, a decrease in ^{3}H-1,2-DAG level was observed in heated cells after 10 min at 45°C, but it became similar to control levels after 15 min

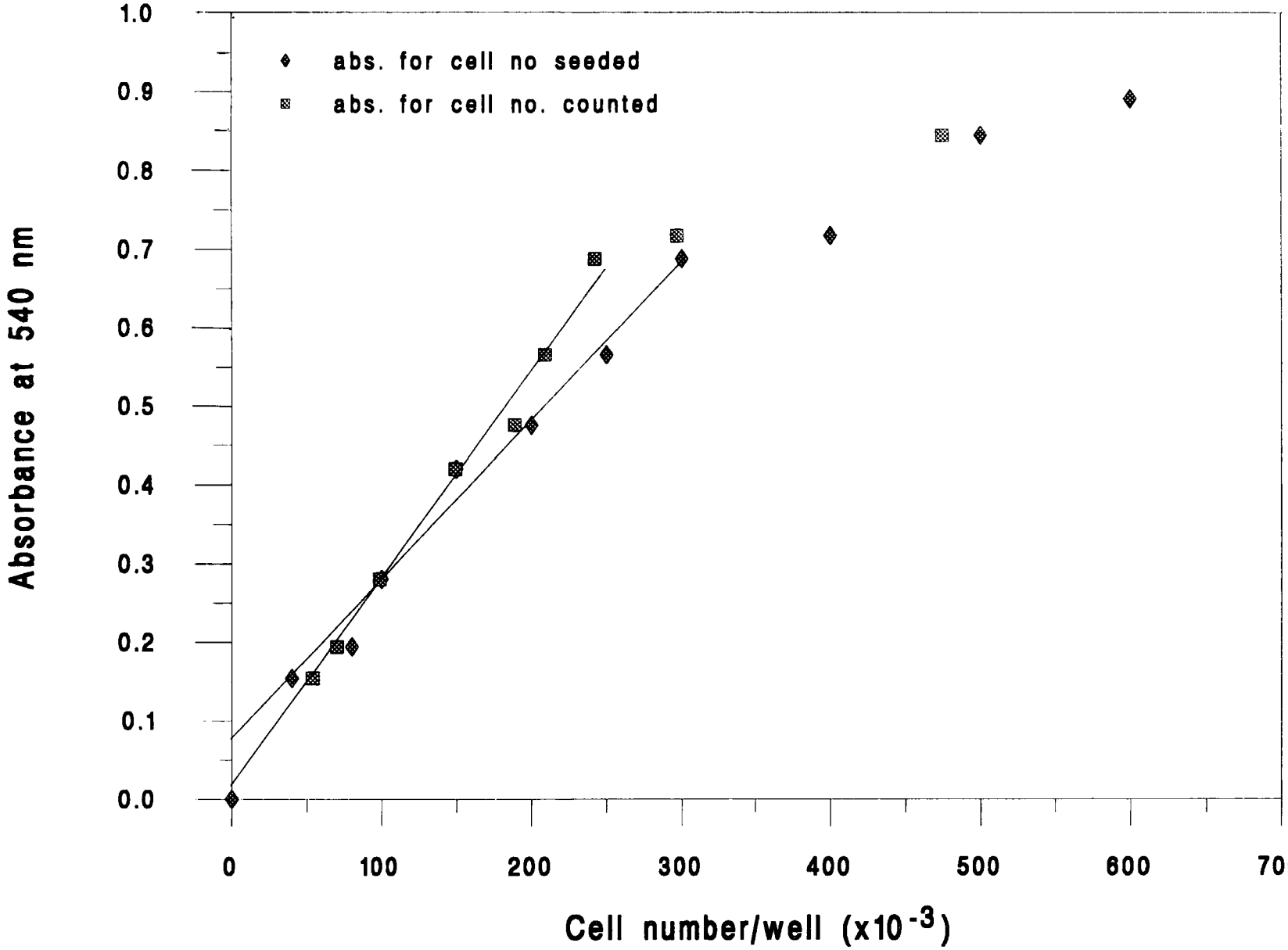
at 37°C. After 2 h at 37°C, the level of ^{3}H-1,2-DAG in cells pre-heated at 45°C was lower than the level in unheated cells (Figure 3.22b). In contrast, an increase in ^{3}H-TAG level immediately after 10 min heating was observed and the ^{3}H-TAG level in heated cells remained higher than the unheated control even after 2 h incubation at 37°C (Figure 3.22c).

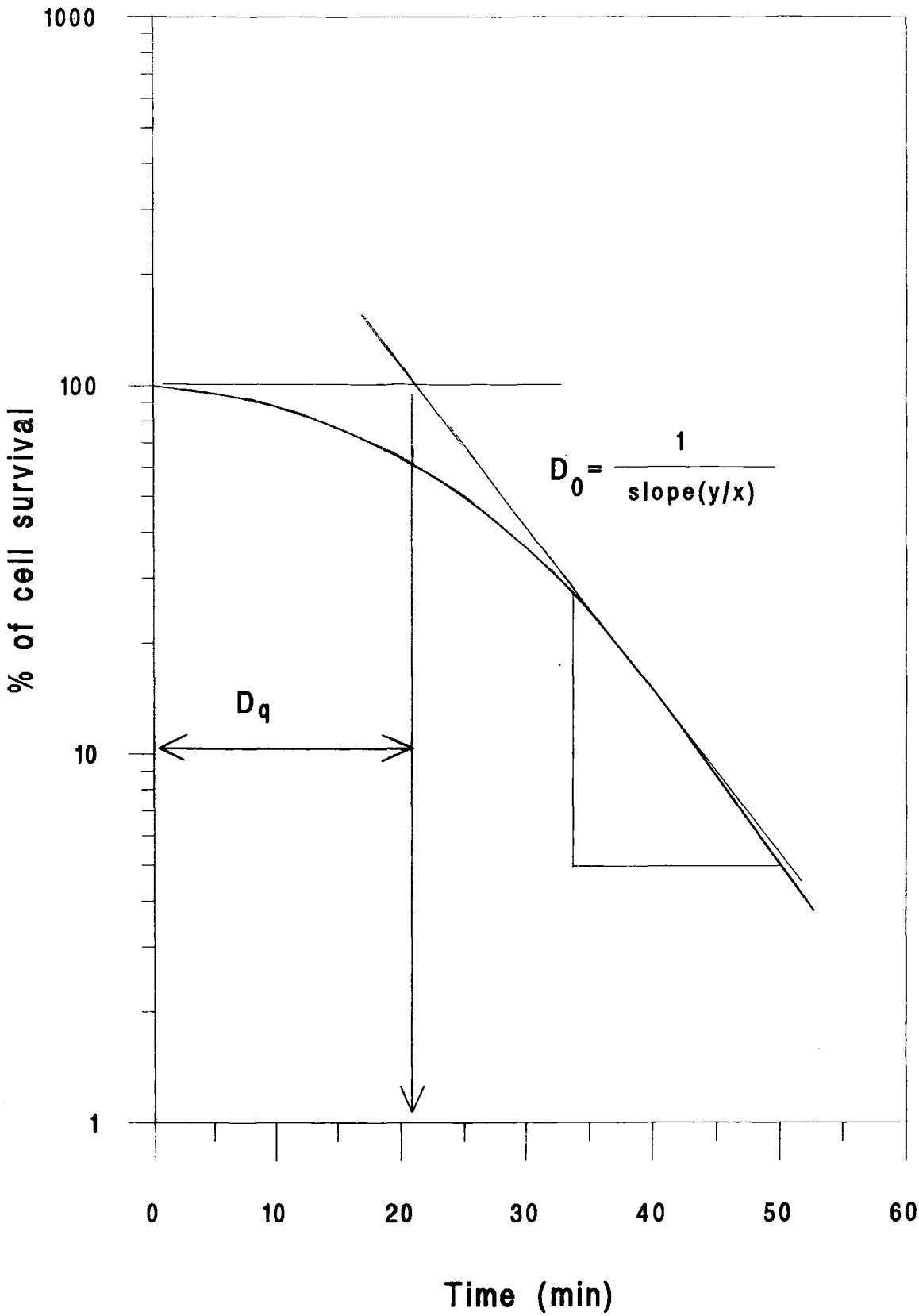
In summary, it was found that (1) exposure of CHO-K1 cells to 45°C for 10 min resulted in a decrease in mean ^{3}H-1,2-DAG levels and a small decrease in ^{3}H-TAG levels at 1-3 min. Heating WRK-1 cells at 45°C for 12.5 min resulted in a decrease in ^{3}H-1,2-DAG which was accompanied by an increase in ^{3}H-TAG level throughout the heating period. (2) Heating CHO-K1 cells for up to 45 min at 45°C resulted in a decrease in the ^{3}H-1,2-DAG level and an increase in ^{3}H-TAG level. Similar findings were observed in WRK-1 cells in which a decrease in ^{3}H-1,2-DAG and an increase in ^{3}H-TAG levels were observed. (3) A decrease in ^{3}H-1,2-DAG level was observed in cells heated at 45°C for 12.5 min in the presence or absence of 100 μM diC_8EG compared with that obtained from cells maintained at 37°C in the presence or absence of diC_8EG, respectively. The levels of ^{3}H-1,2-DAG in cells heated either with or without diC_8EG was similar. Heating in the presence of 100 μM diC_8EG for up to 12.5 min at 45°C resulted in an increase in ^{3}H-TAG level at 6.5 min and 12.5 min compared with cells incubated at 37°C in the presence of diC_8EG. Similarly, when cells were heated in the absence of diC_8EG, an increase in ^{3}H-TAG was observed at 4.5 min, 6.5 min and 12.5 min compared with cells incubated at 37°C in the absence of diC_8EG. (4) Heating WRK-1 cells in the presence of 100 μM diC_8EG at 45°C for up to 45 min resulted in a decrease in ^{3}H-1,2-DAG level throughout the 45 min exposure compared with the level in unheated cells incubated in the presence of diC_8EG. Similarly, a decrease in ^{3}H-1,2-DAG level was observed in cells heated in the absence of diC_8EG compared with diC_8EG-untreated cells maintained at 37°C. However, the levels of ^{3}H-1,2-DAG in cells heated in the presence or absence of diC_8EG was similar. On the other hand, an increase

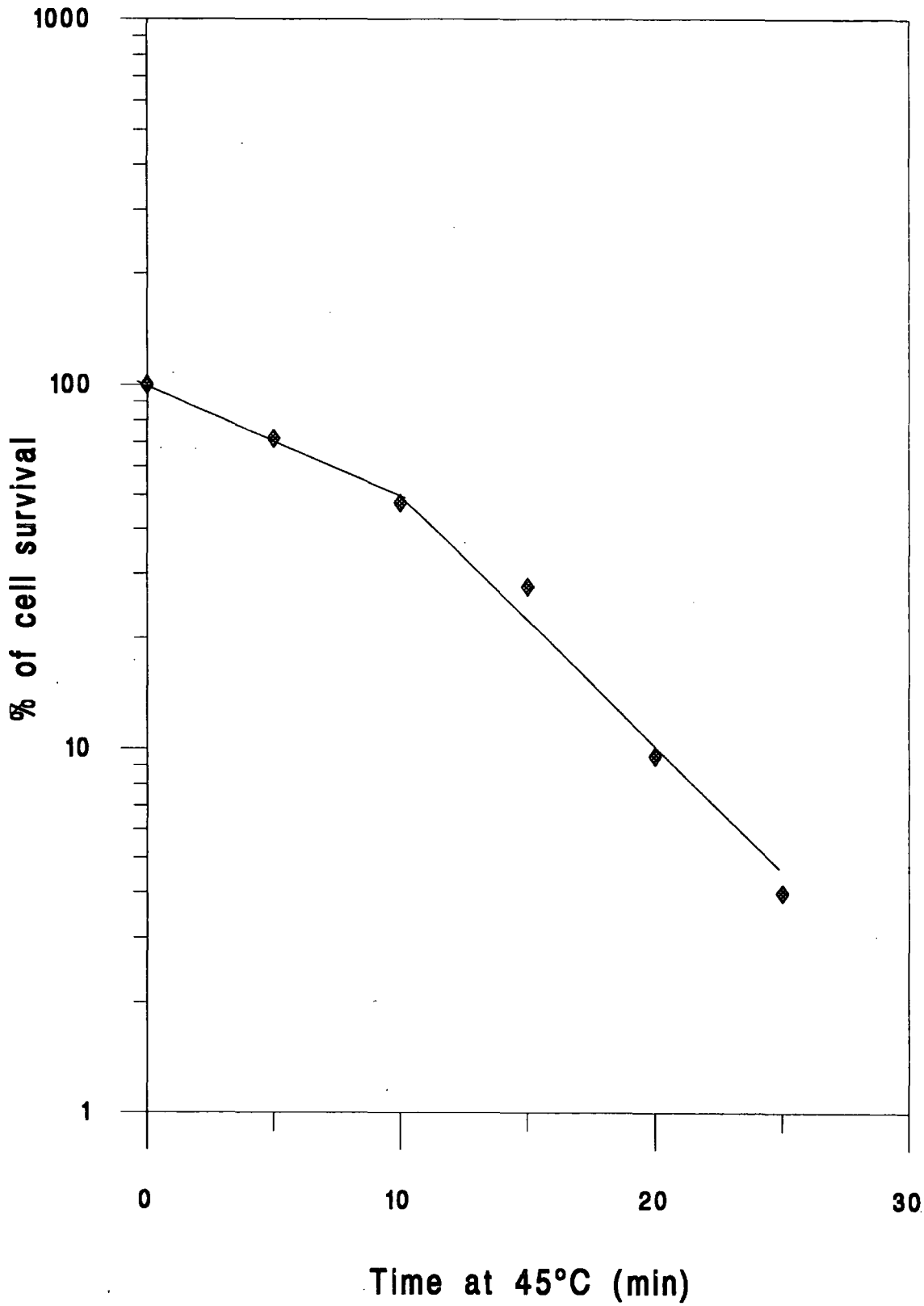
in ^{3}H-TAG level was observed at 20 min and 45 min when heated in the presence of diC$_8$EG compared with the level obtained from diC$_8$EG-treated, unheated cells. When cells were heated in the absence of diC$_8$EG, an increase in ^{3}H-TAG level was observed after 20 min heating compared with the level obtained from unheated cells without diC$_8$EG treatment. (5) After pretreatment of CHO-K1 cells and WRK-1 cells at 45°C for 10 min, followed by a recovery period up to 2 h at 37°C, it was found that ^{3}H-1,2-DAG levels in heated CHO-K1 cells returned gradually to the control level and after 2 h the levels between heated and unheated cells were similar, whereas an increase in ^{3}H-TAG level was observed in heated cells after 2 h at 37°C. In the case of WRK-1 cells, a decrease in ^{3}H-1,2-DAG level was observed after 2 h at 37°C, though after the 30 min post-heating period the levels in heated and unheated cells were similar. In addition, an increase in ^{3}H-TAG level was observed in heated WRK-1 cells and the level remained higher than that in unheated cells even after 2 h post-heating.


Absorbance at 540nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
Incubation period (h)


0.4
0.3
0.2
0.1
0.0
Absorbance at 540nm
0
1
2
3
4
5
MTT concentration (mg/ml)


abs. for cell no seeded
abs. for cell no. counted
Absorbance at 540 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
Cell number/well (x10 -3)


1000
100
10
1
% of cell survival
$D_0 = \frac{1}{\text{slope}(y/x)}$
D_q
0
10
20
30
40
50
60
Time (min)


1000
100
10
1
% of cell survival
0
10
20
30
Time at 45°C (min)

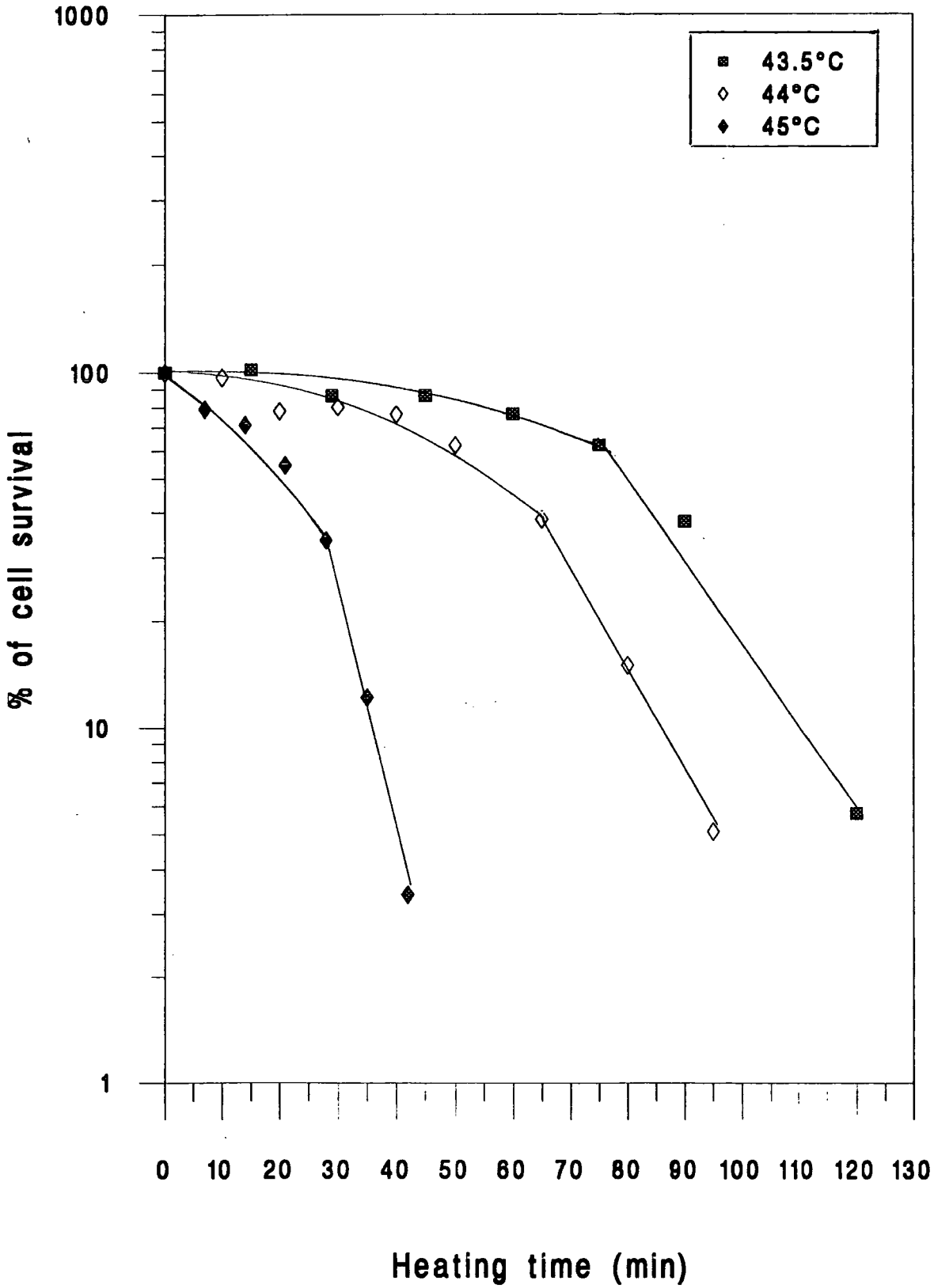

1000
100
10
1
% of cell survival
43.5°C
44°C
45°C
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Heating time (min)

Table 3.1. Cell survival parameters calculated from Figure 3.8 and Figure 3.9

The survival parameters of CHO-K1 cells were calculated from the linear part of the killing curve from Figure 3.8. Values represent estimate ± SEM from linear portion part of the survival curve.

survival parameter	At 45°C (min)
D_q	5.52±1.11
D_0	14.5±0.69
LD_{50}	10.0±0.39
LD_{90}	20.1±0.48

The survival parameters of WRK-1 cells were calculated from the linear part of the killing curve from Figure 3.9. Values represent estimate ± SEM from linear portion part of the survival curve.

survival parameter	At 43.5°C (min)	At 44°C (min)	At 45°C (min)
D_q	68.3±1.44	53.8±2.38	21.4±0.45
D_0	42.4±0.65	38.4±2.20	14.5±0.22
LD_{50}	81.8±1.61	60.0±2.42	21.0±0.47
LD_{90}	110.6±1.48	89.2±2.39	35.9±0.46

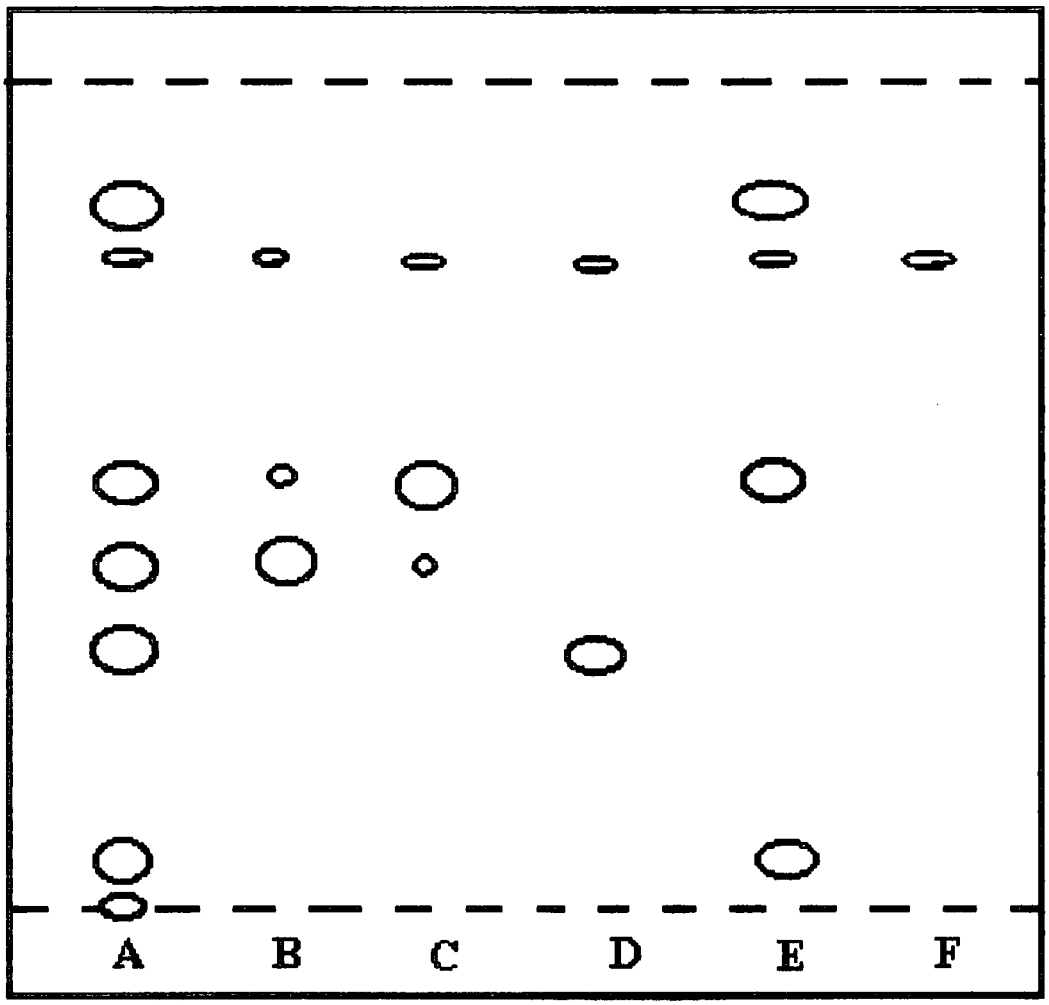

A
B
C
D
E
F

Figure 3.11a. Effect of heat on MAG level

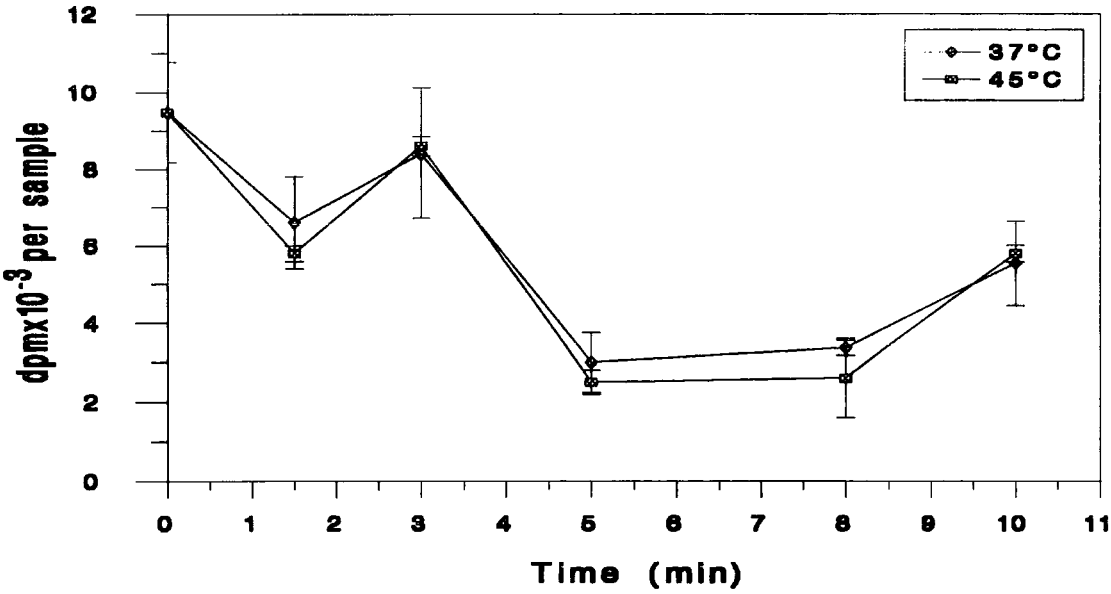


Figure 3.11b. Effect of heat on 1,2-DAG level

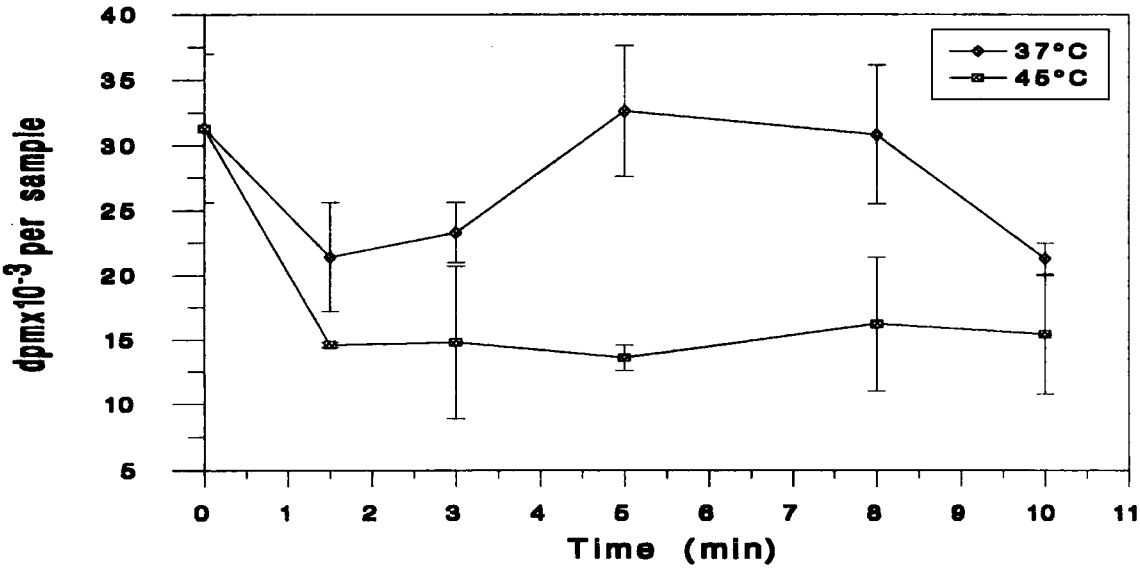


Figure 3.11c. Effect of heat on TAG level

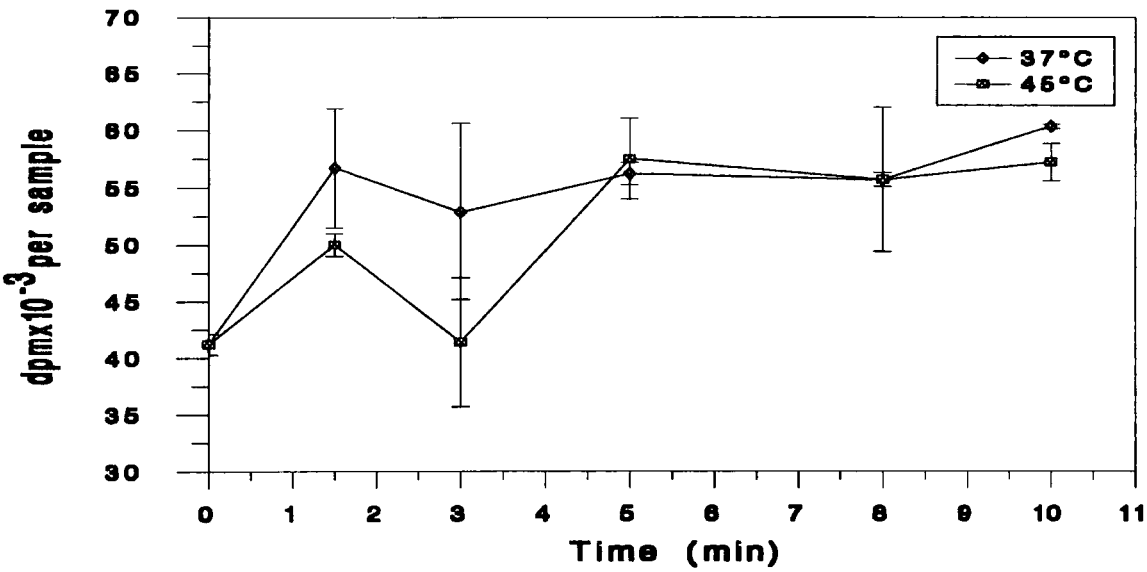


Figure 3.12a. Effect of heat on MAG level

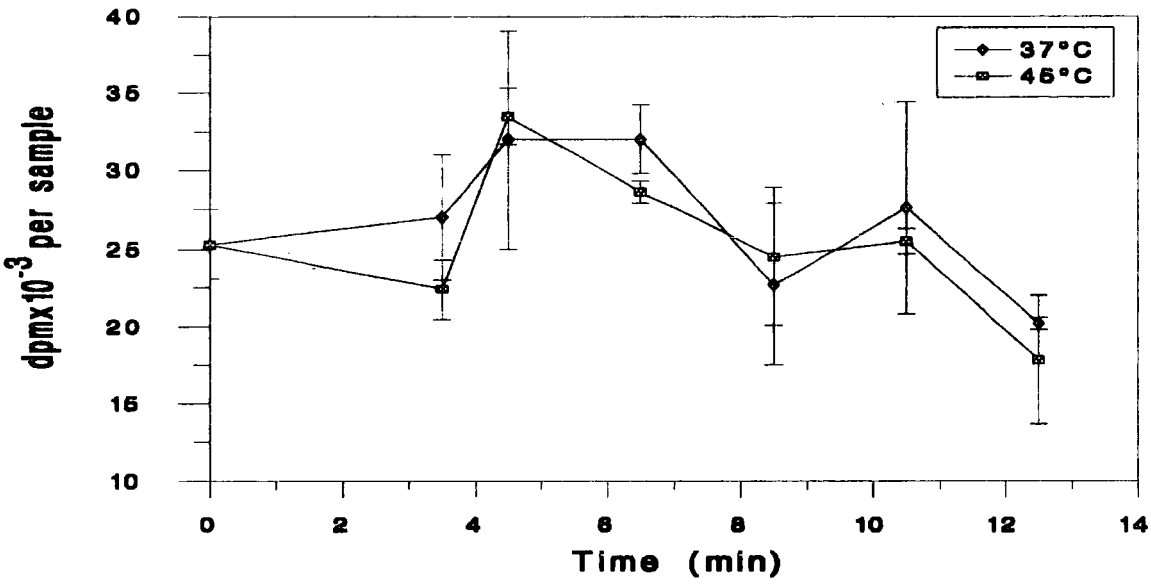


Figure 3.12b. Effect of heat on 1,2-DAG level

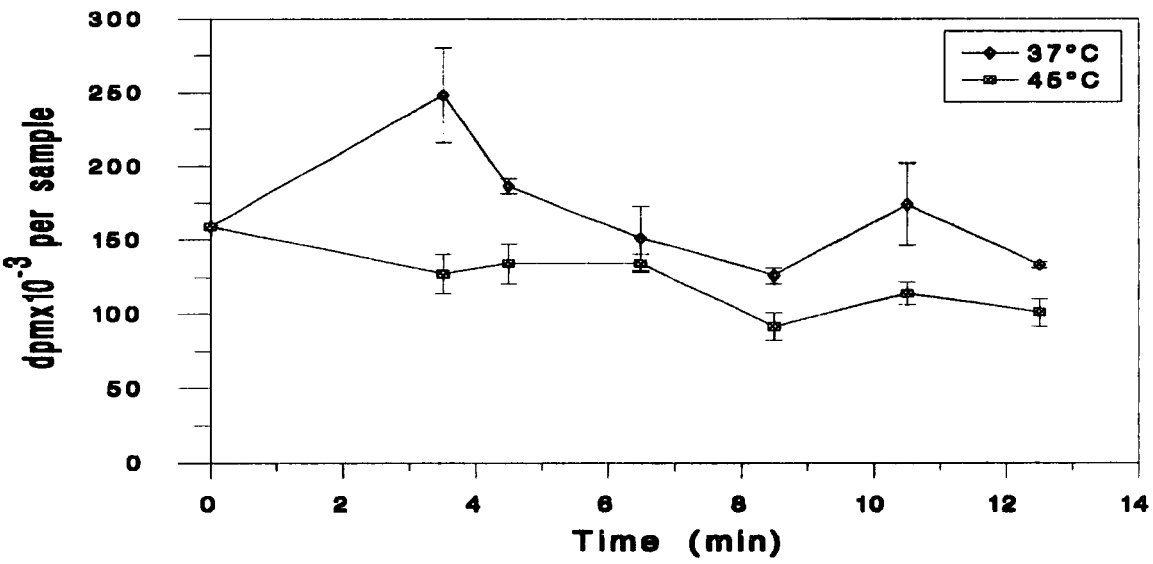


Figure 3.12c. Effect of heat on TAG level

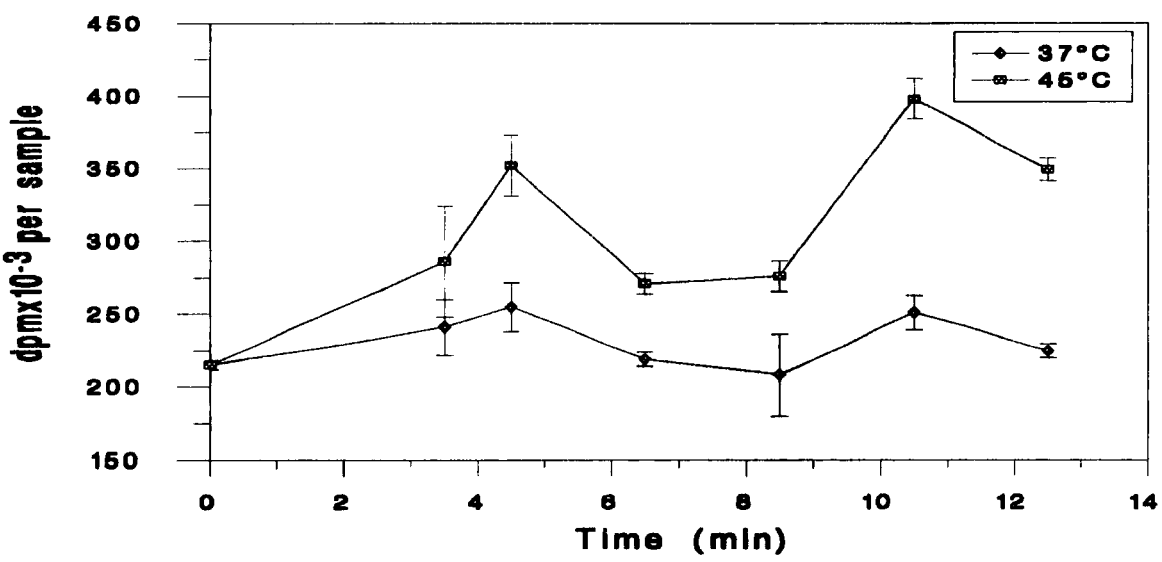


Figure 3.13a. Effect of heat on MAG level

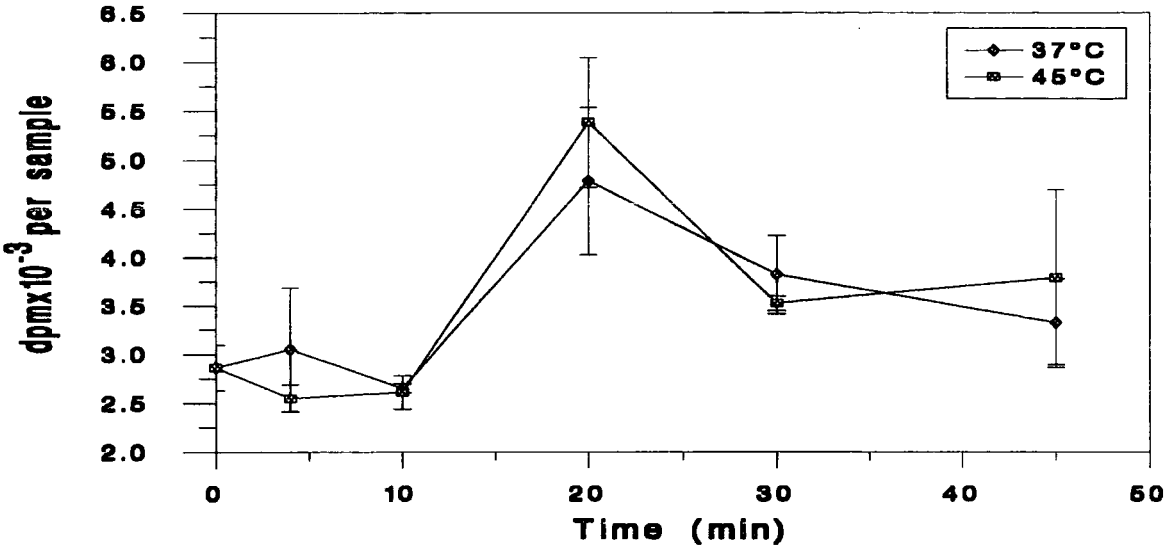


Figure 3.13b. Effect of heat on 1,2-DAG level

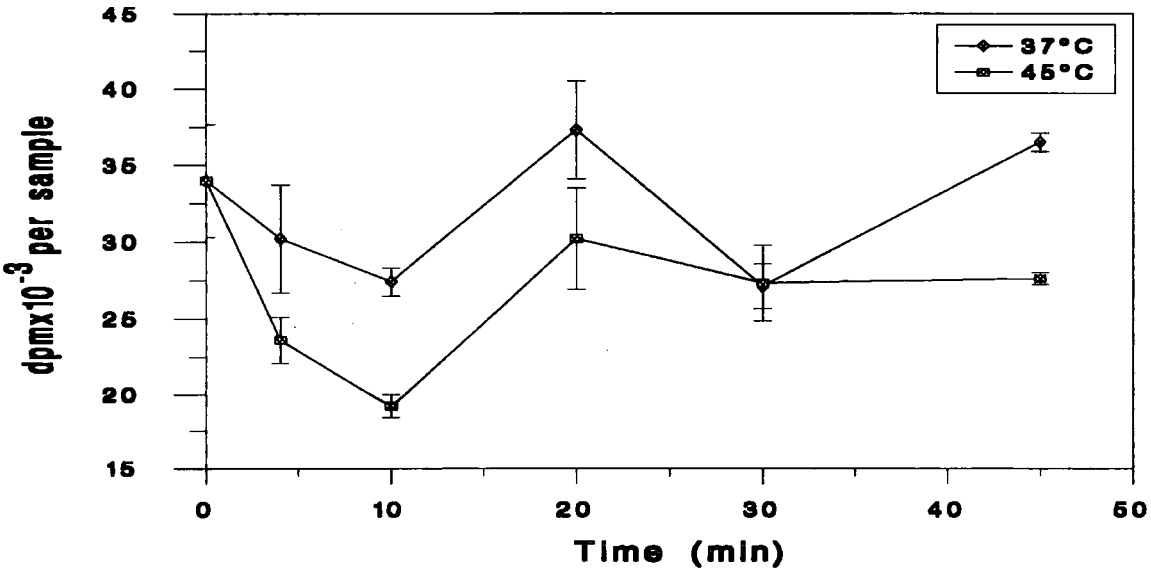


Figure 3.13c. Effect of heat on TAG level

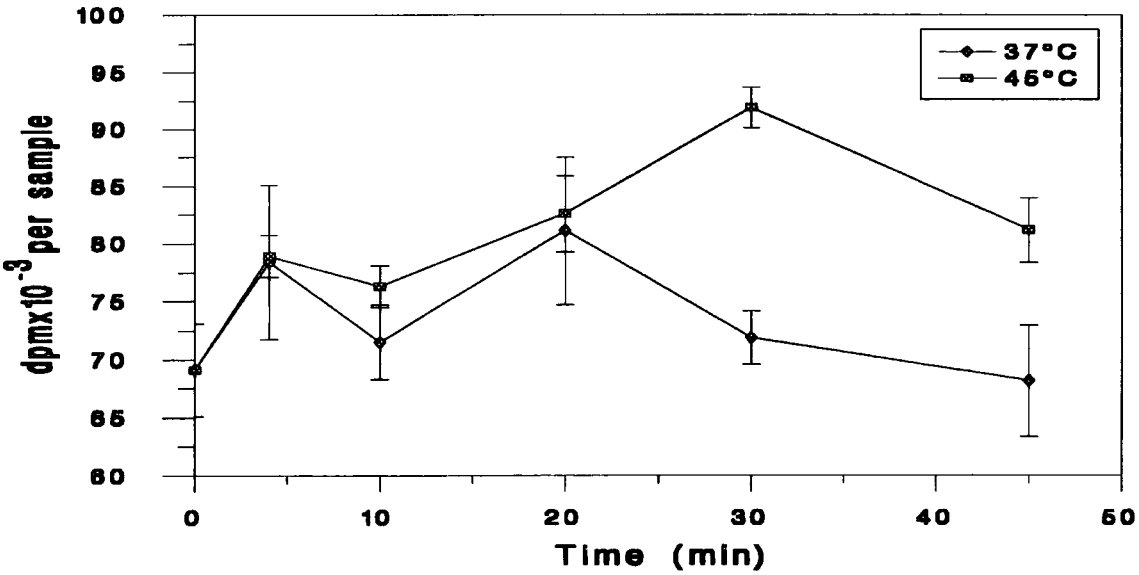


Figure 3.14a. Effect of heat on MAG level

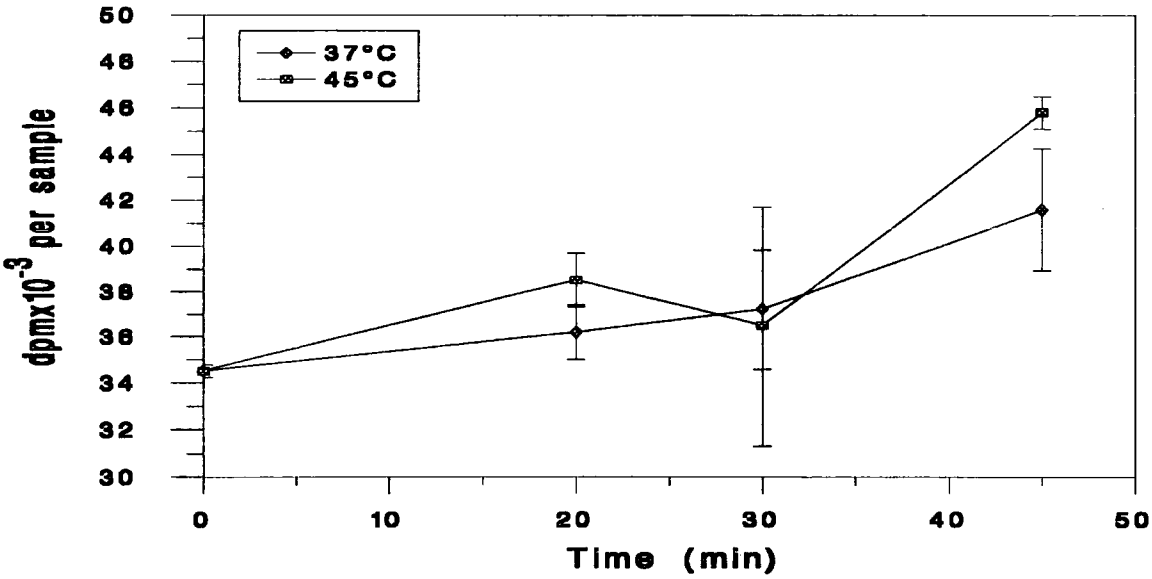


Figure 3.14b. Effect of heat on 1,2-DAG level

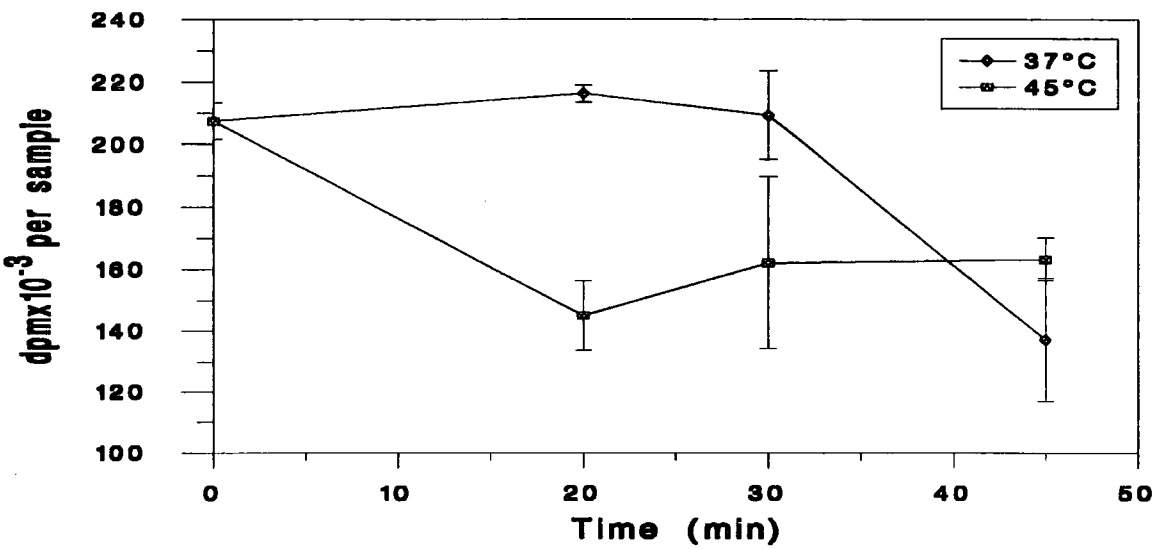


Figure 3.14c. Effect of heat on TAG level

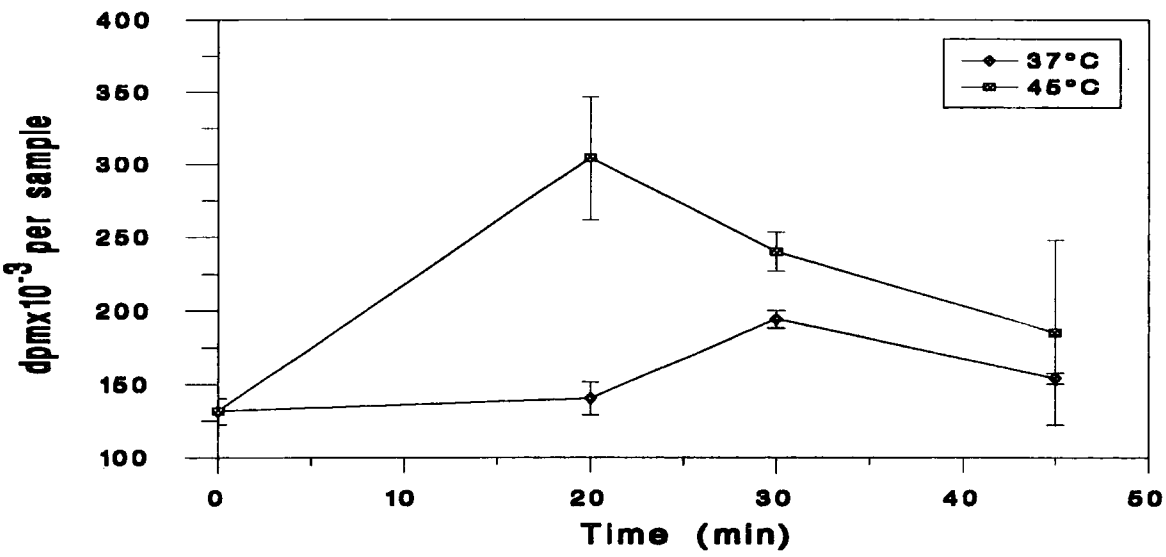


Figure 3.15a. Effect of heat on MAG level

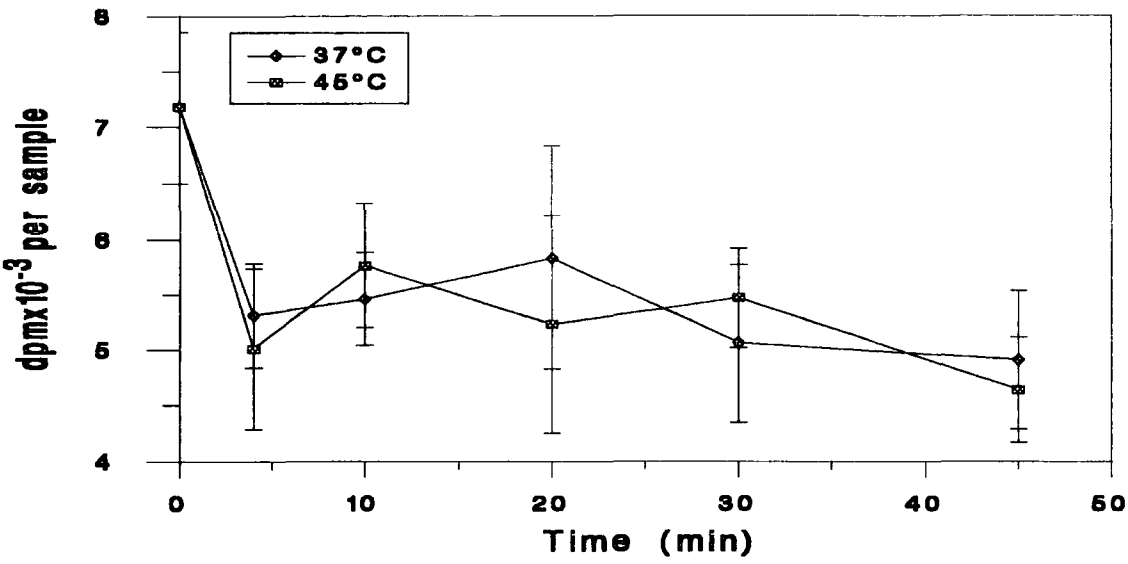


Figure 3.15b. Effect of heat on 1,2-DAG level

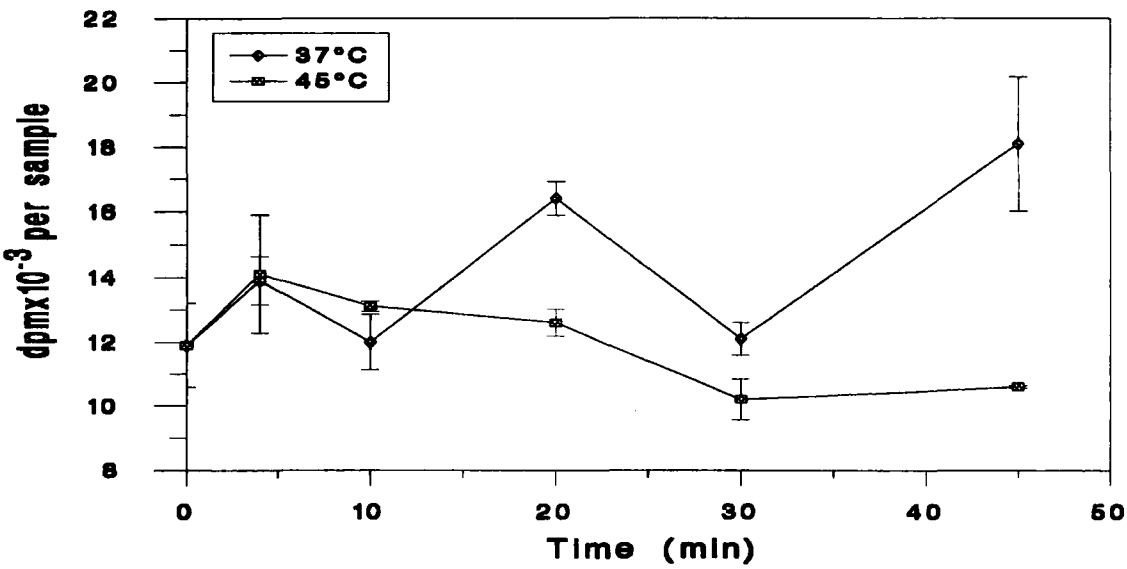


Figure 3.15c. Effect of heat on TAG level

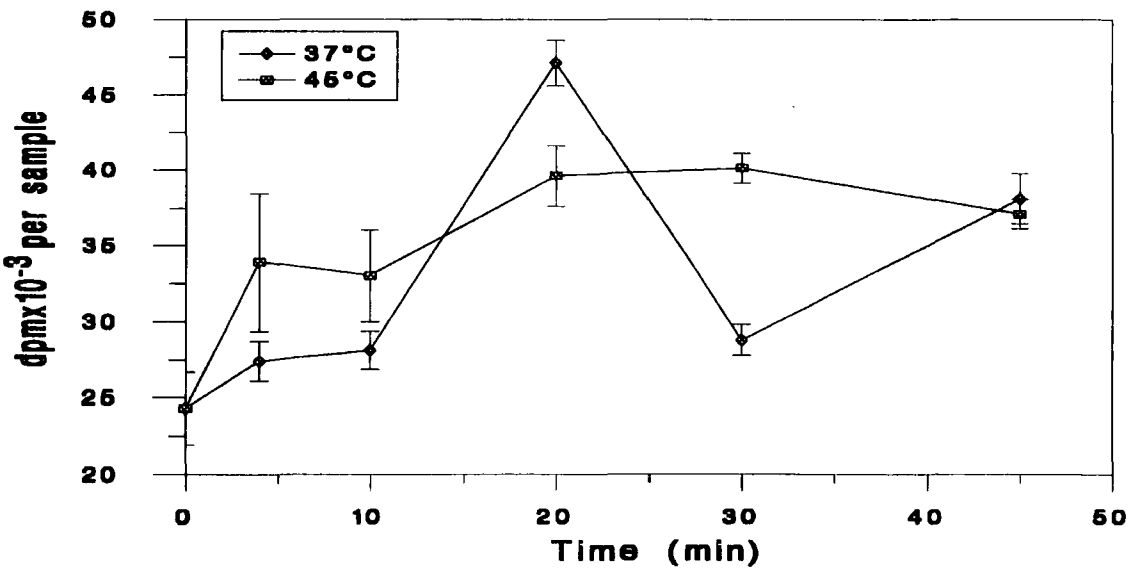


Figure 3.16a. Effect of heat on MAG level

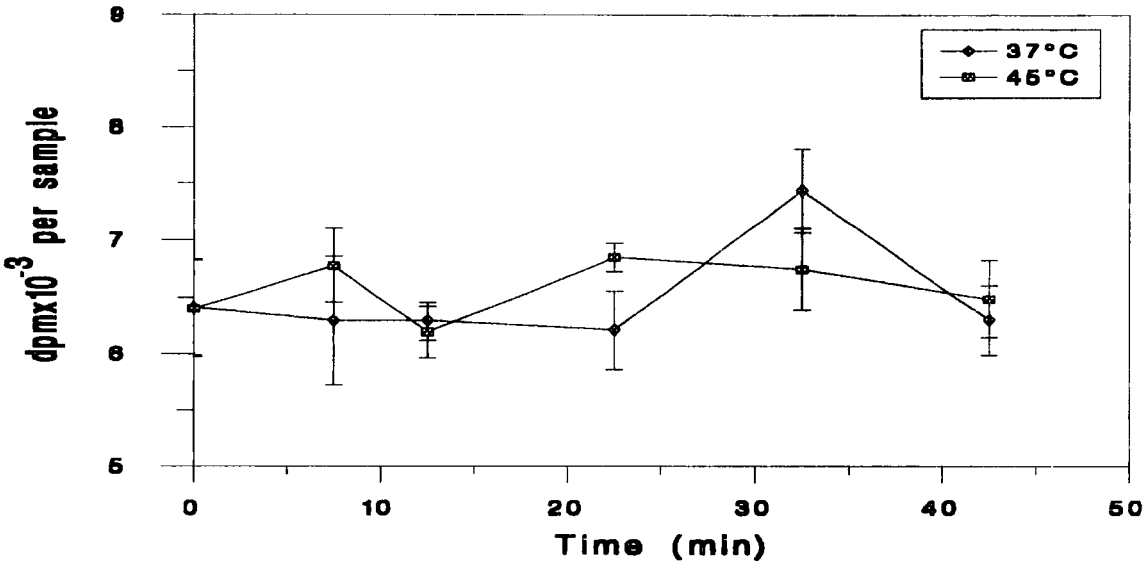


Figure 3.16b. Effect of heat on 1,2-DAG level

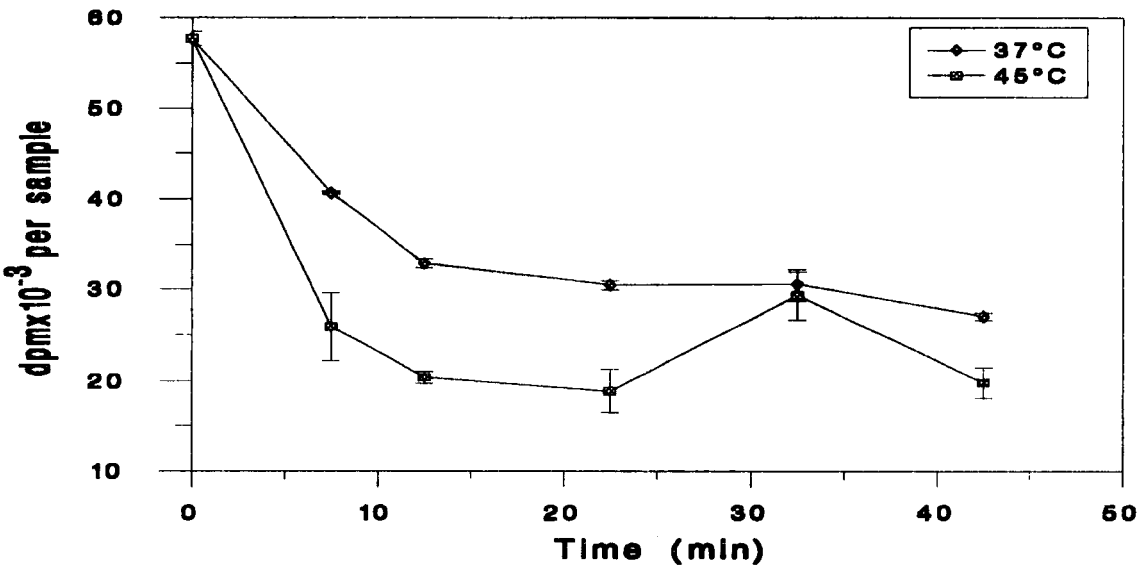


Figure 3.16c. Effect of heat on TAG level

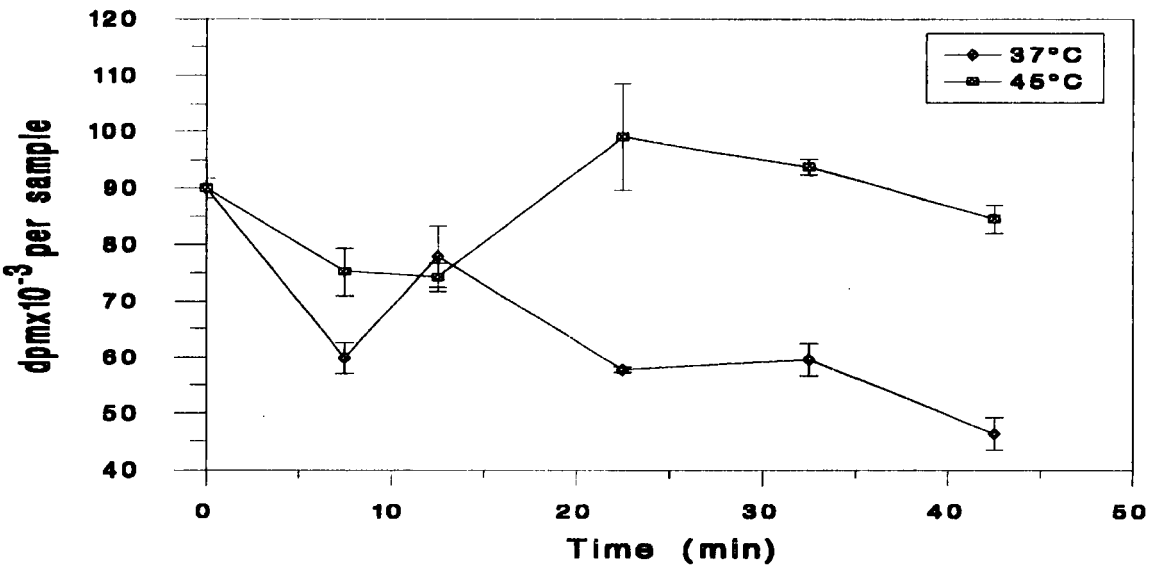


Figure 3.17a. Effect of heat on MAG level

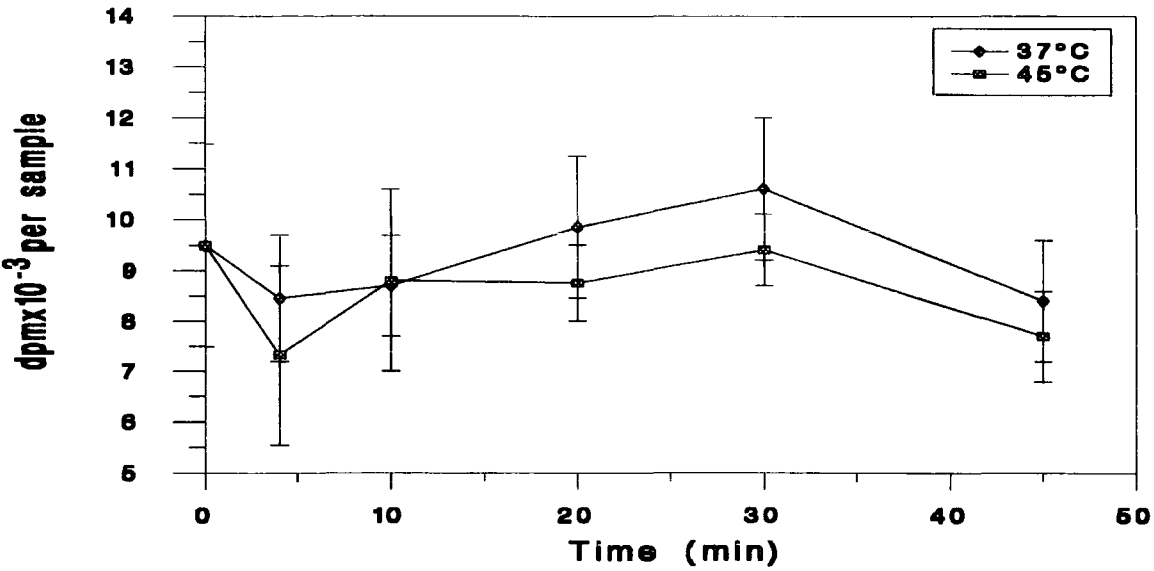


Figure 3.17b. Effect of heat on 1,2-DAG level

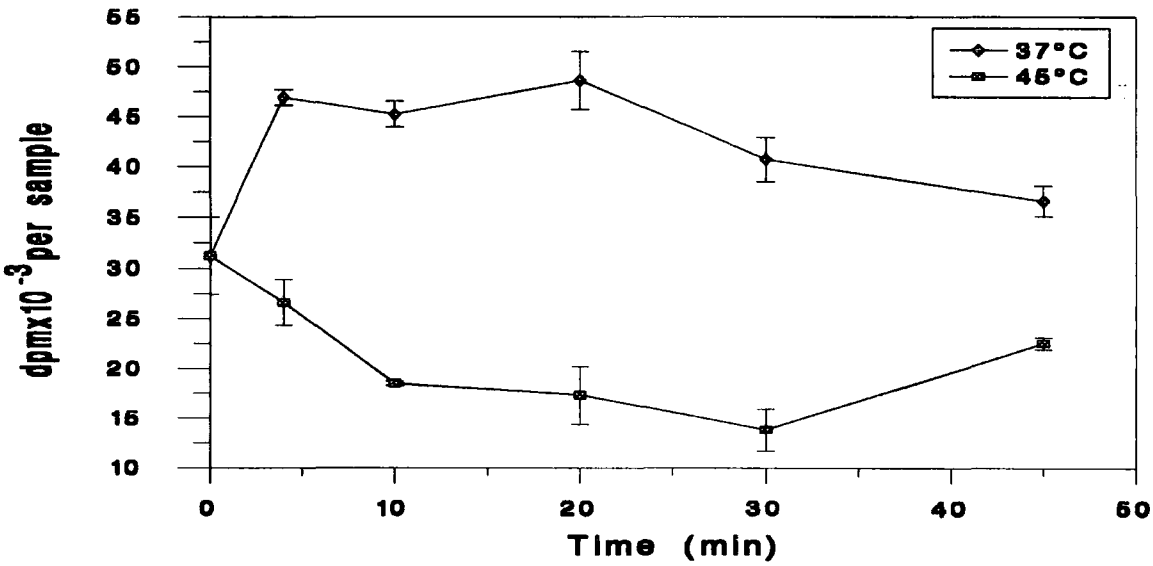


Figure 3.17c. Effect of heat on TAG level

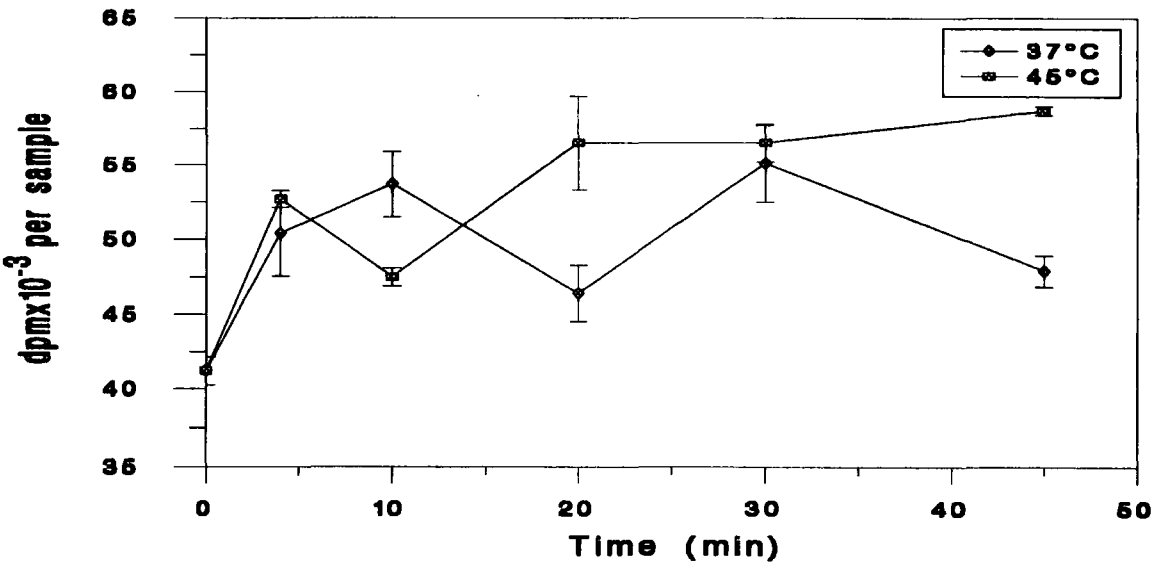


Figure 3.18a. Effect of heat on MAG level

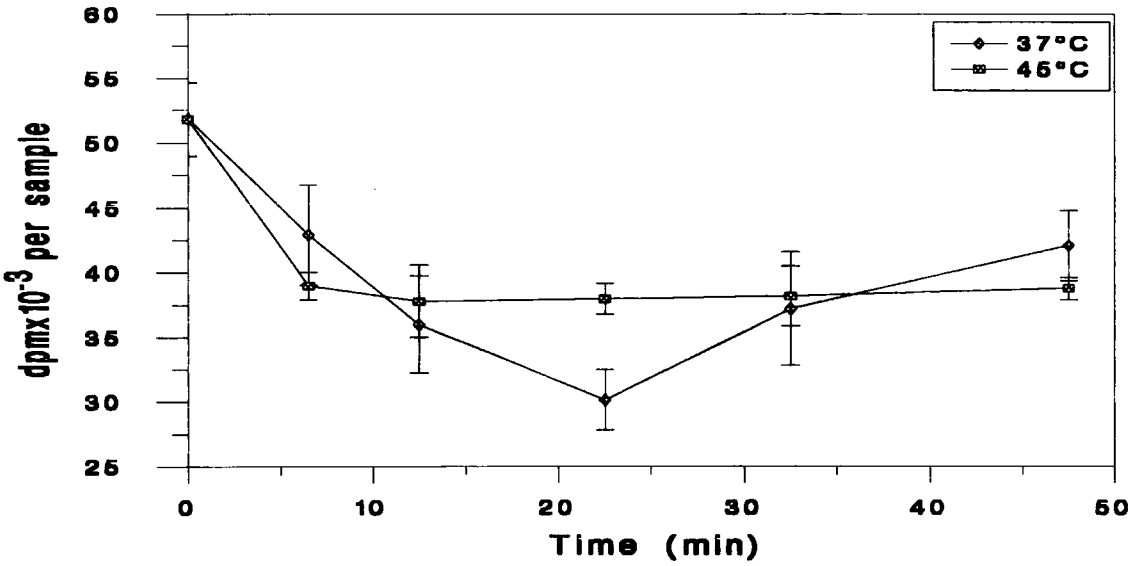


Figure 3.18b. Effect of heat on 1,2-DAG level

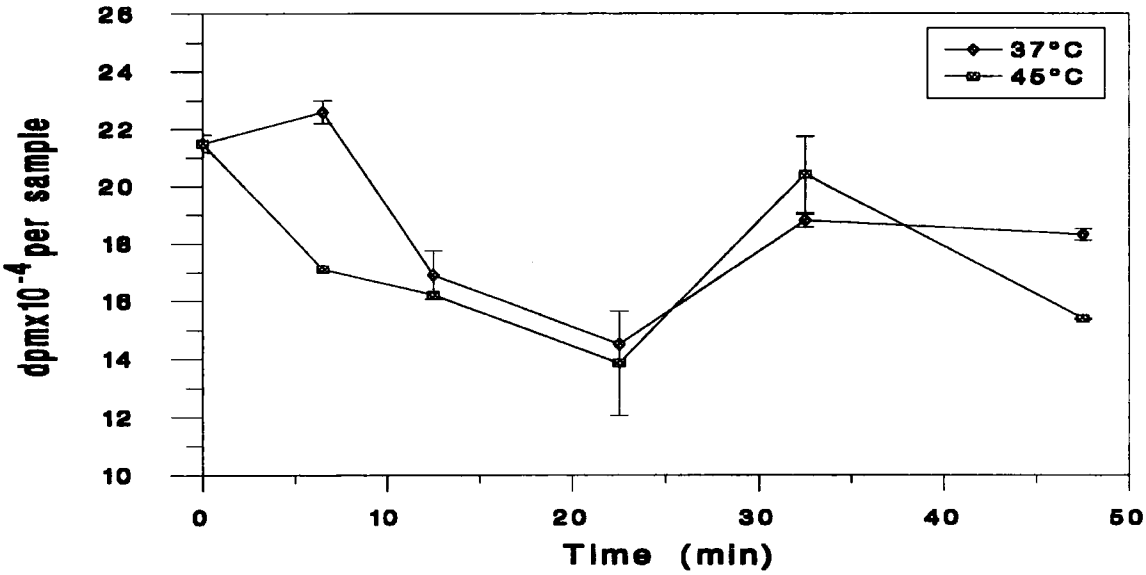


Figure 3.18c. Effect of heat on TAG level

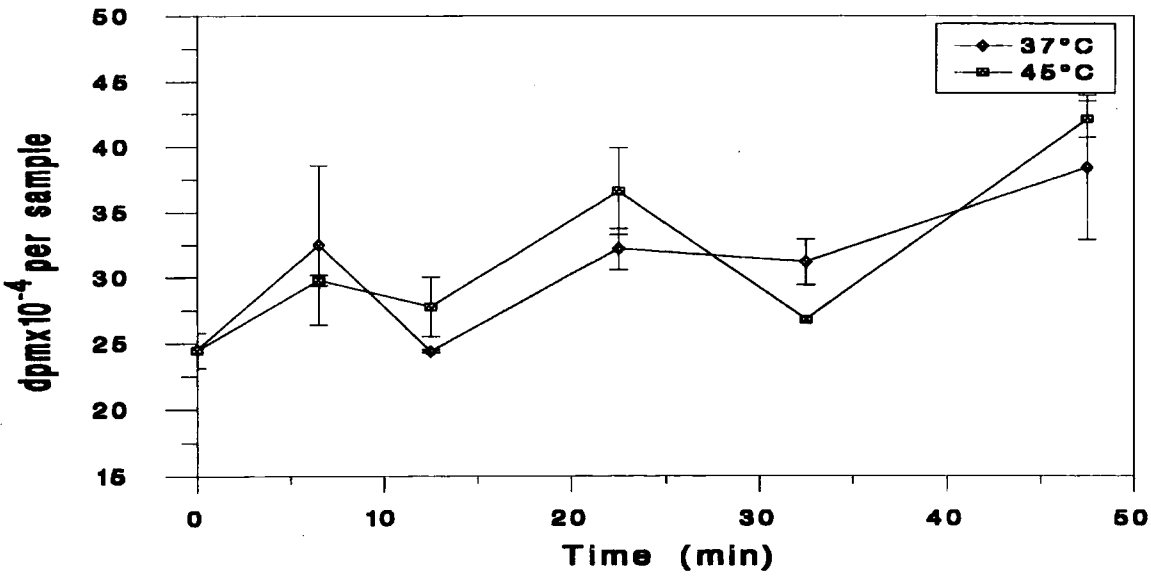


Figure 3.19a. Effect of heat on MAG level

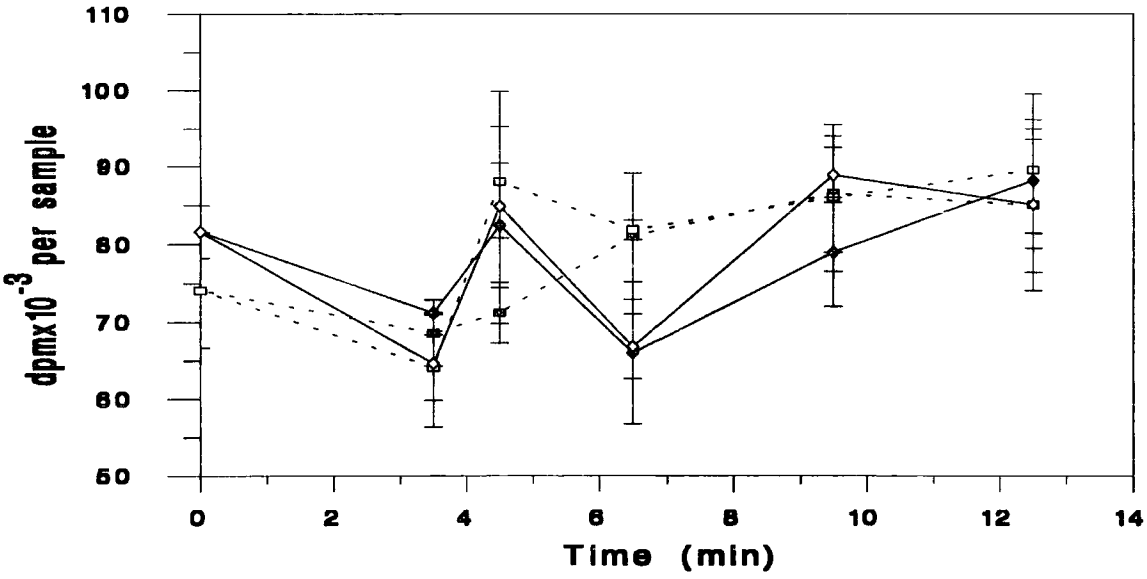


Figure 3.19b. Effect of heat on 1,2-DAG level

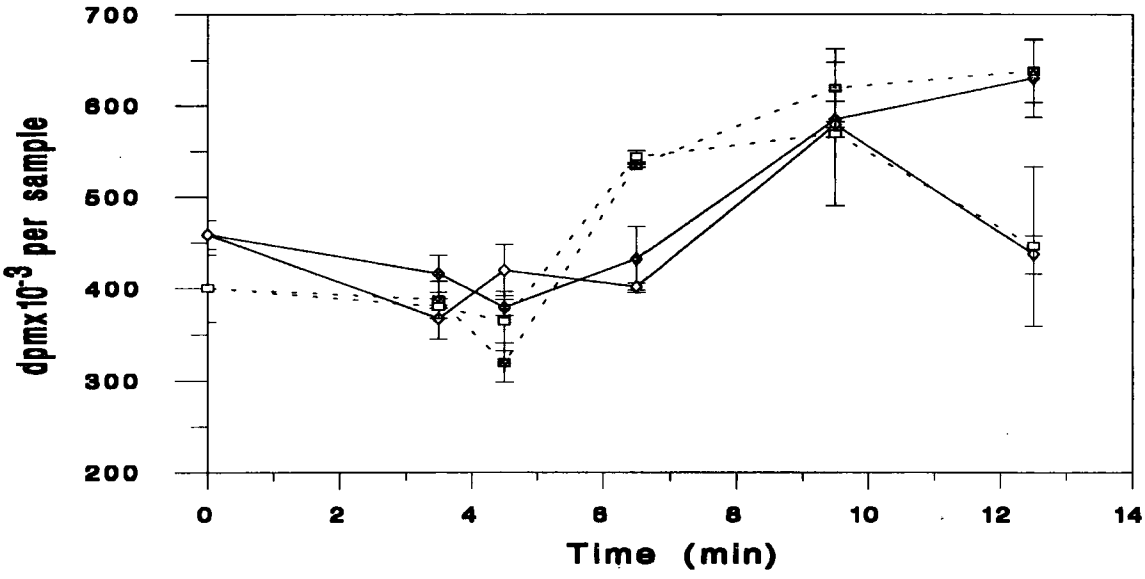


Figure 3.19c. Effect of heat on TAG level

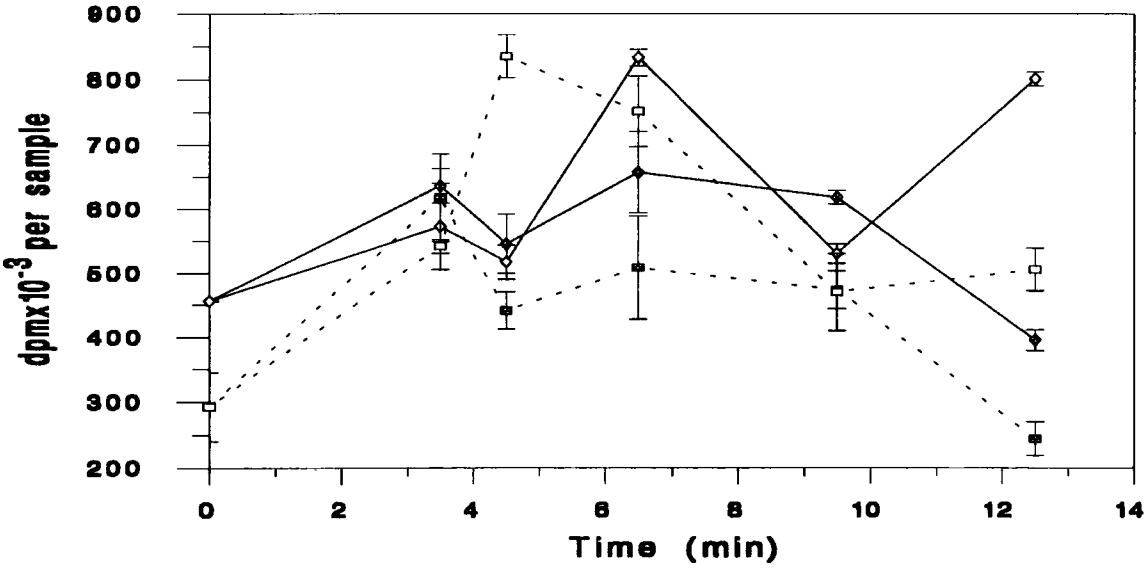


Figure 3.20a. MAG level

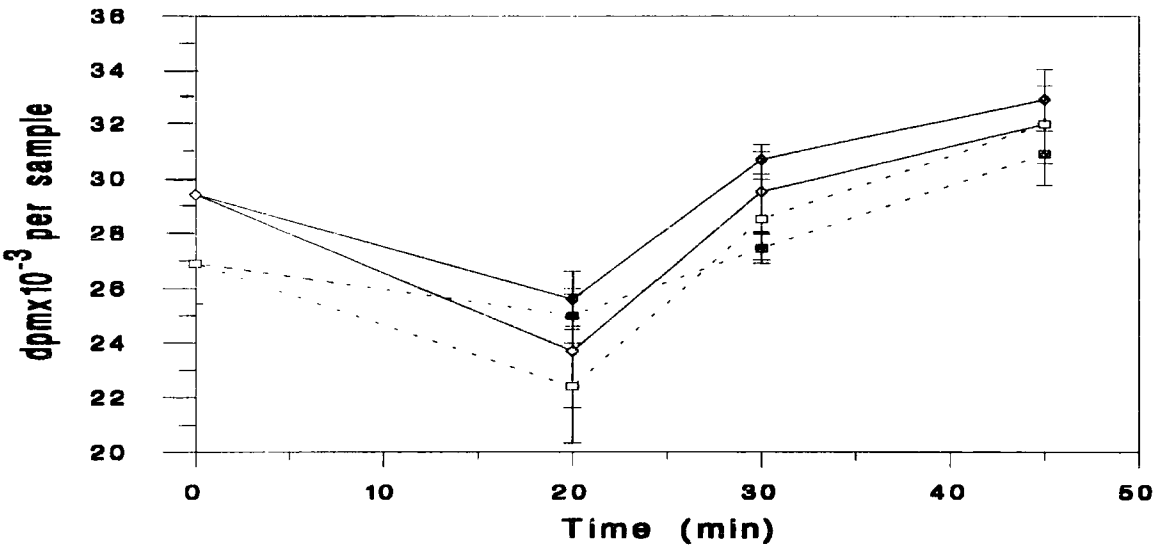


Figure 3.20b. 1,2-DAG level

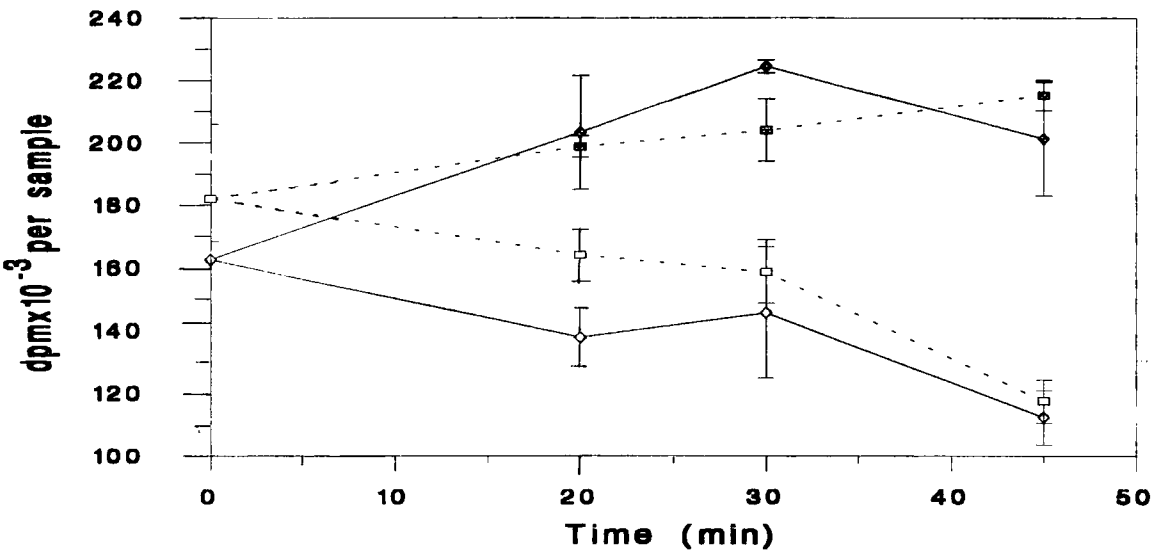


Figure 3.20c. TAG level

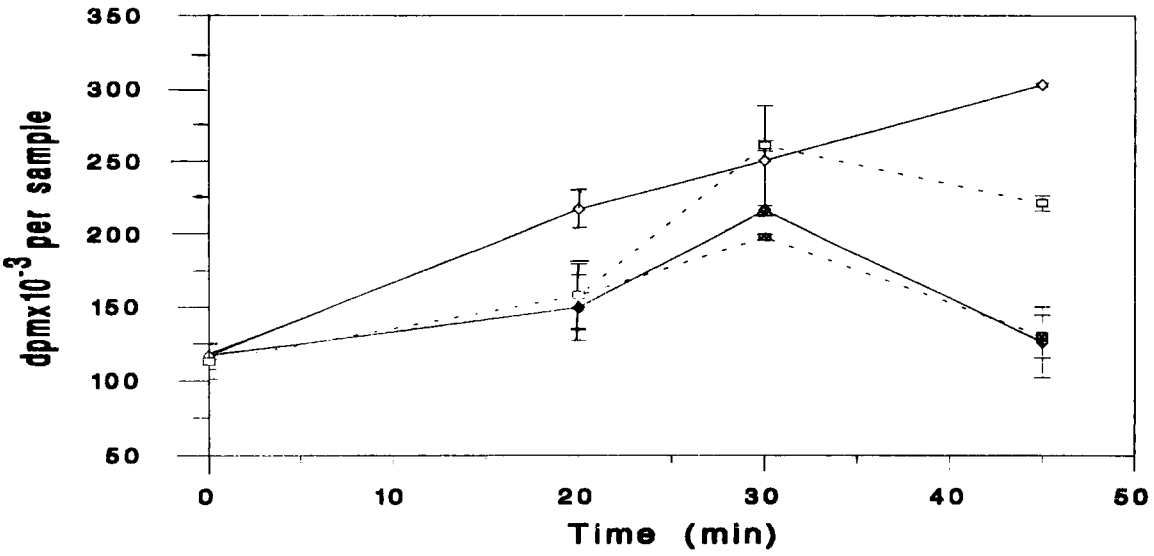


Figure 3.21a. Level of MAG measured at 37°C after 10 min heating at 45°C

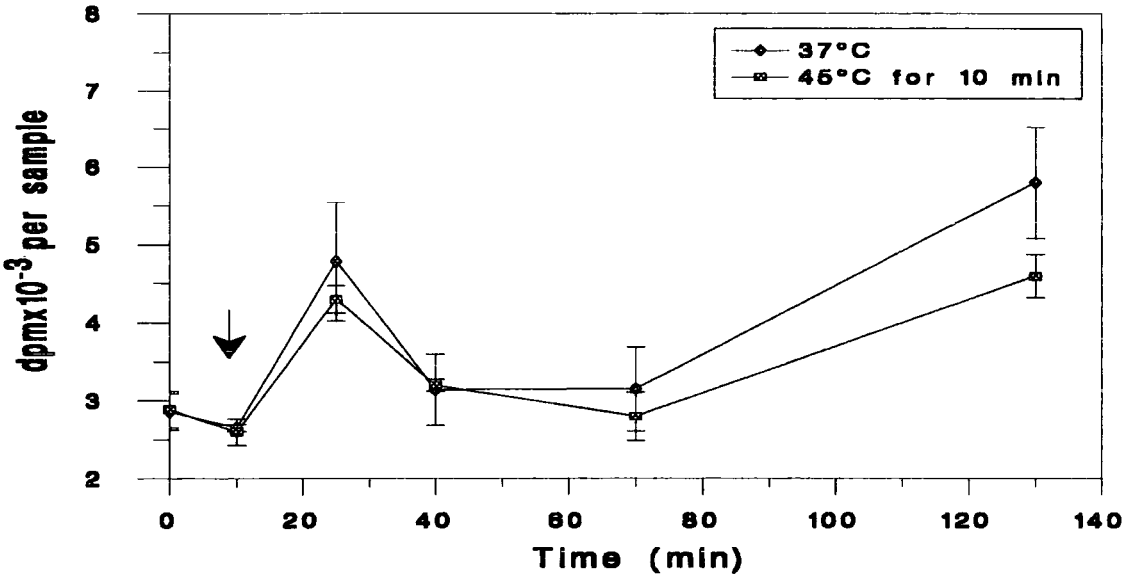


Figure 3.21b. Level of 1,2-DAG measured at 37°C after 10 min heating at 45°C

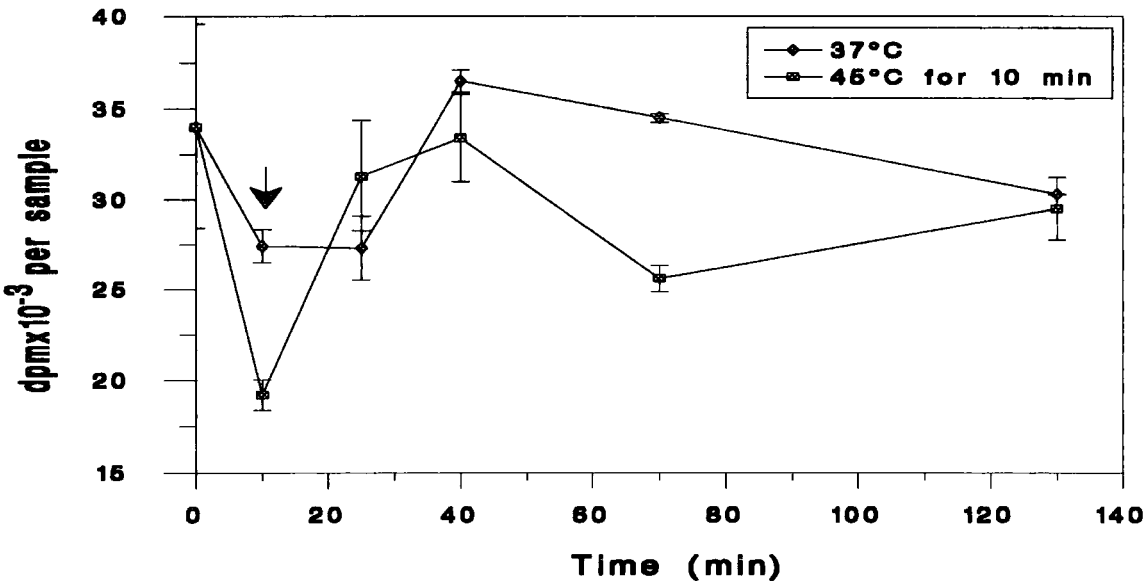


Figure 3.21c. Level of TAG measured at 37°C after 10 min heating at 45°C

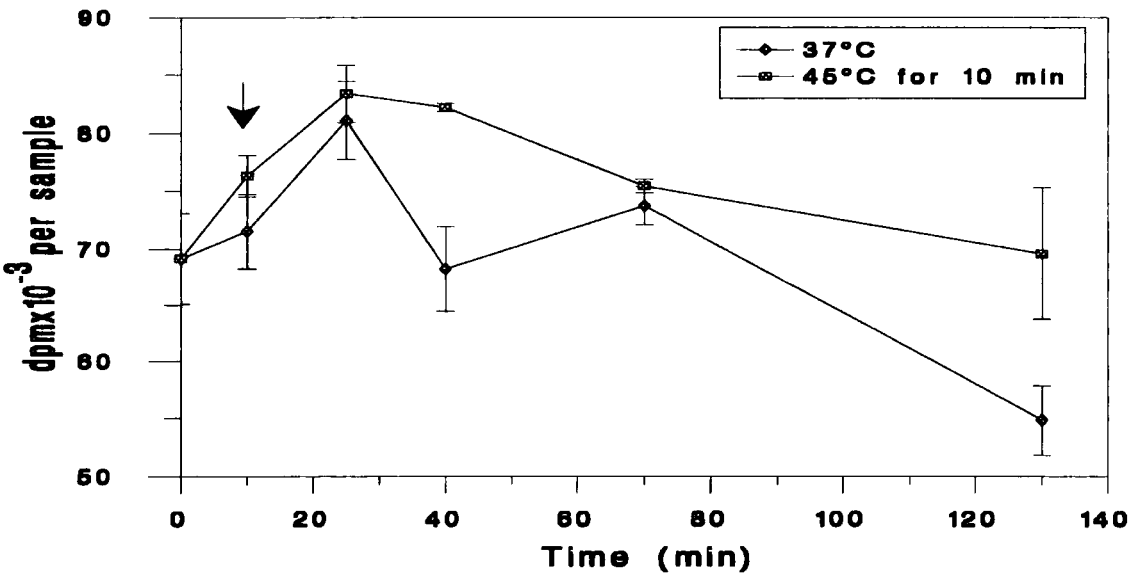


Figure 3.22a. Level of MAG measured at 37°C after 10 min heating at 45°C

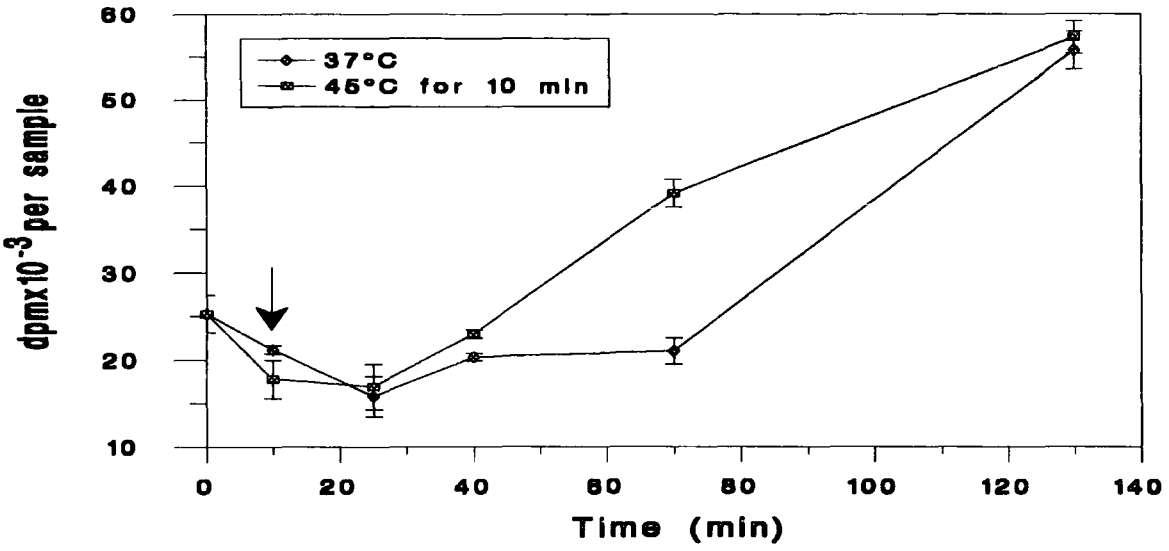


Figure 3.22b. Level of 1,2-DAG measured at 37°C after heating at 45°C for 10 min

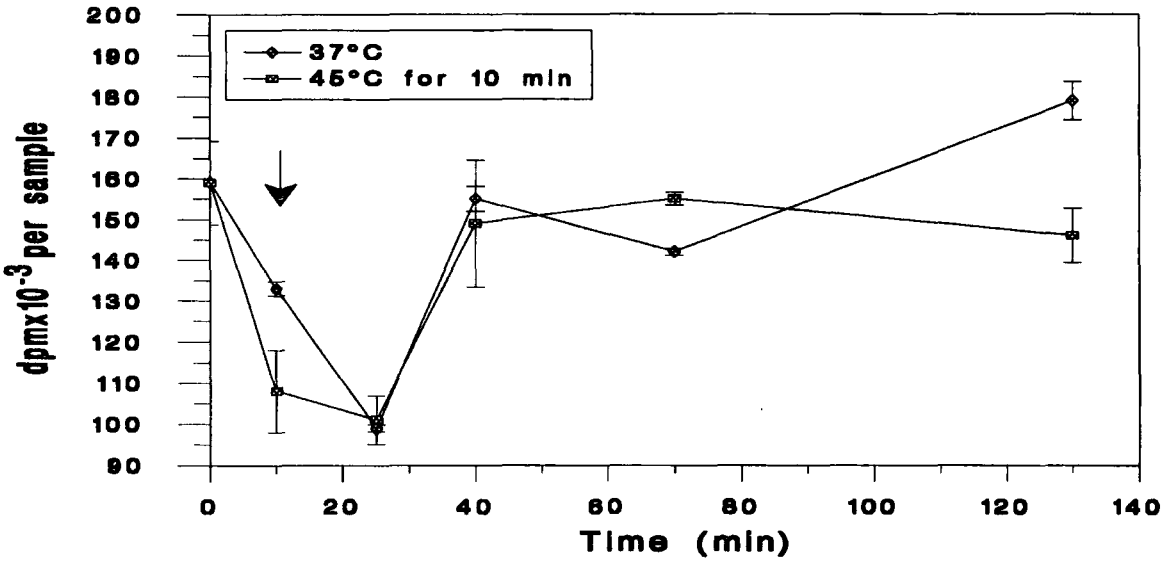
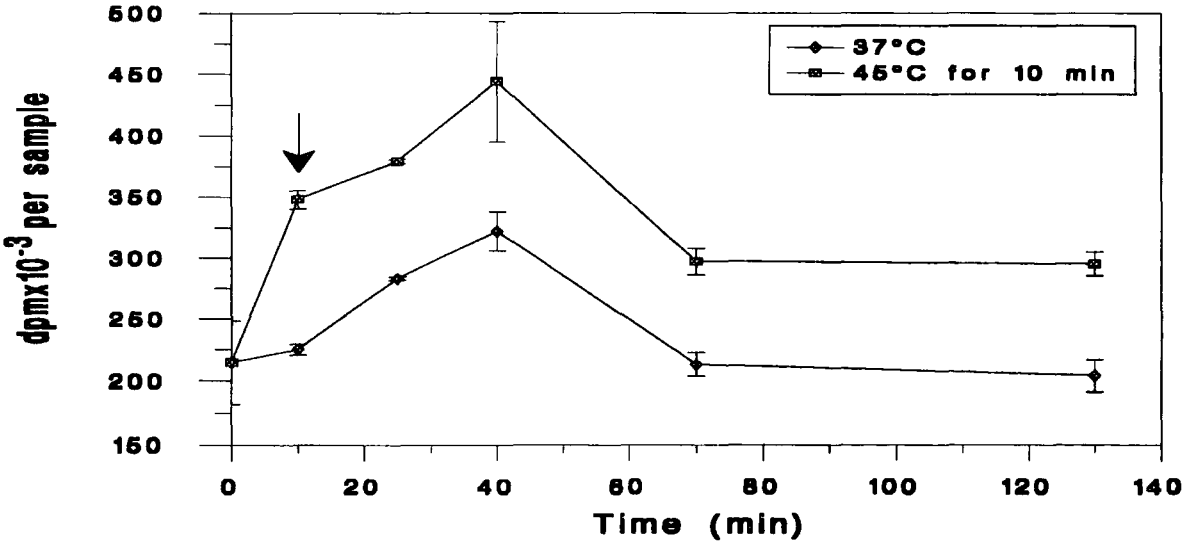


Figure 3.22c. Level of TAG measured at 37°C after 10 min heating at 45°C



3.4 Discussion

Studies on the mechanism(s) underlying heat-induced cell death have been carried out extensively at the single cell level in cell culture. Despite the uncertainty of the primary target of heat-induced cell damage, the plasma membrane has long been suggested to be a likely candidate. Agents such as local anaesthetics and aliphatic alcohols, that are known to act specifically at the membrane, all act synergistically with heat. More supportive evidence for the plasma membrane being affected by heat come from morphological alterations upon heat treatment (bleb formation) (Borrelli *et al.*, 1986; Kapiszewska and Hopwood, 1988). Experimental alterations of membrane composition *in vivo* or *in vitro*, has also been shown to alter the thermosensitivity. For example, P388 ascites cells derived from animals fed a diet high in polyunsaturated fatty acids were found to be more heat sensitive than cells grown in animals fed a diet high in saturated fatty acids (Mulcahy *et al.*, 1981). Similarly, L1210 murine leukaemia cells and LM mouse fibroblasts became more thermosensitive if grown in medium supplemented with highly polyunsaturated fatty acid and more thermoresistant if grown in medium supplemented with highly saturated fatty acid (Guffy *et al.*, 1982). However, using different probes to measure membrane fluidity, it was found that the change in fluidity was observed at 2 different temperature which are 8°C and a border transition between 23°C and 36°C, centred at about 30°C (Lepock, 1982). In another study, using mouse lymphoma cells, the diffusion coefficient of the lipid probe, DII, indicated that no perturbation of lipid fluidity occurred at 41-45°C (Mehdi *et al.*, 1984). There was no evidence for lipid phase transition at temperatures associated with the onset of cell killing e.g at 41.5°C or higher, though the possibility that heterogeneous alteration may occur in microregions in the plasma membrane cannot be excluded. Thus some workers have suggested that it is unlikely that effects on the lipid components of the plasma membrane are responsible for heat-induced cell killing. This is in contrast to the proposal that dramatic changes in membrane fluidity may be a primary cause of heat-induced cell

death (Yatvin, 1977). On the other hand, an irreversible protein transition was observed in membrane at 40-41°C when alteration of membrane proteins were investigated in CHL V79 cells by measuring intrinsic protein fluorescence and energy transfer from membrane proteins to trans-paranaric acid. Similar results were obtained using ESR spin label probe 2N14 (Lepock *et al.*, 1983).

Alterations in membrane proteins are associated with alteration in plasma membrane function and may be related to hyperthermic cell death. In fact, it was found that membrane permeability to K^+, Na^+ and Ca^{2+} alter during or after hyperthermia (Anghileri *et al.*, 1985a; Ruifrok *et al.*, 1985; Calderwood *et al.*, 1988). Furthermore, it was also found that the components of signalling systems were affected by heat. For example, exposure of Rat-1 fibroblasts to 45°C for 30 min resulted in inhibition of binding of EGF to its receptor as a result of a decrease in affinity of receptor (Magun and Fennie, 1981). On exposure of CHO HA-1 cells to 43-45°C for various lengths of time, it was found that binding of insulin to its receptor decreased as temperature and/or heating time increased. This decrease in binding was due to a reduction in receptor number (Calderwood and Hahn, 1983). Heat also caused an accumulation of inositol phosphates and an increase in $[Ca^{2+}]_i$ in several cell lines, ranging from rat, mouse and human, possibly as a result of activation of the components (G proteins and/or PI-PLC) of the phosphoinositide signalling pathway (Stevenson *et al.*, 1986; Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993; Kiang and McClain, 1993). Given the importance of this signalling pathway in the regulation of a variety of cellular responses, it has been postulated that disruption of the phosphoinositide signalling system may play a role in regulation of the stress response of cells. Furthermore, Caldwerwood and co-workers (1987) found that heating CHO HA-1 cells at 45°C for longer than 15 min resulted in the reduction of the cellular content of PtdIns(4)P and PtdIns(4,5)P_2. Since it has been reported that PtdIns(4,5)P_2 is responsible for anchoring microtubules to the cell surface (Lassing and Lindberg, 1985), it was suggested that depletion in

polyphosphoinositide levels in prolonged heating may be related to cell damage (Calderwood *et al.*, 1987).

In the present study, the effect of heat on the phosphoinositide signalling pathway was investigated in two tumour cell lines — CHO-K1 cells and WRK-1 cells. Before the effect of heat on the phosphoinositide signalling pathway was investigated in these cell lines, it was important to determine the effect of temperature on cell survival for the two cell types, since the thermal sensitivity of particular cell types can vary enormously. This information would also aid in the selection of a suitable temperature at which to perform future studies. A common method for determining hyperthermic cell survival is the clonogenic assay which relies on the ability of viable cells to divide and form colonies. This method has been established since 1955 (Puck *et al.*, 1955) and has been used in determining hyperthermic cell survival of many cell types, including CHO HA-1 cells (Calderwood *et al.*, 1987), HTC cells (Kingston *et al.*, 1989), murine L1210 leukaemia cells (Guffy *et al.*, 1982) and NIH3T3 fibroblasts (Stege *et al.*, 1993a). This method was used in this study to determine the hyperthermic cell survival of CHO-K1 cells. The tendency of WRK-1 cells to aggregate eliminated the use of this method in determination of cell survival of this cell line, and a colorimetric assay (MTT assay), based on the ability of a metabolically viable cell to reduce yellow 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product, was used for determining survival of WRK-1 cells. Since MTT is taken up by cells and reduced to blue formazan by succinate dehydrogenase, this assay monitors metabolic viability, rather than reproductive potential, of any surviving cells.

The MTT assay was developed by Mosmann (1983) and modified by Denizot and Lang (1986) to improve the performance. The final method adopted was the same as described by Kingston *et al.* (1989). It has been pointed out that optimal MTT assay conditions vary with cell type and the optimal condition for a particular cell type has to

be evaluated (Carmichael *et al.*, 1987; Plumb *et al.*, 1989). In the present study, it was found that MTT concentrations ranging from 0.5-3 mg/ml and 3-5 h incubation gave a near maximal absorbance value, hence 1 mg/ml MTT concentration and 4 h incubation was chosen as standard assay condition for WRK-1 cells in the subsequent assay.

When hyperthermic cell death of WRK-1 cells was determined, the cells were seeded for 3 days before heating was performed and cells were grown for another 3 days to allow any cell death (and, thus, loss of succinate dehydrogenase activity) to occur. Initially, it was necessary to define the relationship between absorbance reading and cell number, and to ensure that the cell number on the day of the MTT assay lay on the linear portion of this curve. If the cells in control conditions were allowed to reach stationary phase, whereas the heat-treated cells continued to grow in log phase because of their depleted number, the cell survival (which is calculated by dividing absorbance obtained from heated cells by absorbance from control) would be overestimated. It was found that the relationship between cell number and absorbance value remained linear for cell densities up to 3×10^5 cells/well in a 24-well plate by the time of assay. This cell density range is similar to those found in ESH-5L cells (Green *et al.*, 1984), L929 cells (Green *et al.*, 1984) and HTC cells (Ladha, 1990). Since cells would be grown for 6 days before the MTT assay was performed, 7×10^3 cells per well in a 24-well plate was chosen as the seeding density, as this would produce approximately 2×10^5 cells after 6 days growth. Once optimal conditions for MTT assay had been established, hyperthermic cell death of WRK-1 cells was determined. On the other hand, the clonogenic assay was used for determination of hyperthermic death of CHO-K1 cells.

The thermal survival curves obtained from both cell types show a characteristic shoulder region followed by a linear portion. The reason(s) for the shoulder region is/are unclear but it has been suggested that it determines the ability of the cell to sustain sublethal damage (Hahn, 1982). From the survival curve, survival parameters D_q and D_0

can be calculated. D_q measures the width of the shoulder region and was obtained from back-extrapolation of the linear portion of the curve to 100% survival. In other words, the higher the D_q value, the higher the ability of the cell to sustain sublethal cell damage. The exponential part of the curve can be described by the D_0 value, which determines the time required to reduce the cell survival to 1/e times its initial value. Hence the higher the D_0 value, the higher the thermal resistance of a particular cell line. On the other hand, the time taken to kill 90% of cells (LD_{90}) reflects both shoulder region and linear part of the thermal survival curve (Bhuyan, 1979).

The D_q, D_0 and LD_{90} values obtained from CHO-K1 cells heated at 45°C were 5.52 min, 14.5 min and 20.1 min, respectively. The D_0 and LD_{90} values reported previously for CHO cells at 45°C were 2.3 min and 13.3 min, respectively (Westra and Dewey, 1971). The D_q values obtained in WRK-1 cells at 43.5°C, 44°C and 45°C were 68.3 min, 53.8 min and 21.4 min, respectively. D_0 values for WRK-1 cells obtained at 43.5°C, 44°C and 45°C were 42.4 min, 38.4 min and 14.5 min, respectively. It was observed that the D_0 value decreases as the temperature increases. Previous work has shown that an increase of one degree Celsius in temperature is associated with a 50% decrease in the D_0 value (Hahn, 1982). LD_{90} values for WRK-1 cells were 110.6 min, 89.2 min and 35.9 min at 43.5°C, 44°C and 45°C, respectively. However, it should be noted that the clonogenic and colorimetric assays produced different kinetics of cell death in response to heat in HTC cells. The clonogenic assay was characterised by a higher D_q value and lower D_0 value compared to the colorimetric assay, when the HTC cells were heated at 45°C (Kingston *et al.*, 1989).

Once the hyperthermic cell death had been characterised in CHO-K1 cells and WRK-1 cells, the effect of heat on the phosphoinositide signalling pathway was investigated. Hydrolysis of inositol lipids during agonist-stimulation will give rise to 2 second messengers which are *sn*-1,2-diacylglycerol (1,2-DAG) and inositol 1,4,5-

trisphosphate ($Ins(1,4,5)P_3$) (Berridge, 1984; Downes, 1989; Majerus, 1992; Exton, 1994). 1,2-DAG remains in the lipid matrix of the membrane and activates protein kinase C whereas $Ins(1,4,5)P_3$ releases to the cytosol and mobilises Ca^{2+} from internal stores such as from ER and calciosomes. In order to clarify the effect of heat on the phosphoinositide signalling pathway, it is necessary to measure the changes in 1,2-DAG and $Ins(1,4,5)P_3$ and its metabolites and also the change in cytosolic free calcium ($[Ca^{2+}]_i$) resulting from the mobilisation of calcium from internal stores and/or Ca^{2+} influx. This chapter reports the effect of heat on 1,2-DAG and other neutral lipid classes in CHO-K1 and WRK-1 cells. The effect of heat on inositol phosphates and heat-induced changes in $[Ca^{2+}]_i$ are reported in Chapter 4 and Chapter 5, respectively.

Investigation of the effect of heat on CHO-K1 cells prelabelled for 24 h with [^{3}H]arachidonic acid suggested that heating at 45°C for 10 min resulted in a transient decrease in the ^{3}H-1,2-DAG level at 5 min and 8 min heating time, but this returned to the unheated control level by 10 min. On longer heating time (for up to 45 min), a decrease in ^{3}H-1,2-DAG level was observed except at 30 min at 45°C. Levels of ^{3}H-TAG in heated CHO-K1 cells were similar to control levels for up to 10 min heating at 45°C. However, on prolonged heating (up to 45 min) at this temperature, an increase in ^{3}H-TAG level was observed at 30 and 40 min heating. Exposure of WRK-1 cells, prelabelled with [2-^{3}H]glycerol for 4 days, to 45°C for 12.5 min resulted in a consistent decrease in ^{3}H-1,2-DAG levels throughout the heating period (apart from 6.5 min). On prolonged heating at this temperature, a decrease in ^{3}H-1,2-DAG was observed in heated WRK-1 cells, but the level of ^{3}H-1,2-DAG returned to control levels by 45 min at 45°C. An increase in ^{3}H-TAG level was observed during the 10 min heating period at 45°C. On proloned heating an increase in ^{3}H-TAG level was observed for up to 30 min heating, which then returned to control levels by 45 min heating time.

Long term labelling methods, employed in the initial experiments, increase the

chance of isotopic equilibrium labelling of the lipid pool, so that changes in radioactivity of ^{3}H-1,2-DAG represent the changes in 1,2-DAG mass. However, this method may eliminate the detection of the turnover of small, metabolically active pools that have been reported to be present in WRK-1 cells (Monaco, 1982; Koréh and Monaco, 1986). To investigate if heat exerted an effect on such lipid pools, a short term labelling protocol was used, in which cells were labelled with [^{3}H]arachidonic acid (in case of CHO-K1 cells) or with [2-^{3}H]glycerol (in case of WRK-1 cells) for only 2 h. Upon 45°C heat treatment of CHO-K1 cells, a decrease in ^{3}H-1,2-DAG level was observed for heating times equal to or longer than 20 min. On the other hand, an increase in ^{3}H-TAG level was observed in heated cells at 4 min, 10 min and 30 min heating time whereas the level of ^{3}H-TAG was same as the control level at 45 min. Exposure of short-term labelled WRK-1 cells to 45°C for 42.5 min resulted in a decrease in ^{3}H-1,2-DAG levels (apart from 32.5 min). This decrease in ^{3}H-1,2-DAG was accompanied by an increase in ^{3}H-TAG level. The ^{3}H-TAG became higher than the control level by 22.5 min at 45°C and remained higher for up to 42.5 min heating. None of the conditions employed showed a difference in ^{3}H-MAG level between unheated and heated cells of either cell type. Thus, the short-term labelling method, which labels metabolically active pools (Farese and Cooper, 1990), resulted in a decrease in ^{3}H-1,2-DAG levels accompanied by an increase in ^{3}H-TAG levels in heated cells, a result similar to that observed in long-term labelled cells. This suggests that the effect of heat on any small, metabolically active pools that may exist is essentially similar to the effect of heat on the bulk of 1,2-DAG in these cells.

The possibility that addition of fresh serum-containing medium prior to heat treatment could result in the hydrolysis of inositol lipid, thus disturbing the heat-induced effect on the phosphoinositide signalling system was excluded by heating long-term labelled CHO-K1 cells or WRK-1 cells in serum-free medium. This resulted in a

decrease in ^{3}H-1,2-DAG and increase in ^{3}H-TAG levels in heated cells, similar to those observed upon heat treatment in serum-containing medium.

In summary, when long term or short term labelled CHO-K1 cells and WRK-1 cells were heated at 45°C for up to 10-12.5 min, a heat dose that resulted in about 50% cell death and 20% cell death respectively, a decrease in ^{3}H-1,2-DAG and an increase in ^{3}H-TAG levels were observed. Chronic exposure to 45°C for up to 45 min, a heat dose that killed greater than 95% of both cell types, also resulted in a decrease in ^{3}H-1,2-DAG level and an increase in ^{3}H-TAG levels in both cell types, thus arguing against the possibility of 1,2-DAG accumulation as a result of activation of the phosphoinositide signalling pathway by heat.

However, it is possible that the decrease in 1,2-DAG levels, which was observed consistently in these experiments, may be related to the experimental protocol. In the long-term labelling experiments, care was taken to ensure that the 1,2-DAG pool was labelled to a steady state by growth of WRK-1 cells in [2-^{3}H]glycerol for 4 days and growth of CHO-K1 cells in [^{3}H]arachidonic acid for 24 h. In both cases, however, the radioactive medium was replaced by nonradioactive medium before the cells were heated, giving a 'cold-chase' period of 2 h in WRK-1 cells and 15 min in CHO-K1 cells. It is therefore conceivable that a rapidly turning-over pool (e.g. hormone-sensitive pool) of radioactive lipid could be diluted by production of nonradioactive lipid before and during the heating period in these experiments, so that the increased level of radioactive 1,2-DAG reported by other workers could have been missed. Furthermore, if lipid turnover increased during heating, the radioactive 1,2-DAG may be diluted (by nonradioactive lipid) to a greater extent in heated compared to control cells, giving an apparent decrease in levels of ^{3}H-1,2-DAG in heated cells.

The results obtained in this study are in contrast to those reported previously by other workers (Stevenson *et al.*, 1986; Calderwood *et al.*, 1987). The effect of heat on the level of diacylglycerol has been investigated in CHO HA-1 cells (Calderwood *et al.*, 1987), a different subclone of CHO cells compared to the one used in this study. Following a 2 h label with [^{3}H]arachidonic acid and subsequent washes to remove the unincorporated [^{3}H]arachidonic acid, cells were subjected to hyperthermic treatment at 45°C for up to 15 min, a heat dose that only slightly decreased the cell survival of CHO HA-1 cells when measured by the clonogenic assay (Calderwood *et al.*, 1987). A transient increase in diacylglycerol was observed which reached a peak at 2-3 min at 45°C and returned rapidly to unheated control values by 4 min. However, it was not clear whether the isomers of DAG were separated or not. Given that only 1,2-DAG is the activator of PKC, it is important to determine the effect of heat on this particular isomer. Furthermore a very short (2 h) labelling time was employed by these authors, so it is likely that lipids were not labelled to isotopic equilibrium and that the observed transient increase in 1,2-DAG levels may not reflect changes in the mass of 1,2-DAG.

In the present study, the possibility that heat induced an increase in 1,2-DAG which was followed by rapid phosphorylation of 1,2-DAG to PA (in order to replenish the phosphoinositide lipid pools), thus decreasing 1,2-DAG levels was excluded as well. When WRK-1 cells were heated in the presence of the diacylglycerol kinase inhibitor, dioctanoylethylene glycol (diC$_8$EG), a decrease in ^{3}H-1,2-DAG level was observed at 12.5 min heating and this persisted for up to 45 min at 45°C. This decrease in ^{3}H-1,2-DAG was accompanied by an increase in ^{3}H-TAG level at 12.5 min or longer heat treatment. On the other hand, no difference in ^{3}H-MAG level was found between heated and unheated cells in the presence or absence of 100 μM diC$_8$EG. This concentration has been shown to be sufficient to block the conversion of 1,2-DAG to PA in blood platelets (Bishop *et al.*, 1986). In the experiment performed by Bishop and co-workers (1986), it was found that preincubation of platelets with 100 μM diC$_8$EG for 30 min at 37°C

inhibited the conversion of DAG to PA by 70-100% upon thrombin addition. However the possibility that a higher diC_8EG concentration is necessary to inhibit DAG kinase in WRK-1 cells cannot be excluded. Thus it would be interesting to determine the effect of different concentrations of DAG kinase inhibitor in WRK-1 cells upon heat cell death, as well as 1,2-DAG and PA levels in these cells.

On the other hand, the results obtained in this study were consistent with the study reported by Bagi and Hidvégi (1990) concerning with the activity of PKC following heating. Immediately after heating P388 lymphoid tumour cells to 41-45°C for 1 h, PKC activities in particulate and cytosol fractions from both heated and control cells were determined by their ability to phosphorylate H1 histone. It was found that the protein kinase C activities in both fractions decreased progressively as cells were heated at 42-45°C. Furthermore, it was found that the subcellular distribution of PKC altered after heating at 45°C for 1 h. Following heating, PKC activity was evenly distributed between cytosol and particulate fraction whereas the PKC activity in cytosol and particulate fractions was 58% and 42%, respectively, in extracts from unheated cells, though the statistical significance of these differences was not tested. Although a reduction in PKC activity was observed after 1 h heat treatment at various temperatures, PKC activity was not measured at shorter periods of hyperthermia. The 1,2-DAG exerts its effect by promoting the insertion of inactive membrane-associated PKC into the membrane, so dislodging the pseudosubstrate site from the active site and leading to activation of PKC. Thus, it would be expected that reduction in 1,2-DAG levels could result in a decrease in PKC activity (Ganong *et al.*, 1986; Burns and Bell, 1992), and the heat-induced decrease in 1,2-DAG levels reported in the present study is consistent with the decrease in PKC activity caused by heat in P388 lymphoid tumour cells (Bagi and Hidvégi, 1990). In addition, Bagi and Hidvégi (1990) reported that a translocation of PKC to plasma membrane occurred in heated cells but a reduction in PKC activity was detected, which could possibly be due to a decrease in 1,2-DAG level, as observed in the present study. It

is clear that further studies are required in order to clarify this possibility. However, although heat caused a reduction in PKC activity, Bagi and Hidvégi (1990) also found that PKC selectively phosphorylated 3 cytosol proteins having molecular weights of 14, 25 and 33 kD in heated P388 lymphoid tumour cells.

A role of PKC in the response of cells to hyperthermia has been suggested (Mikkelsen *et al.*, 1991b). When human colon cancer HT-29 cells, human carcinoma MCF-7 cells and Chinese hamster V79 lung fibroblasts were heated in the presence of the PKC inhibitors, tamoxifen or H7, hyperthermic cell death of these cells was potentiated. On the other hand, when cells were heated in the presence of a low affinity PKC inhibitor, HA1004, cells death was similar to cells heated in the absence of this inhibitor. Thus it would be interesting to investigate the relationship between heat cell death and PKC activity in response to heat in the presence and absence of protein kinase activators (e.g. phorbol esters and bryostatin), as well as the phosphorylation of other proteins such as HSP that might be involved in the response of a cell to heat treatment.

The effect of heat on MAG, 1,2-DAG and TAG levels was also investigated after cells were returned to 37°C (the post-heating period) in both cell types. Long-term labelled CHO-K1 cells and WRK-1 cells were subjected to heat (45°C for 10 min) followed by a 2 h recovery period. It was found that the ^{3}H-1,2-DAG level in heat-treated CHO-K1 cells was similar to unheated cells after 2 h incubation in 37°C, thus suggesting that the heat-induced decrease in 1,2-DAG is transient and recovers on subsequent incubation at 37°C. In WRK-1 cells, it was found that ^{3}H-1,2-DAG level in heated cells returned to control level after 30 min incubation in 37°C, again suggesting that the heat-induced decrease in 1,2-DAG is transient and recoverable. In both cell types, increases in ^{3}H-TAG levels in heated cells were observed even after 2 h incubation at 37°C.

The level of diacylglycerol in heated cells during subsequent incubation at 37°C has been investigated in CHO HA-1 cells (Stevenson *et al.*, 1986). Following a 2 h labelling with [^{3}H]arachidonic acid, cells were subjected to 45°C treatment for 10 min and then incubated at 37°C for up to 2 h. The ^{3}H-DAG level increased to 160% of control level after the 2 h post-heating period, and this was accompanied by an increase in ^{3}H-PA level, which has been suggested as a Ca^{2+} ionophore. The increase in ^{3}H-PA level correlated with $^{45}Ca^{2+}$ influx (Stevenson *et al.*, 1986; Calderwood *et al.*, 1988).

In summary, the result obtained in this study is in contrast to results reported in CHO HA-1 cells during acute heating (Calderwood *et al.*, 1987) and in the 2 h recovery period following 10 min heat treatment (Stevenson *et al.*, 1986). On the other hand, the increase in ^{3}H-TAG levels following heating in the present study may reflect increased TAG synthesis, possibly coupled with decreased phospholipid synthesis, from the 1,2-DAG precursor, causing 1,2-DAG levels to fall. Given that inositol lipid is not the only source for 1,2-DAG, it would be interesting to investigate if other phospholipids, especially PtdCho is/are affected by heat. Furthermore, a decrease in 1,2-DAG in heated cells might only be confined to 1,2-DAG with certain fatty acyl chain composition (derived from particular phospholipids). Since 1,2-DAG is an activator of certain isoforms of PKC and the response of these isoenzymes to 1,2-DAG as well as the spatial distribution of these enzymes are different, it might be possible that these isoenzymes respond to hyperthermia differently, together with alterations in phosphorylation of certain cellular proteins, and these changes may contribute to hyperthermic cell death.

Chapter 4

Effect of hyperthermia on inositol phosphate levels

4.1 Introduction

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) generates two second messengers — 1,2-DAG and Ins(1,4,5)P_3 (Berridge, 1984). 1,2-DAG remains in the lipid matrix and activates PKC (Chapter 3) whereas Ins(1,4,5)P_3 is released into the cytosol and mobilises calcium from ER or calciosome stores (Berridge, 1993). The Ins(1,4,5)P_3 formed by agonist-stimulated breakdown of inositol phospholipids is recycled to free inositol by a series of inositol phosphomonoesterases. The current picture of inositol phosphate metabolism is extremely complex. However, it seems to fulfil three purposes: (1) rapid turnover of Ins(1,4,5)P_3 thereby controlling Ins(1,4,5)P_3 level and other cellular responses that are dependent upon calcium; (2) recycling of inositol through the metabolism of inositol phosphates; (3) synthesis of specific inositol phosphates such as InsP_5 and InsP_6 which may have distinct functional roles (Downes, 1989; Shears, 1992).

So far up to 37 distinct inositol phosphate species have been identified. However, the routes of inositol phosphate metabolism represents an assemblage of information from a variety of tissues and there is no single cell type in which all of the reactions have been demonstrated to occur (Downes and MacPhee, 1990).

In order to understand the implications of the role of inositol phosphates as intracellular messengers, the metabolism of these inositol phosphates must be considered (Figure 4.1).

Once Ins(1,4,5)P_3 is formed, it can either be dephosphorylated to Ins(1,4)P_2 via the action of Ins(1,4,5)P_3 5-phosphomonoesterase or phosphorylated to Ins(1,3,4,5)P_4 via the action of Ins(1,4,5)P_3 3-kinase (Irvine *et al.*, 1986). Ins(1,3,4,5)P_4 is then dephosphorylated to Ins(1,3,4)P_3 by the same 5-phosphomonoesterase that dephosphorylates Ins(1,4,5)P_3 to Ins(1,4)P_2 (Irvine *et al.*, 1986). Ins(1,3,4)P_3 is either dephosphorylated to Ins(3,4)P_2 by a 1-phosphatase or to Ins(1,3)P_2 via the action of a 4-phosphatase. Hence it has been demonstrated that both Ins(1,3)P_2 and Ins(3,4)P_2 levels are elevated upon agonist stimulation (Barker *et al.*, 1992).

Ins(1,4)P_2, Ins(1,3)P_2 and Ins(3,4)P_2 are dephosphorylated to Ins(4)P_1, Ins(1)P_1 and Ins(3)P_1, respectively (Shears, 1992). All of the metabolites are then converted to free inositol by the action of a single monophosphatase. The free inositol can be recycled for PtdIns synthesis.

Due to the rapid turnover of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 to replenish the inositol lipid pool, it is virtually impossible to prevent Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 being completely dephosphorylated to inositol. In some experiments, Li^+ is included in order to block the dephosphorylation of Ins(1,3,4)P_3 and Ins(1,4)P_2 to free inositol through the inhibition of Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase and the inositol monophosphatase. However, the presence of Li^+ may distort the pathway of inositol fluxes due to the so called "inositol-depletion hypothesis" (Berridge *et al.*, 1989). Li^+ is an uncompetitive inhibitor and binds preferentially to the enzyme-substrate complex, thus preventing the recycling of inositol in stimulated cells (Nahorski *et al.*, 1991). Hence the presence of Li^+ will lower the inositol level, and as a result the synthesis of inositol lipids will slow down. Furthermore, Li^+ can activate the Ins(1,4,5)P_3 5-phosphatase, thus perturbing the fluxes to other metabolites.

Inositol polyphosphates such as InsP_4, InsP_5 and InsP_6 have been found in animal

cells but the origins of some of these highly phosphorylated inositols are unclear. As far as $InsP_4$ is concerned, at least 3 isomers have been identified in mammalian cells. They are Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 (Balla *et al.*, 1987; Stephens *et al.*, 1988; Barker *et al.*, 1988; Wong *et al.*, 1992). Formation of Ins(1,3,4,6)P_4 has been demonstrated in *Xenopus* oocytes (Ivorra *et al.*, 1991) and SH-SY5Y cells (Gawler *et al.*, 1991) to occur via the rephosphorylation of Ins(1,3,4)P_3. This isomer has been found to be the precursor of $InsP_5$ (Hunyady *et al.*, 1988). Synthesis of Ins(1,3,4,6)P_4 in avian erythrocytes has been shown to occur via the phosphorylation of Ins(1,4,6)P_3, though the significance of this route in other cell types is unclear (Stephens and Downes, 1990). On the other hand, it has been shown that Ins(1,3,4,5)P_4 is not further phosphorylated to $InsP_5$ and $InsP_6$ (Irvine *et al.*, 1986). Although the elevation of Ins(3,4,5,6)P_4 was associated with a receptor-mediated rise in Ins(1,4,5)P_3, evidence suggested that Ins(3,4,5,6)P_4 accumulation is an event that occurs in parallel but independent of PLC activation (Barker *et al.*, 1992; Shears, 1992). [^{3}H]inositol is incorporated rapidly into Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 whereas several days are required to label Ins(1,3,4,5,6)P_5 and Ins(3,4,5,6)P_4 to equilibrium (Menniti *et al.*, 1990). Using short-term, long-term and pulse-chase labelling, it was revealed that Ins(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 always had similar specific activities (Menniti *et al.*, 1990). It was also found that receptor-activated increase in Ins(3,4,5,6)P_4 was directly proportional to the level of Ins(1,3,4,5,6)P_5. When WRK-1 cells were labelled to isotopic equilibrium with [^{14}C]inositol followed by brief labelling with [^{3}H]inositol, it showed that [^{3}H] appeared quickly in Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 but not in Ins(3,4,5,6)P_4, suggesting the synthesis of Ins(3,4,5,6)P_4 involves a different pathway whereas a common precursor was used for the synthesis of both Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 (Kirk *et al.*, 1990a). By using a non-equilibrium [^{32}P] labelling protocol, 2 routes for Ins(1,3,4,5,6)P_5 synthesis in avian erythrocytes have been suggested. It can either be synthesized by Ins(3,4,6)P_3 $\rightarrow$ Ins(3,4,5,6)P_4 $\rightarrow$ Ins(1,3,4,5,6)P_5 (Stephens and Downes, 1990) or alternatively Ins(1,3,4,6)P_4 $\rightarrow$ Ins(1,3,4,5,6)P_5 $\leftrightarrow$ Ins(3,4,5,6)P_4 (Hunyady *et al.*, 1988;

Downes and MacPhee, 1990). In animal cells, the major $InsP_5$ isomer is $Ins(1,3,4,5,6)P_5$ although other isomers including $Ins(1,2,4,5,6)P_5$ and $Ins(1,2,3,4,5)P_5$ have been found in HL60 cells (Pittet *et al.*, 1989) and NG115 cells (Stephens *et al.*, 1991). This $InsP_5$ is further metabolised to 3 compounds: $InsP_6$, $Ins(3,4,5,6)P_4$ or $Ins(1,4,5,6)P_4$ (Downes and MacPhee, 1990, Menniti *et al.*, 1993b).

The amount of $InsP_5$ and $InsP_6$ that are present in cells are much higher than the other inositol phosphates (Shears, 1992). Given the ubiquitous occurrence of these compounds in eukaryotic cells and the complex pathways concerning their synthesis and degradation, it is likely that these compounds are functionally significant but their cellular roles remain obscure at present. $Ins(1,3,4,5,6)P_5$ is involved in regulating the affinity of avian haemoglobin for O_2 (Isaacks and Harkness, 1980) and $InsP_6$ has been proposed to function as a phosphorus and/or inositol reserve in plant seeds (Downes and MacPhee, 1990). Both $InsP_5$ and $InsP_6$ have been proposed to be extracellular agonists that stimulate neuronal excitability and reduce blood pressure (Vallejo *et al.*, 1987).

When separating inositol phosphates using HPLC, 2 peaks were eluted after $InsP_6$ (more polar than $InsP_6$) and were identified as inositol pyrophosphates ($InsP_5P$ and $InsP_6P$). The presence of inositol pyrophosphate was first observed in slime-mould (Stephens and Irvine, 1990) and they were also found in mammalian cells such as AR4-2J (Stephens *et al.*, 1993). These 2 compounds are formed by ATP-dependent phosphorylation of inositol phosphates and are rapidly dephosphorylated back to their precursors (Menniti *et al.*, 1993a). The turnover of these compounds is very rapid, so it is very difficult for them to be detected. Recently, it was found that F^- is an inhibitor of pyrophosphatase, so it can be used to facilitate the analysis of pyrophosphates (Mennitti *et al.*, 1993a). When cells were incubated in 1-10 mM F^-, the levels of $InsP_5P$ and $InsP_6P$ increased dramatically, at the expense of $Ins(1,3,4,5,6)P_5$ and $InsP_6$. In AR4-2J cells, it was demonstrated that approximately 50% of the $Ins(1,3,4,5,6)P_5$ pool and 20%

of the $InsP_6$ pool is cycled through pyrophosphates every hour (Oliver *et al.*, 1992). Given the relatively large cellular pool of $InsP_5$ and $InsP_6$, the energy required for the synthesis of pyrophosphates and the fast turnover rate of these compounds suggests that they may have an important role in cell physiology. In fact, Stephens *et al.* (1993) suggested that these compounds may be a new form of high-energy phosphate.

In stimulated WRK-1 cells, 3 cyclic inositol phosphates have been identified. They are Ins(1:2cyc,4,5)P_3 together with its breakdown products Ins(1:2cyc)P_1 and Ins(1:2cyc,4)P_2 which is itself a minor product of PLC attack upon PtdIns(4,5)P_2 (Wong *et al.*, 1988). All of the cyclic inositol phosphates are acid-labile, thus the acid-quench technique usually employed to study inositol phosphates metabolism will convert the cyclic compounds to their corresponding non-cyclic 1-phosphate and 2-phosphate counterparts, leading to the appearance of Ins(2)P, Ins(2,4)P_2 and Ins(2,4,5)P_3. Thus the neutral-quench extraction is employed if the turnover of cyclic inositol phosphates are studied. These compounds are metabolised very slowly and accumulate following a prolonged stimulation. Ins(1:2cyc,4,5)P_3 is not a substrate of Ins(1,4,5)P_3 3-kinase and the only metabolic route is via 5-phosphatase action (Connolly *et al.*, 1987). Thus the metabolism of this compound is much simpler than the Ins(1,4,5)P_3. Furthermore, this compound does not seem to play a messenger role because it is at least 10 times less potent in promoting calcium mobilisation (Lee and Hokin, 1989). The Ins(1:2cyc,4,5)P_3 is dephosphorylated to Ins(1:2cyc,4)P_2 which is dephosphorylated sequentially to Ins(1)P_1 and free inositol as follows (Dawson and Clarke, 1972; Connolly *et al.*, 1986):

Ins(1:2cyc,4,5)P_3 → Ins(1:2cyc,4)P_2 → Ins(1:2cyc)P_1 → Ins(1)P_1 → Ins

It is worth mentioning that the phosphohydrolase that converts Ins(1:2cyc)P_1 to Ins(1)P_1 can also use glycerophosphoinositol (GroPIns) as substrate, so as to form glycerol and Ins(1)P_1 (Ross and Majerus, 1991). The affinity of this enzyme towards GroPIns increases in transformed cells, resulting in the accumulation of Ins(1:2cyc)P_1.

Overexpression of this enzyme in NIH3T3 cells (by transfection) results in a lower Ins(1:2cyc)P_1 level and lower cell density (Ross and Majerus, 1991). Hence it has been proposed that the relative amount of Ins(1:2cyc)P_1 and GroPIns are important in the control of cell growth (Ross and Majerus, 1991).

The complex metabolism of inositol phosphates in intact mammalian cells has prompted the speculation that some of these metabolites may function as intracellular signals. Ins(1,4,5)P_3 is responsible for the hormone-stimulated release of calcium from intracellular stores. The role of Ins(1,3,4,5)P_4 has been shown to act synergistically with Ins(1,4,5)P_3 to control calcium influx (Irvine and Moore, 1987; Irvine, 1992; Irvine and Cullen, 1993) and Ins(1,4)P_2 can activate the low-affinity form of DNA polymerase α (Sylvia *et al.*, 1988). There is lack of evidence that the other metabolites may have functional roles other than being intermediates in the recovery of inositol.

The action of Ins(1,4,5)P_3 on calcium mobilisation was tested by adding Ins(1,4,5)P_3 to permeabilised cells or by injecting Ins(1,4,5)P_3 into cells (Berridge and Irvine, 1984; Berridge and Irvine, 1989). Both conditions led to calcium release from non-mitochondrial stores presumed to be a part of ER. The increase in $[Ca^{2+}]_i$ was transient if cells were stimulated with agonist in calcium-free medium. In the presence of calcium, a prolonged $[Ca^{2+}]_i$ elevation was observed which varied in magnitude depending on the receptor being stimulated (Putney, 1986). A possible role for Ins(1,3,4,5)P_4 in regulating calcium-influx was first suggested following experiments in which the putative signal molecule was injected into sea urchin oocytes (Irvine and Moore, 1987). It has been shown that egg activation appeared to depend upon extracellular calcium. Egg activation occurred in response to co-injection of Ins(1,3,4,5)P_4 and Ins(2,4,5)P_3 (this latter compound is able to mimic the calcium-releasing activity of Ins(1,4,5)P_3 without being an effective substrate for the 3-kinase). Injection of either substance alone was ineffective. Thus it was suggested that

$Ins(1,3,4,5)P_4$ acts in conjunction with $Ins(1,4,5)P_3$ to sustain the agonist-stimulated calcium signal, presumably by opening Ca^{2+} channels in the plasma membrane. However this proposal was challenged by the results obtained by microinjection of $Ins(2,4,5)P_3$ to liver (Burgess *et al.*, 1991) or to acinar cells (Bird *et al.*, 1991) in which it was shown that $Ins(2,4,5)P_3$ on its own was sufficient to cause calcium entry. When the role of $InsP_4$ in calcium entry was investigated in acinar lacrimal cells by three different groups, one group reported that $Ins(2,4,5)P_3$ or $Ins(1,4,5)P_3$ readily caused calcium entry on its own (Bird *et al.*, 1991) whereas this never occurred in experiments performed by Morris *et al.* (1987) and Changya *et al.* (1989). This contradictory result was resolved later by Smith (1992) who found that a sufficiently high dose of $InsP_3$ was able to cause calcium entry on its own, although $InsP_4$ still gave a profound additional effect on this process. It has been pointed out by Irvine (1992) that it is possible that a high level of $InsP_3$ (resulting from using supraphysiological agonist doses) alone is sufficient to cause calcium entry in intact cells, but *in vivo* with physiological agonist doses, $InsP_4$ is essential, otherwise evolution would have removed it and its receptor years ago.

The effect of hyperthermia on inositol phosphates has been investigated in several mammalian cell lines. When HeLa cells were heated at 41°C or at higher temperatures, an elevation in $InsP_1$, $InsP_2$ and $InsP_3$ levels were observed and the levels of these inositol phosphates increased progressively with temperature (Calderwood and Stevenson, 1993). $InsP_1$ and $InsP_2$ liberation reached a maximal level at 42°C and declined slightly when heated at temperatures higher than 42°C. $InsP_3$ accumulation was observed at 43°C and 45°C, and the level of $InsP_3$ began to level off after 10 min heating at 43°C whereas for those cells heated at 45°C, the $InsP_3$ level continued to increase throughout 30 min heating. When CHO HA-1 cells were heated at 42°C, a gradual increase in $InsP_3$ was observed with levels increasing to 5- to 6-fold of the zero control value by 120 min (Calderwood and Stevenson, 1993). When these cells were heated at

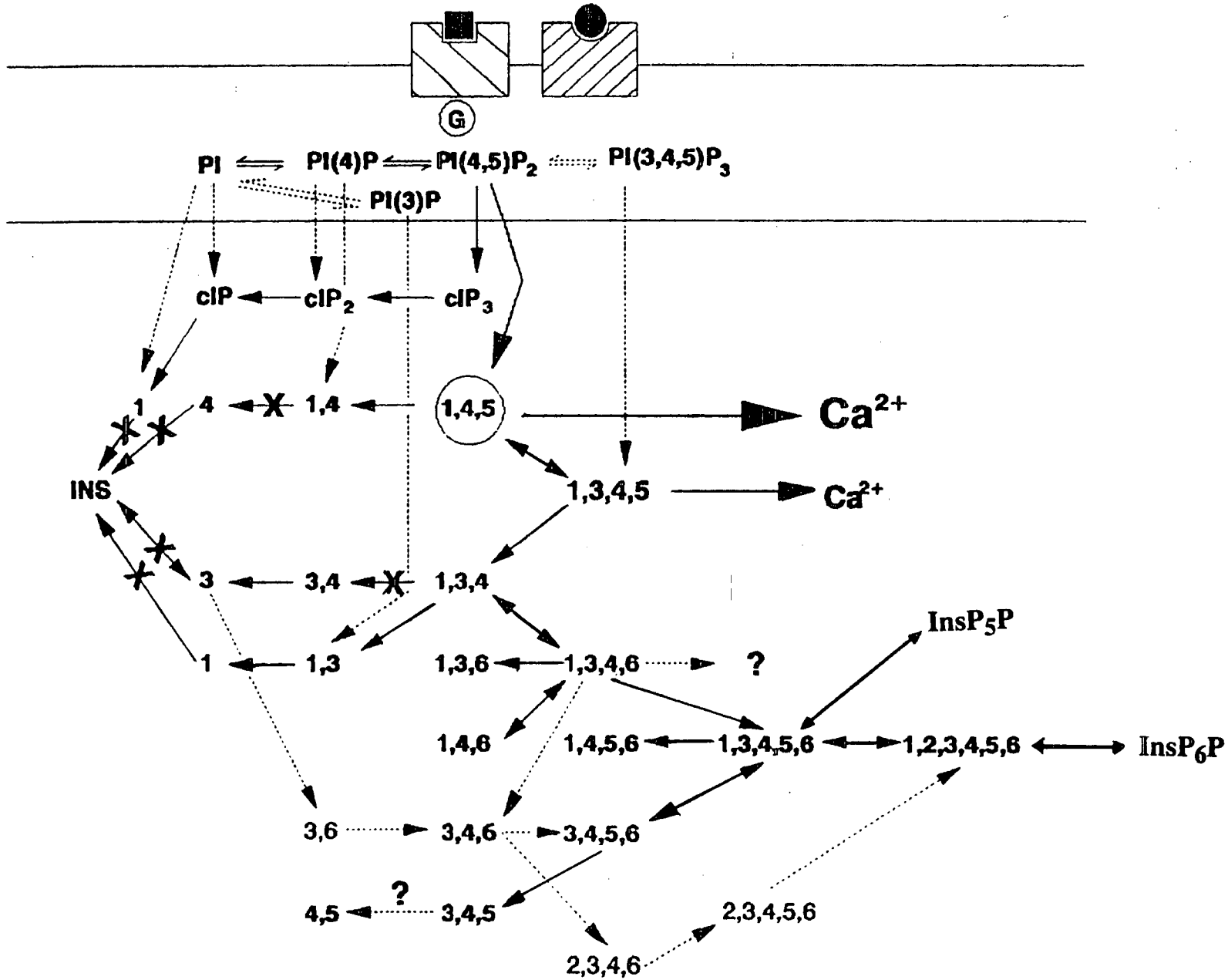
45°C, the $InsP_3$ level reached maximum values after 15 min heating and resulted in a 6-fold increase compared with the control value. A heat-induced increase in $InsP_3$ was also detected in NIH3T3 cells. When these cells were heated at 45°C, $InsP_3$ concentration increased 9-fold, reaching maximal levels after 5 min heating and then declined to a level 2-3-fold higher than the control by 10-15 min (Calderwood *et al.*, 1987). The $InsP_4$ level in heated and unheated cells was similar in all these cell types. The effect of heat on the phosphoinositide signalling pathway was also investigated in human epidermoid A-431 cells (Kiang and McClain, 1993). A significant increase in $InsP_1$ and $InsP_3$ were observed after heating at 42°C for 20 min whereas increase in $InsP_2$ level was not observed unless cells were heated at 45°C for 20 min. Exposure of these cells to higher temperature (45°C and 48°C) resulted in a further increase in both $InsP_1$, $InsP_2$ and $InsP_3$ levels. When cells were heated at 45°C, increases in $InsP_1$, $InsP_2$ and $InsP_3$ levels reached maximum after 20 min and the levels then returned to basal level by 30 min. In most of these studies, the inositol phosphate classes were separated by simple ion exchange chromatography, so it was not possible to measure changes in individual isomers of $InsP_3$ or other inositol phosphates (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993). However, using HPLC to separate inositol phosphate isomers extracted from control and A-431 cells heated at 45°C for 20 min, Kiang and McClain (1993) observed a slight increase in Ins(1,3,4)P_3, a 2.4-fold increase in Ins(1,4,5)P_3 and a 3.6-fold reduction in Ins(1,3,4,5)P_4 levels. Although most studies have suggested that heat causes an elevation of $InsP_3$, Liu and Carpenter (1992) showed that heat did not have any effect on inositol phosphate ($InsP_1$, $InsP_2$ and $InsP_3$) formation in A-431 cells when these cells were heated at 46°C for 30 min. However, these authors only investigated the changes in $InsP_1$, $InsP_2$ and $InsP_3$ levels at a single time point. Given that the change in inositol phosphate levels upon heating is both temperature- and heating time-dependent, this may explain the discrepancy between the results reported by Liu and Carpenter (1992) and those reported by Calderwood and Stevenson (1993) and Kiang and McClain (1993).

The magnitude of PLC stimulation by heat and growth factors have been investigated in CHO HA-1 cells and NIH3T3 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993). The rise in $InsP_3$ during heat treatment at 45°C for 3 min or during stimulation with serum alone for 3 min at 37°C was similar in magnitude. Combination of both agents showed an additive effect on $InsP_3$ formation in both CHO HA-1 (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993) and Balb C 3T3 cells (Calderwood *et al.*, 1987). $InsP_3$ formation was additive when CHO HA-1 cells were treated with heat shock and thrombin (Calderwood and Stevenson, 1993). The results imply that heat shock may be converted to chemical messengers by a route similar to those employed by hormones or growth factors.

The exact mechanism of heat-induced increase in $InsP_3$ level is uncertain. When [^{3}H]inositol labelled CHO HA-1 cells were permeabilised by digitonin and were heated in the presence or absence of non-hydrolysable GTP analogues (GTPγS), it was found that the level of $InsP_3$ and other inositol phosphates increased upon 45°C treatment provided that greater than 10^{-8} M GTPγS was present, suggesting the involvement of a G protein in this heat-induced $InsP_3$ formation (Calderwood *et al.*, 1993). To further investigate the type of G protein that is involved in this response, CHO HA-1 cells or NIH3T3 cells were incubated with pertussis toxin (PTX) for 3 h before being heated at 45°C for 20 min or challenged by thrombin at 37°C for the same length of time. Inositol phosphates were then extracted from these cells and were separated by anion exchange chromatography. It was found that PTX significantly inhibited the release of inositol phosphates ($InsP_1$, $InsP_2$ and $InsP_3$) from thrombin-stimulated cells whilst the level of inositol phosphates released from heated cells were similar to those obtained from cells without pre-treatment by PTX. Thus the authors suggested that a PTX-insensitive G-protein such as G_q is involved in the response of cells to heat treatment. The mechanism of heat-induced $InsP_3$ formation has been postulated as follows: heat may provide the activation energy required to stimulate GTP-GDP exchange by a G-protein. Upon

binding to GTP, the G protein subunits dissociate and the α-subunit then activates a PLC, most likely PLCβ1, which causes the hydrolysis of PtdIns(4,5)P_2. Since Ins(1,4,5)P_3 is responsible for calcium mobilisation, the accumulation of Ins(1,4,5)P_3 during heating may in turn lead to an abrupt increase in $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ may be associated with some toxic effects and may contribute to cell death. The role of $[Ca^{2+}]_i$ in hyperthermic cell death will be discussed in more detail in Chapter 5.

In this study, the effect of heat on inositol phosphate levels and the response of WRK-1 cells to agonist during and following heating was investigated.


G
PI
PI(4)P
PI(4,5)P2
PI(3,4,5)P3
PI(3)P
cIP
cIP2
cIP3
1
4
1,4
1,4,5
Ca2+
INS
1,3,4,5
Ca2+
3
3,4
1,3,4
InsP5P
1
1,3
1,3,6
1,3,4,6
?
1,4,6
1,4,5,6
1,3,4,5,6
1,2,3,4,5,6
InsP6P
3,6
3,4,6
3,4,5,6
4,5
?
3,4,5
2,3,4,5,6
2,3,4,6

4.2 Materials and Methods

4.2.1 Effect of inositol concentration on cell growth

WRK-1 cells were harvested from a 75 cm^2 flask and sedimented by centrifugation at 80 g (r_{av} = 11 cm) at 20°C for 3 min. The pellet was then resuspended in low inositol medium (containing 1 μM inositol) together with 2% (v/v) dialysed rat serum and 5% (v/v) dialysed FBS. Cells were then disaggregated and counted by haemocytometer and diluted to give 1 x 10^6 cells/ml. Then 0.1 ml of the diluted suspension was seeded into a number of 6-well plates with each well containing 1.5 ml of either 1 μM inositol (with dialysed sera) or 11 μM inositol medium (with dialysed sera). The medium was changed the following day and every two days thereafter. Every 24 h, cells grown under both conditions were harvested and counted via haemocytometer to determine the number of live and dead cells.

4.2.2 Separation of inositol phosphate standards

(i) Separation of inositol phosphates standards by BioRad anion exchange column chromatography

The column was constructed by inserting a bolus of plastic wool into a plastic pipette. Column was packed with 2 cm (0.6 cm i.d.) of AG1-X8, 200-400 mesh, formate form resin (BioRad Lab, Herts) which was then linked to a 10 ml reservoir (a plastic syringe barrel). The resin was then washed with 20 ml of water. The mixture of ^3H-inositol phosphate standards, which contained 0.01 μCi each of [^3H]Ins(1)P_1, [^3H]Ins(1,4)P_2, [^3H]Ins(1,4,5)P_3, [^3H]Ins(1,3,4,5)P_4 and [^3H]InsP_6, was loaded onto the column. The column was washed with 20 ml of H_2O to elute any free inositol. Then different classes of inositol phosphates were eluted sequentially as described by

Maccallum *et al.* (1989):

20 ml	60 mM ammonium formate/5 mM sodium tetraborate (GroPIns)
20 ml	150 mM ammonium formate/5 mM sodium tetraborate ($InsP_1$)
20 ml	0.4 M ammonium formate/0.1 M formic acid ($InsP_2$)
20 ml	0.8 M ammonium formate/0.1 M formic acid ($InsP_3$)
20 ml	1.05 M ammonium formate/0.1 M formic acid ($InsP_4$)
20 ml	2 M ammonium formate/0.1 M formic acid ($InsP_5$ and $InsP_6$)

0.5 ml fractions were collected into Pico hang-in vials and 3 ml of Ultima-flo AF scintillation fluid (Packard, Berks) was added to each vial. The vials were then wiped with methanol to prevent static problems and remove salts that formed on the surface of the vials during fraction collection. Radioactivity was then determined by Packard Scintillation Counter (model no. 300). [^{3}H]Ins(1)P_1, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 were purchased from Tocris Neuramin, whereas [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]$InsP_6$ were purchased from NEN.

(ii) Separation of inositol phosphate standards by HPLC

Separation of inositol phosphate standards was performed on a 25 cm x 4.6 mm Partisphere 5-SAX column (Whatman, Kent) fitted with a pellicular anion exchange guard column (Whatman, Kent). The column was flushed with water at least for 45 min at a flow rate of 1 ml/min before use. Then a mixture of labelled inositol phosphate standards, containing 0.05 µCi each of [^{3}H]Ins(1)P_1, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]$InsP_6$ and 0.025 µCi of [^{3}H]Ins(1,3,4)P_3, was made up to 2 ml with filter-sterilised H_2O, applied to the HPLC column, and separated using the gradient profile described by French *et al.* (1991) with slight modification. The gradient was built up using a BioRad HPLC system (gradient module no. 800), fitted with 2

pumps, with pump A delivering H_2O and pump B delivering 0.1 M or 1 M diammonium hydrogen phosphate ($(NH_4)_2HPO_4$ (pH adjusted to 3.8 with phosphoric acid). At 65 min, 0.1 M $(NH_4)_2HPO_4$ was changed to 1 M $(NH_4)_2HPO_4$. Buffers and H_2O were filtered through a 0.45 μm filter (Millipore, Herts) and degassed under vacuum before applying to the system.

The gradient used was as follow:

Time (min)	Pump A (%)	Pump B (%)
0	100	0
2	100	0
35	70	30
35.1	0	100
65	0	100
65.1	55	45
95	55	45
95.1	30	70
110	30	70
110.1	20	80
120	20	80
120.1	0	100
145	0	100
145.1	100	0

The flow rate was 1 ml/min and 0.5 min fractions were collected into Pico hang-in vials by Gilson fraction collector (Model no. 202). To each vial, 3 ml of Uniscint BD scintillation fluid (National Diagnostics) was added and mixed well. The vials were then

wiped with methanol and radioactivity was then determined by scintillation counting. [^{3}H]Ins(1,3,4)P_3 was purchased from NEN.

(iii) Separation of $InsP_3$ isomers by HPLC

Separation of [^{3}H]inositol trisphosphate standards was performed on a 12.5 cm x 4.6 mm Partisphere WAX column (Whatman, Kent) equipped with a pellicular anion exchange guard column. The column was equilibrated with H_2O at least for 45 min at a flow rate of 1 ml/min before a mixture of the standards which contained 0.1 µCi of [^{3}H]Ins(1,3,4)P_3 and 0.05 µCi of [^{3}H]Ins(1,4,5)P_3 was applied. The gradient profile described by Wong *et al.* (1992) was used. The gradient was built up by a 2-pump system with pump A delivering H_2O and pump B delivering 0.5 M $(NH_4)_2HPO_4$, (pH adjusted to 3.2 with phosphoric acid). Buffer and H_2O were filtered through a 0.45 µm filter and degassed by vacuum before applying to the system.

The gradient used was as follow:

Time (min)	Pump A (%)	Pump B (%)
0	100	0
5	100	0
15	88	12
70	88	12
90	0	100
110	0	100

The flow rate was 1 ml/min and 0.5 min fractions were collected into Pico hang-in vials by a Gilson fraction collector. To each vial, 3 ml of Uniscint BD scintillation fluid

was added, mixed well and the radioactivity was determined by a Packard Scintillation Counter.

4.2.3 Equilibrium labelling of inositol lipids

(i) Labelling of WRK-1 cells

WRK-1 cells were harvested from two 25 cm^2 flasks and the cell number was determined via haemocytometer. The cell suspension was further diluted to give 1 x 10^5 cells/ml in growth medium. Then 1 ml of the diluted cell suspension was added to 2 ml of growth medium in a number of 6-well plates (day 0). After 24 h (day 1), the growth medium in 3 wells from each plate was replaced by 1.5 ml of radioactive medium containing 2 μCi/ml myo-[2-^{3}H]-inositol (16-20 Ci/mmol) in growth medium and this medium was replaced every 2 days thereafter. For the other 3 wells, the growth medium was replaced by 1.5 ml of non-radioactive growth medium after 24 h (day 1) and every 2 days thereafter. Every day a plate was used either for lipid extraction (for those wells that contained labelled cells) or for cell number determination (for unlabelled cells).

(ii) Lipid extraction

Lipids were extracted from the radioactively-labelled cells by the method described by Creba *et al.* (1983). The medium in the well was discarded and the cells were killed by adding 0.5 ml of ice-cold 5% (w/v) TCA. Plates were left on ice for 10 min. Cells were then scraped off from the well using a rubber policeman. The TCA cell suspension was then transferred to a pyrex tube. The well was washed with 0.5 ml of ice-cold 1% (w/v) TCA and these two TCA suspensions were combined and centrifuged at 2000 g (r_{av}= 22.3 cm) at 4°C for 10 min. The supernatant was discarded and the precipitate was washed with 1 ml of ice-cold 1% (w/v) TCA containing 1 mM EDTA and then

centrifuged as before. The supernatant was discarded and the pellet was washed with 1 ml of acidified H_2O (pH 4.5-5 using HCl) and was centrifuged as before. The supernatant was discarded and lipids were extracted from the pellet by adding 1.5 ml of chloroform: methanol: conc HCl (100:100:1, by volume). The mixture was mixed with a vortex mixer for 30 sec and left at room temperature for 10 min. It was then centrifuged at 2000 g (r_{av}= 22.3 cm) at room temperature for 10 min. This extraction was repeated once and the supernatants from the two extractions were combined. The pellet was further extracted by 1 ml of chloroform: methanol: conc HCl (200:100:1, by volume), mixed well and centrifuged as before. The three lipid extracts were combined and to this extract was added 1.5 ml chloroform and 1.1 ml 0.1 M HCl. The mixture was mixed for 30 sec and centrifuged as before. The upper (aqueous) phase was discarded and the lower phase was then washed with 2.1 ml of 'synthetic upper phase' (see later), mixed for 30 sec and centrifuged as before. The lower phase was transferred to a clean tube and the remaining upper phase was washed once with 'synthetic lower phase' (see later) and centrifuged as before. The two bottom phases were then combined and dried down under a stream of O_2-free N_2.

(iii) Deacylation of extracted lipids

Deacylation of the dried lipids was done as described by Creba *et al.* (1983). To the dried lipid, 1 ml of chloroform was added to redissolve the lipid and duplicate samples (50 µl) were transferred to 20 ml glass scintillation vials using a syringe. The lipid in the scintillation vials was then dried down under O_2-free N_2 and 5 ml of Ecoscint A scintillation fluid (National Diagnostic) was added and the radioactivity was determined. To the remaining 0.9 ml lipid solution, 100 µl of $CHCl_3$ was added followed by the addition of 0.2 ml methanol and 0.4 ml of 0.5 M NaOH (in methanol/water, 19:1, v/v). The mixture was vortex mixed for 30 sec and was left at room temperature for 20 min. After 20 min, 1 ml of chloroform, 0.6 ml methanol and 0.6 ml distilled water were

added to it and mixed with a vortex mixer for 30 sec. The suspension was then centrifuged at 2000 g (r_{av}= 22.3 cm) for 10 min at room temperature. Of the upper phase, 1 ml was removed and was neutralised with 600 µl of 0.1 M boric acid. This sample was then diluted with distilled water to 5 ml, together with the addition of 0.1 M sodium tetraborate so as to give a final concentration of 5 mM sodium tetraborate. The deacylated lipids were then separated by BioRad anion exchanger.

(iv) Separation of deacylated lipids on anion exchanger

BioRad anion exchange resin (AG 1-X8, 200-400 mesh, formate form) was packed into a plastic pipette (2 cm x 0.6 cm i.d.) containing a small plug of plastic wool. The column was washed with 20 ml of H_2O. The deacylated lipids were then applied to the column. After complete drainage of the sample through the column, the deacylated lipids were eluted as described by Creba *et al.* (1983):

20 ml	0.18 M ammonium formate/5 mM sodium tetraborate (elute glycerophosphoinositol (GroPIns) from deacylation of PtdIns)
10 ml	0.35 M ammonium formate/0.1 M formic acid (elute glycerophosphoinositol 4-phosphate (GroPIns4P) from deacylation of PtdInsP)
10 ml	0.8 M ammonium formate/0.1 M formic acid (elute glycerophosphoinositol 4,5-bisphosphate (GroPIns 4,5-P_2) from deacylation of PtdIns(4,5)P_2)

The eluates were collected and duplicate 1.5 ml samples were transferred to 20 ml plastic scintillation vials. To the sample 1.5 ml of methanol/water (1:1, v/v) and 15 ml of Ecoscint A were added for radioactivity determination.

(v) Preparation of synthetic lower and upper phases

To a large separating funnel, chloroform, methanol and 0.1 M HCl were mixed in a 10:5:3 proportion (by volume). Mixtures were mixed well and left overnight to allow equilibration. The 2 phases were then separated and stored in brown bottles at room temperature.

4.2.4 Effect of heat on inositol phosphate levels

(i) Labelling of WRK-1 cells

Cells were harvested from two 25 cm^2 flasks, resuspended in a modified growth medium, comprising EMEM (containing 11 µM inositol), 5% (v/v) dialysed FBS and 2% (v/v) dialysed rat serum, 2 mM L-glutamine, penicillin (100 i.u/ml), streptomycin (100 µg/ml), tylosin (10 µg/ml) and non-essential amino acids (1%, v/v) and the medium was buffered to pH 7.4 with 24 mM sodium bicarbonate (designated as inositol-depleted medium) at a density of 1 x 10^5 cells/ml. Then 1 ml of the diluted suspension was added to each well of a number of 6-well plates containing 2 ml of inositol-depleted medium (day 0). After 24 h (day 1), medium was replaced by 1.5 ml of inositol-depleted medium supplemented with myo[2-^{3}H]inositol at 2 µCi/ml (when samples were going to be analysed by HPLC, 10 µCi/ml of myo[2-^{3}H]inositol was used instead). The cells were grown in radioactive medium for 4 days (until day 5) with one medium change on day 3. One extra plate was set up and cells were grown in non-radioactive inositol-depleted medium, which would be used for cell number determination on the day of the experiment.

(ii) Effect of temperature on inositol phosphate levels

WRK-1 cells were harvested, seeded and labelled as described in section 4.2.4 (i).

On day 5, radioactive medium was removed and cells were washed 3 times with inositol-depleted medium containing 20 mM Hepes, pH 7.4 and 1 ml of the same medium was added to each well. Plates were returned to the 37°C incubator for 1 h. After 1 h, the cells were washed once with EMEM + 10 mM LiCl + 20 mM Hepes, pH 7.4 (designated as medium A) and then 1 ml of medium A was added to each well. Plates were incubated at 37°C for 10 min. Medium A was then replaced with 1 ml of normal growth medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (designated as medium B) before sealing plate and plates were then submerged in a water bath at 37°C water bath for 5 min before rapid transfer and submersion in water baths at various temperatures. Reactions were terminated after 30 min (including 1.5 min lag) by addition of 1 ml of ice-cold 10% (v/v) perchloric acid (PCA). Inositol phosphates were extracted as described in section 4.2.5(i) and were separated by BioRad anion exchanger as described in section 4.2.2 (i).

(iii) Effect of heating duration on inositol phosphate levels

Experiments were performed either in presence or absence of serum. Cells were seeded and labelled as described in section 4.2.4 (i). On the day of the experiment (day 5) cells were washed as described in section 4.2.4 (ii). Following 1 h incubation at 37°C, medium was removed and cells were washed once with medium A and 1 ml of medium A was added to each well. Plates were then returned to 37°C incubator for 10 min. Medium was then replaced by 1 ml of medium A or medium B (serum-containing medium). Plates were sealed and equilibrated under the surface of a 37°C water bath for 5 min before rapid transfer and submersion in a 47°C bath for 40 sec. A control plate containing the same volume of medium and fitted with thermocouple was used in order to monitor the temperature and when the medium reached 44°C, the temperature of the water bath was rapidly adjusted to 45°C by replacing 1.2 l of water at 47°C by 1.2 l of water at 9°C. By doing so, the lag period required to achieve 45°C reduced to about 1.5 min. Some plates were maintained at 37°C as parallel controls. At various times,

reactions were terminated by addition of 1 ml of ice-cold 10% (v/v) PCA and inositol phosphates were extracted as described in section 4.2.5 (i). Different classes of inositol phosphate were separated by BioRad anion exchanger as described in section 4.2.2 (i).

(iv) Effect of heat shock inducers and serum on inositol phosphate levels

Cells were seeded and labelled as described in section 4.2.4 (i). On the day of the experiment (day 5) cells were washed as described in section 4.2.4 (ii). Following 1 h incubation at 37°C, cells were washed once with medium A and 1 ml of medium A was added to each well. Plates were then returned to 37°C incubator for 10 min, then the medium was replaced by 1 ml of one of the following media: (1) medium A (section 4.2.4(ii)), (2) 50 µM sodium orthovanadate (made up in medium A), (3) 5 mM NaF and 10 µM $AlCl_3$ (made up in medium A), (4) 5% (v/v) ethanol (made up in medium A) (5) 5% (v/v) non-dialysed FBS and 2% (v/v) non-dialysed rat serum in medium A (medium B).

All of the media were filter-sterilised through a 0.22 µm filter (Sartorius) before applying to the cells. The plates were then returned to 37°C incubator and reactions were terminated by addition of 1 ml of ice-cold 10% (v/v) PCA after 30 min. For the plate to be heated at 45°C, 1 ml of medium A or medium B was added to the well, and it was equilibrated by submersion in a 37°C bath for 5 min and then heated as described in section 4.2.4 (iii). Reactions were terminated after 30 min by addition of 1 ml of ice-cold 10% (v/v) PCA. Inositol phosphates were extracted as described in section 4.2.5(i) and were separated by BioRad anion exchanger as described in section 4.2.2 (i).

(v) Effect of calcium on inositol phosphate levels

Cells were seeded and labelled as described in section 4.2.4 (i). On the day of the

experiment (day 5) the cells were washed as described in section 4.2.4(ii). Plates were returned to 37°C incubator for 1 h. After 1 h, the medium was removed and cells were washed once with either medium A (section 4.2.4(ii)) or calcium-free buffer (138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 5.5 mM glucose, 10 mM LiCl and 20 mM Hepes, pH 7.4) and 1 ml of the appropriate medium was added to each well. Plates were then returned to 37°C for 10 min. The medium was then replaced by 1 ml of either medium A or calcium-free buffer. Plates were sealed and equilibrated under the surface of a water bath at 37°C for 5 min and then heated as described in section 4.2.4(iii). Reactions were terminated at various times by addition of 1 ml of ice-cold 10% (v/v) PCA. Inositol phosphates were extracted as described in section 4.2.5(i) and were then separated by BioRad anion exchanger as described in 4.2.2(i).

(vi) Response of cells to vasopressin at elevated temperature

Cells were seeded and labelled as described in 4.2.4(i). On the day of the experiment (day 5), cells were washed as described in section 4.2.4(ii) and returned to culture. After 1 h, cells were washed once with medium A and 1 ml of medium A was added to each well. Plates were returned to 37°C incubator for 10 min. The medium was then replaced by 0.5 ml of medium A and plates were sealed. Plates were submerged in a water bath at 37°C for 5 min before submersion in a water bath at 45°C. After 2 min incubation at 45°C water bath (including 1.5 min lag period), 1 ml of 600 nM vasopressin (made up in medium A) at 45°C was injected into the well whilst keeping the plate submerged at 45°C. Control cells were taken through an identical procedure, except that all treatments were performed at 37°C. Reactions were terminated at various times by addition of 0.5 ml ice-cold 20% (v/v) PCA. Inositol phosphates were extracted as described in section 4.2.5(i) and were separated by BioRad anion exchanger as described in section 4.2.2(i).

(vii) Response of cells to vasopressin post-heating

Cells were seeded and labelled as described in section 4.2.4(i). On the day of the experiment (day 5), cells were washed as described in 4.2.4(ii) and returned to culture. After 1 h, cells were washed once with medium A and 1 ml of medium A was added to each well. Plates were returned to the 37°C incubator for 10 min, then medium was replaced by 0.5 ml of medium A. Plates were sealed and thermoequilibrated by submerging in a water bath at 37°C for 5 min before rapid transfer and submersion in a water bath at 45°C. After heating for various times the plates were returned to the 37°C water bath. After 2 min at 37°C 1 ml of 600 nM vasopressin (made up in medium A) at 37°C was injected into the well while keeping the plates submerged at 37°C. Reactions were terminated after 30 sec by addition of 0.5 ml of 20 % (v/v) ice-cold PCA. Inositol phosphates were extracted as described in section 4.2.5(i) and were separated by BioRad anion exchanger as described in 4.2.2(i).

(vii) Dialysing sera

Sera were dialysed against 70 volumes of 154 mM NaCl and 5 mM Hepes, pH 7.4 using a dialysis tubing with molecular weight cut-off at 3500 (Medicell, London) at 4°C for 48 h with continuous stirring. Dialysed sera were filter-sterilised by passing through a 0.22 μm filter (Millipore, Herts) and were kept at -20°C until used.

4.2.5 Extraction and analysis of inositol phosphates

(i) Quenching and extraction of inositol phosphates

After addition of PCA to terminate the reaction, phytic acid hydrolysate (equivalent to 25 μg of phosphorus), phytic acid solution (equivalent to 1 mg) and 2 mg

of BSA (fraction 5) (BSA was added only when cells were heated in serum-free medium) were added to each well. Plates were left on ice for 10 min and the cells were scraped off from the well with a rubber policeman. The PCA extract was transferred to a polystyrene centrifuge tube (Sarstedt, Leicester). Wells were washed once with 1 ml of ice-cold 1% (v/v) PCA. The two PCA extracts were combined and then left on ice for 10 min. The protein was pelleted by centrifugation at 3000 g (r_{av}= 9 cm) at 4°C for 10 min. The supernatant was then transferred to another polystyrene tube and was neutralised with a solution containing 1.5 M KOH, 75 mM Hepes and 60 mM EDTA in the presence of 10 µl of universal indicator. The potassium perchlorate was left to precipitate at 4°C for 2 h, followed by centrifugation at 3000 g (r_{av}= 9 cm) at 4°C for 10 min. The supernatant was then transferred to a siliconised pyrex tube and was diluted to 20 ml with 5 mM sodium tetraborate-0.5 mM EDTA. The extracts were stored at -20°C before separation by BioRad anion exchange column.

(ii) Preparation of samples for HPLC analysis

Cells were seeded as described in section 4.2.3(i). After 24 h, medium was replaced by 1.5 ml of inositol-depleted medium (section 4.2.4(i)) supplemented with myo[2-^{3}H]inositol at 10 µCi/ml. The cells were grown in radioactive medium for 4 days (until day 5) with one medium change on day 3. On day 5, cells were washed 3 times with inositol-depleted medium containing 20 mM Hepes, pH 7.4. Then 1 ml of the same medium was added to each well and cells were returned to culture. After 1 h cells were washed once with medium A and 1 ml of medium A was added to each well. Plates were then returned to 37°C incubator for 10 min. Medium A was then replaced with 1 ml of medium B, plates were sealed and thermoequilibrated at 37°C for 10 min (by submerging in a 37°C water bath) before rapid transfer and submersion in a 45°C water bath. Reactions were terminated after 30 min (including 1.5 min lag period) by addition of 0.2 ml of ice-cold 30% (w/v) trichloroacetic acid (TCA). Plates were left on ice for 10

min, then the cells were scraped off by a rubber policeman. The suspension was transferred to a polystyrene tube. The well was washed with 0.5 ml of ice-cold 1% (w/v) TCA and the remaining cells were scraped off. The 2 TCA extracts were combined and left on ice for 10 min. The protein was precipitated by centrifugation at 3000 g (r_{av}= 9 cm) at 4°C for 10 min. The supernatant was extracted 5 times with 2 volumes of H_2O-saturated diethyl ether. The pH of the extract was checked with pH paper to ensure that it was neutralised. To the extract 0.1 ml of 180 mM EDTA was added, the samples were kept at -20°C until used. The whole extract was used for HPLC analysis as described in section 4.2.2(ii).

(iii) Preparation of inositol trisphosphate isomers for HPLC analysis

Cells were seeded and labelled as described in section 4.2.5(ii). On day 5, cells were treated exactly as described in section 4.2.4(iii). Inositol phosphates were extracted as described in section 4.2.5(i). An aliquot (2 ml out of the 20 ml extract) of inositol phosphate extract obtained as described in section 4.2.5(i) was separated by BioRad anion exchange column chromatography as described in section 4.2.2(i). Samples that showed a marked change in $InsP_3$ level were then used for HPLC analysis. Firstly, the remaining 18 ml extracts were separated by BioRad anion exchanger and the $InsP_3$ fraction was collected and passed through a 10 ml wet Dowex column (50W-X8, 20-50 mesh, H^+ form). After the sample drained through the column, 2 x 20 ml of H_2O was added to the column to wash off the remaining $InsP_3$. The eluant was collected in a siliconised pear-shaped flask and was then freeze-dried. To redissolve the freeze-dried sample, flasks were washed sequentially with 3 x 0.7 ml H_2O, the solutions were combined and $InsP_3$ isomers were separated by HPLC using the Partisphere WAX column as described in section 4.2.2(iii).

(iv) Preparation of phytic acid hydrolysate (Wreggrett *et al.*, 1987)

Sodium phytate (1 g) was dissolved in 10 ml of 0.1 M sodium acetate/acetic acid buffer (pH 4) in a stoppered flask and incubated in a boiling water bath for 8 h. It was then cooled and passed down an Amberlite 1R-120 column (BDH) in the H^+ form to convert the mixed inositol phosphates to free acids. The effluent was then freeze-dried and redissolved in 1 ml of H_2O and its phosphorus content was determined (Rouser *et al.*, 1970). Then, the inositol phosphate composition of the hydrolysate was determined on a portion containing 25 µg phosphorus. Phytic acid hydrolysate was separated by anion exchange chromatography using buffers as described in section 4.2.2(i). The eluants were then freeze-dried and the phosphorus content was determined as described in Rouser *et al.* (1970). It was found that phytic acid hydrolysate contained 3.94% $InsP_1$, 4.7% $InsP_2$, 9.4% $InsP_3$, 25.5% $InsP_4$ and 56.7% $InsP_{5/6}$ (assuming no cross over of $InsP_5$ to $InsP_4$ fraction).

4.3 Results

Before investigating effects of heat on inositol phosphate levels in WRK-1 cells, it was important to carry out preliminary experiments in order to optimise the labelling and separation of the inositiol phosphates. Ideally, it would be an advantage to grow cells in the presence of a low concentration of inositol, in order to maximise specific radioactivity of inositol phosphates when cells were labelled with myo[2-^{3}H]inositol. Thus, in a preliminary experiment, WRK-1 cells were grown in the presence of 1 μM or 11 μM inositol, in growth medium supplemented with 5% (v/v) dialysed FBS and 2% (v/v) dialysed rat serum, such that the growth characteristics of WRK-1 cells in low inositol medium could be investigated (Figure 4.2). There was a 24 h lag period before the cells started to multiply. Cells grown in low-inositol medium (containing 1 μM inositol) started to die after 3 days, despite changing the medium 24 h after seeding and every 2 days thereafter. For those grown in growth medium containing 11 μM inositol, the cells reached confluence after 3 to 4 days with a density of about 8 x 10^5 cells/well. Since cells could not survive in low inositol medium for longer than 2-3 days, this medium was not suitable for longer term (4-5 days) labelling which was necessary to achieve isotopic equilibrium labelling of inositol lipids. Thus, subsequent experiments used growth medium containing 11 μM inositol together with 7% (v/v) dialysed sera for cell labelling in order to ensure that sufficient time was available for the inositol lipid pool to reach equilibrium labelling in cells that were in a healthy state at the time of the analysis.

In order to investigate the effect of heat and agonist on inositol phosphate levels, it was necessary to develop a protocol that can separate different classes of inositol phosphates. Ion-exchange chromatography which separates inositol phosphates according to their degree of phosphorylation, provided a quick and easy method. Several separation protocols are available (Dean and Beaven, 1989) and the method described by

Maccallum *et al.* (1989), enabling the separation of inositol phosphates up to $InsP_6$, was adopted. It is known that batch variation in the ion exchange resin exists, so it was important to evaluate the elution profiles with inositol phosphate standards for each new batch of resin. A typical separation of standards is shown in Figure 4.3 and it was found that this elution profile resulted in > 90% recovery of the appropriate standard in the appropriate eluant.

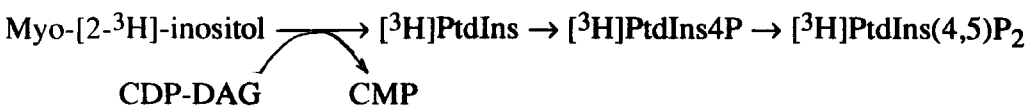
Simple anion-exchange chromatography only separates individual classes of inositol phosphate, but it provides a quick way to analyse a large number of samples. However, in order to resolve the isomers within a class, HPLC is required. The method described by French *et al.* (1991) using a Partisil SAX column was able to separate $InsP_2$ isomers and $InsP_5$ isomers as well as partially separating $InsP_3$ isomers. However, it was found that $InsP_3$ and $InsP_4$ standards coeluted using the gradient profile described by French *et al.* (1991). When the gradient profile for eluting $InsP_3$ was changed from 30% to 45% of 1 M $(NH_4)_2HPO_4$ between 65.1 to 95 min, the coelution problem was solved and this modified gradient profile was used in this study. A typical separation of inositol phosphate standards with a modification of the gradient profile described by French *et al.* (1991) is shown in Figure 4.4. Using this gradient profile, the recovery of any particular inositol phosphate standard was about 95%.

Upon agonist stimulation, Ins(1,4,5)P_3 is generated from hydrolysis of PtdIns(4,5)P_2. Due to the rapid turnover of Ins(1,4,5)P_3, the increase in Ins(1,4,5)P_3 is transient and accompanied by an increase in Ins(1,4,5)P_3 metabolites such as Ins(1,4)P_2 and Ins(1,3,4,5)P_4. Three $InsP_3$ isomers have been identified in WRK-1 cells (Wong *et al.*, 1992) and in many other cell types (Shears, 1992). Since only the Ins(1,4,5)P_3 plays a messenger role, a method enabling the separation of these isomers was sought. The method described by Wong *et al.* (1992) using a Partisphere WAX column was found to separate 3 $InsP_3$ isomers extracted from WRK-1 cells upon vasopressin stimulation. The

lack of Ins(3,4,5)P_3 standard did not allow the determination of the retention time of this isomer in this study. Nevertheless, Ins(1,3,4)P_3 and Ins(1,4,5)P_3 standards were successfully separated by the gradient profile described by Wong *et al.* (1992) (Figure 4.5).

Following the establishment of the methods for inositol phosphate separation, it was necessary to determine the time required for inositol lipids to achieve equilibrium labelling for WRK-1 cells growing in the presence of myo[2-^{3}H]inositol. This is important to ensure that the changes in radioactivity in [^{3}H]inositol phosphates, formed from inositol lipid hydrolysis upon agonist stimulation or heat treatment, are a true reflection of the mass of the inositol phosphates.

There are at least three types of inositol lipids present in the membrane, including phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Once the [^{3}H]inositol enters the cells, it can be incorporated into these three inositol lipids via the following route:



In order to determine the kinetics of labelling of these inositol lipid pools, WRK-1 cells were exposed to myo[2-^{3}H]inositol for up to 6 days and the incorporation of ^{3}H into total inositol lipids and individual classes of inositol lipids was determined everyday. Various methods have been established for extracting polyphosphoinositides from tissues and cells, but only acidified solvents achieve a substantial extraction of PtdIns(4,5)P_2 (Creba *et al.*, 1983). Thus the method described by these authors was used for phospholipid extraction from the WRK-1 cells, previously labelled with myo[2-^{3}H]inositol. The phosphoinositides could have been separated directly by affinity

chromatography on neomycin beads (Kirk *et al.*, 1990b), thin-layer chromatography (Simpson *et al.*, 1992) or HPLC. However, an alternative separation method which involved the conversion of these lipids to the water soluble glycerophosphoinositol derivatives, followed by the separation of these derivatives on anion exchange columns (Creba *et al.*, 1983; Simpson *et al.*, 1992) was adopted in this study. Following deacylation:

PtdIns gives rise to glycerophosphoinositol (GroPIns)
PtdIns(4)P gives rise to glycerophosphoinositol 4-phosphate (GroPIns4P)
PtdIns(4,5)P_2 gives rise to glycerophosphoinositol 4,5-bisphosphate (GroPIns 4,5-P_2)

The time-course of incorporation of myo[2-^{3}H]inositol into total inositol lipids of WRK-1 cells is shown in Figure 4.6a. A biphasic incorporation was observed, with an initial plateau at 3-5 days, followed by a large increase when cells were labelled for 6 days. This large increase in radioactivity could be due to an expansion in cell volume (C.J. Barker, personal communication) as the cells reached confluence (deduced from the growth curve on the same Figure). Thus it can be concluded that at least 3 days labelling is required for the cells to reach equilibrium labelling as far as total inositol lipid was concerned.

As mentioned earlier, at least 3 different inositol lipids are present, and the rate of incorporation of [^{3}H]inositol may vary in different classes. For example it would be expected that PtdIns precursor would be more rapidly labelled than the polyphosphoinositide products derived from it. Thus it was necessary to analyse time-dependent changes in labelling of the individual inositol lipid classes (Figure 4.6b-4.6d). A biphasic incorporation was observed in all 3 different classes. For [^{3}H]inositol incorporation into PtdIns and PtdIns(4)P, an initial plateau was observed at 3-5 days followed by a larger increase when cells were labelled for 6 days. A biphasic

incorporation into PtdIns(4,5)P_2 was observed and gave a maximal incorporation after 3 days and slight decrease in radioactivity after 4 and 5 days labelling. The level of radioactivity started to increase again after 6 days. Thus at least 3 days labelling is required for all 3 classes of inositol lipid to reach the initial phase of equilibrium labelling. It must be pointed out here that the equilibrium labelling experiment was done in growth medium containing 11 μM inositol (EMEM medium) but with non-dialysed sera. Thus the results obtained from this experiment was only a guideline for the time required for cells to achieve equilibrium labelling with myo[2-^{3}H]inositol. Since the inositol content was depleted in dialysed serum, inositol-depleted medium, containing dialysed serum, was employed during labelling in order to maximise the incorporation of myo[2-^{3}H]inositol into inositol lipids.

Since cells in each well would be processed separately during future experiments, it was important to ensure that there was sufficient radioactive material to enable the analysis of inositol phosphates in each sample. Therefore 4 days labelling was chosen as an optimum labelling period for future work. At this time, cells were approaching confluence and had achieved the initial phase of equilibrium labelling.

Several methods are available for the extraction of inositol phosphates from cells. However one point worth mentioning here is that loss of $InsP_4$ has been observed during extraction owing to the binding of this compound to the plastic tubes frequently used in this work (Wreggett *et al.*, 1987). The loss of this inositol phosphate may distort the picture of inositol phosphate fluxes and lead to misinterpretation of the effect of heat-induced changes in inositol phosphate formation. It has been reported that this loss of $InsP_4$ can be overcome by increasing the mass of this metabolite, by addition of phytic acid hydrolysate, in thymocytes during extraction (Wreggett *et al.*, 1987). Thus in this study, phytic acid hydrolysate (equivalent to 25 μg of phosphorus) and phytic acid solution (equivalent to 1 mg of phytic acid) were included in the samples to prevent the

loss of $InsP_4$ during extraction.

Once the methods for labelling, extraction and separation of inositol phosphates had been established, the effect of heat on the phosphoinositide signalling pathway of WRK-1 cells was investigated.

Due to the rapid turnover of $Ins(1,4,5)P_3$ to replenish the inositol pool, it is virtually impossible to prevent the $Ins(1,4,5)P_3$ being completely dephosphorylated to inositol. Thus, in all the heating experiments 10 mM LiCl was included in the heating medium, in order to avoid total dephosphorylation of $Ins(1,3,4)P_3$ and $Ins(1,4)P_2$ to free inositol through the inhibition of $Ins(1,3,4)P_3$/$Ins(1,4)P_2$ 1-phosphatase and inositol monophosphatase (Berridge *et al.*, 1989). Heating WRK-1 cells at various temperatures for 30 min resulted in a statistically significant increase ($p< 0.05$) in $InsP_1$ level in cells heated at 39°C but no difference was observed for temperatures above 39°C (Figure 4.7a). Conversely, an increase in $InsP_2$ level became significance at or above 42°C ($p< 0.05$) compared with 37°C control (Figure 4.7b). On the other hand, a significant increase ($p< 0.05$) in $InsP_3$ was only observed in cells heated at 45°C for 30 min (Figure 4.7c). Separation of $InsP_4$ and $InsP_{5/6}$ on anion exchange columns with buffers described in section 4.2.2(i) resulted in a relatively high radioactivity count in the $InsP_4$ fraction (data not shown). Since it has been reported that $InsP_5$ accounts for a relatively large proportion of the inositol phosphates in many cell types, including WRK-1 cells (Barker *et al.*, 1992), this led to the suspicion that $InsP_5$ may have contaminated the $InsP_4$ fraction. However, the lack of an $InsP_5$ standard made it difficult to identify the position of $InsP_5$ elution. Due to this reason, $InsP_4$, $InsP_5$ and $InsP_6$ fractions were pooled together for analysis. A fluctuation in the $InsP_{4/5/6}$ fraction was observed as temperature changed, but no significant difference in $InsP_{4/5/6}$ was detected for cells heated at various temperatures when compared with cells incubated at 37°C (Figure 4.7d).

In order to determine the effect of heat on inositol phosphate isomers in WRK-1 cells, the ^{3}H-labelled inositol phosphates were extracted from cells heated at 45°C for 30 min then the isomers were separated by HPLC. WRK-1 cells were labelled to high specific activity by growing the cells in 10 µCi/ml of myo[2-^{3}H]inositol in inositol-depleted medium for 4 days, followed by heating at 45°C for 30 min in normal growth medium containing 10 mM LiCl, 20 mM Hepes, pH 7.4. A typical HPLC separation of inositol phosphates and some of the isomers from these cells is shown in Figure 4.8. The radioactivity present in individual peaks was added together (Table 4.1). Statistical analysis of individual inositol phosphate fractions by Student's t-test suggested that only Ins(1,3)P_2 and Ins(?)P_2 fractions showed a significant increase in heated cells ($p<0.05$) compared with unheated control levels. Although the HPLC method allowed separation of $InsP_4$, $InsP_5$ and $InsP_6$, this was a time-consuming method and was not suitable for multiple routine analyses. Thus, simple anion exchange chromatography was used for future routine analyses but, since this sometimes resulted in coelution of $InsP_4$ and $InsP_5$, the $InsP_{4/5/6}$ fractions were pooled together and treated as a single fraction.

To investigate the effect of heating time on inositol phosphate levels, myo[2-^{3}H]inositol labelled WRK-1 cells were heated in the presence of serum at 45°C for various times (up to 45 min). No significant difference was found in levels of ^{3}H-$InsP_1$ (Figure 4.9a), ^{3}H-$InsP_3$ (Figure 4.9c) and ^{3}H-$InsP_{4/5/6}$ levels (Figure 4.9d) between heated and unheated cells. On the other hand, a significant increase in ^{3}H-$InsP_2$ was found in heated cells at 5 min onwards ($p<0.05$) (Figure 4.9b).

Heating in the presence of sera can mimic the physiological environment, but addition of serum-containing medium just before heating may trigger the hydrolysis of inositol lipids and may alter the response of the phosphoinositide signalling system to heat. In addition, using serum-free medium enabled investigation of the response of cells to vasopressin during or following heating without the complications of serum-triggered

turnover of inositol lipids. Thus, the rest of the experiments described below were performed in serum-free conditions unless specified.

Heating myo[2-^{3}H]inositol labelled WRK-1 cells at 45°C in serum-free medium for up to 20 min resulted in an elevation in ^{3}H-$InsP_2$ which was observed after heating for 8 min and the level remained significantly elevated for up to 20 min ($p<0.05$) (Figure 4.10b). On the other hand, a transient but significant increase ($p<0.05$) in $InsP_3$ was observed at 11 min heating (Figure 4.10c). Heat did not produce any significant changes in $InsP_1$ and $InsP_{4/5/6}$ levels (Figure 4.10a & 4.10d).

However, anion exchange chromatography can only separate different classes of inositol phosphate and is unable to resolve the isomers. In WRK-1 cells, 3 $InsP_3$ isomers have been identified upon vasopressin-stimulation (Barker *et al.*, 1992; Wong *et al.*, 1992). Hence it is necessary to separate the isomers in order to determine which $InsP_3$ isomer(s) are affected by heat.

The experiment was performed by labelling WRK-1 cells to higher specific activity by using 10 μCi/ml myo[2-^{3}H]inositol. The previous experiment (Figure 4.10c) demonstrated an increase in $InsP_3$ after approximately 11 min at 45°C but other similar experiments showed that this optimum time varied slightly (results not shown). In order to determine the time at which the biggest increase in $InsP_3$ occurred, cells were heated at 45°C for various periods from 8 min to 12.5 min. An aliquot from the PCA extract was then separated on anion exchange columns. It was found that cells heated for 11 min at 45°C gave the biggest and most significant elevation ($p<0.05$) in $InsP_3$ level, resulting in a 33% increase in $InsP_3$ level compared with unheated control level (Figure 4.11c). Thus the remaining extract corresponding to this heating time was separated on a simple anion exchange column, and the eluant corresponding to the $InsP_3$ fraction was desalted, freeze-dried and separated on HPLC. A typical separation of $InsP_3$ isomers is shown in

Figure 4.12. The identification of different $InsP_3$ isomers was based on the separation of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ standards, but the lack of $Ins(3,4,5)P_3$ standard did not allow the determination of the retention time of this isomer, and its identification was based on the chromatogram shown in Wong *et al.* (1992). Although there was an increase in mean dpm count in heated cells in all 3 isomers, the difference in levels of these isomers was statistically insignificant when analysed by Student's t-test analysis (Table 4.2).

It has been reported that the activation of PLC required an elevated level of cytosolic free calcium (Rhodes *et al.*, 1983; Gallo-Payet and Payet, 1989). The dependency of the activation of PLC on calcium upon heating has been investigated in A-431 cells (Kiang and McClain, 1993). It was reported that heat caused an elevation of $[Ca^{2+}]_i$ and this increase in $[Ca^{2+}]_i$ preceded the increase in $InsP_3$ levels. Furthermore, the increase in $InsP_3$ upon heating was not detected when cells were heated in calcium-free buffer. This result led to the proposal that a heat-induced increase in $[Ca^{2+}]_i$ activates a calcium-dependent PLC, which then hydrolyses $PtdIns(4,5)P_2$, to increase $InsP_3$ (Kiang and McClain, 1993). Similarly, Calderwood and Stevenson (1993) found that incubation of CHO HA-1 cells in calcium-free (EGTA containing) buffer for 1-3 h before heating at 43°C for 30 min abolished the heat-induced increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels in these cells. In order to investigate the effect of calcium on the heat-induced increase in $InsP_3$ in WRK-1 cells, these cells were heated at 45°C in either calcium-free buffer (together with 0.5 mM EGTA) or EMEM medium. At the same time, some plates with cells incubated in either buffer were kept in a 37°C water bath as parallel controls. It was found that the magnitude of the increase in the ^3H-$InsP_3$ level was similar for cells heated in either calcium-free buffer or EMEM medium, though the $InsP_3$ reached maximal levels at a different time (Figure 4.13c). An $InsP_3$ peak occurred at 11 min when cells were heated in EMEM medium, whereas for cells heated in calcium-free buffer the $InsP_3$ reached a maximum after 9.5 min, both heating conditions

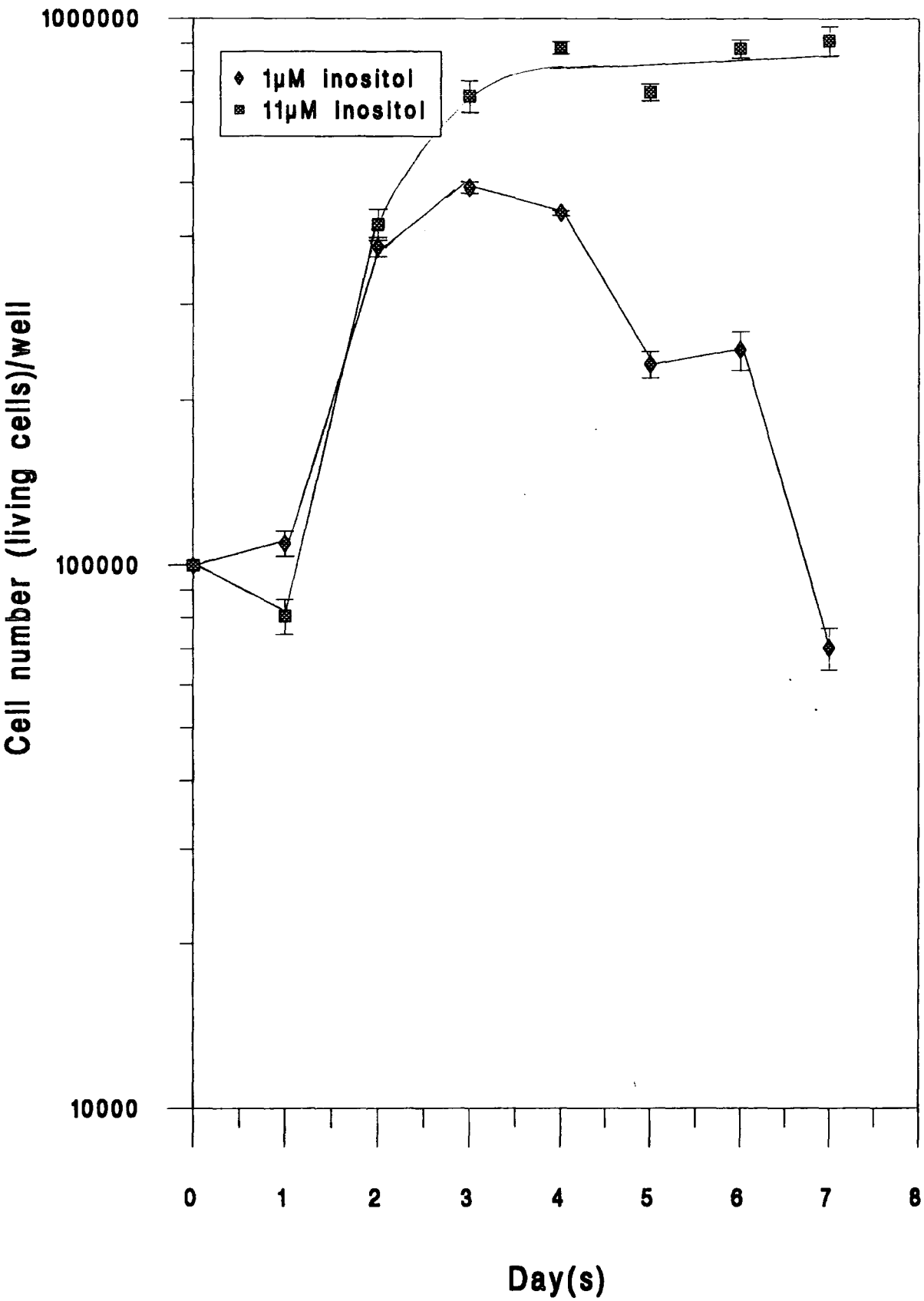
resulting in a 50% increase compared with the parallel unheated control. ^{3}H-InsP_3 levels then declined to control levels after 12.5 min heating (Figure 4.13c). When levels of other inositol phosphates were determined, a 20% increase in InsP_2 levels was observed in cells heated in EMEM medium compared with the parallel control at 8 and 9.5 min heating whereas only 5% increase in InsP_2 level was found in cells heated in calcium-free buffer compared with the parallel control at those time points (Figure 4.13b). The magnitude of the increase in InsP_1 levels was similar in cells heated in either condition compared with the parallel controls, except at 9.5 min when the increase in InsP_1 was higher in cells heated in EMEM compared with cells heated in calcium-free buffer (Figure 4.13a). On the other hand, Ins$P_{4/5/6}$ levels declined slightly in cells heated in EMEM or calcium-free buffer compared with parallel controls throughout the 12.5 min heating period (Figure 4.13d). Thus extracellular calcium was unnecessary for the heat-induced changes in inositol phosphates, at least within the 8 to 12.5 min heating period at 45°C.

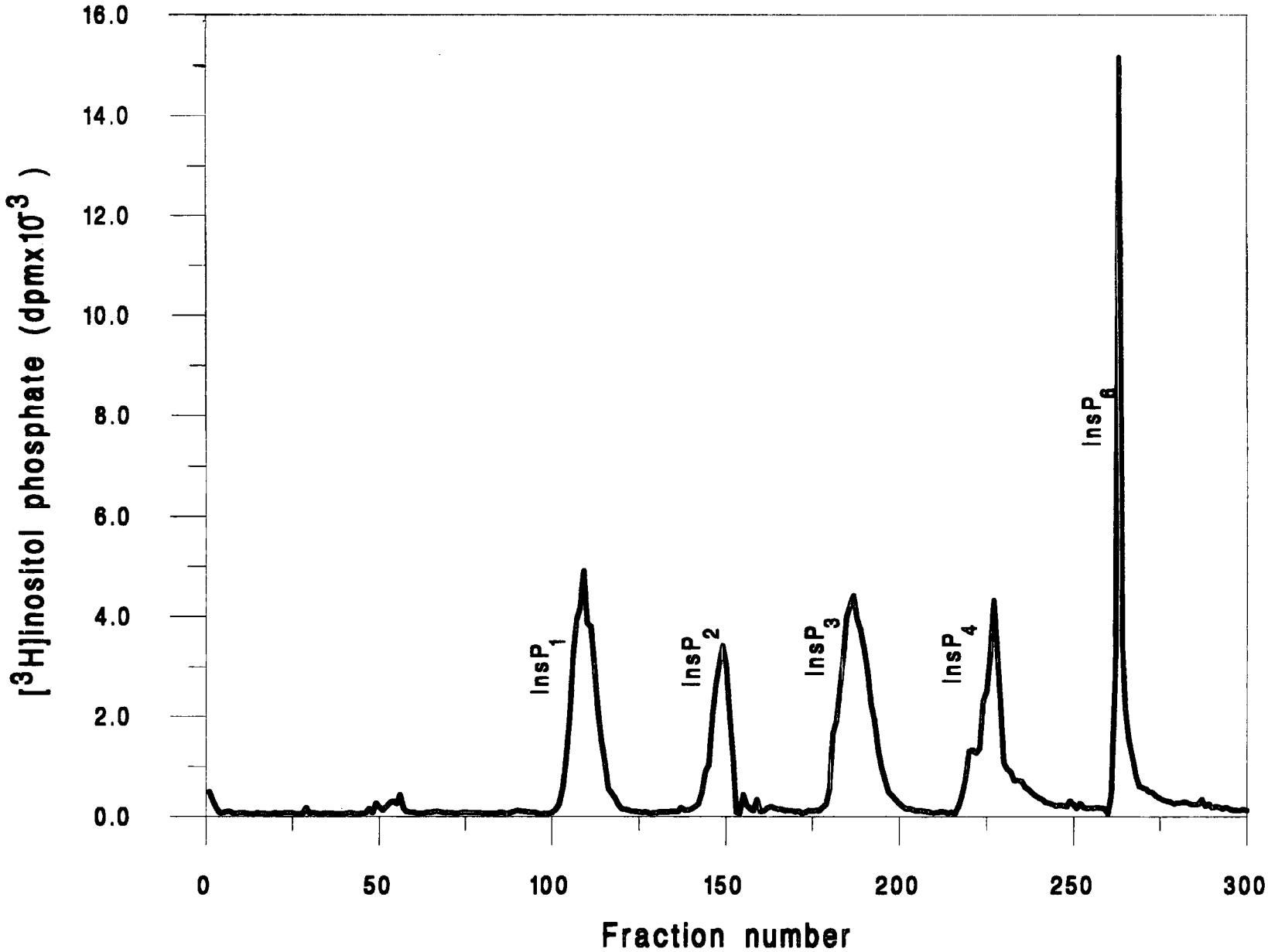
In order to investigate the effect of a variety of agents, including heat, ethanol, sodium orthovanadate, AlF_4^- and sera, on the phosphoinositide signalling pathway, [^{3}H]inositol-labelled WRK-1 cells were incubated at 37°C in an air/CO_2 (19:1, v/v) atmosphere with the appropriate substance for 30 min (Table 4.3). No significant difference was found in InsP_1, InsP_2 and Ins$P_{4/5/6}$ fractions when cells were incubated in ethanol, sodium orthovanadate, AlF_4^- and sera or when heated at 45°C when compared with levels obtained from cells incubated at 37°C with EMEM for the same period. A statistically significant increase in ^{3}H-InsP_3 level was found in cells heated in EMEM at 45°C for 30 min or cells incubated in AlF_4^- at 37°C for 30 min when compared with unheated control (incubated in serum-free EMEM medium at 37°C for 30 min) ($p<0.05$). On the other hand, the difference in ^{3}H-InsP_3 level was insignificant in cells incubated in 5% (v/v) ethanol or sodium orthovanadate at 37°C for 30 min compared to control cells. When cells were incubated in the presence of 7% (v/v) sera in

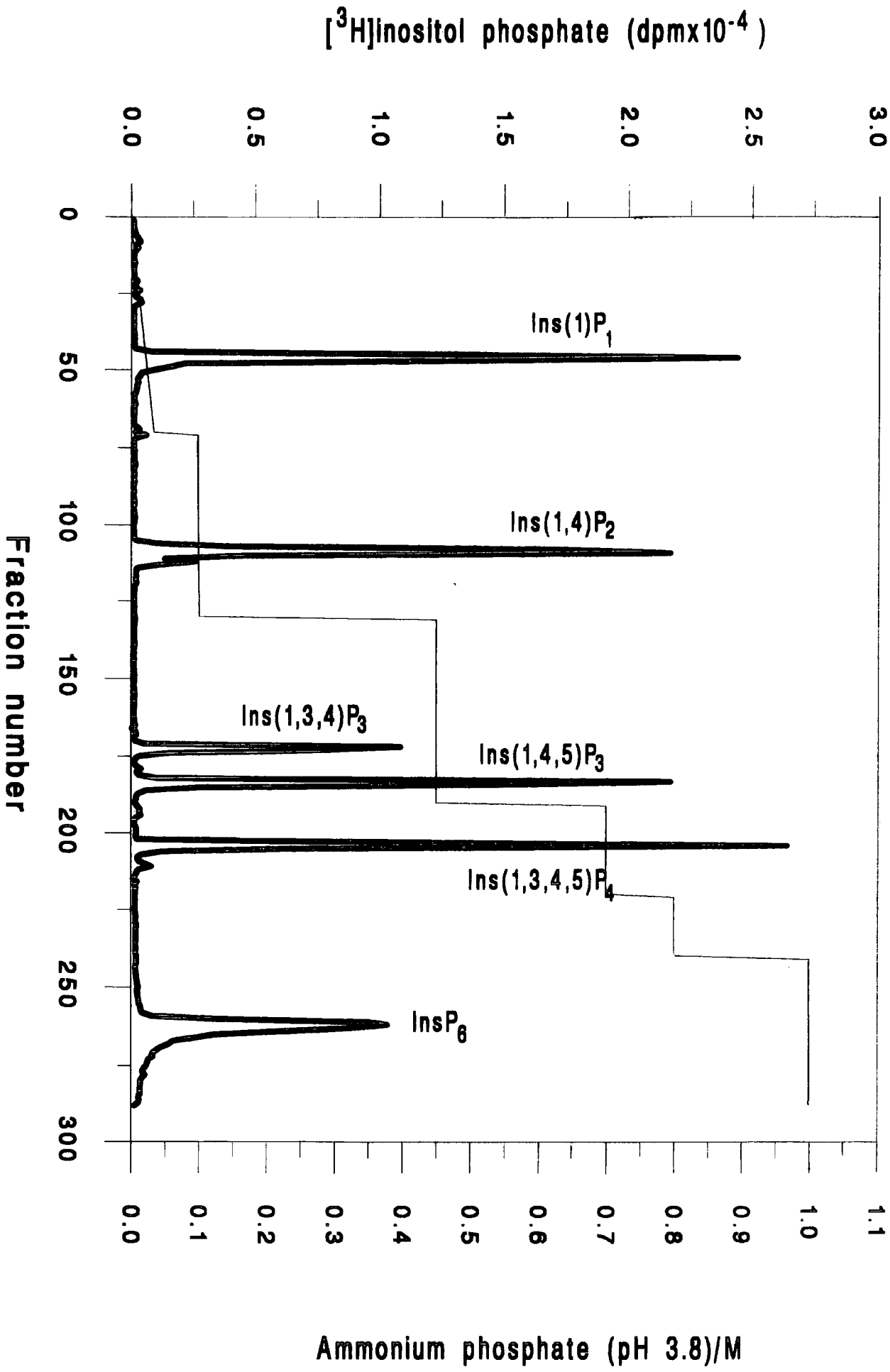
EMEM + 10 mM LiCl at 37°C for 30 min, an increase in the mean dpm values of ^{3}H-$InsP_3$ was observed compared to cells incubated at 37°C in EMEM only, but this difference was insignificant. However, ^{3}H-$InsP_3$ levels in cells heated at 45°C for 30 min in serum-containing medium were significantly higher ($p<0.05$) than in cells incubated in serum-free EMEM medium at 37°C for the same period, but were not significantly different from the ^{3}H-$InsP_3$ level in cells incubated at 37°C for 30 min in serum-containing medium (Table 4.3).

In order to determine the influence of heat on a hormone-stimulated signalling system, the response of WRK-1 cells to vasopressin under hyperthermic conditions (at 45°C) was investigated. Cells were incubated at either 37°C or 45°C for 2 min (including 1.5 min lag period), then vasopressin, in EMEM medium (containing 10 mM LiCl and 20 mM Hepes, pH 7.4), at either 37°C or 45°C was injected into the well quickly to give a final concentration of vasopressin of 400 nM. For unchallenged cells, 1 ml of the same medium without vasopressin at either 37°C or 45°C was added to the well as parallel control. By using this protocol the medium in the well after vasopressin addition remained at the desired temperature. Accumulation of $InsP_1$ and $InsP_2$ were observed about 10 s after addition of vasopressin to unheated cells, whereas accumulation of $InsP_1$ was not detected in heated cells throughout the 2 min vasopressin stimulation period (Figure 4.14a). Accumulation of $InsP_2$ in heated cells was more gradual compared to unheated cells throughout the 2 min vasopressin stimulation (Figure 4.14b). On the other hand, a 5-6 fold increase in $InsP_3$ was observed in heated cells whereas only a 2-fold increase in $InsP_3$ was observed in unheated cells during 2 min vasopressin stimulation (Figure 4.14c). An increase of approximately 4-fold was observed in the $InsP_{4/5/6}$ fraction following vasopressin stimulation of heated cells, whereas no change occurred in the $InsP_{4/5/6}$ level in control cells exposed to vasopressin (Figure 4.14d).

It has been suggested that heat may alter the response of cells to growth factors and hormones (Mikkelsen *et al.*, 1991a). To address this possibility, WRK-1 cells were heated at 45°C for up to 40 min and then returned to 37°C for 2 min to allow the equilibration of the medium back to 37°C. The cells were then stimulated with vasopressin for 30 sec at 37°C, while the serum-free EMEM vehicle was added to control wells. In the absence of vasopressin, the levels of $InsP_1$ in both heated and unheated cells were similar (Figure 4.15a). Upon vasopressin stimulation, the mean dpm in $InsP_1$ levels in both unheated and heated cells were higher than those without stimulation but the difference was statistically insignificant. Heat alone led to a significant increase ($p<0.05$) in $InsP_2$ level at 30 min and 40 min compared with unheated cells (Figure 4.15b). Upon vasopressin stimulation, a significant increase ($p< 0.05$) in $InsP_2$ levels in both heated and unheated cells compared with unchallenged cells was observed. However, the levels of $InsP_2$ in heated, vasopressin-stimulated cells was insignificantly different from those obtained in unheated, vasopressin-stimulated cells (Figure 4.15b). Similarly, the level of $InsP_3$ in cells heated at 45°C was higher than unheated cells, but a significant difference ($p<0.05$) was observed at 30 min heating only (Figure 4.15c). The $InsP_3$ levels in heated or unheated cells upon vasopressin stimulation were significantly higher than unchallenged cells ($p<0.05$), even after cells were heated for 40 min before challenging. However, the difference between heated and unheated, stimulated cells was insignificant. On the other hand, no difference was observed in $InsP_{4/5/6}$ levels in vasopressin-stimulated and unchallenged cells or heated and unheated cells (Figure 4.15d). The results found in this study showed that generally there was an increases in $InsP_1$, $InsP_2$ and $InsP_3$ fractions in cells challenged with vasopressin at 37°C compared to unchallenged cells, but upon vasopressin stimulation, both heated and unheated cells gave a similar $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_{4/5/6}$ levels (Figure 4.15a-d). These results imply that the vasopressin receptor-mediated signalling system remained intact following treatment at 45°C for up to 40 min, a treatment that would eventually kill more than 95% of WRK-1 cells, determined by the MTT assay (Figure 3.8).


1000000
100000
10000
1µM inositol
11µM inositol
Cell number (living cells)/well
0
1
2
3
4
5
6
7
8
Day(s)


16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
[3H]Inositol phosphate (dpmx10-3)
InsP1
InsP2
InsP3
InsP4
InsP6
0
50
100
150
200
250
300
Fraction number


[3H]inositol phosphate (dpmx10-4)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
50
100
150
200
250
300
Fraction number
Ins(1)P1
Ins(1,4)P2
Ins(1,3,4)P3
Ins(1,4,5)P3
Ins(1,3,4,5)P4
InsP6
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Ammonium phosphate (pH 3.8)/M

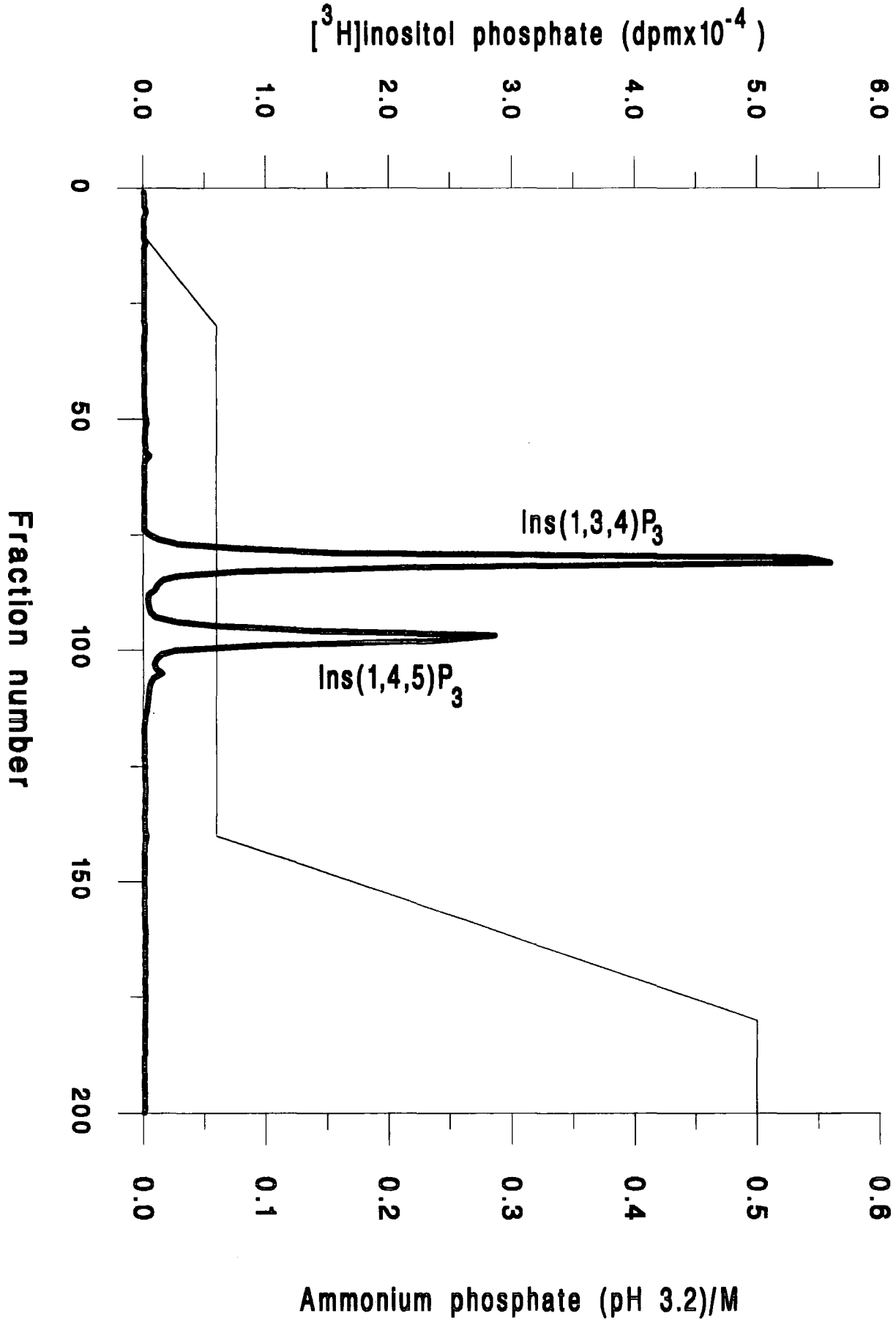

[3H]inositol phosphate (dpmx10-4)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
0
50
100
150
200
Fraction number
Ins(1,3,4)P3
Ins(1,4,5)P3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Ammonium phosphate (pH 3.2)/M

Figure 4.6a. Incorporation of [^{3}H]inositol into inositol lipids

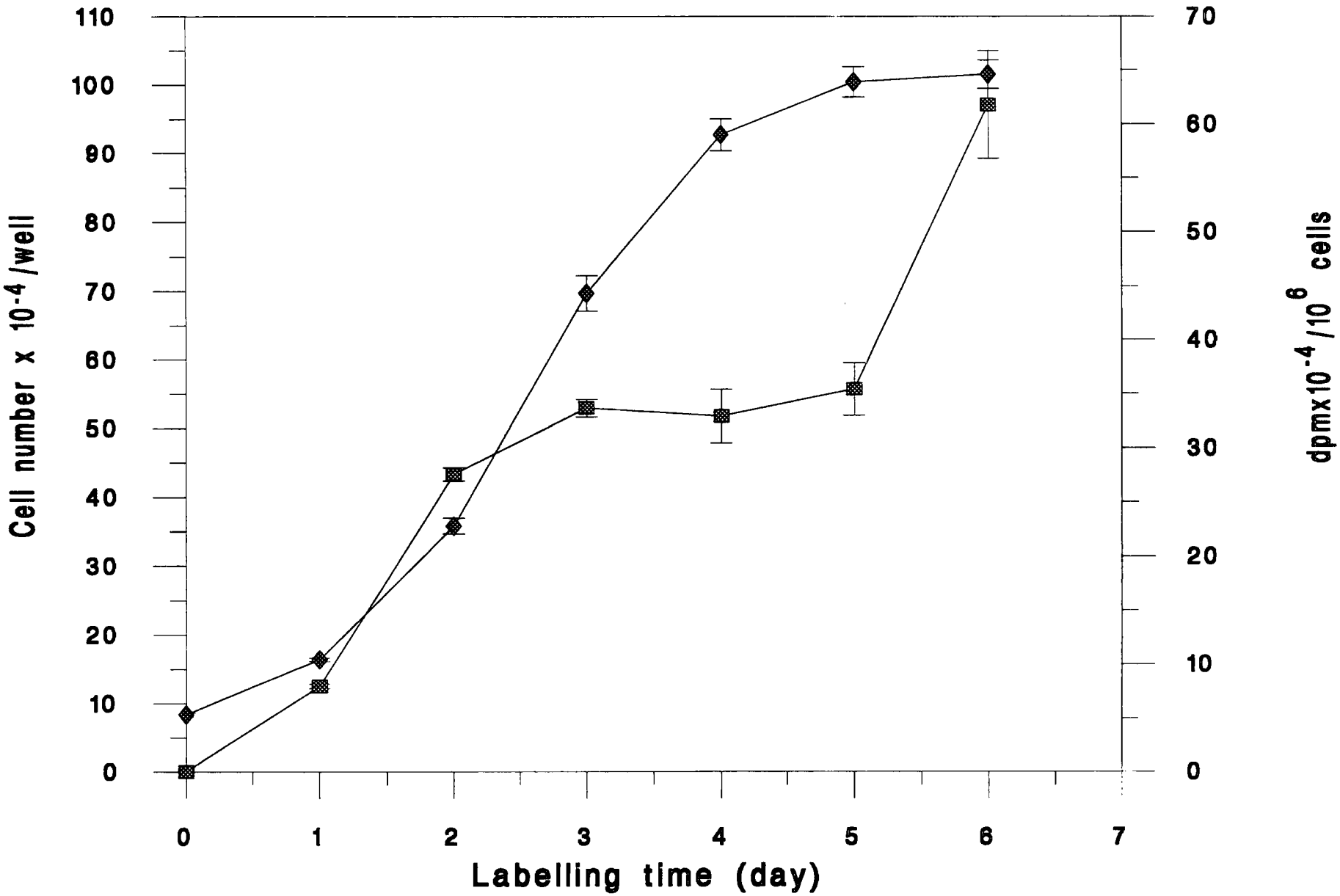


Figure 4.6b. Incorporation of [^{3}H]inositol into phosphatidylinositol

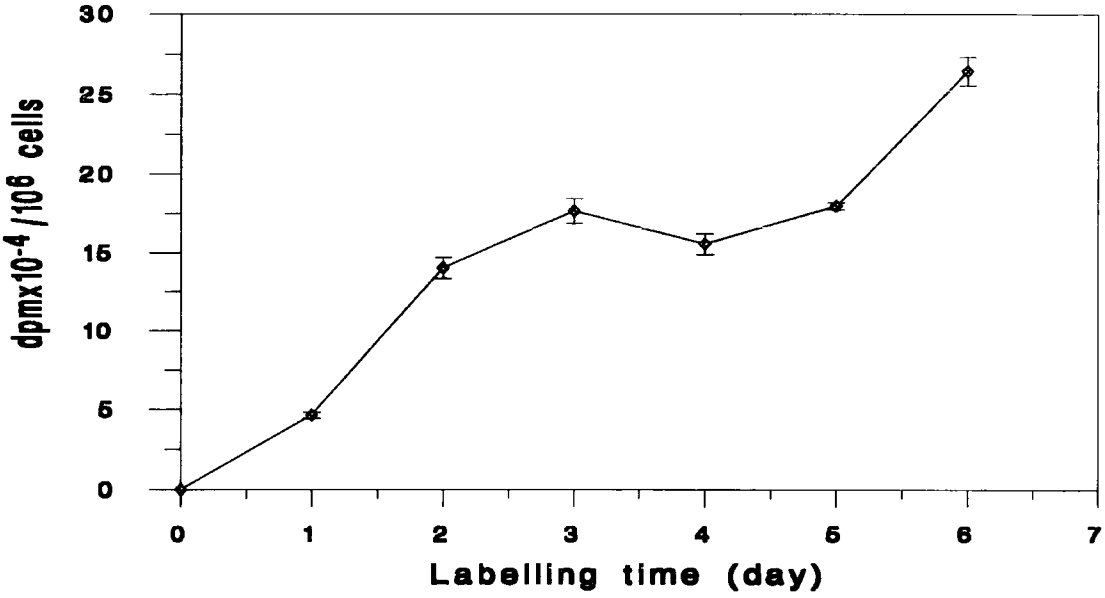


Figure 4.6c. Incorporation of [^{3}H]inositol into phosphatidylinositol 4-phosphate

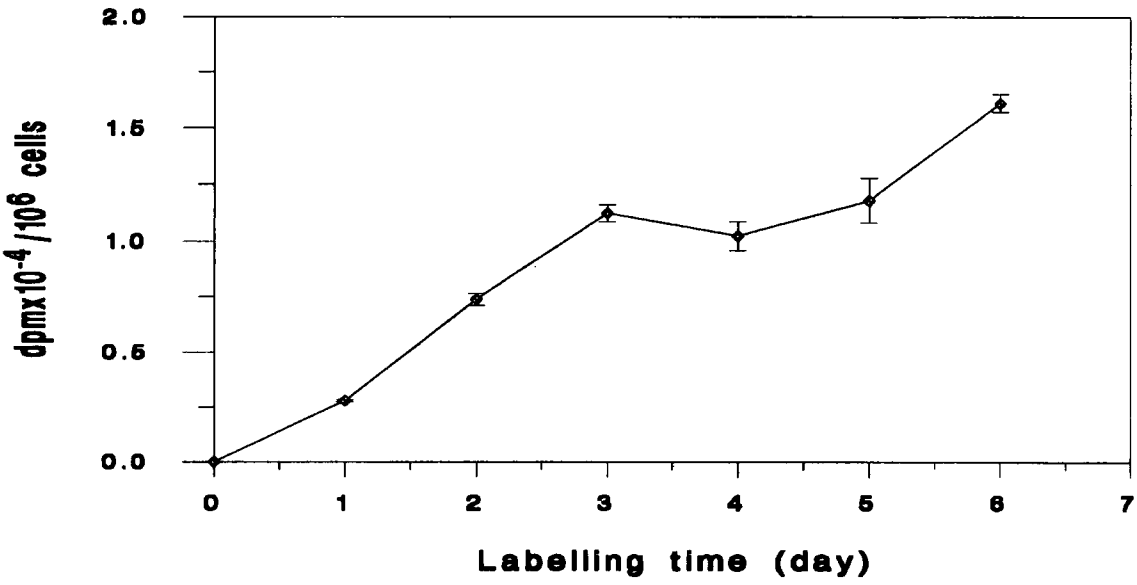


Figure 4.6d. Incorporation of [^{3}H]inositol into phosphatidylinositol 4,5-bisphosphate

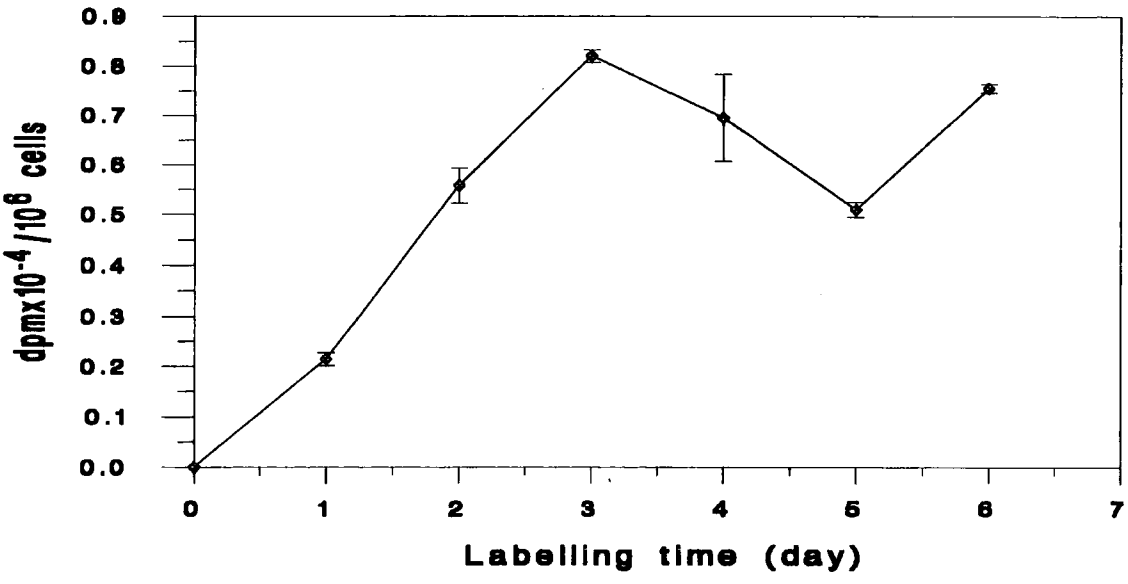


Figure 4.7. Effect of different temperatures on inositol phosphate levels

Cells were harvested and seeded in a no. of 6-well plates at a density of 100,000 cells with each well containing 3 ml of inositol-depleted medium. On the following day (day 1), cells were labelled with radioactive medium (2 μCi/ml of myo[2-^{3}H]inositol made up in inositol-depleted medium and 1.5 ml per well was used) and the medium was changed on day 3. On the day of the experiment (day 5) radioactive medium was removed and cells were washed 3 times with non-radioactive inositol-depleted medium containing 20 mM Hepes, pH 7.4 and 1 ml of this medium was added to each well, plates were then returned to culture. After 1 h, medium was removed and cells were washed with 1 ml of EMEM medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium A) and then 1 ml of medium A was added to each well. The plates were incubated at 37°C for 10 min then medium was replaced with 1 ml of growth medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium B) before sealing plates. Plates were then equilibrated by submersion in a 37°C water bath for 5 min before rapid transfer and submersion in a water bath at varying temperatures. The reactions were terminated after 30 min (including 1.5 min lag period) by addition of 1 ml ice-cold 10% (v/v) PCA. Inositol phosphates were then extracted and separated by BioRad anion exchange columns.

Values represent mean ±SEM from three independent measurements (n=3).

The 1.5 min lag period was included in the heating time reported.

*found to be significantly different ($p<0.05$) from control value (37°C) by using Student's t-test analysis.

Figure 4.7a. Effect of heat on $InsP_1$ level

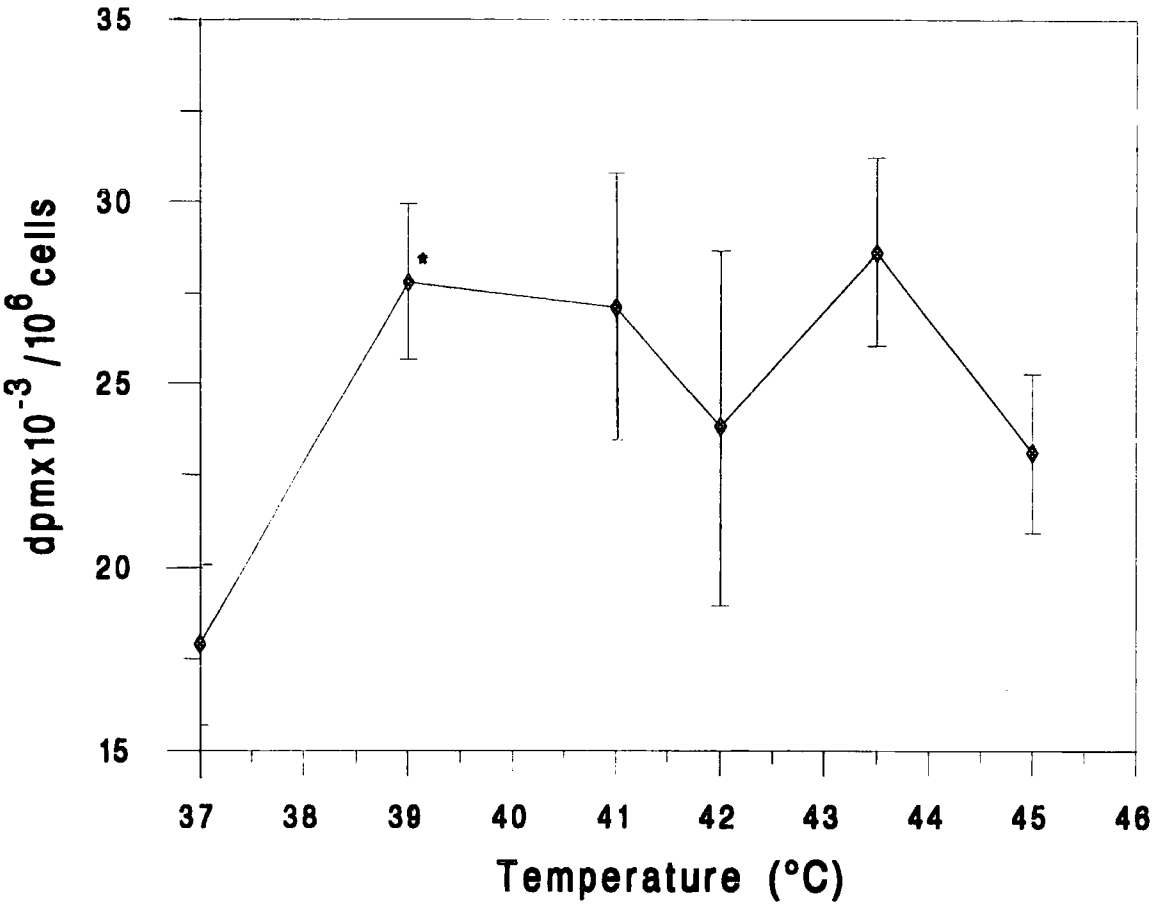


Figure 4.7b. Effect of heat on $InsP_2$ level

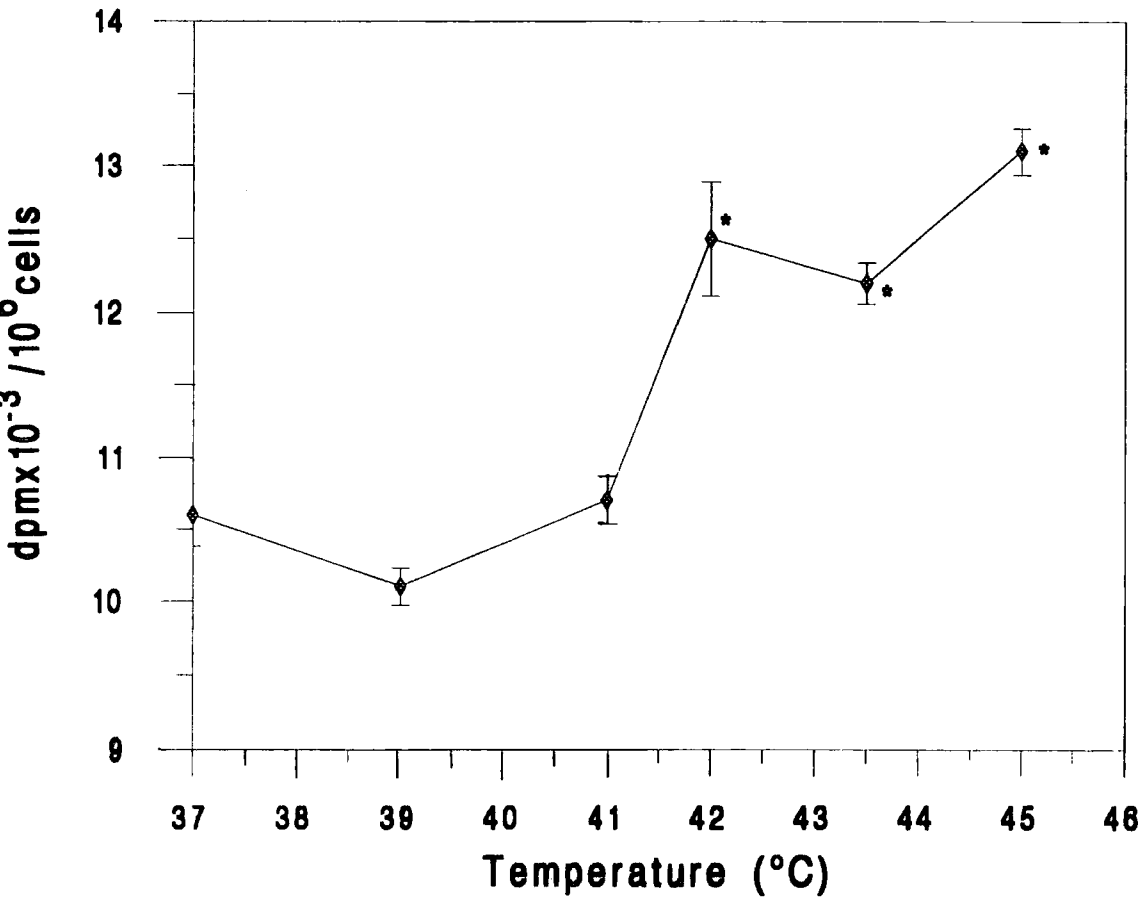


Figure 4.7c. Effect of heat on $InsP_3$ level

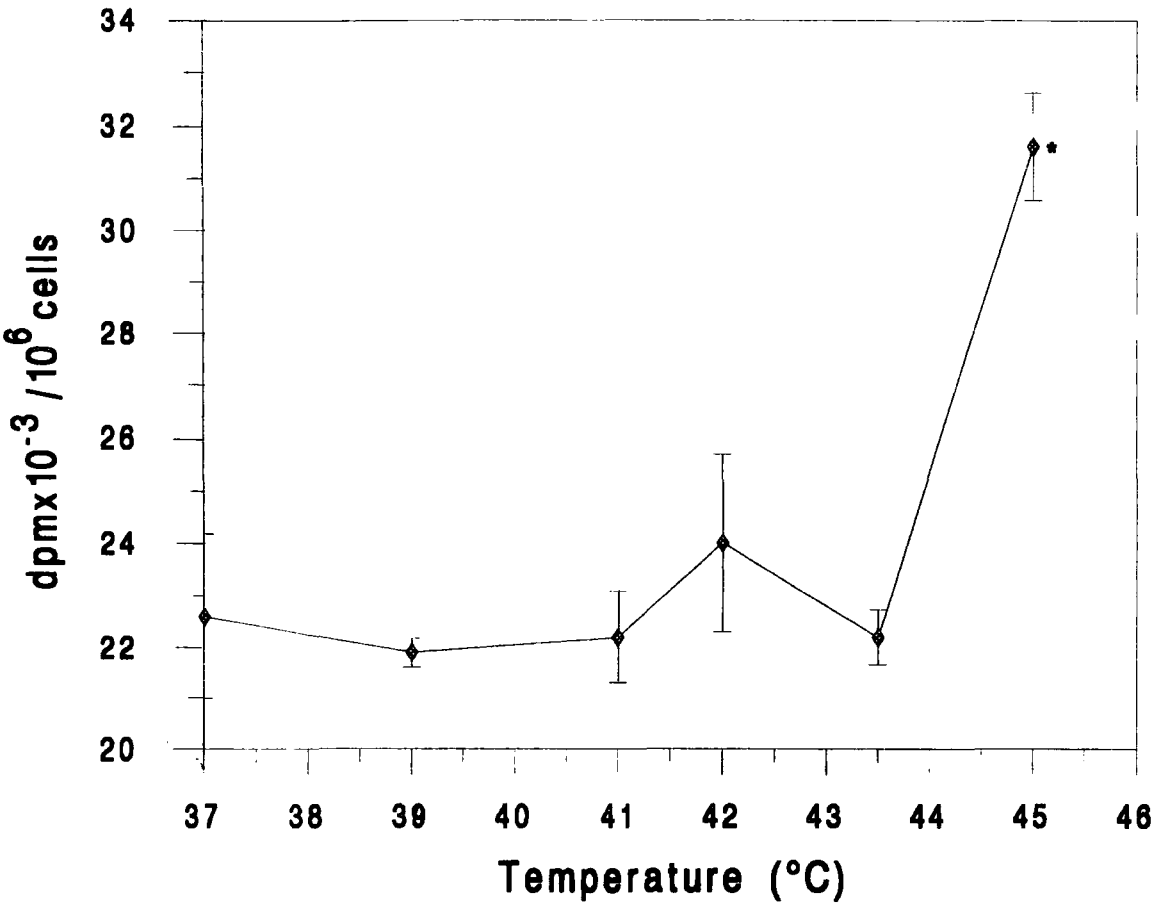


Figure 4.7d. Effect of heat on $InsP_{4/5/6}$ level

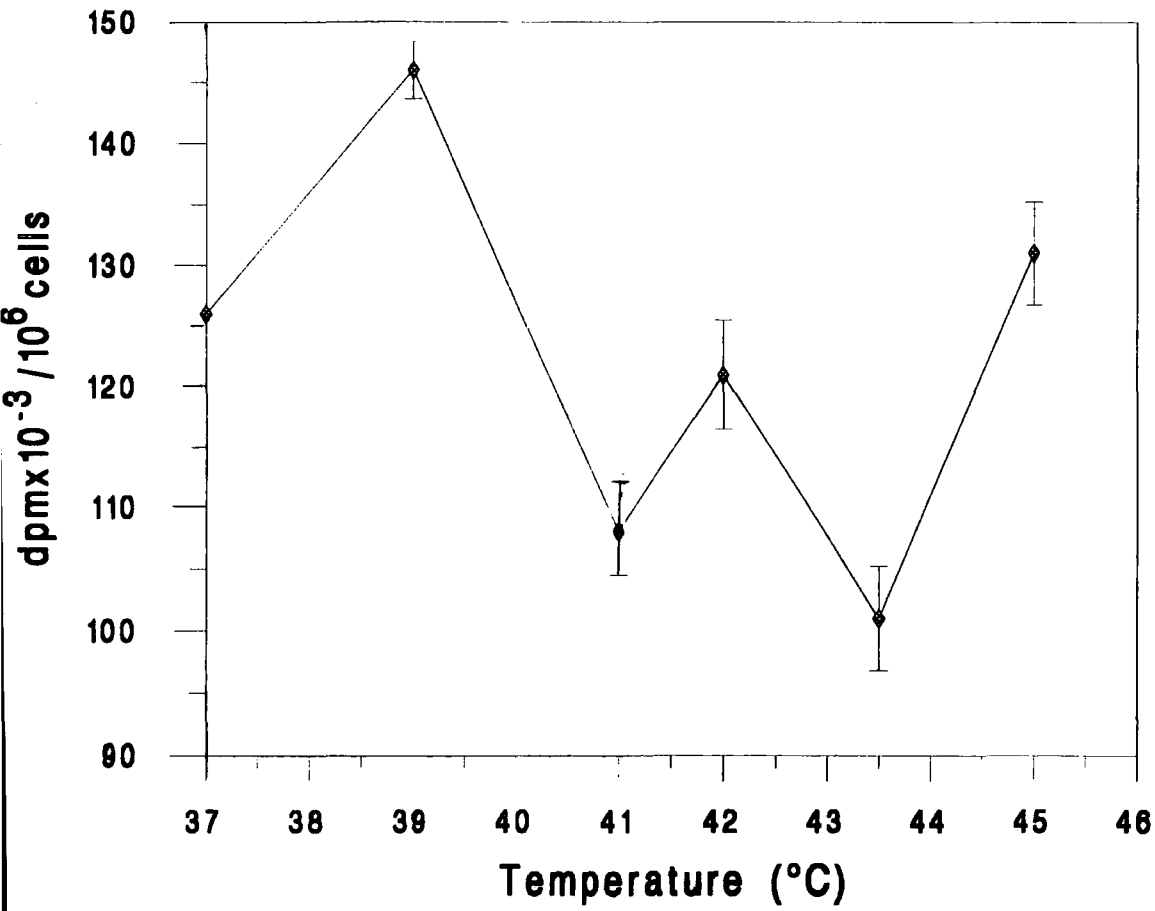
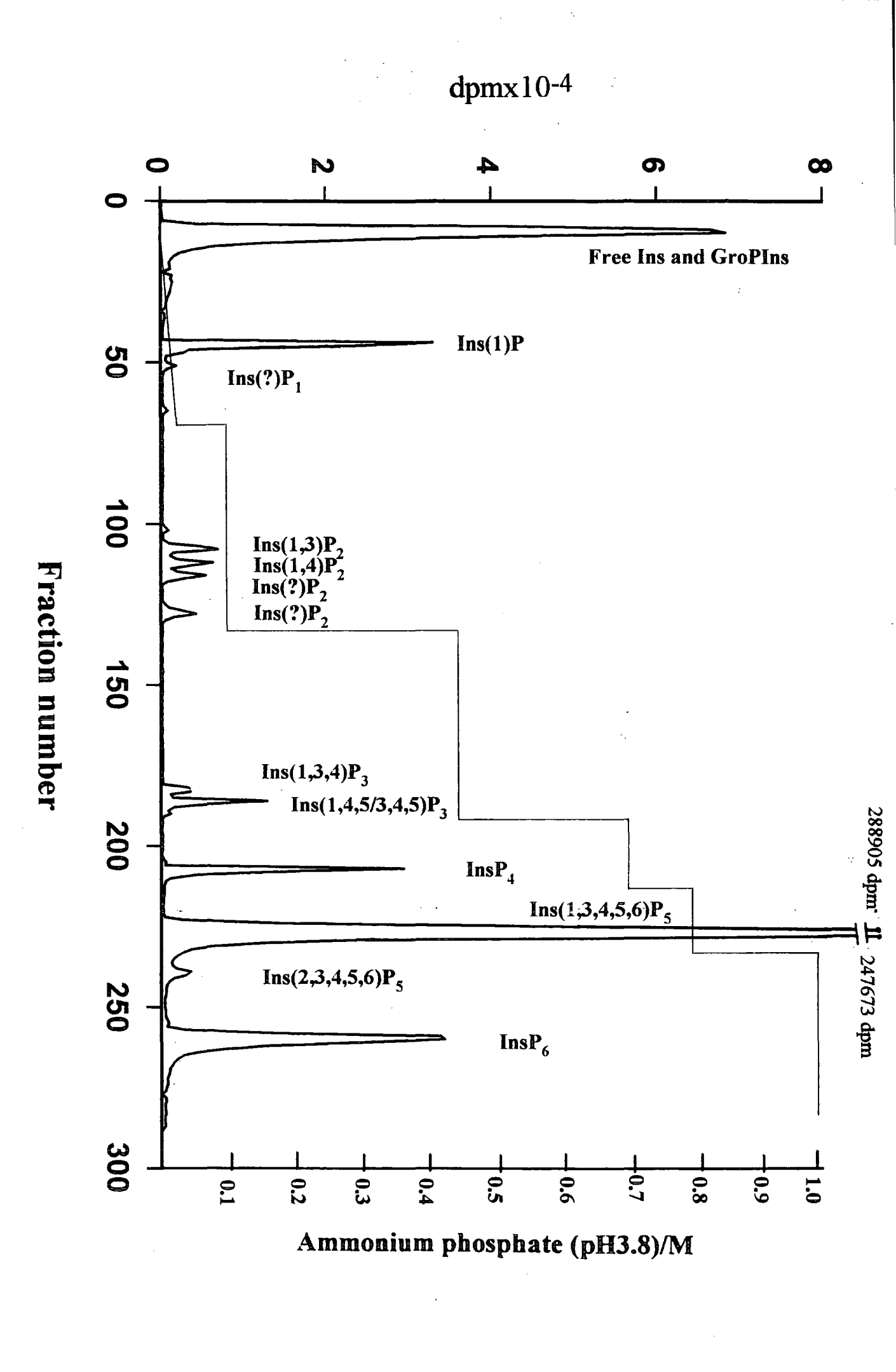


Figure 4.8. HPLC separation of inositol phosphates from heat-treated WRK-1 cells

WRK-1 cells were seeded into a no. of 6-well plates at a density of 100,000 cells with each well containing 3 ml of inositol-depleted medium. After 24 h, cells were labelled with radioactive medium (10 μCi/ml of myo[2-^{3}H]inositol made up in inositol-depleted medium and 1.5 ml of the medium was used per well) and the medium was changed on day 3. On the day of the experiment (day 5) radioactive medium was removed and cells were washed 3 times with non-radioactive inositol-depleted medium and 1 ml of this medium was then added to each well. The plates were returned to the 37°C incubator for 1 h. After 1 h, medium was removed and cells were washed once with 1 ml of EMEM medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium A) and then 1 ml of medium A was added to each well. The plates were incubated at 37°C for 10 min then the medium was replaced with 1 ml of normal growth medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium B) before sealing. Plates were then equilibrated by submersion in a 37°C water bath for 5 min before rapid transfer and submersion in a water bath at 45°C for 30 min (1.5 min lag period was included). The reactions were terminated by addition of 0.5 ml ice-cold 1% (v/v) TCA. Inositol phosphates were then extracted and the whole extract was separated on HPLC using a Partisphere SAX column together with a pellicular guard column. The gradient profile used was described in section 4.2.2(ii).

Diagram represents a typical HPLC separation of inositol phosphates of a sample.


dpmx10-4
0
2
4
6
8
Free Ins and GroPIns
Ins(1)P
Ins(?)P1
Ins(1,3)P2
Ins(1,4)P2
Ins(?)P2
Ins(?)P2
Ins(1,3,4)P3
Ins(1,4,5/3,4,5)P3
InsP4
Ins(1,3,4,5,6)P5
Ins(2,3,4,5,6)P5
InsP6
288905 dpm
247673 dpm
Fraction number
0
50
100
150
200
250
300
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Ammonium phosphate (pH3.8)/M

compound	unheated	heated	% of control
Ins(1)P_1	66833±2302	67174±2951	101
Ins(?)P_1	3452±285	4512±1566	131
Ins(1,3)P_2	15033±266	18910±810*	126
Ins(1,4)P_2	13155±875	13369±797	102
Ins(?)P_2	10639±437	11132±531	105
Ins(?)P_2	9857±536	13146±213*	133
Ins(1,3,4)P_3	8153±372	8004±161	98.2
Ins(1,4,5/3,4,5)P_3	23308±305	30957±2195	133
$InsP_4$	55225±1722	49862±2710	90.3
Ins(1,3,4,5,6)P_5	720702±49552	692350±87021	96.1
Ins(2,3,4,5,6)P_5	14207±735	12513±335	88.1
$InsP_6$	156646±3692	117879±8485	75.4
total $InsP_1$	70283±1753	71686±2442	102
total $InsP_2$	48684±1660	56557±1849	117
total $InsP_3$	31461±541	38961±1918	123
total $InsP_5$	734909±40723	704863±71253	95.9

Figure 4.9. Effect of heating duration on inositol phosphate levels in serum-containing medium

Cells were harvested and seeded in a no. of 6-well plates at a density of 100,000 cells with each well containing 3 ml of inositol-depleted medium. On the following day (day 1), cells were labelled with radioactive medium (2 µCi/ml of myo[2-^{3}H]inositol made up in inositol-depleted medium and 1.5 ml per well was used) and the medium was changed on day 3. On the day of the experiment (day 5) radioactive medium was removed and cells were washed 3 times with non-radioactive inositol-depleted medium containing 20 mM Hepes, pH 7.4 and 1 ml of this medium was added to each well, plates were then returned to culture. After 1 h, medium was removed and cells were washed with 1 ml of EMEM medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium A) prewarmed at 37°C and then 1 ml of medium A was added to each well. The plates were incubated at 37°C for 10 min and medium was replaced with 1 ml of normal growth medium + 10 mM LiCl + 20 mM Hepes, pH 7.4 (medium B) before sealing plates. Plates were then equilibrated by submersion in a 37°C water bath for 5 min before rapid transfer and submersion in a 45°C water bath. The reactions were terminated at various times by addition of 1 ml ice-cold 10% (v/v) PCA. Inositol phosphates were then extracted and separated by BioRad anion exchange columns.

Values represent mean ±SEM from three independent measurements (n=3).

The 1.5 min lag period was included in the heating time reported.

Figure 4.9a. Inositol monophosphate formation during heating

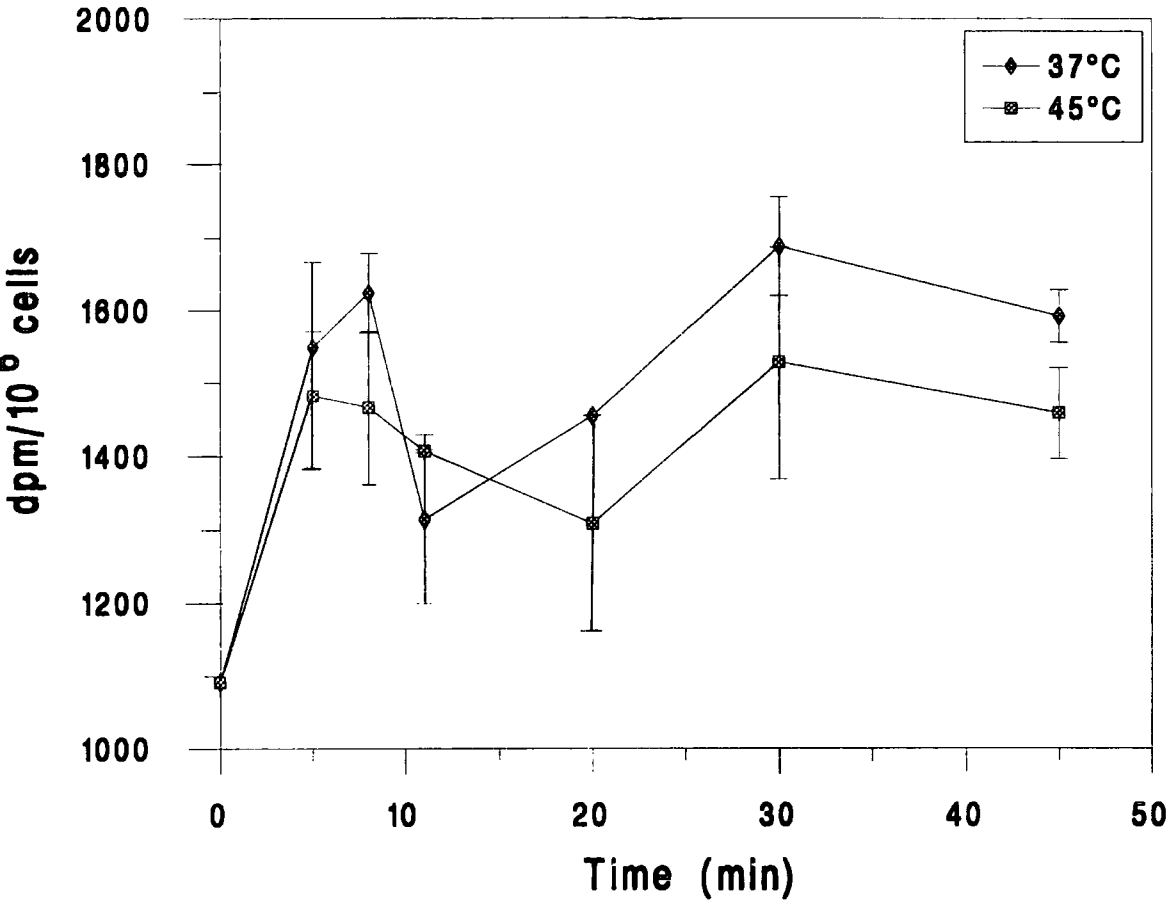


Figure 4.9b. Inositol bisphosphate formation during heating

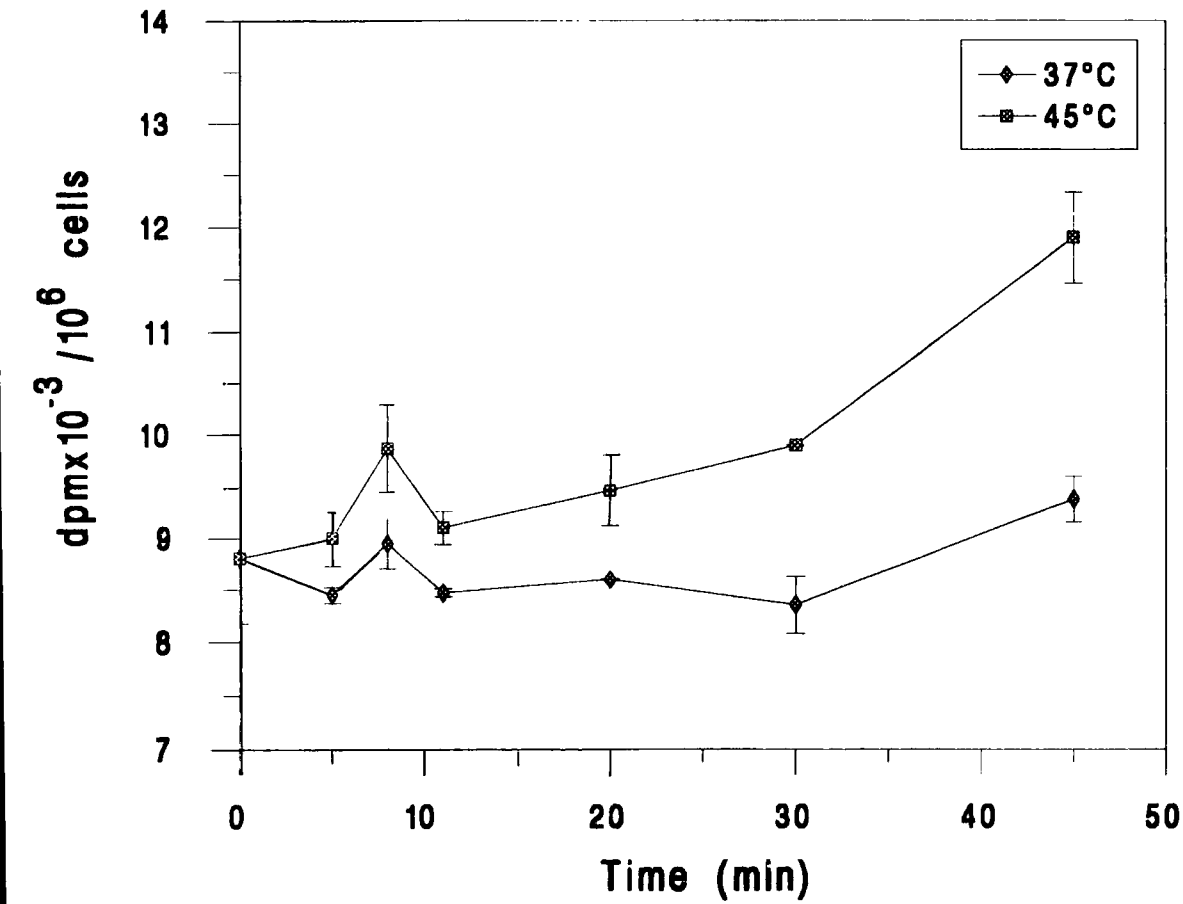


Figure 4.9c. Inositol trisphosphate formation during heating

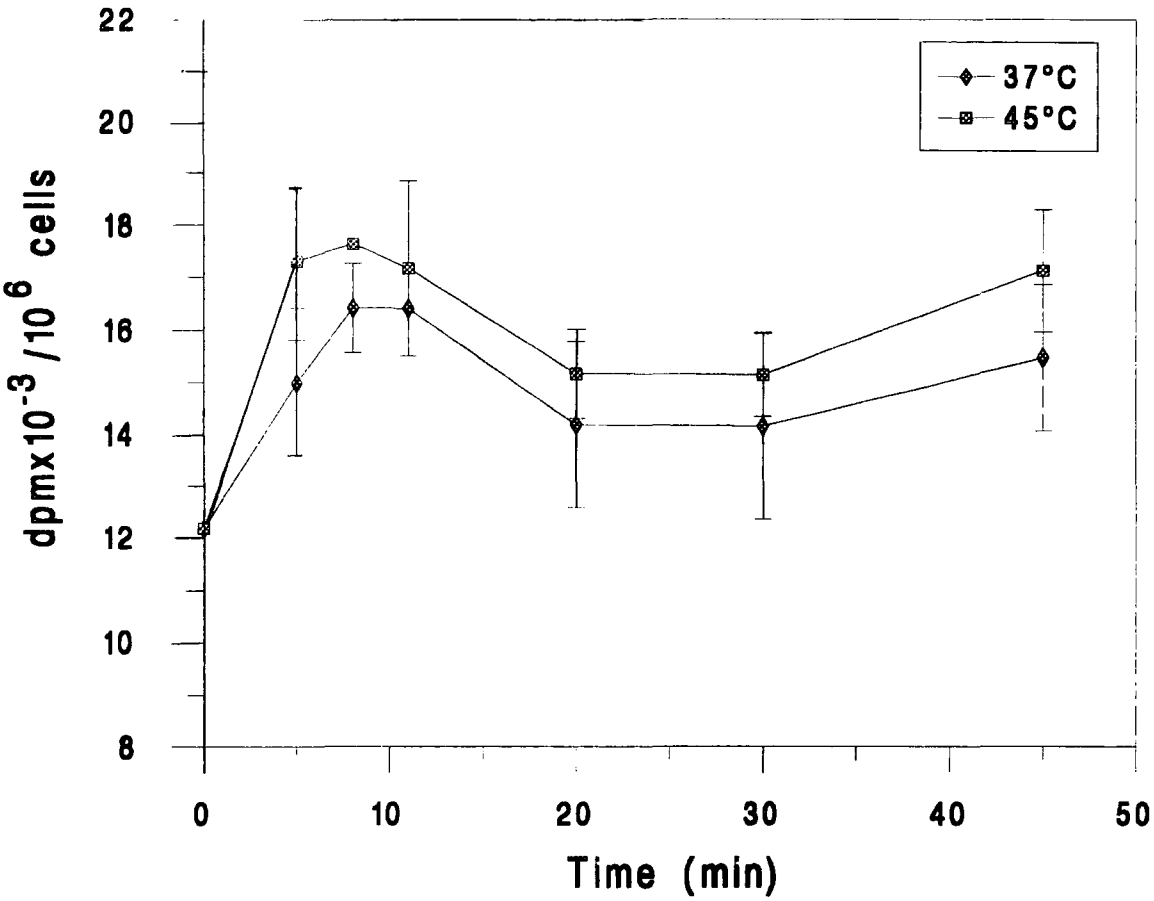


Figure 4.9d. $InsP_{4/5/6}$ formation during heating

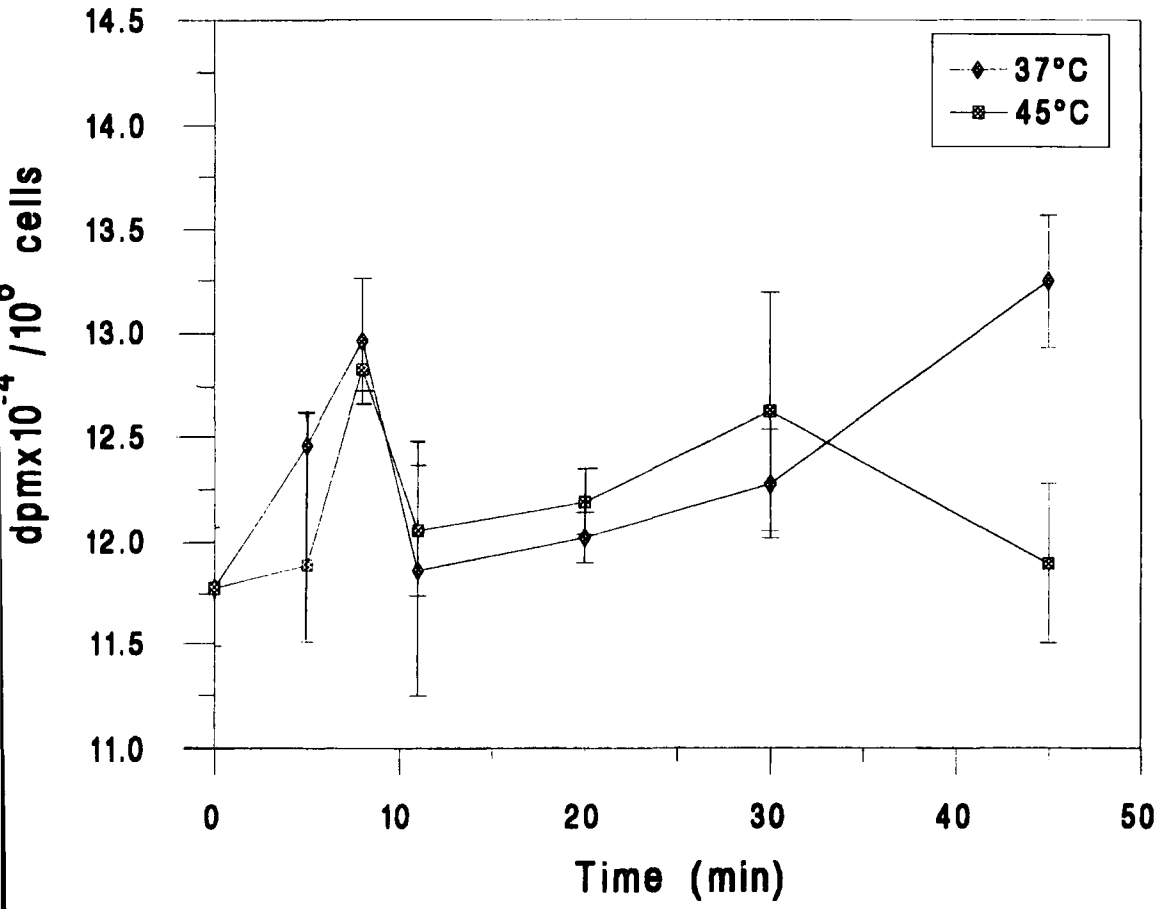


Figure 4.10a. Inositol monophosphate formation during heating in serum-free medium

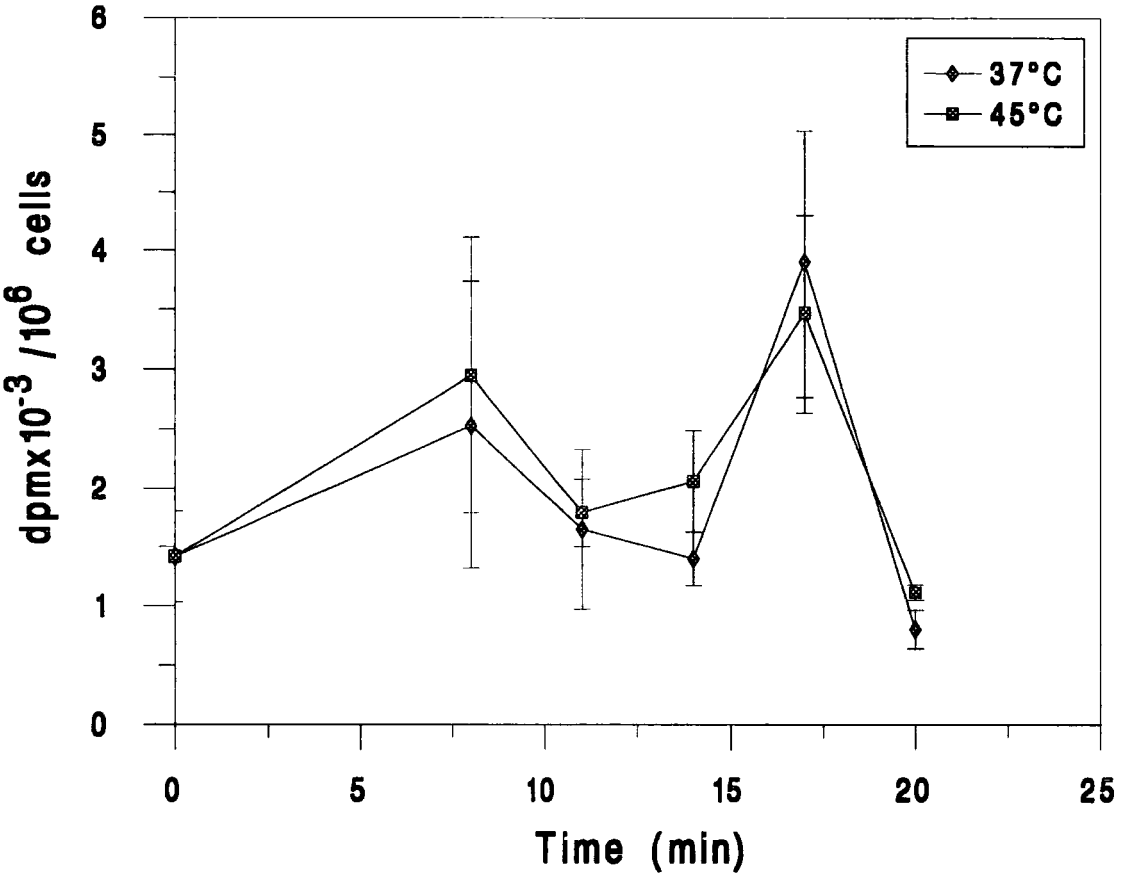


Figure 4.10b. Inositol bisphosphate formation during heating in serum-free medium

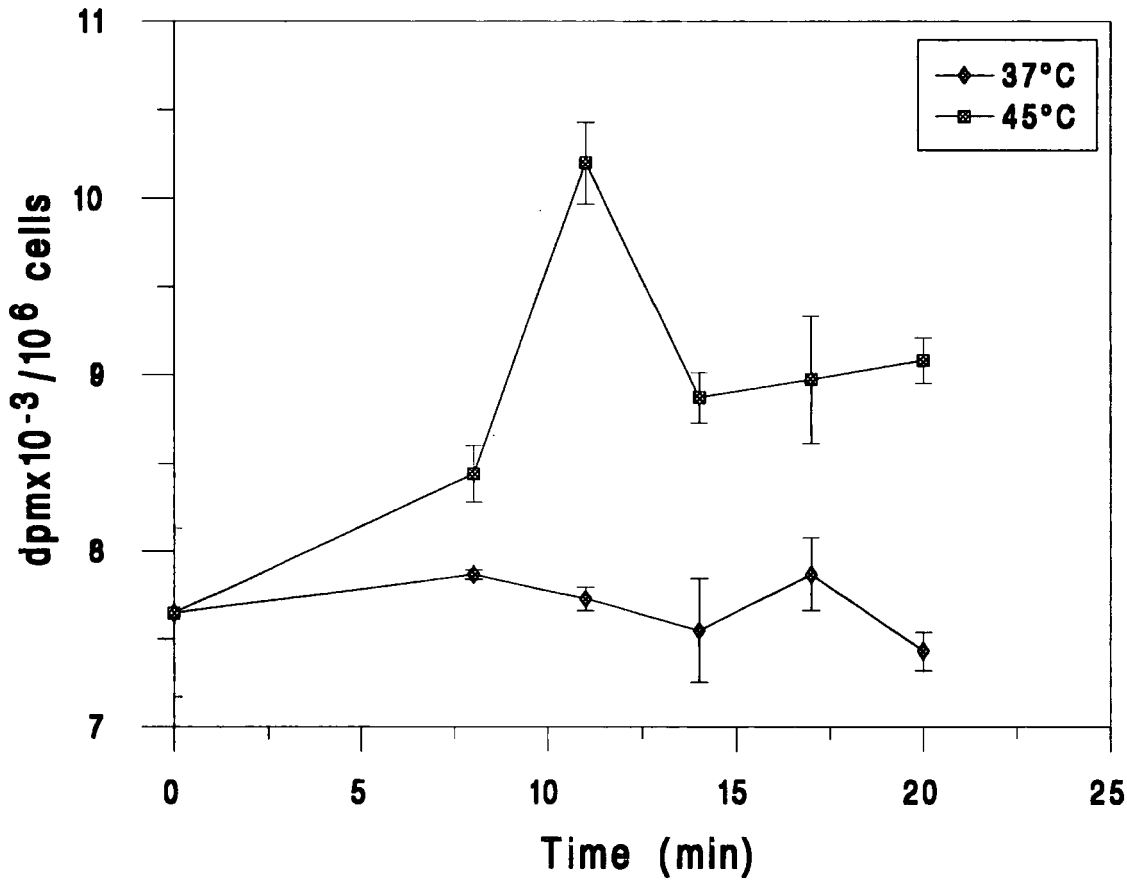


Figure 4.10c. Inositol trisphosphate formation during heating in serum-free medium

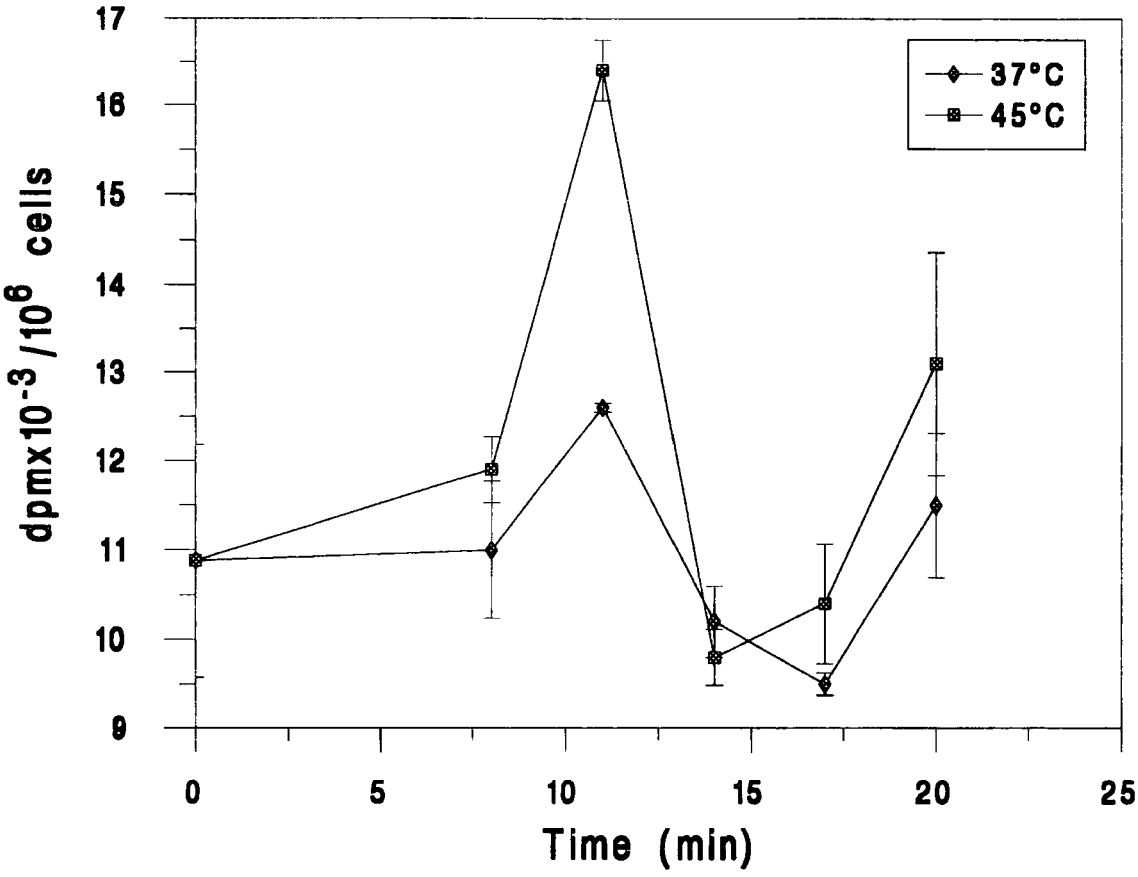


Figure 4.10d. $InsP_{4/5/6}$ formation during heating in serum-free medium

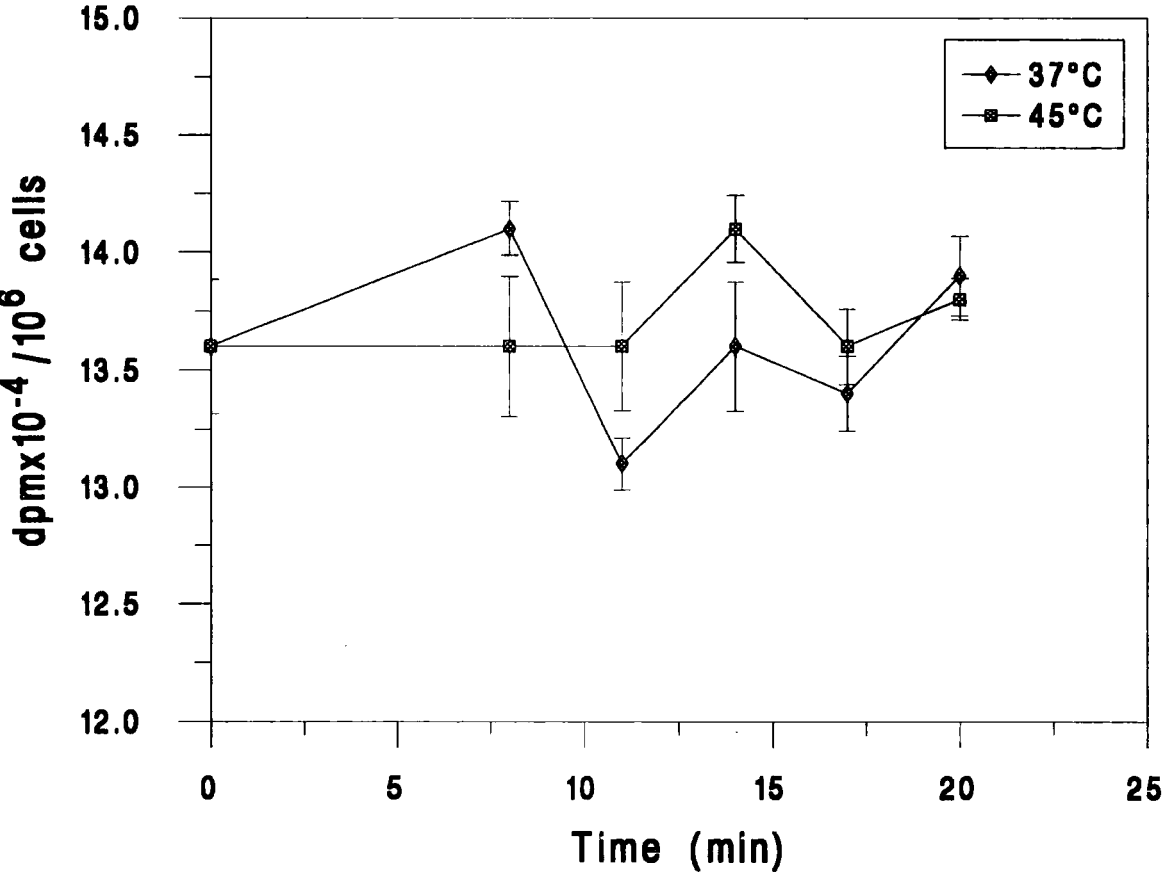


Figure 4.11a. Inositol monophosphate formation during heating in serum-free medium

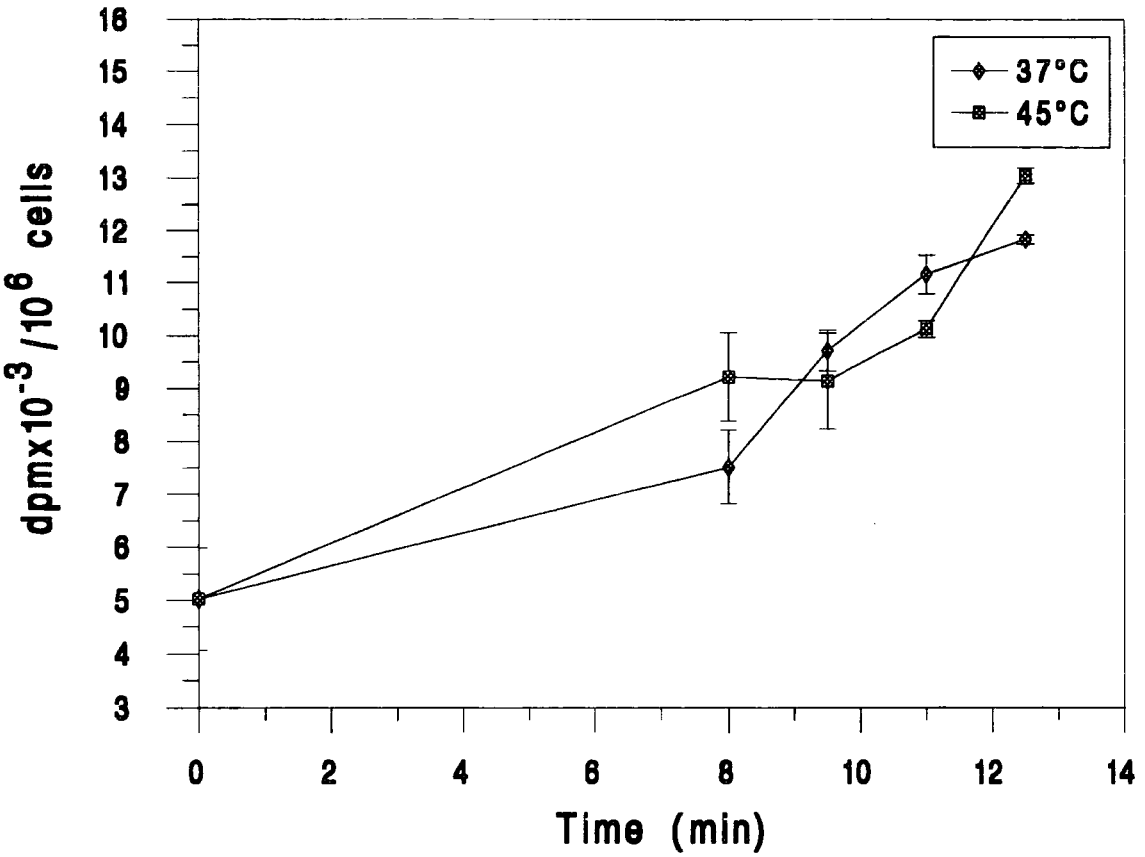


Figure 4.11b. Inositol bisphosphate formation during heating in serum-free medium

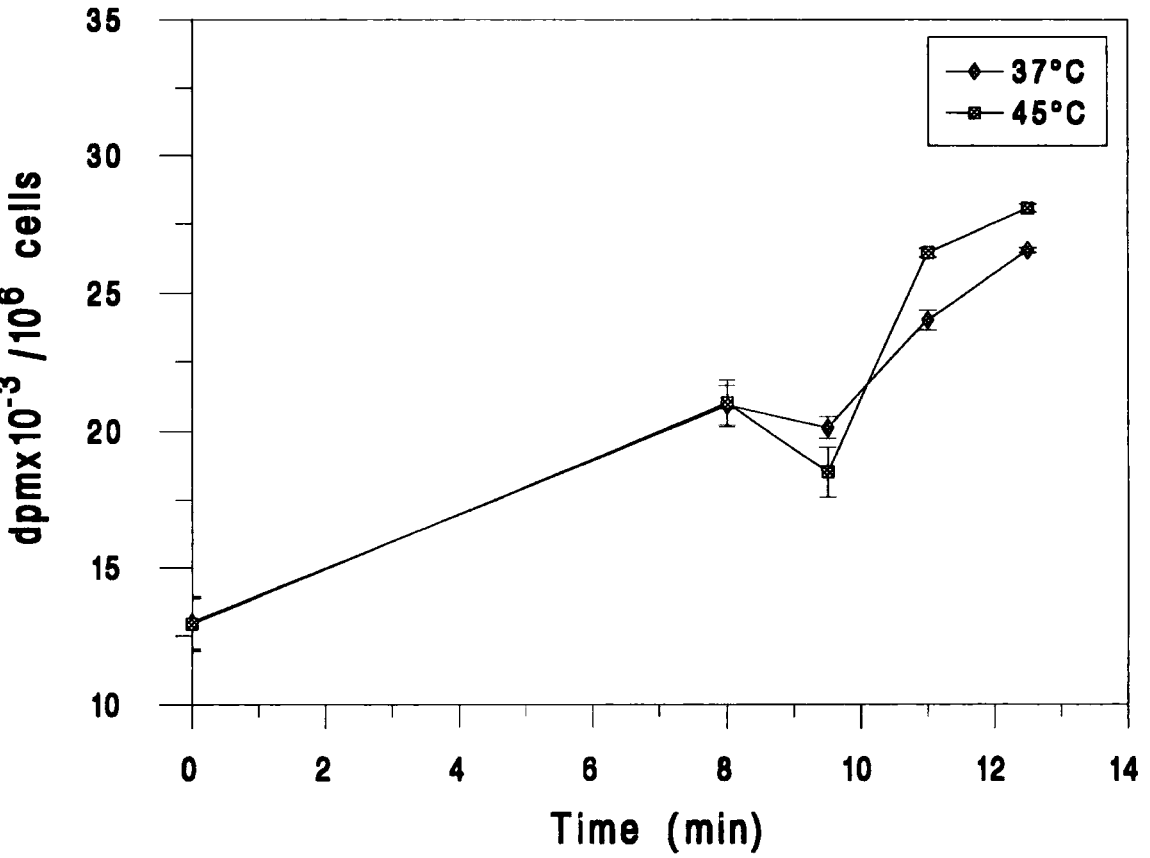


Figure 4.11c. Inositol trisphosphate formation during heating in serum-free medium

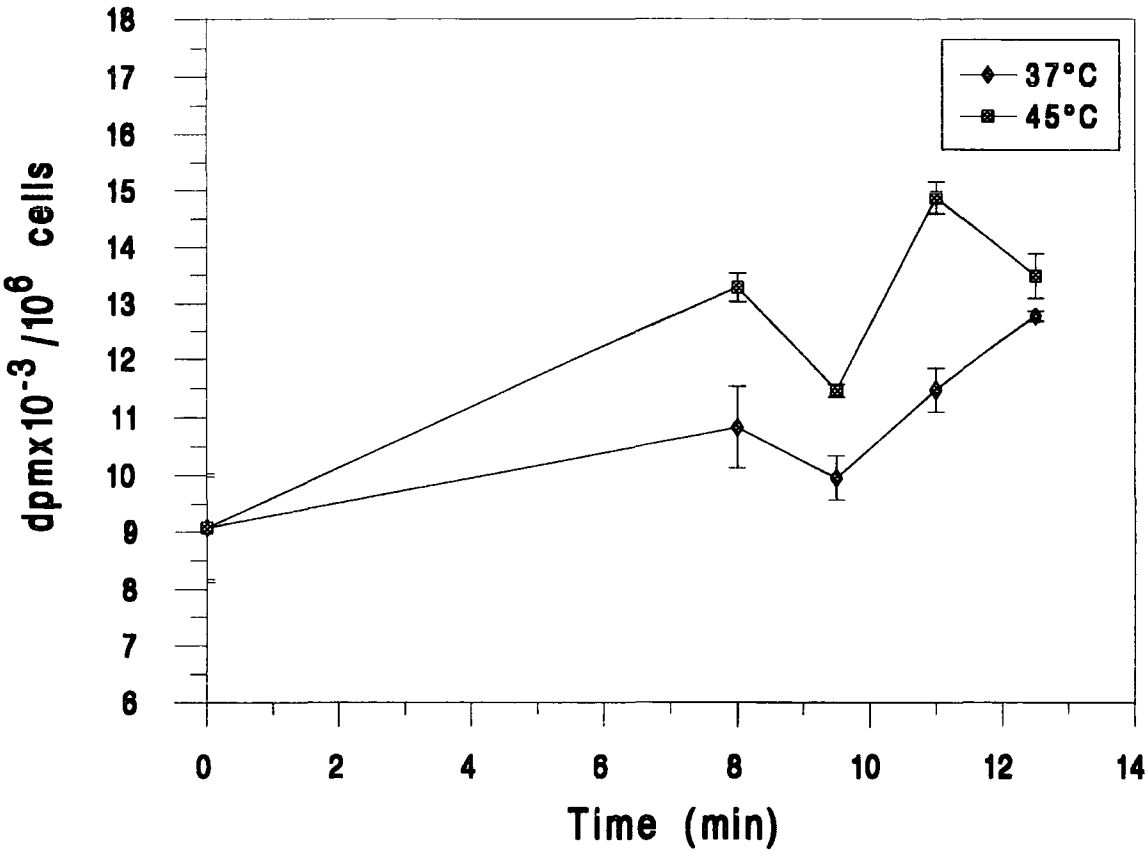
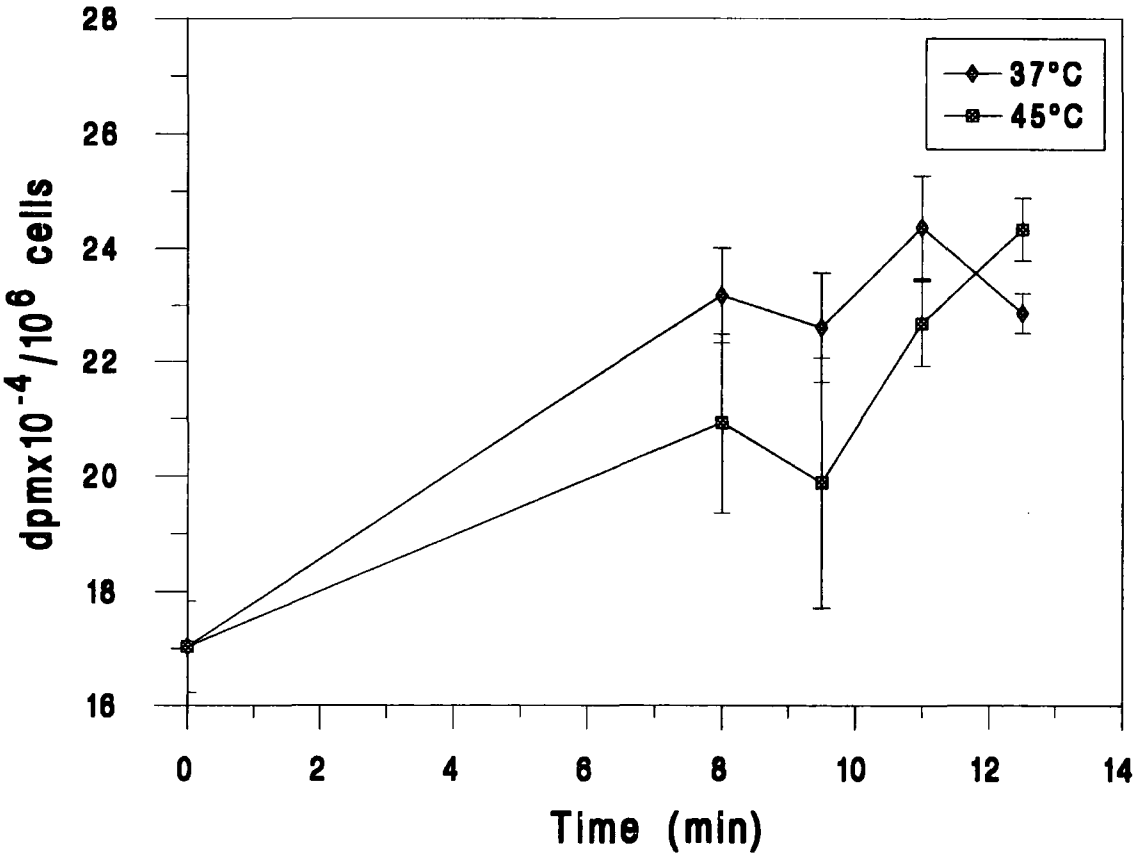


Figure 4.11d. InsP$_{4/5/6}$ formation during heating in serum-free medium



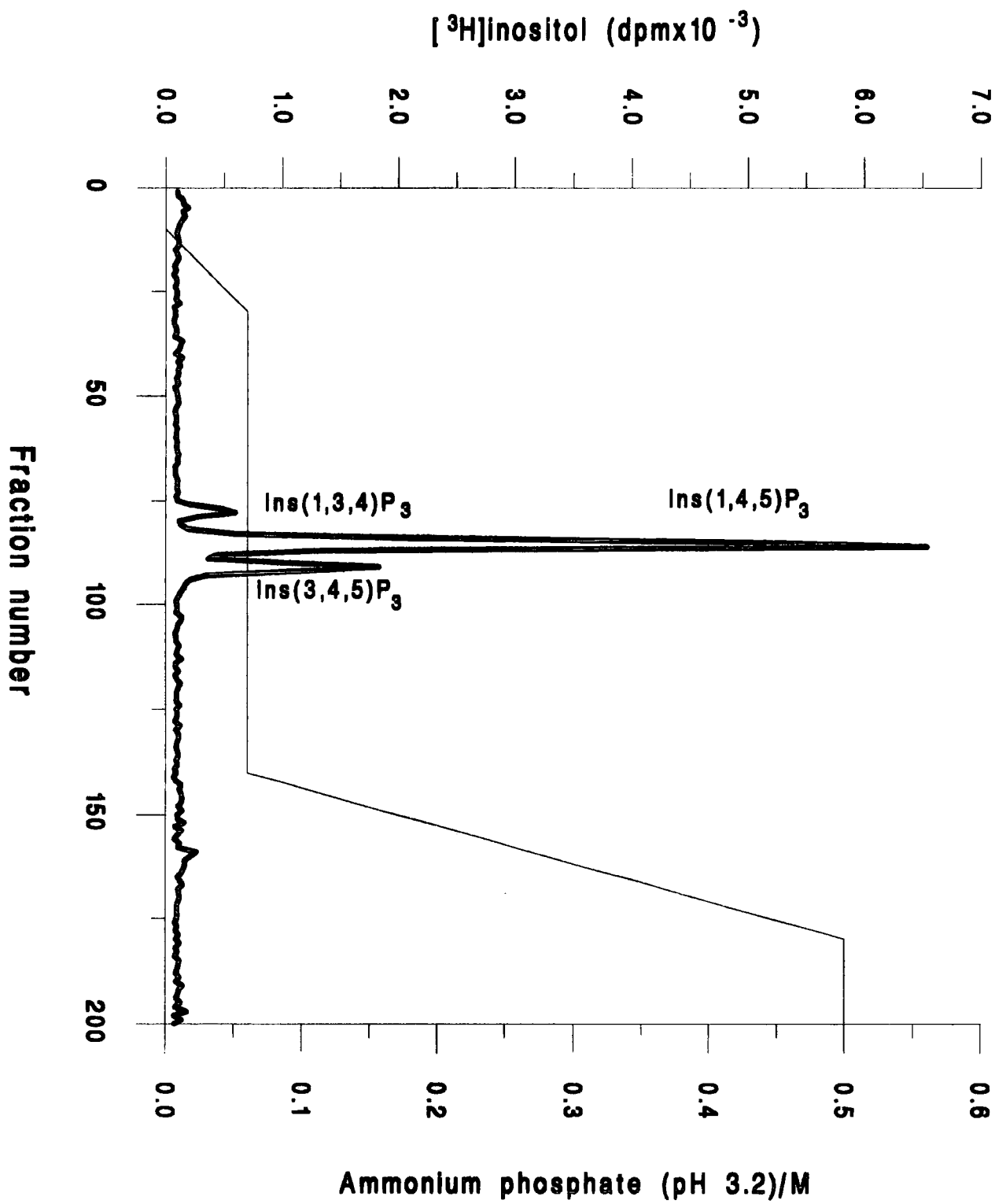

[3H]inositol (dpmx10 -3)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0
50
100
150
200
Fraction number
Ins(1,3,4)P3
Ins(1,4,5)P3
Ins(3,4,5)P3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Ammonium phosphate (pH 3.2)/M

Table 4.2. Separation of inositol trisphosphate isomers using HPLC

The values represent the total radioactivity in each inositol trisphosphate peak and correspond to the mean dpm count ± SEM from three independent measurements (n=3). Values were then analysed by Student's t-test.

isomer	unheated	heated	% of control
$Ins(1,3,4)P_3$	1359±156	1554±149	114
$Ins(1,4,5)P_3$	13521±1195	15112±2410	112
$Ins(3,4,5)P_3$	3869±235	4038±189	104
total $InsP_3$	18749±718	20704±2100	110

No significant difference was found between corresponding isomers from heated and unheated cells when analysed by Student's t-test.

Figure 4.13a. Effect of extracellular calcium on inositol monophosphate level at 45°C

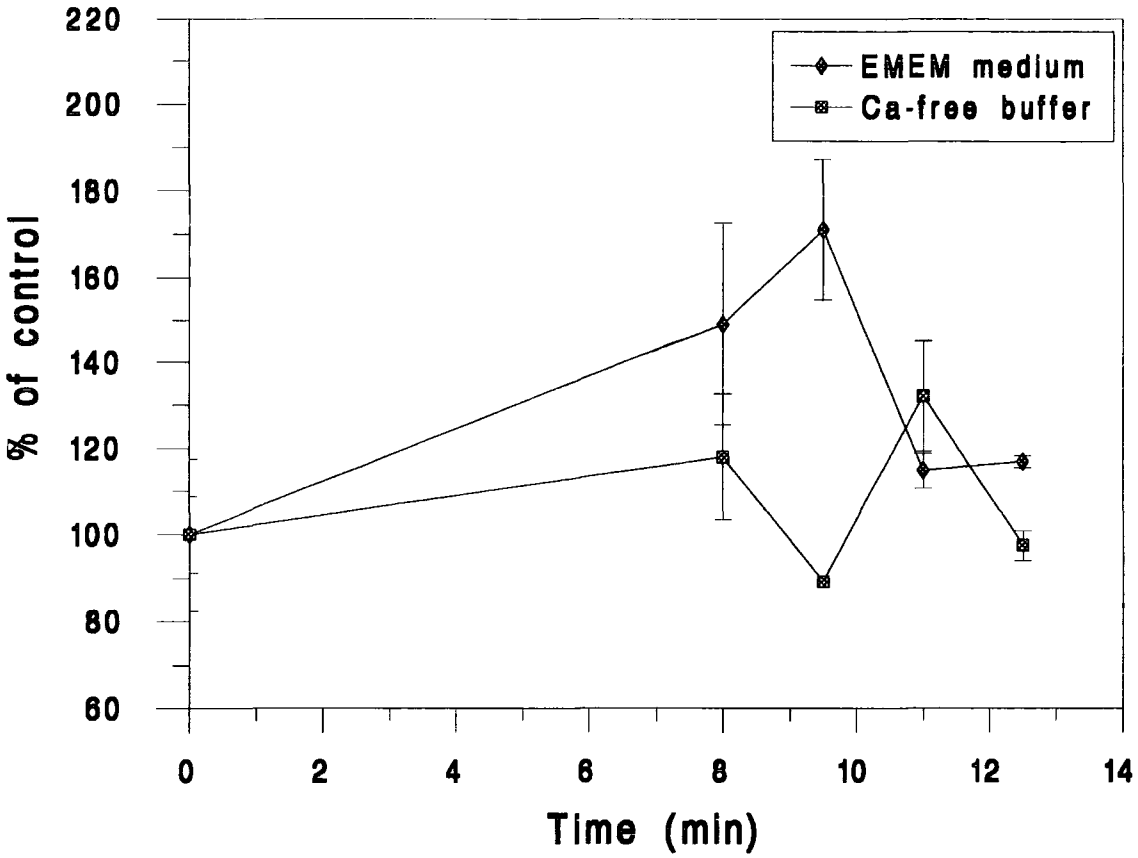


Figure 4.13b. Effect of extracellular calcium on inositol bisphosphate level at 45°C

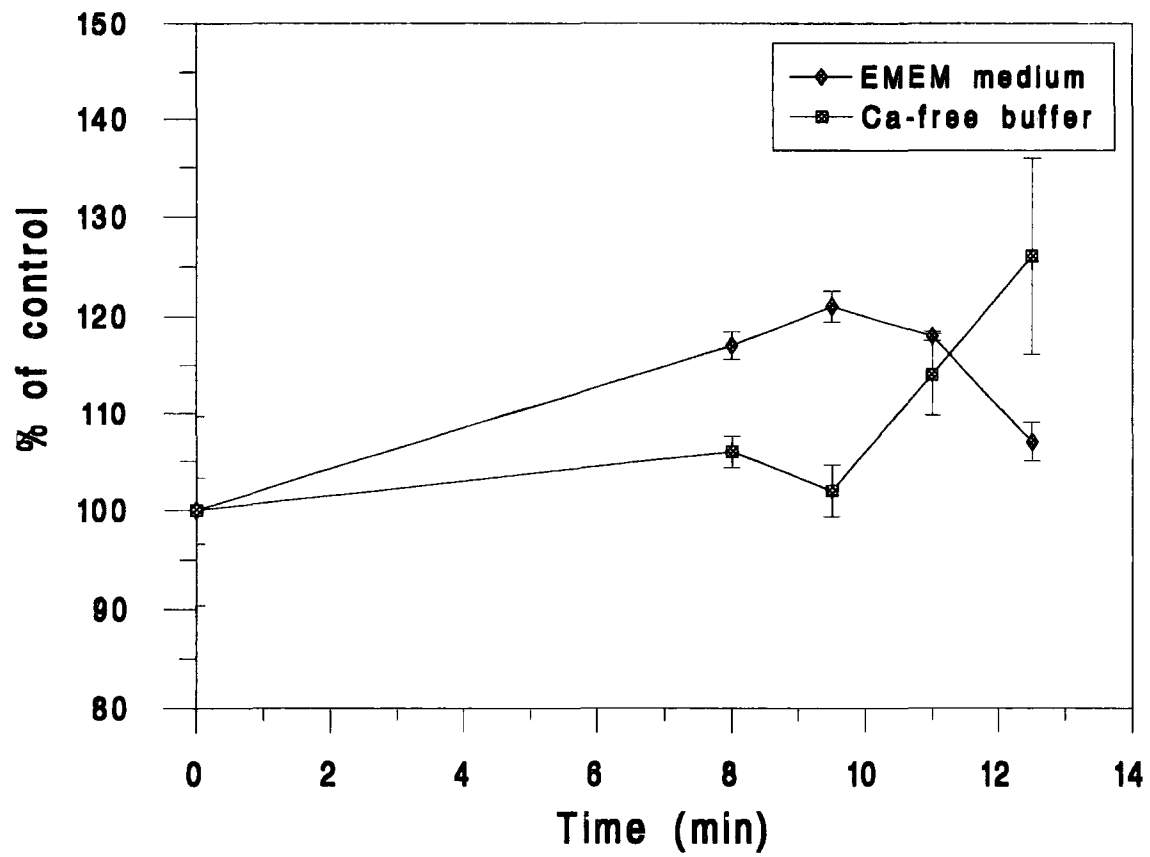


Figure 4.13c. Effect of extracellular calcium on inositol trisphosphate level at 45°C

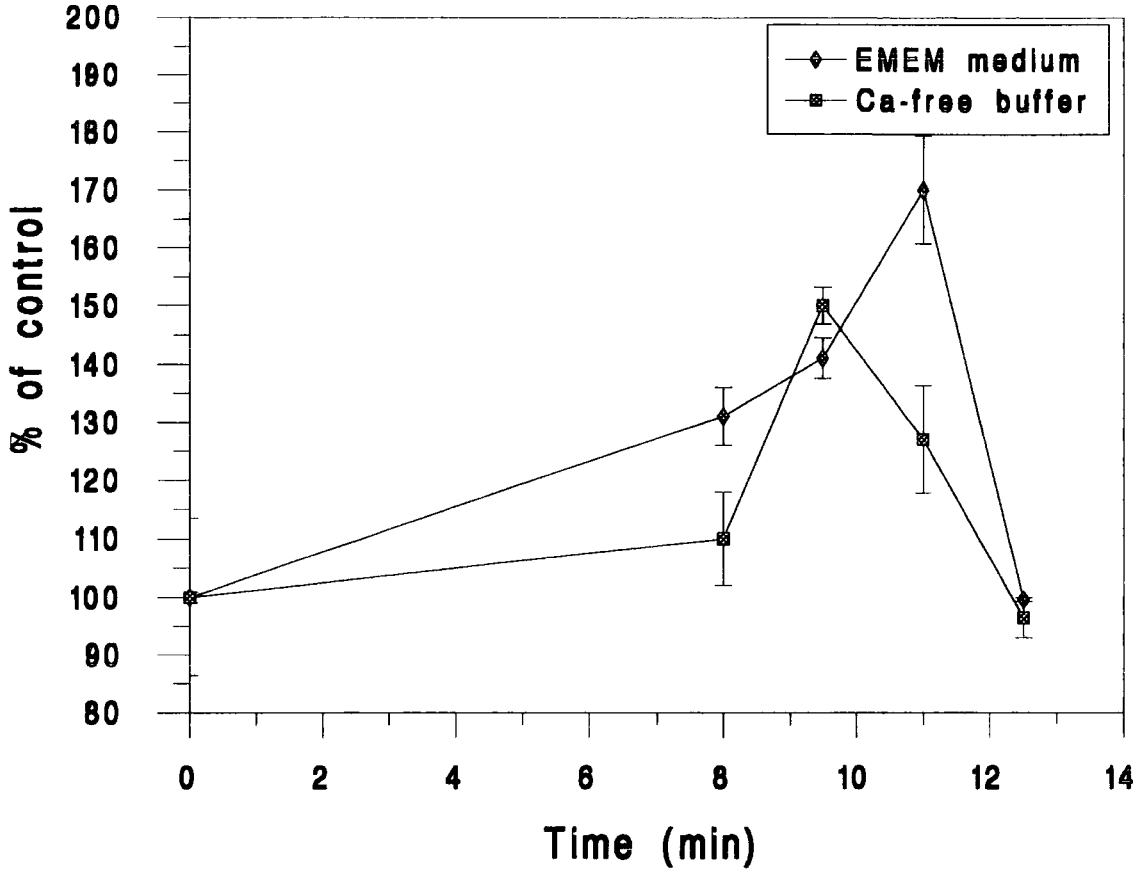
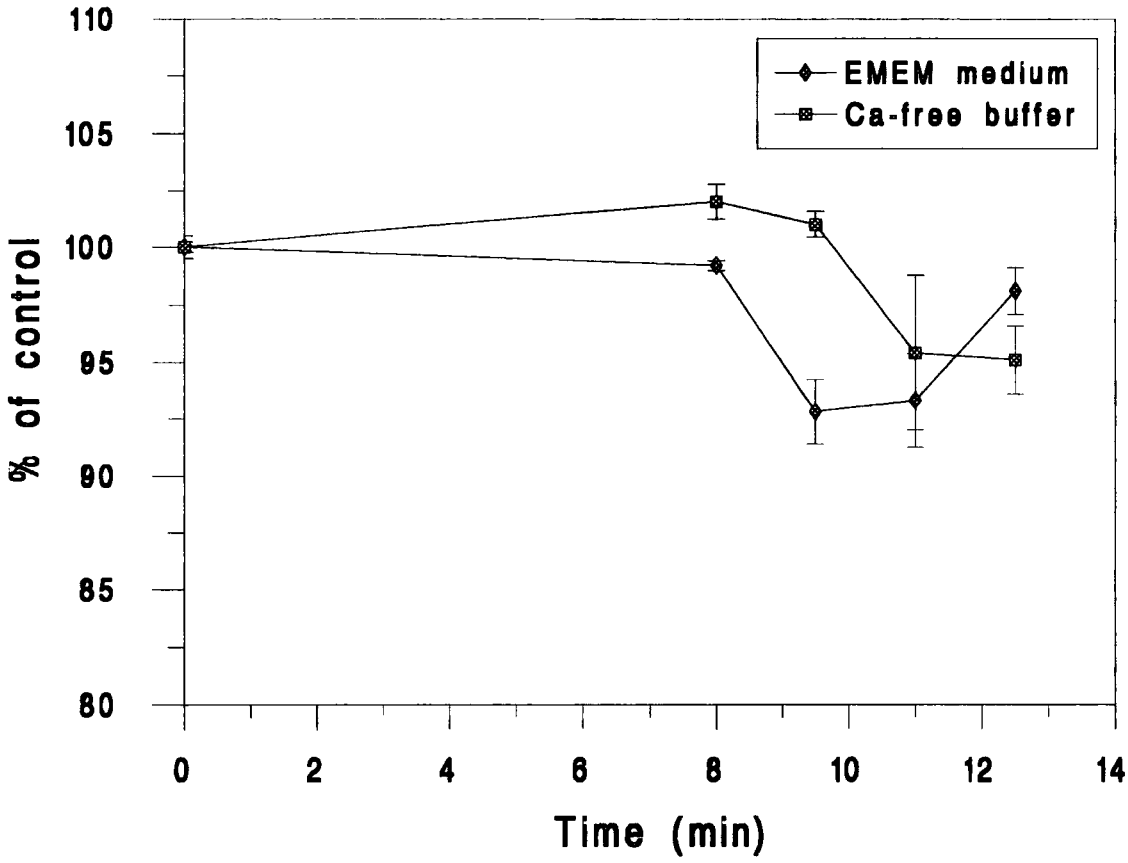


Figure 4.13d. Effect of extracellular calcium on $InsP_{4/5/6}$ level at 45°C



Treatment	$InsP_1$	$InsP_2$	$InsP_3$	$InsP_{4/5/6}$
EMEM (37°C)	1017±25.0	7621±131	8213±498	163128±5137
Ethanol (5%) (37°C)	873±125	8362±524	9933±937	160339±1188
sodium orthovanadate (50 µM) (37°C)	821±52.0	7657±312	8952±477	162791±1386
AlF_4^- (5 mM NaF, 10 µM $AlCl_3$)(37°C)	741±38.8	8203±389	9907±442*	159243±1166
EMEM (45°C)	939±52.6	8729±684	9912±385*	163747±5418
EMEM+7%sera (37°C)	1301±51.9	8014±789	10398±1622	173029±7844
EMEM+7%sera (45°C)	1114±14.2†	7154±81.7	17044±236*	180489±7088

Figure 4.14a. Formation of inositol monophosphate

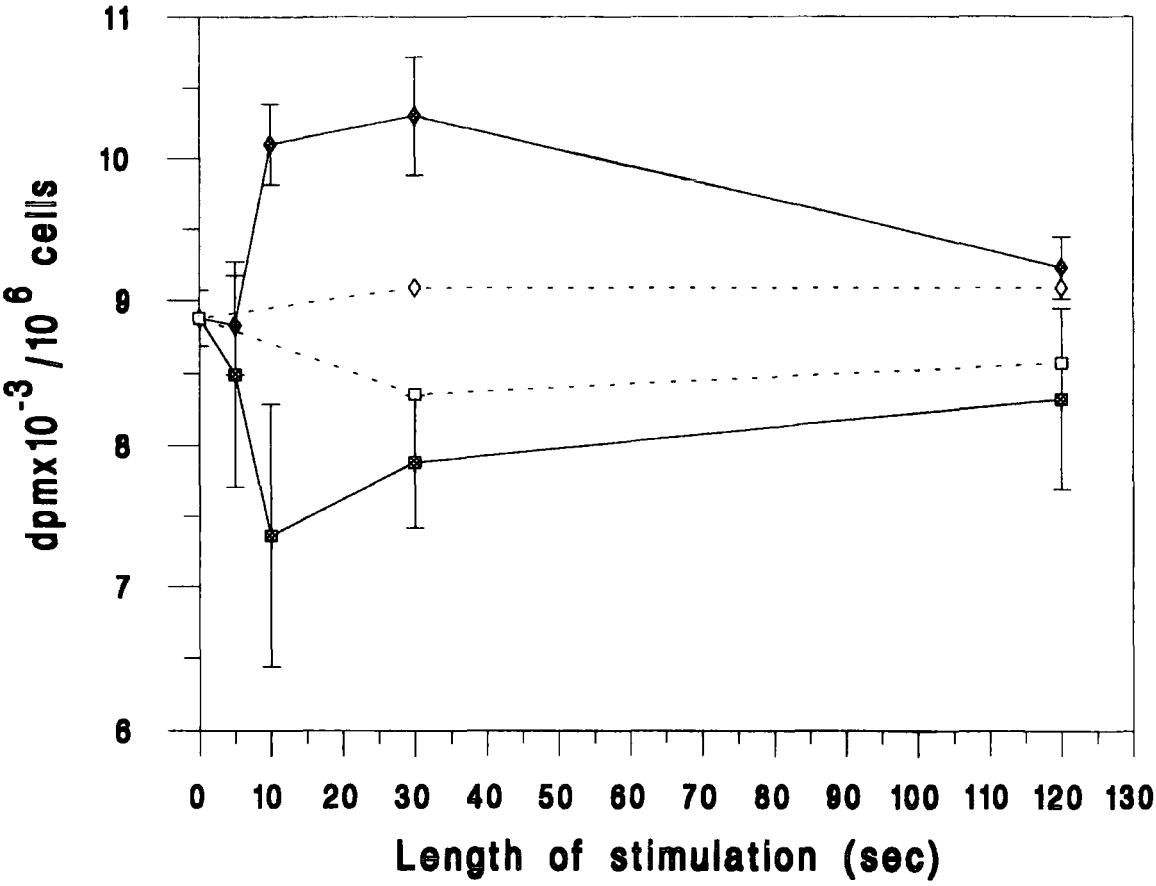


Figure 4.14b. Formation of inositol bisphosphate

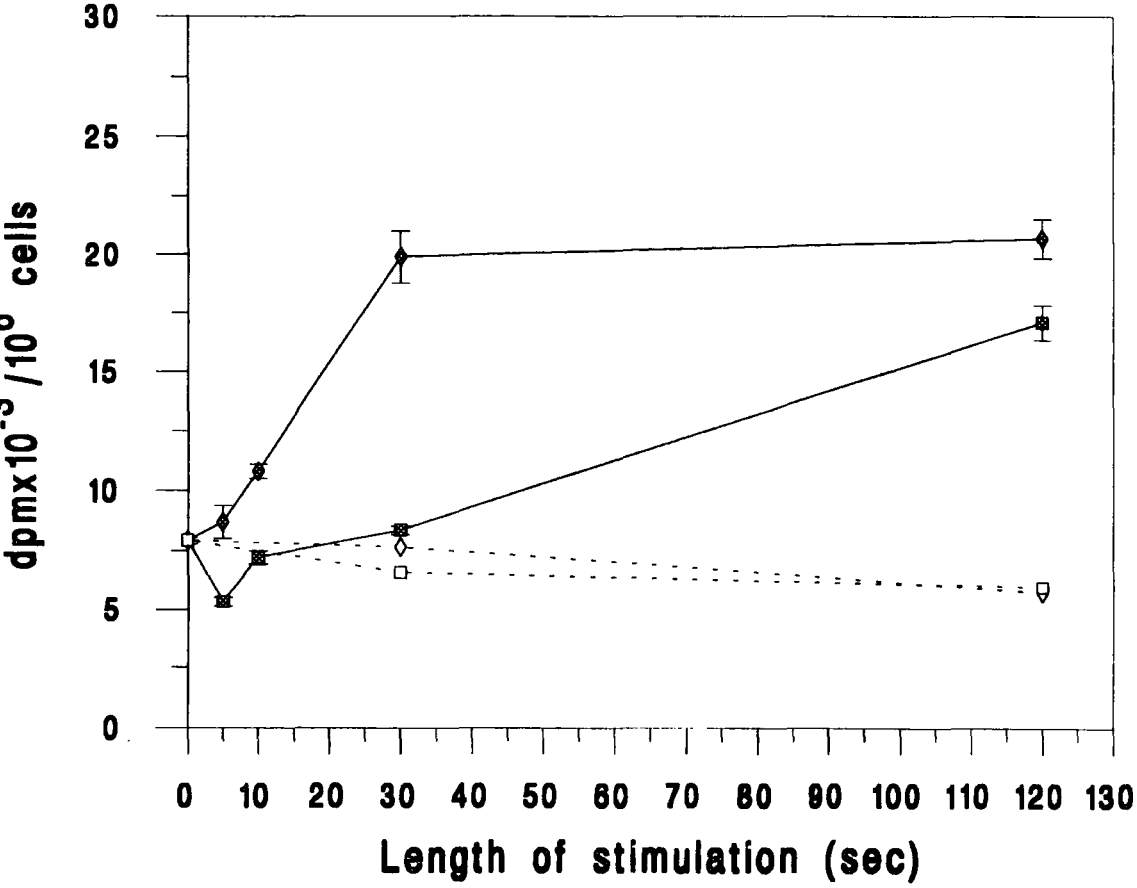


Figure 4.14c. Formation of inositol trisphosphate

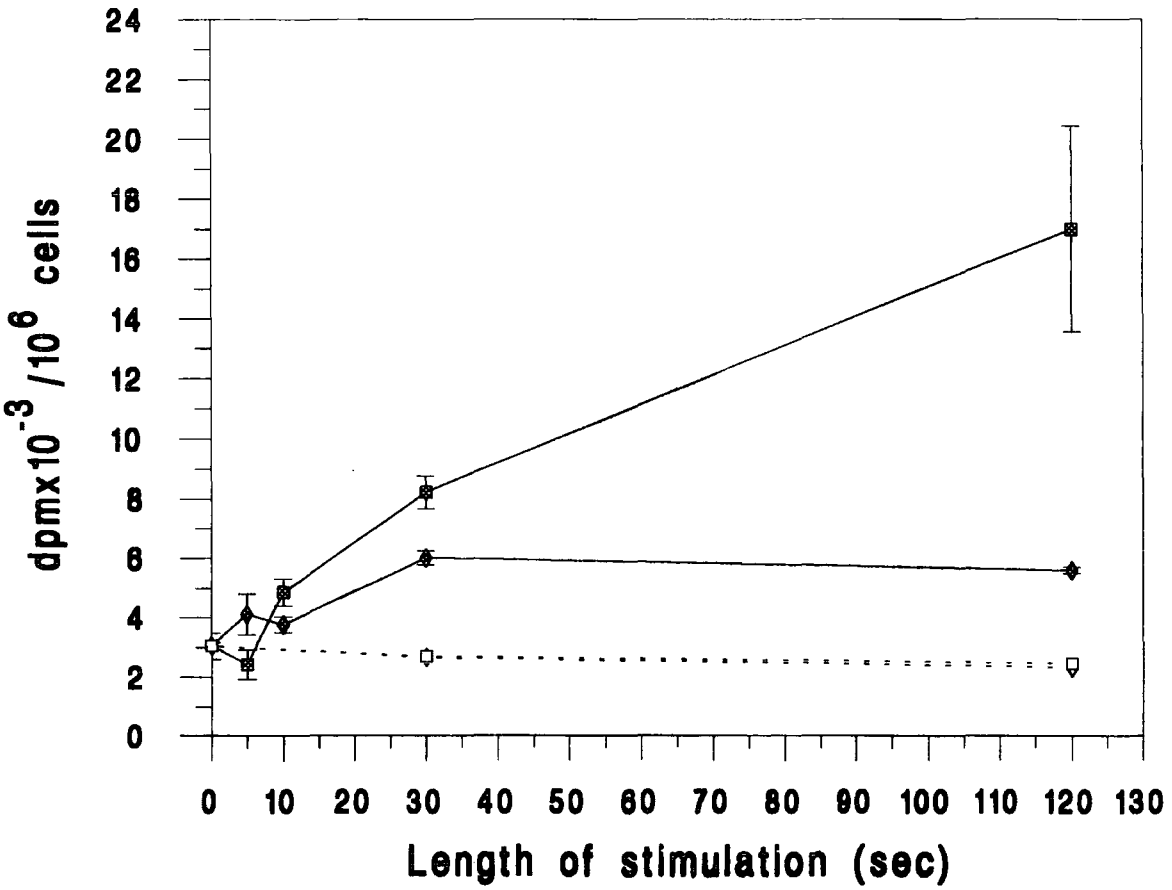


Figure 4.14d. Formation of $InsP_{4/5/6}$

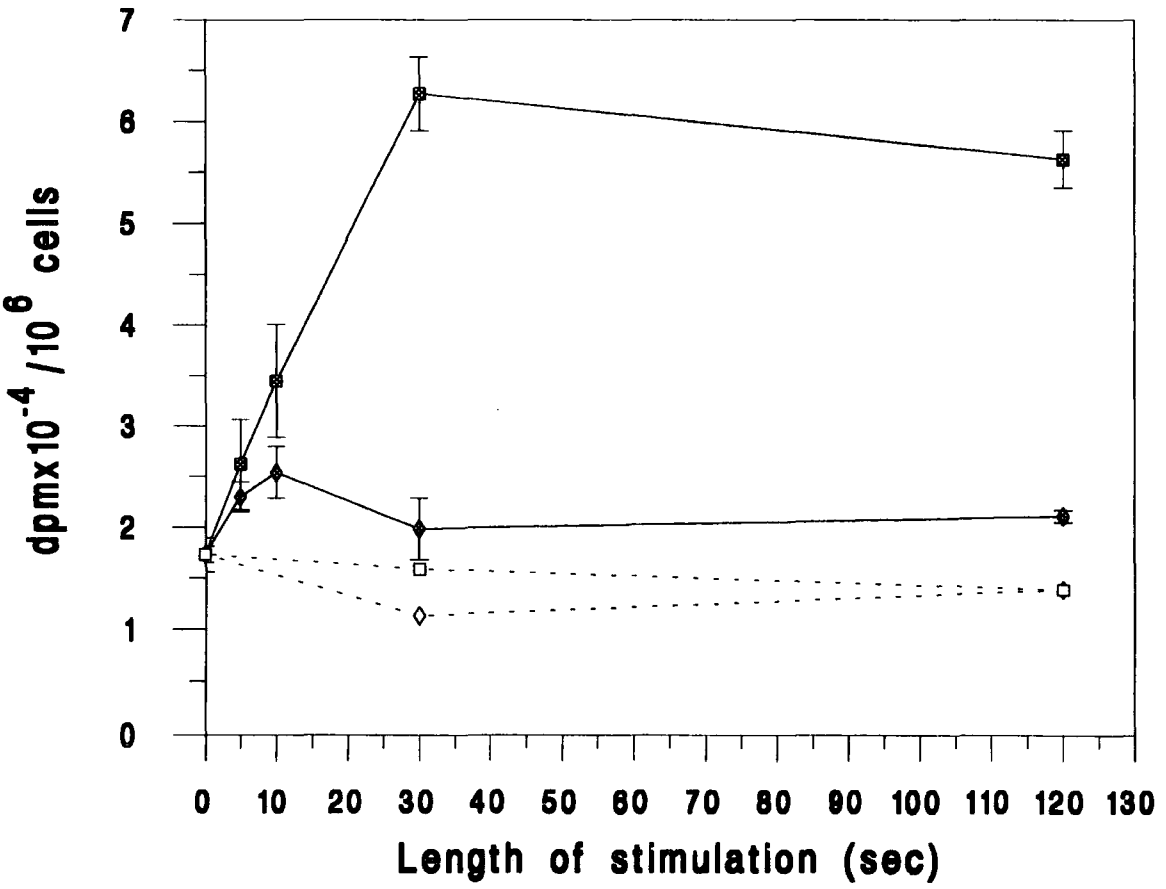


Figure 4.15a. Inositol monophosphate formation in heated WRK-1 cells upon vasopressin stimulation

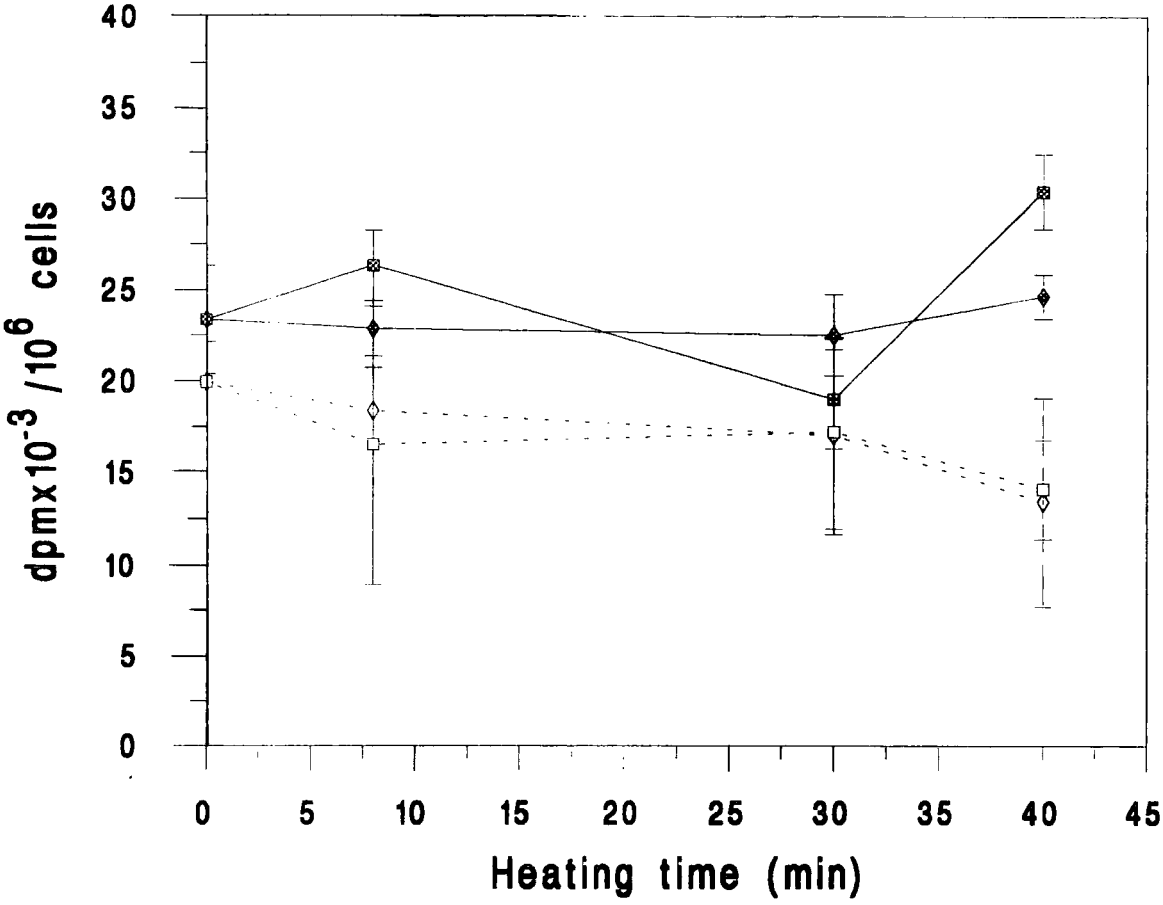


Figure 4.15b. Inositol bisphosphate formation in heated WRK-1 cells upon vasopressin stimulation

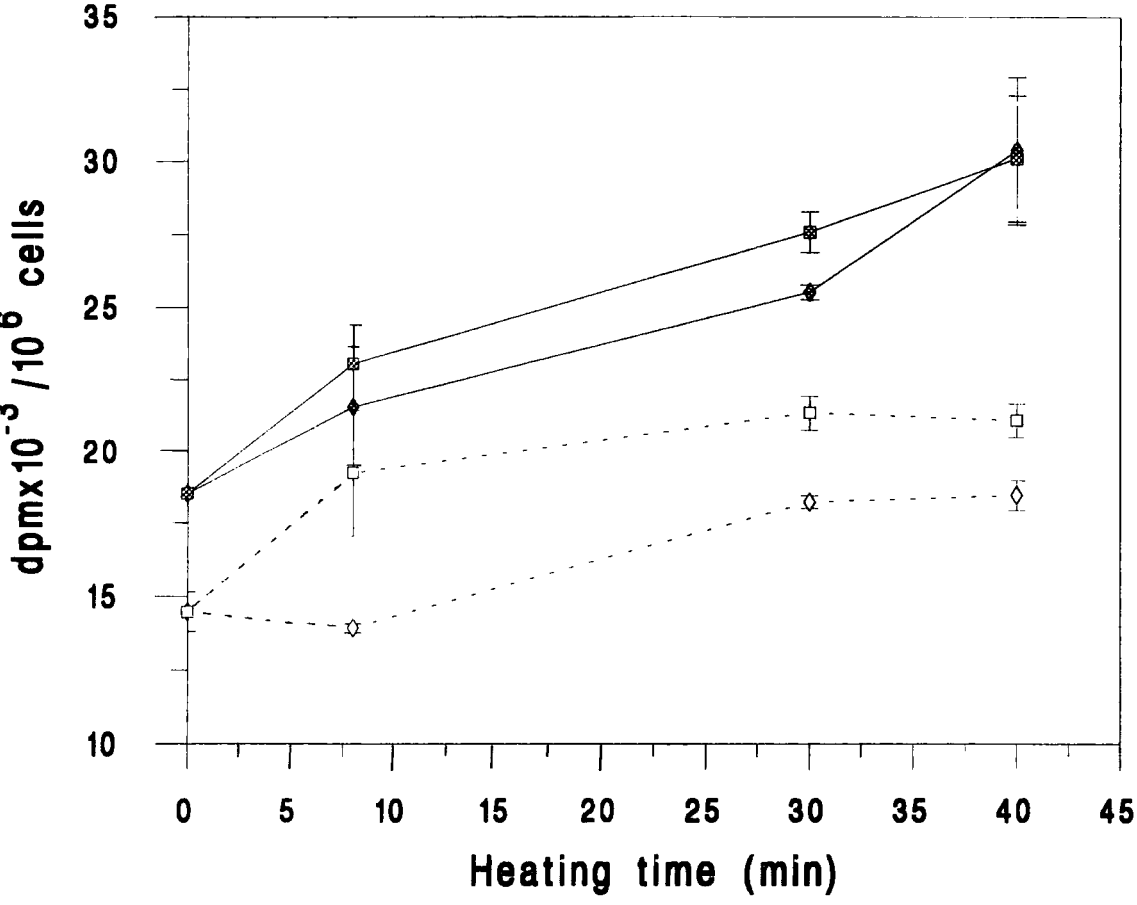


Figure 4.15c. Inositol trisphosphate formation in heated WRK-1 cells upon vasopressin stimulation

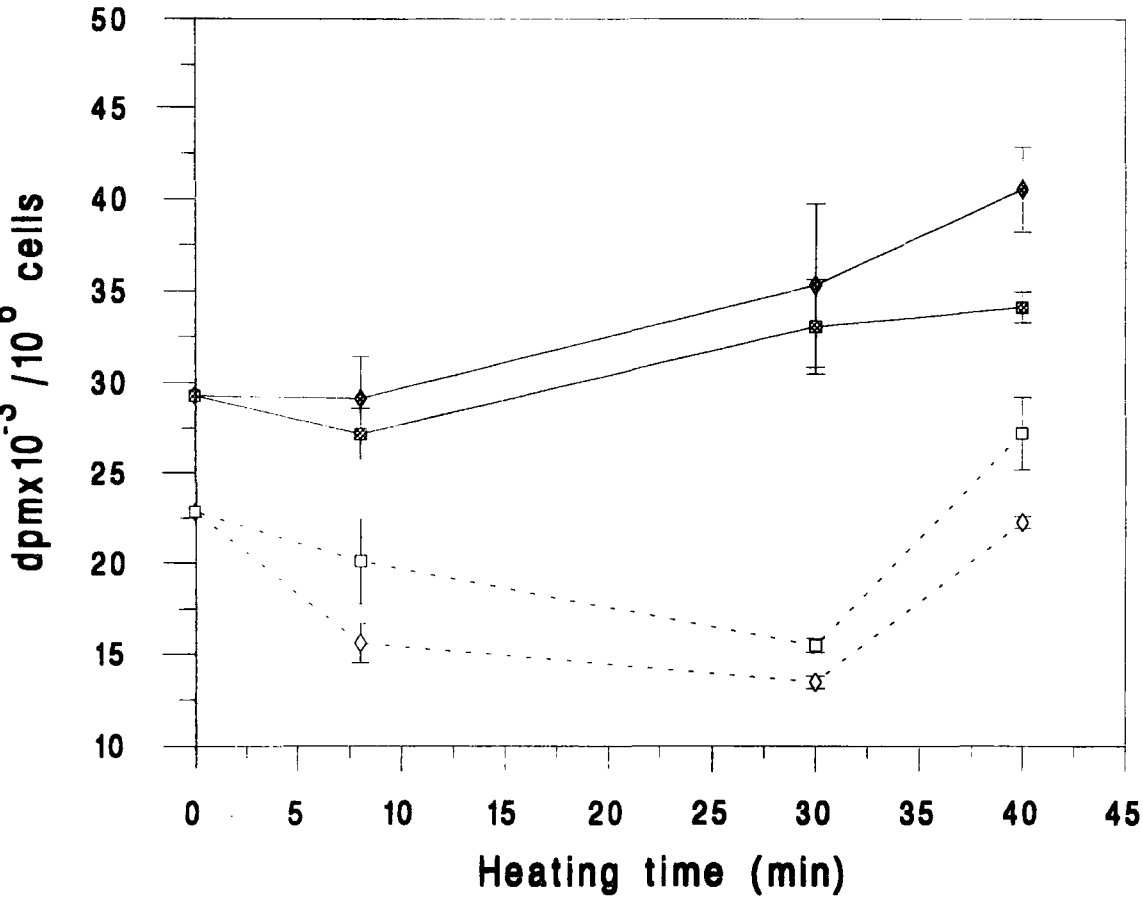
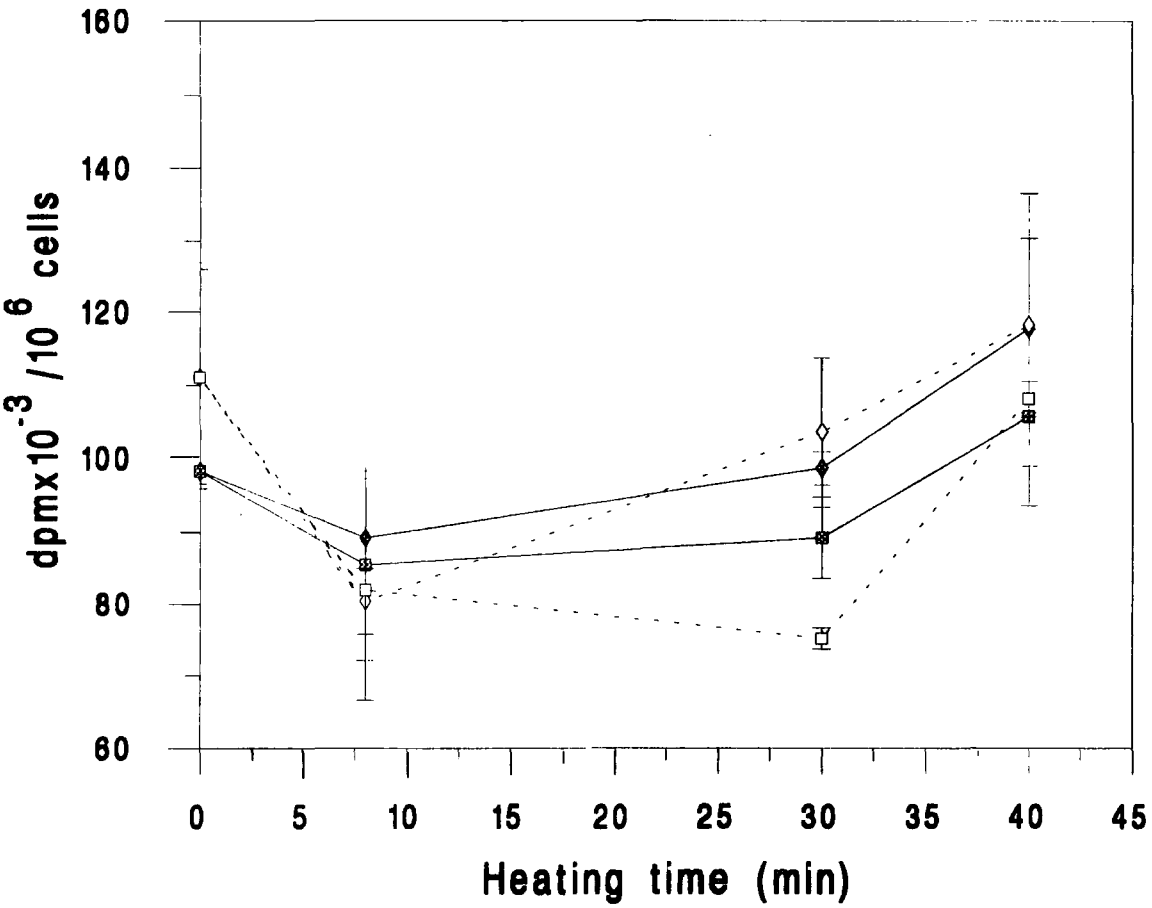


Figure 4.15d. $InsP_{4/5/6}$ formation in heated WRK-1 cells upon vasopressin stimulation



4.4 Discussion

The effect of heat on the phosphoinositide signalling pathway can be determined by measuring inositol lipids levels or by measuring the levels of its products such as inositol phosphates generated from hydrolysis of inositol lipids. In this study the effect of heat on this signalling pathway was investigated by measuring the levels of inositol phosphates generated from myo[2-^{3}H]inositol-labelled WRK-1 cells in both heated and unheated cells. The relatively small amount of inositol lipids that are present in membranes and the rapid turnover of inositol trisphosphate and its metabolites means that methods that can efficiently detect the changes are necessary. Radioactive isotopes such as [^{3}H]inositol and [^{32}P]orthophosphate have proved to be valuable tools for the study of this signalling pathway. In this way, the degradation of inositol lipids and the formation of inositol phosphates in the cell pre-labelled with the radioactive precursor can be followed. As long as the inositol lipid pools have been labelled to equilibrium, the changes in radioactivity reflect the changes in mass.

In order to maximise the incorporation of myo[2-^{3}H]inositol precursor into the inositol lipids, inositol-depleted medium should be used during labelling. Many cell types have been labelled in inositol-depleted medium. For example, labelling of WRK-1 cells was performed in medium containing 1 μM inositol, together with dialysed sera (Wong *et al.*, 1988), while CHO HA-1 cells were labelled in medium containing 5 μM inositol and 0.2% FBS (instead of 10% FBS in growth medium) (Calderwood and Stevenson, 1993). HL-60 cells were labelled with medium containing inositol (5 μM) and dialysed serum (French *et al.*, 1991). In the preliminary experiment of this study, the growth characteristics of WRK-1 cells in growth medium containing dialysed sera (made up in inositol-free EMEM medium), supplemented with 1 μM or 11 μM inositol were investigated. When cells were grown in medium containing 1 μM inositol, the cell number decreased after 3 days, whilst for cells grown in medium containing 11 μM

inositol, the cell density reached a plateau (at 8 x 10^5 cells) after 3 days and was maintained at about 8 -9 x 10^5 cells/well for up to 6 days. It has been shown previously that WRK-1 cells grown in low inositol medium (less than 0.7 μM inositol) resulted in ill-health after 3 days, even when the medium was changed on day 3 (Maccallum *et al.*, 1989). By using [U-^{3}H]-glucose, Maccallum and co-workers (1989) further demonstrated that at least 95% of the inositol used for lipid synthesis in these cells was obtained from exogenous sources. Since it was important to maintain cell growth during labelling procedures, it was decided that, in subsequent experiments, cells should be labelled with myo[2-^{3}H]inositol in the presence of 11 μM inositol and dialysed sera. This inositol concentration was sufficient to allow healthy cell growth, coupled with substantial labelling of inositol lipids and the related inositol phosphates, during a 4 day period of labelling.

The effect of heat on the phosphoinositide signalling pathway was investigated in WRK-1 cells. Due to the rapid turnover of Ins(1,4,5)P_3 and its metabolites, any newly formed inositol is uncounted for in such flux analysis. Thus 10 mM LiCl was added to all of the samples in this study to prevent complete dephosphorylation of inositol phosphates through the inhibition of Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase and inositol monophosphatase by Li^+ (Berridge *et al.*, 1989). When WRK-1 cells were heated at various temperatures (39°C-45°C) for 30 min in serum-containing medium, increases in the levels of ^{3}H-InsP_2 and ^{3}H-InsP_3 were observed. Furthermore, the magnitude of the increase in ^{3}H-InsP_2 and and ^{3}H-InsP_3 was temperature-dependent. Elevation in ^{3}H-InsP_2 was observed at 42°C and increased as temperature increased, to 124% of parallel control (30 min at 37°C) after 30 min at 45°C. On the other hand, the elevation in ^{3}H-InsP_3 was observed only in cells heated at 45°C, resulting in 141% of parallel control values (30 min at 37°C). No significant difference was observed in ^{3}H-InsP_1 and ^{3}H-Ins$P_{4/5/6}$ fractions in cells heated at various temperatures compared with the 37°C control. When cells were heated at 45°C for up to 45 min in serum-containing medium,

an increase in ^{3}H-InsP_2 level was observed at 5 min and the level increased as the heating duration increased, whereas no significant changes in any other inositol phosphate levels were observed. This could be due to a rapid turnover of inositol trisphosphate to its metabolites. This finding was consistent with the HPLC separation of inositol phosphates extracted from cells heated at 45°C for 30 min in serum-containing medium, in which a significant increase in Ins(1,3)P_2 and Ins(?)P_2 were observed ($p< 0.05$). The increase in the Ins(1,3)P_2 fraction could be explained as heat-induced increase in Ins(1,4,5)P_3, which was then phosphorylated to Ins(1,3,4,5)P_4 by 3-kinase, then dephosphorylated to Ins(1,3,4)P_3 followed by further dephosphorylation to Ins(1,3)P_2 and Ins(3,4)P_2. It is likely that the presence of 10 mM Li^+, being an inhibitor of the Ins(1,3,4)P_3 1-phosphatase, potentiated the accumulation of Ins(1,3)P_2 but suppressed the level of Ins(3,4)P_2, thus resulting in an increase in Ins(1,3)P_2. It has been shown in WRK-1 cells that the presence of 10 mM Li^+ led to an accumulation of Ins(1,3)P_2 accompanied by a suppression of Ins(3,4)P_2 levels upon 10 min vasopressin stimulation, compared with Ins(1,3)P_2 and Ins(3,4)P_2 levels obtained from cells challenged with vasopressin in the absence of Li^+. When 30 mM Li^+ was used, the level of Ins(1,3)P_2 increased further together with a further suppression in Ins(3,4)P_2 (Barker *et al.*, 1992). The results obtained from HPLC analysis also suggested that there was indeed a cross-over of InsP_5 into the InsP_4 fraction when analysed on simple anion exchange columns, as a relatively high dpm count was obtained in the 1.05 M ammonium formate/0.1 M formic acid eluate from these columns. Furthermore, HPLC analysis and results reported previously (Wong *et al.*, 1992) also suggested that InsP_5 accounted for the highest inositol phosphate level in WRK-1 cells. No significant differences were observed in other inositol phosphate isomers when analysed by HPLC.

It is possible that the addition of serum-containing medium prior to heating may trigger the hydrolysis of inositol lipids, which may perturb the fluxes of inositol phosphates during heating as well as distort the effect of heat on this signalling system.

To eliminate this possibility, labelled cells were heated in serum-free medium. By using serum-free medium, the response of these cells to vasopressin during and following heat treatment can be investigated without the effect of other agonists, present in serum, on the hydrolysis of inositol lipids prior to heat treatment.

When myo[2-^{3}H]inositol labelled WRK-1 cells were heated at 45°C in serum-free medium for up to 20 min, a transient increase in ^{3}H-InsP_3 was observed at 11 min with a level of 132% of control values, and this returned to the control level by 14 min. An increase in ^{3}H-InsP_2 level was observed after 8 min, with a peak at 11 min (at 133% of control level), then the level declined to 117% of control level and remained at this level for up to 20 min heating. On the other hand, ^{3}H-InsP_1 and ^{3}H-Ins$P_{4/5/6}$ levels were similar in heated and unheated cells throughout 20 min heating.

In summary, the heat-induced increases in InsP_2 and InsP_3 were temperature-dependent. Heating at 45°C for 30 min in serum-containing medium resulted in a significant increase in ^{3}H-Ins(1,3)P_2 and ^{3}H-Ins(?)P_2, but insignificant differences in other fractions were found. Heating at 45°C in serum-free medium resulted in a 30% transient increase in ^{3}H-InsP_3 at 11 min, whereas a sustained increase in ^{3}H-InsP_2 was observed over the 20 min heating period. The magnitude of the heat-induced increases in ^{3}H-InsP_1, ^{3}H-InsP_2 and ^{3}H-InsP_3 in the present study were lower than those reported previously in human epidermoid A-431 cells (Kiang and McClain, 1993) and in CHO HA-1 cells, HeLa cells, NIH3T3 cells, Balb C 3T3 cells and PC 12 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993).

The effect of heat on the phosphoinositide signalling pathway has been investigated in human epidermoid A-431 cells by Kiang and McClain (1993). A significant increase in levels of ^{3}H-InsP_1 and ^{3}H-InsP_3 were observed after heating at 42°C for 20 min, and higher temperatures induced further increases in InsP_1 and resulted

in 160% of control level after heating for 20 min at 45°C. An increase in $InsP_2$ was not detected unless cells were heated at 45°C for 20 min. Both $InsP_2$ and $InsP_3$ reached maximal levels after heating at 45°C for 20 min, resulting in 300% and 180% of control levels, respectively. The increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels also depended upon the duration of heating. When A-431 cells were heated at 45°C for various times up to 30 min, an increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels were observed after 10 min which reached maximal levels, i.e. 160%, 300% and 180% of control levels respectively, after 20 min heating. Then the levels of $InsP_1$, $InsP_2$ and $InsP_3$ declined and resulted in levels similar to the control at 30 min. On the other hand, a 3.6-fold decrease in Ins(1,3,4,5)P_4 level was detected in these cells after 20 min at 45°C (Kiang and McClain, 1993).

The effect of heat on the phosphoinositide signalling pathway has also been investigated in CHO HA-1 cells, HeLa cells, NIH3T3 cells, Balb C 3T3 cells and PC 12 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993). Upon heat treatment of CHO HA-1 cells at 42°C, a gradual increase in $InsP_3$ was observed, and the level increased to 5-fold the control value after 120 min heating, whilst heating at 45°C for 15 min resulted in a 6-fold increase in $InsP_3$. In HeLa cells, no alteration in $InsP_1$, $InsP_2$ and $InsP_3$ levels were observed when cells were heated below 41°C. When HeLa cells were heated at 45°C, a 40% increase in $InsP_1$ level was detected after 10 min heating and the level declined slightly on heating for longer periods, to levels about 30% greater than control values after 30 min. $InsP_2$ and $InsP_3$ levels increased as heating proceeded, reaching about 40% and 80% greater than control levels, respectively, after 30 min at 45°C. On the other hand, a transient increase in $InsP_3$ was detected in NIH3T3 cells upon heat treatment at 45°C, with a 9-fold increase in the $InsP_3$ level after 5 min, and the level returned rapidly to 2 to 3-fold of the control level by 10-15 min (Calderwood and Stevenson, 1993). A transient increase in $InsP_3$ level was also detected in Balb C 3T3 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993). The $InsP_3$ level of these cells reached 160% of the control level after 5 min heating at 45°C and decreased

slightly at 10 min, resulting in 140% of control level. Increases in $InsP_1$ and $InsP_2$ levels were also observed, which were 105% and 120%, respectively, of control levels. When PC 12 cells were heated at 45°C for 5 min, increases in $InsP_1$, $InsP_2$ and $InsP_3$ were observed, resulting in 120%, 180% and 140% of control values, respectively (Calderwood and Stevenson, 1989; Calderwood and Stevenson, 1993). None of the cell lines mentioned above showed an increase in $InsP_4$ level (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993).

A possible correlation between the heat-induced increase in $InsP_3$ and an increase in cytosolic free calcium ($[Ca^{2+}]_i$) has been suggested by Stevenson *et al.* (1986). When $[Ca^{2+}]_i$ was monitored in quin2-loaded CHO HA-1 cells during 45°C heat treatment, it was found that an increase in $InsP_3$ preceded the increase in $[Ca^{2+}]_i$ and was observed as early as 1 min heating. The source of the heat-induced increase in $[Ca^{2+}]_i$ was suggested to come from internal stores since heating in medium containing less than 1 μM extracellular calcium did not abolish this effect (Stevenson *et al.*, 1986). This result led to the proposal that heat induced an increase in $InsP_3$ (from $PtdIns(4,5)P_2$ hydrolysis) which then mobilised Ca^{2+} from an $InsP_3$-sensitive store, though the possibility that heat may perturb other intracellular stores was not ruled out (Stevenson *et al.*, 1986). Disruption of calcium homeostasis has been found to be the cause of cell injury during ischaemia and toxin treament (Farber, 1981) and the putative role of a heat-induced increase in $[Ca^{2+}]_i$ in hyperthermic cell death has drawn a lot of attention (Stevenson *et al.*, 1987; Vidair *et al.*, 1990; Mikkelsen *et al.*, 1991a; Stege *et al.*, 1993a,b). In order to investigate the effect of heat on the $[Ca^{2+}]_i$ level and its relation to hyperthermic cell death, the effect of heat on $[Ca^{2+}]_i$ in WRK-1 cells was investigated and the results are reported in Chapter 5.

The results reported by Calderwood and co-workers (Calderwood *et al.*, 1987; Calderwood *et al.*, 1988; Calderwood and Stevenson, 1993) (described above) did not

address the effect of heat on particular isomers of inositol phosphates. Given the complexity of the inositol phosphate fluxes, and the fact that only the Ins(1,4,5)P_3 isomer has significant calcium mobilising ability, the link between $InsP_3$ elevation and increases in $[Ca^{2+}]_i$ observed in heated cells will remain obscure unless the $InsP_3$ isomers are separated.

Consequently, the effect of heat on $InsP_3$ isomers was investigated in WRK-1 cells. $InsP_3$ levels were maximum in WRK-1 cells heated at 45°C for 11 min in serum-free medium, so total $InsP_3$ was isolated from these cells by simple anion exchange chromatography then separated into isomers by HPLC. Results suggested that in both heated and unheated cells, Ins(1,3,4)P_3 acounts for the smallest fraction in $InsP_3$. On the other hand, Ins(1,4,5)P_3 is the major isomer in both heated and unheated cells. The levels of Ins(1,3,4)P_3 and Ins(1,4,5)P_3 obtained in unheated cells in the present study was comparable to those reported previously in the same cell line (Wong *et al.*, 1988). Although the mean dpm in heated cells in all three isomers showed a higher level compared with unheated cells, the difference in the levels of any of the inositol trisphosphate isomers was insignificant when analyed by Student's t-test. More dramatic changes in $InsP_3$ isomers were observed in A-431 cells after 20 min heating at 45°C, in which a slight increase in Ins(1,3,4)P_3, a 2.4-fold increase in Ins(1,4,5)P_3 and a 3.6-fold reduction in Ins(1,3,4,5)P_4 were observed (Kiang and McClain, 1993).

In summary, the effect of heat on the phosphoinositide signalling pathway in WRK-1 cells was less dramatic than effects observed in several other mammalian cell lines (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993; Kiang and McClain, 1993). This differential effect of heat on this signalling pathway may possibly be related to the differential thermal sensitivity of different cell types since it was found that in CHO HA-1 cells, 15 min heating at 45°C did not result in any clonogenic cell death (Calderwood *et al.*, 1987). Similarly, cell death of A-431 cells was not observed until

cells were heated at 45°C for 10 min and by 30 min heating viability only decreased by 5-10% (examined by trypan blue exclusion or ethidium bromide-acridine orange staining) (Kiang *et al.*, 1990). In the case of WRK-1 cells, 20 min heating already resulted in 50% cell death (Figure 3.8).

The possible requirement for calcium in the production of $InsP_3$ from $PtdIns(4,5)P_2$ by activation of PLC is equivocal. For example, when hepatocytes were incubated in calcium-depleted buffer (together with EGTA) the vasopressin-induced inositol lipid hydrolysis was inhibited (Rhodes *et al.*, 1983). In rat glomerulosa cells, activation of corticortropin receptor activates adenylyl cyclase and stimulates calcium influx via L-type calcium channels. Simultaneously an accumulation of inositol phosphates was also observed in these cells. This observation led to the proposal that PLC was activated as a result of calcium influx since the activation of PLC was blocked by $CoCl_2$ (a calcium channel blocker) (Gallo-Payet and Payet, 1989). Alternatively, other workers found that a minimum level of $[Ca^{2+}]_i$ is necessary for hormone action and when $[Ca^{2+}]_i$ was reduced to that below the physiological resting level, the inositol lipid turnover is reduced (Creba *et al.*, 1983). On the other hand, results obtained by Renard *et al.* (1987) showed that when hepatocytes were treated with ionomycin, the $[Ca^{2+}]_i$ increased from 0.2 to 1 μM, but the PLC activity was unaffected. In WRK-1 cells, it has been shown that vasopressin-stimulated $InsP_3$ formation was unaltered in either calcium-containing or calcium-free conditions (Mouillac *et al.*, 1990).

In the present study, heat treatment (45°C) of WRK-1 cells either in the presence (EMEM medium) or absence of extracellular calcium (calcium free buffer together with 0.5 mM EGTA), resulted in a 50% increase in $InsP_3$ in both conditions, though the maximal $InsP_3$ level was achieved at different times i.e. at 9.5 min and 11 min in calcium-free and EMEM medium, respectively. The results imply that the heat-induced increase in $InsP_3$ in WRK-1 cells was independent of extracellular calcium, at least

within 12.5 min heat treatment at 45°C. This result is in contrast to those reported previously, in which it was found that heat-induced $InsP_3$ formation was inhibited when A-431 cells (Kiang and McClain, 1993) were heated in the absence of extracellular calcium. It has been proposed that $InsP_3$ accumulation in WRK-1 cells upon vasopressin-stimulation results from 2 mechanisms, a direct vasopressin-stimulated PLC activation which is independent of extracellular calcium followed by a calcium-mediated PLC activation which is related to $InsP_3$-induced intracellular calcium mobilisation (Mouillac *et al.*, 1990). Thus it may be possible that heat mimics the calcium-independent PLC activation observed in vasopressin-stimulated WRK-1 cells, producing a transient heat-induced increase in $InsP_3$ formation (within the 12.5 min heating time). However it is clear that further study is necessary to clarify this possibility.

The effect of agents such as ethanol, sodium orthovanadate and AlF_4^-, that have been shown to affect the components such as G proteins or PI-PLC of the phosphoinositide signalling pathway, was investigated in WRK-1 cells. Ethanol has been reported as a heat sensitiser, possibly by fluidising the plasma membrane (Li and Hahn, 1978). Ethanol has been shown to lead to activation of PI-PLC in hepatocytes, possibly due to the disordering effect of ethanol on the membrane, thus affecting the interaction of PLC with plasma membrane or by altering the intramembrane receptor-PLC coupling mechanism (Hoek *et al.*, 1987). Sodium orthovanadate has been shown to lead to $InsP_3$ formation in Chinese hamster lung fibroblast (CCL 39) cells (Paris and Pouysségur, 1987). AlF_4^- has been demonstrated to induce $InsP_3$ formation in hepatocytes (Blackmore *et al.*, 1985), parotid cells (Taylor *et al.*, 1986), CCL 39 cells (Paris and Pouysségur, 1987) and WRK-1 cell membranes (Guillon *et al.*, 1986a) and this effect of AlF_4^- has been proposed to result from activation of G proteins by this agent.

No significant difference in any of the inositol phosphate levels were found when cells were incubated in ethanol or sodium orthovanadate at 37°C for 30 min compared

with cells incubated in EMEM for the same time period. On the other hand, a significant increase in $InsP_3$ level was observed in cells heated at 45°C for 30 min or when cells were incubated in AlF_4^- at 37°C for 30 min compared with $InsP_3$ level in cells incubated in EMEM at 37°C for 30 min. The increase in $InsP_3$ in both conditions were similar in magnitude (121% of unheated control). However whether heating and AlF_4^- stimulation share a common mechanism or whether they exert their effect on different components of the signalling system remains unclear. It is clear that further investigation is necessary before any conclusions concerning the mechanisms of heat-induced inositol phosphate formation are drawn.

The precise role of the activation of the phosphoinositide signalling pathway during heating remains unclear. However, the transient increase in $InsP_3$ observed in WRK-1 cells may have a protective role in heat-induced cell death, possibly through the action of $InsP_3$-induced increase in $[Ca^{2+}]_i$ leading to induction of heat shock protein (HSP) production. The induction of heat-shock protein synthesis has been shown to play a role in protecting cells from thermal injury and it was shown that an increase in $[Ca^{2+}]_i$ is required for the binding of heat shock factor to heat shock element in the promoter region, and causes the expression of heat shock genes (Price and Calderwood, 1991). Evidence supporting the view that HSP synthesis has a protective role in hyperthermic cell death comes from study using thermoresistant variants of CHL cells (CL6 cells) which were found to have an elevated HSP28 level (Landry *et al.*, 1988). By transfection of human HSP70 to rat cells, it was found that the intermediate filament remained intact even after 30 min heating at 45°C and cells were more resistant to heat (Li *et al.*, 1991). Furthermore, it was found that the expression of HSP70 in A-431 cells required $InsP_3$-induced $[Ca^{2+}]_i$ elevation since the expression of this protein is attenuated by treatment with U-73122 (an inhibitor of $InsP_3$ production) (Kiang and McClain, 1992). To further probe the role of $[Ca^{2+}]_i$ in hyperthermic cell death, heat-induced changes in $[Ca^{2+}]_i$ in WRK-1 cells were analysed at single cell level by a fluorescent microscopic technique

and this work is reported in Chapter 5.

Many altered plasma membrane functions, induced by hyperthermia, are those which are presumably mediated in part, if not entirely, by the protein components of the membrane. For example it has been shown that binding of EGF to its receptor in Rat-1 fibroblasts was inhibited by heat, as a result of the reduction in the affinity of the receptor (Magun and Fennie, 1981). Hyperthermia also inhibits the binding of monoclonal antibody to histocompatibility antigens on the surface of murine lymphoma cells in suspension culture (Mehdi *et al.*, 1984). Insulin binding was decreased when CHO HA-1 cells were heated at 43-45°C, owing to the decrease in the number of cell-surface insulin receptors (Calderwood and Hahn, 1983). Thus, the effect of hyperthermia on membrane proteins may depend upon the cell types and the membrane protein in question. In order to investigate the possibility that heat may exert an effect on the receptor proteins, thus altering the response of the cells to the agonist, the response of WRK-1 cells to vasopressin at 45°C and response to vasopressin of cells pre-heated at 45°C for various times were investigated. Heat alone did not show any changes in inositol phosphate levels throughout 2 min compared with control levels (37°C without vasopressin). When cells were challenged with vasopressin for up to 2 min at 45°C, the magnitude of increase in $InsP_1$ and $InsP_2$ were much lower than those challenged at 37°C. On the other hand, higher $InsP_3$ and $InsP_{4/5/6}$ levels were observed in cells stimulated with vasopressin at 45°C. Although the exact mechanism that lead to a heat-induced increase in $InsP_3$ and $InsP_{4/5/6}$ levels during vasopressin stimulation at 45°C is unclear, several possibilities exist. (1) Provided that the heat dose is not sufficient to denature enzymes, the elevated temperature will increase the kinetic energy of reacting molecules, thus increasing enzyme activity. Thus, if PI-PLC is active under these conditions, heat will cause an increase in $Ins(1,4,5)P_3$ production. The metabolism of this compound is very complex and the level of a particular inositol phosphate metabolite will depend on the balance of competing metabolic pathways. Thus if the heat-

stimulated activity of the kinase for a particular isomer is higher than the activity of phosphatase for the same isomer, it is obvious that a higher level of the phosphorylated form of the isomer will be produced and this would explain why elevated $InsP_{4/5/6}$ levels were observed on heating. (2) It could be possible that heat (together with vasopressin) has a more pronounced stimulation on the formation of Ins(3,4,5,6)P_4. It has been reported that stimulation of WRK-1 cells with vasopressin resulted in the formation of 3 $InsP_4$ isomers, which were Ins(3,4,5,6)P_4, Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 (Barker *et al.*, 1988; Barker *et al.*, 1992). It is known that Ins(1,3,4,5)P_4 is formed by the phosphorylation of Ins(1,4,5)P_3 whereas it has been suggested that Ins(1,3,4,6)P_4 is a phosphorylated product of Ins(1,3,4)P_3 which is generated by dephosphorylation of Ins(1,3,4,5)P_4. On the other hand, the origin of the Ins(3,4,5,6)P_4 isomer is obscure, but it is the most abundant $InsP_4$ isomer in unstimulated WRK-1 cells and its level increase on vasopressin stimulation (Barker *et al.*, 1992), so it is possible that it becomes particularly abundant on heating in the presence of vasopressin. Since the isomers were not separated in this study, this possibility needs to be clarified. (3) Vasopressin can induce approximately 5 to 10-fold increase in $[Ca^{2+}]_i$ in WRK-1 cells (see Chapter 5) and heat also led to an increase in $[Ca^{2+}]_i$, possibly due to the thermal inactivation of some of the components that are involved in regulation of calcium homeostasis. Thus, it is possible that vasopressin stimulation at elevated temperature leads to an accumulation of cytosolic free Ca^{2+} which in turn activates Ins(1,4,5)P_3 3-kinase through the action of the calcium calmodulin complex (CaCaM), leading to elevated levels of Ins(1,3,4,5)P_4. Since the $InsP_4$ isomers were not separated in the present study, the above possibilities remain to be clarified.

When WRK-1 cells were heated at 45°C for up to 40 min, a heat dose that killed more than 95% of the cells measured by MTT assay, then challenged with vasopressin at 37°C, the formation of $InsP_3$ in heated cells was comparable to that in vasopressin-challenged control cells (maintained at 37°C throughout experiment). This result

suggests that the vasopressin-sensitive phosphoinositide signalling pathway remained intact following extreme heat treatment.

The exact mechanism of heat-induced inositol phosphate formation is unclear. In a recent study, it was found that a G protein may participate in the heat-induced increase in inositol phosphates (Calderwood and Stevenson, 1989; Calderwood *et al.*, 1993). When digitonin-permeabilised CHO HA-1 cells were heated at 45°C for 20 min in the absence of a non-hydrolysable GTP analog (GTPγS), the levels of $InsP_3$ were similar between heated and control cells. When cells were heated in the presence of 10^{-7} to 10^{-6} M GTPγS, about 50% increase in $InsP_3$ was observed in heated cells but not in cells incubated at 37°C. As the concentration of GTPγS was increased to 10^{-5} M, the $InsP_3$ level reached maximal values at both 37°C and 45°C, resulting in a 3-fold increase in the $InsP_3$ level obtained in the absence of GTPγS. (Calderwood and Stevenson, 1989; Calderwood *et al.*, 1993). These results suggested that the heat-induced increase in $InsP_3$ is guanyl nucleotide-dependent. Since G proteins can be classified as pertussis toxin (PTX)-sensitive or PTX-insensitive, the type of G protein that was involved in the heat-induced increase in inositol phosphates was studied in CHO HA-1 cells and NIH3T3 cells (Calderwood and Stevenson, 1989; Calderwood *et al.*, 1993). When either cell type was incubated in 200 ng of PTX for 3 h before cells were heated at 45°C for 20 min or challenged by thrombin for the same length of time, it was found that the presence of PTX significantly inhibited the release of inositol phosphates ($InsP_1$, $InsP_2$ and $InsP_3$) in thrombin-stimulated cells whilst the levels of inositol phosphates in heated cells were similar to those obtained from cells without pre-treatment with PTX. Thus the authors suggested that a PTX-insensitive G-protein (possibly G_q) is involved in the response of cells to heat treatment. However, in contrast to the results reported by Calderwood and Stevenson (1993), Kiang and McClain (1993) found that a PTX-sensitive G protein was involved in heat-induced formation of $InsP_3$ in A-431 cells. When A-431 cells were treated with 30 ng/ml of PTX for 24 h at 37°C, the basal level of $InsP_3$ increased, but the

level of $InsP_3$ did not increase any further after heating at 45°C for 10 min (Kiang and McClain, 1993).

On the basis of their observations, Calderwood and co-workers have suggested a possible mechanism of heat-induced changes in inositol phosphates and $[Ca^{2+}]_i$ (Stevenson *et al.*, 1986; Calderwood *et al.*, 1987; Calderwood *et al.*, 1988; Calderwood and Stevenson, 1993; Calderwood *et al.*, 1993). They suggest that heat may provide the activation energy required to stimulate GTP-GDP exchange of a G protein. Upon GTP binding, subunits of the G protein dissociate and the α-subunit then binds to and activates a PLC, likely to be PLCβ1 as this enzyme has been shown to be activated by G_q, and hydrolyses $PtdIns(4,5)P_2$, resulting in the formation of $InsP_3$ (Calderwood and Stevenson, 1993). Heat-induced elevation in $InsP_3$ may then release calcium from internal stores, leading to an elevation in $[Ca^{2+}]_i$, though the possibility that heat disturbed other mechanism that resulted in the release of calcium from internal store was not excluded by Calderwood and co-workers (Stevenson *et al.*, 1986; Calderwood *et al.*, 1988)

In summary, the present study showed that heat led to a transient increase in the $InsP_3$ level and a sustained increase in $InsP_2$ levels. This heat-induced increase in $InsP_3$ was comparable to those obtained when cells were incubated in AlF_4^-, an agent that is known to exert an effect on the G-protein component of the phosphoinositide signalling system. However, whether heat and AlF_4^- acted on the same component in phosphinositide signalling pathway is unknown and requires further investigation. Finally, the magnitude of the vasopressin-stimulated increase in $InsP_3$ in cells heated at 45°C for up to 40 min (a heat dose that killed more than 95% of the cells determined by MTT assay), was comparable to that in unheated cells, suggesting that the phosphoinositide signalling pathway (at least the one employed by vasopressin) remained intact. However it cannot be deduced from these experiments whether (1) the

action was confined to a discrete pool of phosphoinositides such as a hormone sensitive pool; (2) a G-protein is involved and, if so, whether it is pertussis-toxin sensitive or not; (3) single or multiple isoform(s) of PLC are involved and finally (4) the transient increase in $InsP_3$ is significant in terms of hyperthermic cell death.

Chapter 5

Changes in cytosolic free calcium during hyperthermia

5.1 Introduction

Elucidation of the role of Ca^{2+} as an intracellular messenger began over 100 years ago with the observation by Ringer (1883) that the contraction of cardiac muscle required the presence of extracellular calcium. It is now known that a change in cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) is involved in the regulation of a variety of cellular processes such as DNA replication and transcription, phospholipid turnover, regulation of cytoskeletal structure and modulation of the activities of some enzymes such as protein kinases, phospholipases and calcium-dependent proteases (Trump *et al.*, 1980; Orrenius *et al.*, 1989). In order to fulfil these roles, $[Ca^{2+}]_i$ must be tightly controlled.

In an animal cell, the value of $[Ca^{2+}]_i$ is about 0.1 μM whereas the concentration of free Ca^{2+} in the interstitial fluid is greater than 1 mM, so a large Ca^{2+} gradient exists between the extracellular fluid and the cytoplasmic space. There are at least 2 reasons for maintaining $[Ca^{2+}]_i$ at such a low value. Firstly, by keeping the $[Ca^{2+}]_i$ low, only a few ions are sufficient to produce a significant fluctuation in $[Ca^{2+}]_i$. Secondly, as phosphorylated compounds are continuously degraded to liberate energy, and resynthesized to store it, a significant concentration of inorganic phosphate is always present in the cell. If $[Ca^{2+}]_i$ is high, phosphate and Ca^{2+} will combine to form a precipitate of calcium phosphate. By keeping the $[Ca^{2+}]_i$ low, the cell is able to make use of phosphate-containing compounds, such as ATP, as metabolic fuels.

Cells have a network of channels and transporter proteins in membranes in order to maintain $[Ca^{2+}]_i$ and keep a constant concentration of free Ca^{2+} in intracellular stores (Nicholls, 1986; Carafoli, 1987) (Figure 5.1). The mechanisms work mainly by controlling the movement of Ca^{2+} across 3 membranes: (a) the plasma membrane, which surrounds the cell, (b) the inner membrane of mitochondria, and (c) the membranes of compartment(s) that contain reserves of Ca^{2+} ions, such as SR in muscle and ER (or calciosomes) in non-muscle cells. In the resting cell, the maintenance of the concentration gradient depends on 2 features of the plasma membrane: its low permeability to Ca^{2+} and the presence of membrane-bound pumps that drive Ca^{2+} out of the cell against the concentration gradient. Basal calcium inflow through the plasma membrane can occur via non-specific leaks or by facilitated diffusion, which is thought to occur via a specific calcium channel protein and the rate of Ca^{2+} influx is similar to the rate of Ca^{2+} being pumped out.

When a cell is stimulated by certain extracellular signals, channels in the plasma membrane open and allow Ca^{2+} (but no other ions) to flow into the cell cytosol. In addition, Ca^{2+} can be released from the ER upon $Ins(1,4,5)P_3$ binding to its receptor which is present in the ER membrane. Ca^{2+} influx across the plasma membrane is facilitated by voltage-gated calcium channels or by receptor-operated calcium channels.

Using the patch clamp technique, 4 different types of voltage-gated calcium channels, namely L-type, T-type, N-type and P-type, have been identified based on the changes in membrane potential required to induce opening of the channel, the rate of inactivation of the channel, and the sensitivity of channels to dihydropyridines and ω-conotoxin (Meldolesi and Pozzan, 1987). L-type channel are opened by strong depolarisation, and are inhibited by dihydropyridines such as nifedipine or activated by dihydropyridines such as Bay K 8644. This type of channel is the predominant voltage-gated calcium channel present in cardiac and skeletal muscle cells (McCleskey *et al.*,

1987). T-type channels are activated by weak depolarisation and are relatively insensitive to dihydropyridines. They are found in GH_3 cells, heart, skeletal and smooth muscle and chick dorsal root ganglion neurons (Carbone and Lux, 1984; Suzuki and Yishioka, 1987). N-type channels are activated by relatively strong depolarisation. They are inhibited by ω-conotoxin but are insensitive to dihydropyridines. They are found in chick dorsal root ganglion neurons and in rat sympathetic neurons (Tsien, 1990). P-type channels are activated by medium depolarisation. They are inhibited by funnel web spider toxin and are insensitive to both dihydropyridines and ω-conotoxin (Llinas *et al.*, 1989). The movement of Ca^{2+} through voltage-gated Ca^{2+} channels is also inhibited by La^{3+} and divalent metal ions (Hallet *et al.*, 1990).

Receptor-operated calcium channels can be classified into 2 types depending on their nature of activation. In the first type, called ionotropic receptors, the calcium channel is an integral part of the receptor protein itself. This channel is opened by the binding of agonist to the receptor which is followed by a conformational change of the receptor protein, which opens the channel and allows Ca^{2+} influx. An example of this type is the nicotine acetylcholine receptor. Although this receptor is responsible for Na^+ influx, it is also considerably calcium permeable. The second type of channel is called a metabotropic receptor and is activated by a second messenger generated within the cells such as calcium and inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$). This type of channel is also known a second-messenger operated calcium channel (Meldolesi and Pozzan, 1987).

Restoration of the $[Ca^{2+}]_i$ to its resting level following stimulation is achieved by Ca^{2+} extrusion out of the cell or Ca^{2+} uptake by intracellular organelles. In the plasma membrane, Ca^{2+} extrusion is carried out by the Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger. The ER also has a Ca^{2+}-ATPase system which is responsible for Ca^{2+} reuptake after agonist-stimulated Ca^{2+} release. In the resting state, Ca^{2+}-ATPase present

in the ER and in the plasma membrane are both responsible for the maintenance of $[Ca^{2+}]_i$ at the basal level of 0.1 μM, as both systems have a K_m value for Ca^{2+} at about 0.2 μM.

The Ca^{2+}-ATPase in the plasma membrane catalyses the exchange of 1 Ca^{2+} (out) for 2 H^+ ions. The energy required to move Ca^{2+} out against the concentration gradient is derived from ATP hydrolysis with Mg^{2+} as cofactor. Ca^{2+}-ATPase is found in all cell types and is activated by the calcium-calmodulin complex. This ATP-driven calcium-pump is a high affinity, low capacity calcium pumping system which responds to a minute increase in intracellular calcium and is responsible for maintaining the $[Ca^{2+}]_i$ at rest.

The Na^+/Ca^{2+} exchanger is found particularly in excitable plasma membranes such as those in nerve and heart cells (Carafoli, 1987). It is also found in non-excitable tissues such as endocrine tissues (Herchuelz *et al.*, 1980) and epithelial cells (Lee *et al.*, 1980). In this system, each Ca^{2+} ion moves out of the cell in exchange for the entry of 3 Na^+ ions. The energy for pumping Ca^{2+} to the exterior is derived from the inwardly directed concentration gradient of Na^+ which is established by the action of Na^+-K^+-ATPase.

For a long time, mitochondria have been regarded as important organelles in the storage and mobilisation of Ca^{2+}, but it is likely that the importance of mitochondria as a cytosolic Ca^{2+} buffer system has been overestimated under physiological conditions (Irvine, 1986; Carafoli, 1987). The reason for this conclusion is based on the following observations. Firstly, it has been shown that when hepatocytes are incubated in elevated levels of extracellular Ca^{2+}, mitochondria continue to absorb this ion and become the major Ca^{2+} store. However, when the experiment was conducted in physiological calcium concentration, it was shown that the ER contained most of the stored Ca^{2+}

(Somylo *et al.*, 1985). Secondly, when Ca^{2+} uptake was monitored in permeabilised cells so that $[Ca^{2+}]_i$ could be altered, it was found that ER was the key organelle responsible for intracellular Ca^{2+} buffering (Burgess *et al.*, 1983). Thirdly, the affinity of mitochondrial Ca^{2+}-ATPase is much too low (in micromolar range) to be consistent with a role for them in buffering $[Ca^{2+}]_i$, except perhaps during agonist stimulation when $[Ca^{2+}]_i$ has risen to the micromolar range (Crompton *et al.*, 1976). Instead its role is mainly concerned with the modulation of calcium concentration in the mitochrondrial matrix which regulates the activity of intra-mitochondrial enzymes such as pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (Irvine, 1986; Carafoli, 1987). Due to its ability to combine with phosphate and precipitate as crystalline hydroxyapatite deposits in the matrix, mitochondria have an insatiable appetite for Ca^{2+} and this is likely to act as a safety device to handle the dramatic increase in $[Ca^{2+}]_i$ during pathological events (Schanne *et al.*, 1979).

Calcium can directly affect the activity of some enzymes such as calcium-dependent proteases that are involved in cytoskeleton organisation or it can exert its effect on a number of enzymes such as PLA_2, adenylyl cyclase and Ca^{2+}-ATPase by acting through a calcium-calmodulin complex (CaCaM). Calmodulin is an abundant protein of ubiquitous distribution and requires calcium for its action by forming CaCaM (Cheung, 1980; Means *et al.*, 1982). It is a high affinity binding protein and is a major, if not a principal, mediator of calcium action. By forming CaCaM it can interact with a large number of cellular enzymes, thus altering their activities. The enzymes that are regulated by CaCaM are listed in Table 5.1. As far as calcium homeostasis is concerned, CaCaM can activate Ca^{2+}-ATPase in the plasma membrane that is responsible for Ca^{2+} extrusion. Thus Ca^{2+}, through the formation of CaCaM constitutes a self-regulation device for maintaining a low steady-state level of $[Ca^{2+}]_i$ (Bolander, 1989; Reeves, 1990).

The importance of Ca^{2+} in the regulation of many cellular processes suggests that $[Ca^{2+}]_i$ must be tightly controlled and failure in calcium homeostasis results in calcium accumulation, disruption of cellular processes and may lead to cell death. In fact, failure in calcium homeostasis has been reported as the cause of cell death in ischaemia (Siesjö, 1989) and cell death induced by some toxins (Schanne *et al.*, 1979; Farber, 1981; Orrenius *et al.*, 1988). The exact mechanism of calcium-induced cell death is uncertain, but it can be due to the disruption of a variety of cellular processes such as cytoskeletal organisation, sustained activation of phospholipases which lead to disruption of phospholipid turnover and activation of calcium-dependent proteases which result in the activation of some enzymes by limited proteolysis (Figure 5.2).

Normal cytoskeletal organisation is essential for cellular processes such as cell division, intracellular transport, receptor turnover and control of cell mobility and cell shape. Disruption of this organisation results in the appearance of surface protrusions known as blebs, which will disrupt the membrane integrity and possibly lead to cell death. Bleb formation is a phenomenon associated with toxic, hyperthermic and ischaemic cell death (Schanne *et al.*, 1979; Farber, 1981; Kapiszewska and Hopwood, 1988; Orrenius *et al.*, 1988; Siesjö, 1989). The involvement of Ca^{2+} in bleb formation has been observed when cells were treated with cyanide (Nicotera *et al.*, 1989) and $HgCl_2$ (Smith *et al.*, 1991). Phospholipase A_2 (PLA_2) is activated by the calcium-calmodulin complex, resulting in the formation of *lyso*phospholipid and fatty acid. Since *lyso*phospholipid has been found to be a toxic substance, its level must be tightly controlled (Siesjö and Wieloch, 1985). Sustained PLA_2 activation will result in accumulation of this toxic product. Furthermore, sustained PLA_2 activation can also alter membrane integrity by affecting the phospholipid turnover and perhaps lead to cell damage. Evidence of PLA_2 activation in cell death came from a study using a PLA_2 inhibitors. It was found that in the presence of a PLA_2 inhibitor, ischaemic cell death in liver and heart were prevented (Trump *et al.*, 1980). Ca^{2+} can also activate calcium-

dependent proteases that are responsible for cytoskeletal and cell membrane remodelling, proteolytic activation of PKC and regulation of cell mitosis. Evidence for calcium-dependent proteases being involved in cell injury was obtained by using an inhibitor of calcium-activated proteases, which prevented the onset of bleb formation and cytotoxicity induced by cystamine (associated with an increase in $[Ca^{2+}]_i$) (Nicotera *et al.*, 1986). Although the substrates for proteases are unidentified, these authors suggested that cytoskeletal proteins are likely to be a major target for calcium-activated proteases when treated by toxins.

Use of $^{45}Ca^{2+}$ enables calcium fluxes and total calcium content to be measured during or after heating. However, it has been known for a long time that $[Ca^{2+}]_i$ rather than the total cell calcium is responsible for cellular signalling and metabolic control, so direct measurement of $[Ca^{2+}]_i$ is important to resolve the relationship of hyperthermic cell death and $[Ca^{2+}]_i$.

Measurement of $[Ca^{2+}]_i$ is complicated by several factors, including the small volume of the cytoplasmic space, the large proportion of calcium bound to proteins and membranes, and the heterogeneous distribution of the bound and free calcium within the cell. Three different approaches have been used successfully in $[Ca^{2+}]_i$ measurement, namely calcium-specific microelectrodes, calcium-sensitive photoproteins and fluorescent indicators.

Calcium-specific microelectrodes are free of artifacts associated with incomplete diffusion of a Ca^{2+}-indicator molecule and can measure a very wide range of $[Ca^{2+}]_i$, but the technique requires cell impalement by the electrode and, therefore, is limited to individual giant cells. Also, some of the unidentified constituents of the cytoplasm may derange the electrode (Tsien and Rink, 1980).

Ca^{2+}-sensitive photoproteins such as aequorin emit light upon binding to calcium. However, the application of this technique to $[Ca^{2+}]_i$ measurement is limited by the fact that microinjection or cell fusion is required to introduce the photoproteins into the cell. The potential problem concerning with the use of aequorin is that it reacts with Ca^{2+} and the reaction is irreversible. As the composition of many kinds of glass includes Ca^{2+}, any leakage of Ca^{2+} from the glass will result in the inactivation of this photoprotein. Hence experiments must be done in Ca^{2+}-free EGTA solution. Furthermore, photoproteins are destroyed rapidly at temperatures above 30°C (Cobbold and Rink, 1987).

The development of fluorescent indicators, together with their membrane permeable analogues, has revolutionised the study of $[Ca^{2+}]_i$ in small mammalian cells (Grynkiewicz *et al.*, 1985). The availability of the ester form (acetoxy methylesters) of the dye permits dye loading without any disruption of the plasma membrane. Since the ester groups are uncharged and hydrophobic, the ester form of the dye can cross the membrane readily. Once in the cytosol, the esters are cleaved by endogenous esterases, giving rise to dye in its free acid form which cannot permeate the membrane and is trapped in the cytosol. When the extracellular dye has been removed, the alteration of cytosolic free calcium can be monitored by changes in fluorescence signals. By using this method, the problems concerning calcium leakage from damaged membranes are eliminated (Grynkiewicz *et al.*, 1985). Nowadays, several fluorescent indicators are commercially available and the most commonly used dyes are quin2, fura-2 and indo-1 (Tsien, 1989)

Quin2, has been used for several years and has revealed vital information about $[Ca^{2+}]_i$ levels in cells under stimulation, but it has some disadvantages (Tsien and Pozzan, 1989). For example, it has a short excitation wavelength (339 nm), so it excites significant autofluorescence from cells. The low extinction coefficient and fluorescence

quantum yield means that high concentrations of the indicator is required to overcome the autofluorescence, but high concentrations can partially buffer $[Ca^{2+}]_i$ transients. Furthermore, upon binding to calcium, there is no shift in both the excitation and emission wavelengths, so ratio imaging is not feasible. Therefore, both dye concentration and cell thickness contribute to the fluorescence intensity. Furthermore, quin2 saturates at about 1-2 μM Ca^{2+} so is not suitable for measuring $[Ca^{2+}]_i$ above 10^{-6} M. Quin2 also binds Mg^{2+} with K_d of 1-2 mM, so it is not absolutely specific for Ca^{2+}-binding. Although Mg/quin2 complexes do not fluorescence, Mg^{2+} does have an effect on the calcium and quin2 binding affinity. Thus, in the absence of Mg^{2+} the K_d of Ca/quin2 is 60 nM, whereas in the presence of Mg^{2+} the K_d value is 150 nM. Finally, heavy metals quench the quin2 fluorescence, so if the cells contains a high level of heavy metals, it will give a false low $[Ca^{2+}]_i$ reading (Tsien and Pozzan, 1989).

Most of the drawbacks of quin2 have been overcome by fura-2 and indo-1 (Grynkiewicz *et al.*, 1985). The K_d of both dyes are not affected by Mg^{2+} since the K_d for Mg^{2+} is about 6-10 mM, hence giving a better Ca/Mg discrimination compared to quin2. Upon binding to Ca^{2+}, fura-2 exhibits a shift in the excitation wavelength (about 30 nm towards shorter wavelength). On the other hand, indo-1 shows a shift in the emission wavelength upon binding to Ca^{2+}. Hence both dyes are suitable for ratio imaging measurements, so the effect of dye concentration and cell thickness on the fluorescent signal can be eliminated. Since fura-2 requires dual excitation wavelengths, it is more useful in microscopic techniques. In the case of indo-1, a single excitation wavelength is adequate, making it more useful for spectrofluorimeter and flow cytometry techniques. With the development of modern technology, a change in $[Ca^{2+}]_i$ upon stimulation can be determined not only at the single cell level but the spatial distribution of the calcium concentration within a single cell can be obtained from digitised imaging microscopy techniques (Grynkiewicz *et al.*, 1985; Tsien, 1989).

Although the fluorescent dyes provide a valuable tool for studying $[Ca^{2+}]_i$, they are not without limitations (Roe *et al.*, 1990). Cellular compartmentalisation of the dye has been reported as a problem, when the sequestered dye is not sensitive to the change in $[Ca^{2+}]_i$ in cytosol (Steinberg *et al.*, 1987). In addition, photodamaging effects can give rise to molecular species that are non-calcium sensitive or only sensitive to mM rather than nM calcium concentration (Becker and Fay, 1987). Furthermore, dye loss from loaded cells during measurement has been reported, and this is a serious problem at high temperatures (Malgaroli *et al.*, 1987). The fluorescence signal is quenched by heavy metals present in cells (Arlsan *et al.*, 1985). Finally, the K_d of the dye depends on viscosity, ionic strength and temperature of the environment, so these parameters are important when comparing and evaluating results (Tsien, 1989).

The problem of dye sequestration can be overcome by loading at lower temperatures, since endocytosis and sequestration can often be significantly slowed down by reduction in temperature e.g. from 37°C to 32°C (Tsien and Pozzan, 1989; Di Virgilio *et al.*, 1990). Due to the hydrophobicity of the esterified form of the dye, it must be dissolved initially in DMSO before diluting the stock solution in an aqueous medium. By doing so, microcrystals can form, which may be endocytosed by the cells. Addition of amphiphilic agents such as albumin, serum or Pluronic F127 in the medium reduces the formation of microcrystals. This is particularly important with cell monolayers, where there is a tendency for precipitates to settle on cells and become endocytosed (Hallett *et al.*, 1990). Photobleaching can be diminished by employing the lowest level of excitation intensity and shortest duration of exposure (Roe *et al.*, 1990). The light intensity can be reduced by passing the light through a neutral density filter. Furthermore, between each measurement the light can be blocked by closing the shutter. Dye leakage can be passive (from damaged cells) or active (by anion transport system) (Di Virgilio *et al.*, 1990). The latter case of leakage is temperature-dependent (Malgaroli *et al.*, 1987). This problem can be prevented by an anion transport inhibitor such as

probenecid or sulfinpyrazone, as well as performing the measurement at lower temperatures (Di Virgilio *et al.*, 1990). The presence of heavy metals can quench fura-2 fluorescence, but this can be prevented by treating cells with N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a chelator of heavy metals (Arslan *et al.*, 1985). Finally, the effects of ionic strength, viscosity and temperature can be eliminated by correcting the K_d of EGTA and Ca^{2+} as well as the K_d for fura-2 and Ca^{2+} to the corresponding temperature and pH before constructing the calibration curve.

The role of calcium in hyperthermic cell death is controversial. In order to explore its role, the effect of altering the extracellular calcium concentration upon heat-induced cell death was determined. When human colon cancer HT-29 cells were heated at different extracellular calcium concentrations, ranging from 0 to 15 mM for up to 2 h at 44°C, cell death was neither potentiated in high extracellular calcium concentration nor protected in low extracellular calcium concentration (Mikkelsen *et al.*, 1991a). Similarly, cell death of CHO cells was neither sensitised nor protected when cells were heated at 45°C for 30 min in 0.2 mM or 10 mM extracellular calcium, a condition that resulted in at least a 5-fold difference in total $^{45}Ca^{2+}$ content between cells heated under these 2 conditions (Vidair and Dewey, 1986). On the other hand, Reuber H35 rat hepatoma cell death at 44°C was enhanced by 7.5 mM extracellular calcium concentration and protected when cells were heated in 0.03 mM extracellular calcium concentration (Wiegant *et al.*, 1984). A similar observation was reported by Landry *et al.* (1988), who found that MH-7777 cells were protected from heat cell death by pre-incubation in low extracellular calcium (achieved by addition of EGTA to the medium) for 90 min before heating at 43°C in the same medium.

Using $^{45}Ca^{2+}$ to study the calcium fluxes and total calcium content during heating, it was found that exposure of Ehrlich ascites cells to 45°C resulted in an increase in total calcium content (Anghileri *et al.*, 1985a,b). When $^{45}Ca^{2+}$ influx and Ca^{2+}-ATPase

activity were measured in heated cells (at 45°C), a 2 stages of $^{45}Ca^{2+}$ uptake were detected. The initial uptake was observed at early heating times (about 5 min), which then declined to control level by 20 min. A second phase of $^{45}Ca^{2+}$ uptake was observed after 45 min heating. This late increase in $^{45}Ca^{2+}$ uptake was related to a decrease in Ca^{2+}-ATPase activity (Anghileri *et al.*, 1984). Vidair and Dewey (1986) also found that total cell calcium content in CHO cells increased and remained elevated at 23 h after heating at 45°C for 15-35 min. This increase in Ca^{2+} content was found to result from a decrease in both influx and efflux rates, by determination of $^{45}Ca^{2+}$ efflux in cells prelabelled with $^{45}Ca^{2+}$ for 48 h, and by monitoring $^{45}Ca^{2+}$ influx when cells were incubated in medium containing $^{45}Ca^{2+}$. The relationship between calcium influx and heat cell death was investigated in hepatocytes (Malhotra *et al.*, 1986) and Chinese hamster lung CHL V79 cells (Malhotra *et al.*, 1987). It was found that $^{45}Ca^{2+}$ influx was related to cell death when hepatocytes or CHL V79 cells were heated in 15 mM, but not when they were heated in buffer at 4 mM extracellular calcium concentration. Hence, these authors suggested that cell death is promoted at higher extracellular calcium but the entry of calcium is not the primary mechanism of heat-induced cell death at normal calcium concentration. An increase in $^{45}Ca^{2+}$ influx due to the alteration of membrane permeability in CHO HA-1 cells during heating has been reported by Stevenson *et al.* (1987). Although the increase in $^{45}Ca^{2+}$ influx did not directly cause cell death, maximal permeability was observed in the shoulder region of the killing curve. Thus, Stevenson and co-workers (1987) suggested that calcium can play a role such as triggering calcium-dependent events that contribute to cell death.

A heat dose-dependent increase in $[Ca^{2+}]_i$ was observed in CHO HA-1 cells. When $[Ca^{2+}]_i$ was monitored in quin2 loaded cells by spectrofluorimetry, an increase in $[Ca^{2+}]_i$ was observed at 42°C and the level reached a plateau at 45°C, resulting in 450-1100 nM $[Ca^{2+}]_i$ (Calderwood *et al.*, 1988). A similar temperature-dependent increase in $[Ca^{2+}]_i$ was found in NIH3T3 mouse fibroblasts (Dynlacht *et al.*, 1993). Following

loading with indo-1, these cells were heated and $[Ca^{2+}]_i$ was monitored by flow cytometry. An increase in $[Ca^{2+}]_i$ was observed as soon as the temperature started to increase and reached a plateau at 42°C, achieving $[Ca^{2+}]_i$ of 300 nM. This $[Ca^{2+}]_i$ level was maintained up to 45.5°C (Dynlacht *et al.*, 1993). When $[Ca^{2+}]_i$ was measured in fura-2 loaded A-431 cells by spectrofluorimetry upon heat treatment, an increase in $[Ca^{2+}]_i$ was observed after 5 min exposure to 40°C or 45°C and reached maximum after 20 min (Kiang *et al.*, 1992). On the other hand, when cells were heated at 50°C, an increase in $[Ca^{2+}]_i$ was observed at 1 min and reached maximum after 20 min heating. For those cells heated at 45°C and 50°C, $[Ca^{2+}]_i$ remained elevated for up to 30 min whereas for cells heated at 40°C $[Ca^{2+}]_i$ declined to the control value after 30 min (Kiang *et al.*, 1992).

As mentioned earlier, Ca^{2+} can either come from external sources or be released from intracellular stores, so some studies have attempted to clarify the source of the heat-induced rise in $[Ca^{2+}]_i$. The elevation in $[Ca^{2+}]_i$ in CHO HA-1 cells (Stevenson *et al.*, 1986) and Drosophila salivary gland (Drummond *et al.*, 1988) was thought to derive from internal stores because the increase in $[Ca^{2+}]_i$ in both cell types was observed when they were heated in medium containing less than 0.1 mM extracellular calcium. However, calcium influx was thought to be the source of the heat-induced rise in $[Ca^{2+}]_i$ in mouse mammary tumour cells (Furukawa *et al.*, 1992) and human epidermoid carcinoma A-431 cells (Kiang *et al.*, 1992), as the heat-induced rise in $[Ca^{2+}]_i$ was abolished when both cell types were heated in low extracellular calcium concentration. Furthermore, using $^{45}Ca^{2+}$ to measure calcium influx in A-431 cells upon heating, Kiang and co-workers (1992) demonstrated that this increase in $[Ca^{2+}]_i$ correlated well with the time course of $^{45}Ca^{2+}$ influx.

The effect of heat on $[Ca^{2+}]_i$ has also been analysed at the single cell level by using fura-2 together with digitised fluorescence microscopy. When $[Ca^{2+}]_i$ was

measured in fura-2 loaded human colon HT-29 cells, an increase in $[Ca^{2+}]_i$ was observed in more than 80% of these cells after heating at 44°C for 1 h (Mikkelsen *et al.*, 1991a). Similarly, by using the same technique, an increase in $[Ca^{2+}]_i$ was observed in mouse mammary carcinoma FM3A cells after heating at 44°C for 1 h (Kondo *et al.* 1993). In contrast, when heating mouse mammary carcinoma MMT060562 cells at 44°C for 20 min an increase in $[Ca^{2+}]_i$ in some of these cells was found, but the mean $[Ca^{2+}]_i$ in heated and control cells was not significantly different (Furukawa *et al.*, 1992). The discrepancies in these studies are likely to be due to different heating times and cell types employed.

The relationship between the heat-induced increase in $[Ca^{2+}]_i$ and heat cell death could be largely dependent on the cell type used. When $[Ca^{2+}]_i$ was monitored in fura-2 loaded Ehrlich ascites tumour cells (EAT), HeLaS3 cells, mouse fibroblast 3T3 cells, and murine lymphoma cell lines (L5178Y-S and L5178Y-R) by spectrofluorimetry, no significant difference in $[Ca^{2+}]_i$ between control and heated cells were observed in EAT, HeLaS3, L5178Y-S or L5178Y-R cells at a heat dose that killed more than 90% of these cells (Stege *et al.*, 1993a,b). On the other hand, a significant increase in $[Ca^{2+}]_i$ was observed in 3T3 fibroblasts. Therefore, these results implied that hyperthermic cell death could occur without any changes in $[Ca^{2+}]_i$.

The determination of $[Ca^{2+}]_i$ post-heating has also given conflicting results. Mikkelsen *et al.* (1991a), using human colon HT-29 cells, demonstrated that those cells heated at 44°C for 1 h which failed to return their $[Ca^{2+}]_i$ to resting level at 4-6 h post-heating were the clonogenically dead cells. However, it was found that calcium homeostasis remained intact in NIH3T3 cells after heating at 45°C for 40 min, a heat dose that resulted in greater that 95% cell death (Wang *et al.*, 1991). Immediately after heat treatment (45°C for 40 min) in buffers containing 1.8 mM extracellular calcium, cells were switched to buffers containing either 0.017 mM or 15 mM extracellular

calcium, and incubated at 37°C for another 2 h before $[Ca^{2+}]_i$ was measured in indo-1 loaded cells by flow cytometry in either buffer. It was found that lowering the extracellular calcium concentration during the 2 h incubation (and during measurement) lowered the $[Ca^{2+}]_i$, while raising the extracellular calcium level resulted in a higher $[Ca^{2+}]_i$. However, in both conditions $[Ca^{2+}]_i$ levels were insignificantly different from those in unheated cells that underwent the same treatment. When the experiment was repeated again but cells were switched to buffers containing different extracellular calcium concentrations at 18 h after heating, it was found that $[Ca^{2+}]_i$ between heated and unheated cells that underwent the same treatment were similar (Wang *et al.*, 1991).

The role of calcium in cell death has been further investigated by means of calcium ionophores, local anaesthetics and calcium channel blockers.

Calcium ionophores such as ionomycin and A23187 have been demonstrated to form a pore in the plasma membrane, allowing the movement of calcium down its concentration gradient into the cell (Truter, 1976). When hepatocytes (Malhotra *et al.*, 1986) were heated at 43°C in the presence of A23187 and external calcium (either 4 mM or 15 mM), hyperthermic cell death was potentiated. However, it was found that Ca^{2+} influx did not correlate well with loss of viability (determined by trypan blue exclusion) for hepatocytes heated in 4 mM extracellular calcium since Ca^{2+} influx was not observed until heat doses that reduced cell viability to less than 1% were achieved. On the other hand, Ca^{2+} influx in 15 mM extracellular calcium preceded loss of viability so, under these conditions, the increase in membrane permeability of the plasma membrane to Ca^{2+} may contribute to loss of viability. The results imply that Ca^{2+} influx, caused by high extracellular calcium or A23187, increases cellular damage caused by supraoptimal temperatures. The effect of heat and ionomycin on $[Ca^{2+}]_i$ and cell death was further investigated in EAT cells and HeLaS3 cells (Stege *et al.*, 1993a,b). The results obtained by these workers suggested that a threshold $[Ca^{2+}]_i$ must be exceeded before the

synergistic effect of ionomycin and $[Ca^{2+}]_i$ on cell death was observed. In HeLaS3 cells, $[Ca^{2+}]_i$ must exceed 700 nM, whereas in EAT cells $[Ca^{2+}]_i$ must exceed 300 nM in order to observe cytotoxic effects of ionomycin.

Local anaesthetics are heat sensitisers, possibly by increasing membrane fluidity (Yatvin, 1977), inducing calcium release from intracellular stores (Chen, 1974), displacing membrane-bound calcium (Kwant and Seeman, 1969) or affecting Ca^{2+} fluxes by interfering with the Na^+/Ca^{2+} exchanger (Garcia-Martin *et al.*, 1990). It has been shown that the presence of lidocaine, procaine or tetracaine sensitised NIH3T3 cells to 45.5°C treatment. The effects of these drugs on $[Ca^{2+}]_i$ during heating were investigated in indo-1 loaded NIH3T3 fibroblasts by flow cytometry (Dynlacht *et al.*, 1993). Heating at 45.5°C for 25 min in the presence of procaine or lidocaine resulted in $[Ca^{2+}]_i$ levels similar to cells heated in the absence of these drugs. On the other hand, an increase in $[Ca^{2+}]_i$ was observed in cells heated in the presence of tetracaine compared with cells heated in the absence of this drug. The heat-induced increase in $[Ca^{2+}]_i$ in the presence of tetracaine was suggested to be due to substantial membrane damage in the presence of this drug at elevated temperature, since cells were unable to exclude trypan blue, whereas this was not the case in the presence of lidocaine and procaine. Hence, the authors concluded that increase in $[Ca^{2+}]_i$ was not involved in potentiation of hyperthermic cell death by local anaesthetics.

Calcium channel blockers such as nifedipine, diltiazem and verapamil have been used to block calcium entry via voltage-gated channels. Using concentrations of verapamil (50-75 μM) and diltiazem (100-250 μM) that did not block calcium influx, cell death of CHO 10B4 cells was potentiated when they were heated at 44°C for up to 80 min (Coss *et al.*, 1989). On the other hand, Mikkelsen *et al.* (1991a) did not observe any effect on cell death when HT-29 cells were heated at 44°C for up to 2 h in the presence of verapamil at concentrations ranging from 0-25 μM. However $[Ca^{2+}]_i$ was

not determined in either case. In a recent study, the heat-induced increase in $[Ca^{2+}]_i$ in the presence of verapamil or diltiazem was determined in fura-2 loaded mouse mammary carcinoma FM3A cells by digitised fluorescent micoscopy (Kondo *et al.*, 1994). It was found that cells heated at 44°C for 1 h in the presence of either 100 μM verapamil or diltiazem displayed a higher $[Ca^{2+}]_i$ compared with those heated in the absence of the Ca^{2+} channel blocker. This increase in $[Ca^{2+}]_i$ was due to Ca^{2+} influx, as determined by measurement of $^{45}Ca^{2+}$ flux. In addition, heating in the presence of these drugs delayed cell growth induced by hyperthermia (Kondo *et al.*, 1994). La^{3+} was also shown to potentiate cell death of EAT cells heated at 43°C for 3 h. This sensitisation was found to be due to enhancement of calcium influx under hyperthermic conditions, associated with alteration of membrane fluidity by La^{3+} (Anghileri *et al.*, 1983).

Although the exact role of changes in $[Ca^{2+}]_i$ in hyperthermic cell death is still uncertain, it may play a secondary role leading to cell death. For example, the increase in $[Ca^{2+}]_i$ following heating can lead to disruption of cytoskeletal organisation or it can activate calcium-dependent enzymes, leading to uncontrolled cellular processes as suggested by Orrenius *et al.* (1988) (Figure 5.2).

Calcium ions have been shown to play a role in the modulation of cytoskeletal structure and function either alone or, more often, associated with the formation of CaCaM complex (Table 5.2). Through their ability to regulate the activities of cytoskeletal binding proteins, calcium ions can control the assembly of actin and tubulin. Alternatively, changes in cytoskeletal structure may be mediated by catabolic enzymes such as cytosol neutral proteases (Trump and Berezesky, 1992).

Disruption of cytoskeletal organisation is associated with surface bleb formation. Bleb formation has been observed in CHO cells when heated above 43°C (Bass *et al.*, 1982; Borrelli *et al.*, 1986). Both groups reported that there was a correlation between

cells covered with blebs and cell survival. By measuring the bleb size, Borrelli *et al.* (1986) reported that cells having blebs greater than 50% of the cell diameter (measured immediately after heating) were those that could not form colonies. However, this correlation only held at G_1 phase. Some evidence supports the suggestion that calcium plays a role in bleb formation. For example, treating hepatocytes with A23187 caused a sustained increase in $[Ca^{2+}]_i$, and bleb formation was observed (Kapiszewska and Hopwood, 1988). On the other hand, pretreatment of hepatocytes with an inhibitor of calcium-regulated cytosolic proteases, such as leupeptin, can prevent bleb formation (Nicotera *et al.*, 1986). When rabbit proximal tubule epithelium was treated with $HgCl_2$ at a concentration of 10-50 μM, a massive increase in $[Ca^{2+}]_i$ was observed, which was followed by bleb formation. By digitised imaging microscopy, it was shown that $[Ca^{2+}]_i$ in the bleb region was at a much higher level (Smith *et al.*, 1991). Such a massive increase in $[Ca^{2+}]_i$ in the bleb region could activate calcium-dependent proteases that cleave actin-binding proteins, thus eliminating the plasma membrane anchor to the cytoskeleton. Disruption of cytoskeleton causes a weakening of the cell surface and leads to membrane blebbing (Kapiszewska and Hopwood, 1988).

The effect of heat on CaM function has been investigated by employing CaM inhibitors (Wiegant *et al.*, 1985; Landry *et al.*, 1988; Evans and Tomasovic, 1989). The presence of CaM inhibitors, such as trifluoperazine, compound 48/80 and calmidazolium, potentiated heat cell death of neuroblastoma N_2A cells and hepatoma H_{35} cells upon heating at 43°C for 30 min or longer (Wiegant *et al.*, 1985). The exact role of CaM inhibitors in sensitisation to heat cell death remains unclear, but it is possible that cytoskeletal reorganisation during heating is inhibited. Using the anti-CaM drug W13 and its non-functional analogue W12, Landry and co-workers (1988) reported that the presence of W13 potentiated hyperthermic cell death of Chinese hamster lung 023 cells upon heating at 43°C whereas W12 had little influence. As W12 possesses 5 to 10-fold less binding affinity for calmodulin than W13 but only has a 10-15% reduction

in hydrophobic index, the authors suggested that the sensitisation to hyperthermic cell death by these agents was not due to their effects on membranes, but was related to inhibition of calmodulin. Potentiation of hyperthermic cell death by CaM inhibitors was also observed in mouse tumour clone C cells (MTC cells) (Evans and Tomasovic, 1989). When MTC cells were incubated in W7 or the less active analogue, W5, for 30 min before heating for up to 3 h, it was found that cell death was enhanced when cells were heated at 43°C in the presence of W7 but slightly protected if heated (and pre-incubated) in W5 compared to those heated in the absence of drug. However, the potentiation of cell death by W7 was not observed when cells were heated at 42°C i.e. the cell death at 42°C was similar between cells heated in the absence or in the presence of either W7 or W5. Based on these observations, Evans and Tomasovic (1989) proposed that it could be possible that the target of heat damage between 42°C and 43°C treatment was different. For example, it could be possible that a W7-sensitive target was involved in cell death at 43°C but not at 42°C. It could also be possible that the uptake and distribution of W7 was temperature-dependent. Although there is no unambiguous evidence that mechanisms of heat cell death may arise predominantly through perturbation of Ca^{2+} or CaM-regulated processes, these results imply that perturbation of these systems may contribute to hyperthermic cell killing.

$[Ca^{2+}]_i$ regulates the activities of phospholipases such as PLA_2 or PLC (Lapetina, 1990). PLA_2 is responsible for the cleavage of the fatty acyl chain in the *sn*-2 position of phospholipid, giving rise to *lyso*phospholipid and free fatty acid. So far only the PLA_2 responsible for cleaving the arachidonic acid moiety has been characterised. It was found that heat shock (42-45°C) led to arachidonic acid accumulation in CHO HA-1 fibroblasts, HeLa cells, Balb C 3T3 fibroblasts and PC 12 rat pheochromocytoma cells (Calderwood *et al.*, 1989). These investigators proposed that this was due to an activation of PLA_2 at elevated temperature because accumulation of *lyso*PC and *lyso*PI were observed at the same time. Accumulation of arachidonic acid in heated CHO HA-1

cells and HeLa cells were both temperature and heating-time dependent and was accompanied by an increase in eicosanoid products. Although the activation of PLA_2 in CHO HA-1 cells at elevated temperature was not mediated by an increase in $[Ca^{2+}]_i$, the dependency of PLA_2 activity on Ca^{2+} levels in other cell types upon heating remains to be evaluated. It has been reported that *lyso*phospholipid itself is toxic, so the level of this substance must be tightly controlled (Siesjo and Wieloch, 1985). Sustained PLA_2 activation can lead to the accumulation of this toxic substance, disruption of phospholipids turnover and to the disruption of membrane integrity.

As indicated in Chapter 3, phosphatidylinositol-specific phospholipase C (PI-PLC) is involved in the phosphoinositide signalling pathway. Upon stimulation, it hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) giving rise to two second messengers, 1,2-diacylglycerol (1,2-DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) (Berridge, 1984). 1,2-DAG together with calcium and phosphatidylserine (PtdSer) activates protein kinase C (Nishizuka, 1984; Chapter 3) while Ins(1,4,5)P_3 is responsible for calcium mobilisation from intracellular stores such as ER (Berridge, 1993; Chapter 4). It has been suggested that heat shock leads to an increase in hydrolysis of polyphosphoinositides (PPI), giving rise to inositol trisphosphate and its metabolites in several cell lines such as CHO HA-1 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993), Balb C 3T3 cells (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993) and A-431 cells (Kiang and McClain, 1993). Furthermore it has been shown that heating at 45°C for longer than 15 min causes a depletion in PPI (Calderwood *et al.*, 1987). Since PPI are involved in microfilament anchoring to the cell surface (Lassing and Lindberg, 1985), depletion of PPI could disrupt the cytoskeletal organisation and may be an important component in the pathway leading to heat cell death.

In this study, heat-induced changes in $[Ca^{2+}]_i$ were investigated under different

conditions in fura-2 loaded WRK-1 cells, using fluorescence microscopy. Fura-2 was chosen as a Ca^{2+}-indicator in this study for the following reasons: (1) The availability of the dual wavelength equipment. (2) The overlapping of wavelengths of indo-1 emission with cellular autofluorescence from pyridine nucleotides is more severe than that of fura-2. Hence the autofluorescence in fura-2 loaded cells is negligible (Aubin, 1979). (3) The rate of photobleaching of fura-2 is several fold slower than indo-1. This is important in this type of study since experiments were performed at elevated temperature, which increases the photobleaching rate (Tsien and Pozzan, 1989).

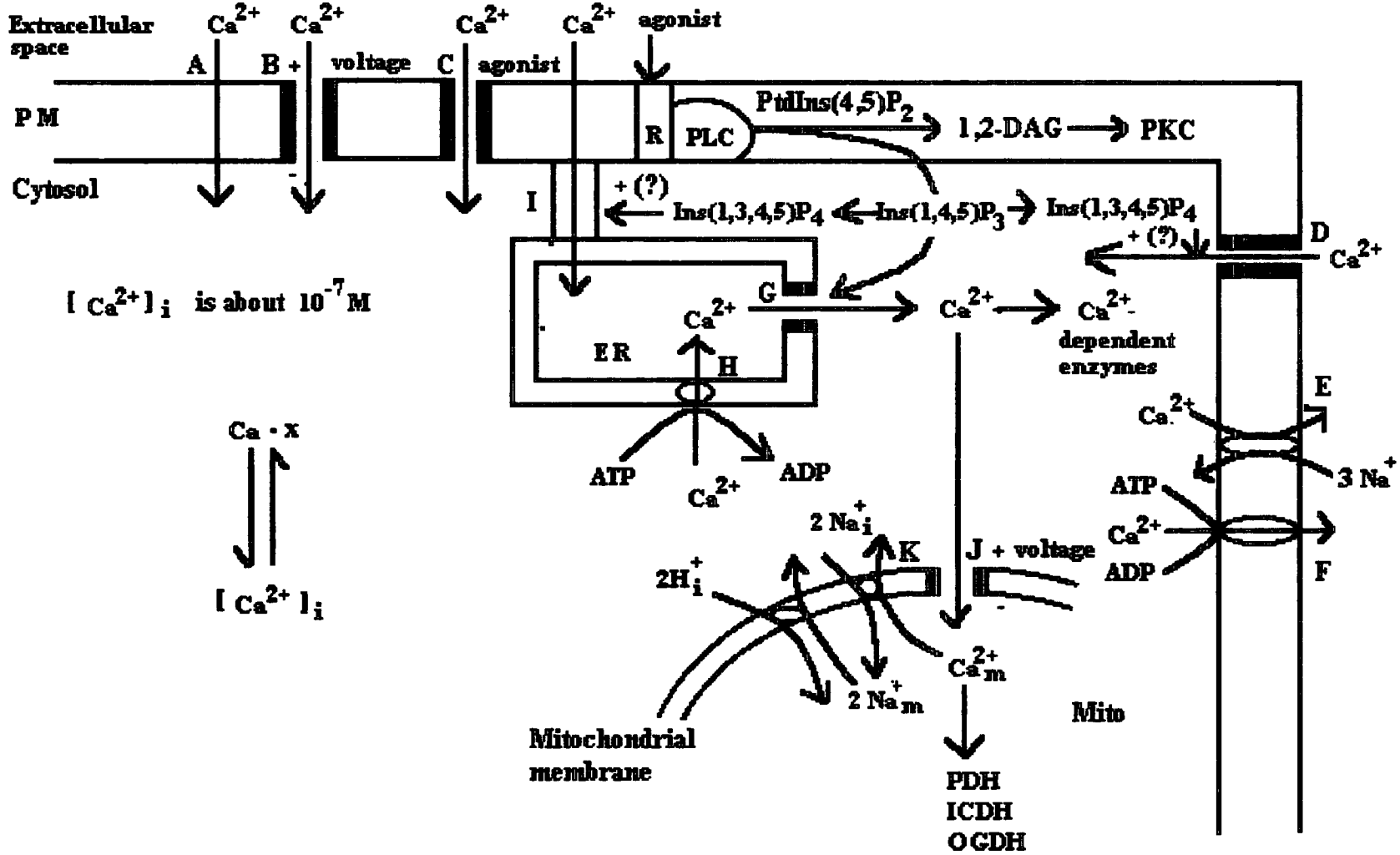

Extracellular free Ca^{2+} is greater than 1 mM
Extracellular space
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
agonist
A
B +
voltage
C
agonist
PM
R
PLC
PtdIns(4,5)P_2
1,2-DAG
PKC
Cytosol
I
+ (?)
Ins(1,3,4,5)P_4
Ins(1,4,5)P_3
Ins(1,3,4,5)P_4
+ (?)
D
Ca^{2+}
$[Ca^{2+}]_i$ is about 10^{-7} M
G
Ca^{2+}
Ca^{2+}
Ca^{2+}-dependent enzymes
ER
H
Ca^{2+}
E
Ca · x
ATP
ADP
Ca^{2+}
Ca^{2+}
3 Na^+
ATP
Ca^{2+}
ADP
F
2 Na^+_i
K
J + voltage
$2H^+_i$
Ca^{2+}_m
2 Na^+_m
Mito
$[Ca^{2+}]_i$
Mitochondrial membrane
PDH
ICDH
OGDH

Table 5.1. Calcium-calmodulin regulated enzymes

Enzymes that are regulated by calcium-calmodulin complex (CaCaM) are:

Cyclic nucleotide phosphodiesterase

Adenylyl cyclase

Guanylyl cyclase

cGMP protein kinase

Myosin light-chain kinase (MLCK)

Ca^{2+}-Mg^{2+}-ATPase in the plasma membrane

Phosphorylase kinase

Glycogen synthase kinase

NAD^+ kinase

Phosphoprotein phosphatase

Phospholipase A_2

Succinate dehydrogenase

Table 5.2. Effect of CaCaM on cytoskeletal organisation

MLCK:

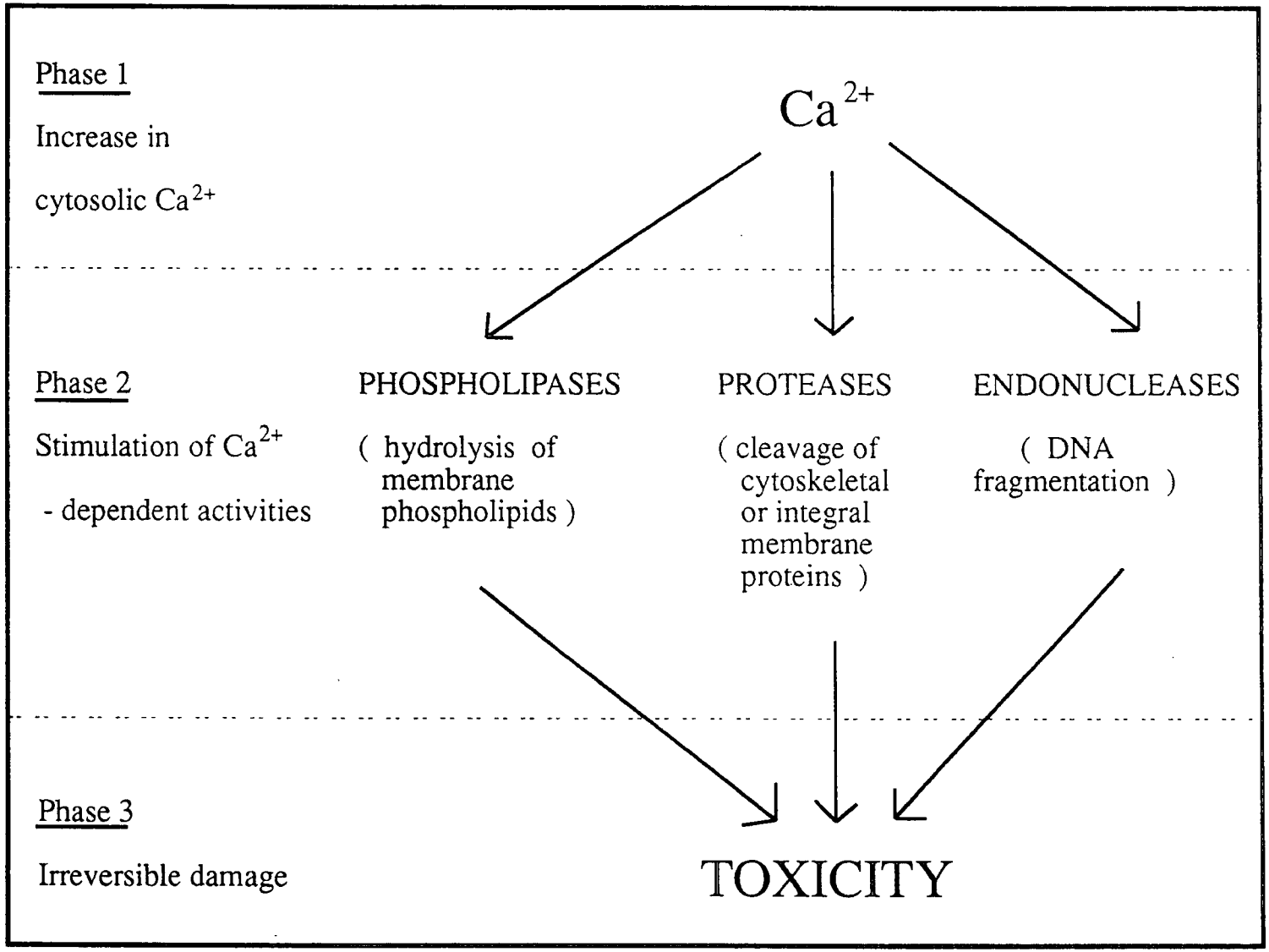
Presence of CaCaM activates this enzyme and promotes the phosphorylation of the light chain myosin molecule, allowing a conformational change in the myosin that allows actin binding and stimulates the ATPase activity. The hydrolysis of ATP provides energy necessary for tension development and contractility

Actomyosin:

Actomyosin may be a calmodulin regulated component of the mitotic apparatus. In interphase phase of nonmuscle cells, actomycin provides the chemical and mechanical basis for contractility

Microtubule-associated proteins (MAP):

CaCaM affects the assembly of microtubules. Upon addition of 11 μM Ca^{2+} the microtubule polymerisation is inhibited through the action of CaCaM, possibly through the action on MAP, although the actual mechanism is unclear


Phase 1
Increase in
cytosolic Ca^{2+}
Ca^{2+}
Phase 2
Stimulation of Ca^{2+}
- dependent activities
PHOSPHOLIPASES
(hydrolysis of membrane phospholipids)
PROTEASES
(cleavage of cytoskeletal or integral membrane proteins)
ENDONUCLEASES
(DNA fragmentation)
Phase 3
Irreversible damage
TOXICITY

5.2 Materials and Methods

5.2.1 Determination of cytosolic free calcium using a fluorescent microscopy technique

(i) Equipment (Figure 5.2)

The system consists of the following accessories:

1. Microscope and objective

A Nikon inverted microscope (Diaphot-TMD) with a Nikon CF Fluor x40 objective were used.

2. Illumination source

A xenon lamp (75W) which gave a more uniform spectral output than a mercury lamp was used in the study.

3. Excitation wavelength selector

This was achieved by filters assembled into a wheel, which was situated between the light source and the microscope. The wheel alternated between 350 and 380 nm filters and the rate of alternation was controlled by a personal computer. The light intensity was attenuated by a 15.2% neutral density filter to prevent photobleaching and photodamage. Between measurements the light was blocked by either closing the shutter or by putting a mirror between the light source and the microscope.

The time required for one complete cycle measurement i.e the time taken to move to each filter and the photon counting time = 2 x (filter change time + photon count time)

The filter change time in this system was 300 ms and the photon count time was 200 ms, so 1 s was required for one complete cycle.

4. Dichroic mirror

A dichroic mirror was used to reflect the excitation light into the objective, but to pass longer wavelength emission light (in the uv region) to the eye-piece or detector.

5. Emission filter

The emitted fluorescence, before feeding into the photomultipliers, was filtered through a 520 nm cut-off filter.

6. Photomultiplier tube (PMT)

The photomultiplier converted photons emitted from the dye to electrons. These electrons were then accelerated and amplified by the pulse amplifier box. The output was then fed into the computer, then analysed and displayed on the screen.

7. Program

Photon counting system count 4 (Newcastle Photometric Systems)

The program enabled the control of the filter changing time, graphic output, data storage and analysis. The average light intensities obtained over the excitation period (total of 8 s) at each of the two wavelengths were used by the computer to calculate the 350/380 nm ratio after background subtraction.

(ii) Temperature controller

A system able to control the temperature at either 37 or 45°C was necessary, and this was achieved by a PDMI-2 open perfusion micro-incubator system together with a TC-202 temperature controller (Medical System Corp, Greenwale, NY). Perfusion was achieved by a peristaltic pump at a flow rate of 1.5 ml/min. As the medium was perfused through the unit, it was warmed up to ±0.2°C of the set temperature.

Continuous perfusion during measurements allowed (1) simple addition of drug or agonist; (2) better temperature control and fast temperature changes; (3) maintenance of a constant volume of medium (no loss through evaporation); (4) removal of any leaked dye during measurement.

However an inevitable temperature gradient existed within the chamber, the

magnitude of which depended on the depth of the medium, position within the chamber, flow rate and the ambient temperature. Consequently, a thermocouple was inserted into the chamber to monitor the temperature of the medium during measurement. In all of the measurements, the cells close to the thermocouple were used.

The focus and the position of the specimen (cell) will alter as temperature changes. Thus, it must be ensured that the specimen is in focus during measurement.

(iii) Cell incubation chamber

The base of the incubation chamber must be made of material that allows the transmission of the required wavelength and gives a good quality image. This was easily done by cutting a hole at the bottom of a plastic petri dish (35 mm dish from Corning) and fixing a glass coverslip to it. In order to reduce heat loss, a polystyrene lid with a small window in the middle (made by cutting the middle of the lid off and attaching small pieces of glass to either side of the lid) was used. By this means, the adjustment of the focus could be carried out without removing the lid.

(iv) Calibration

In vitro calibration was done by using 10 μM fura-2 free acid (Cambridge Bio-science) and the calcium calibration buffer kit which contained 11 calcium buffers at different concentrations of free calcium (Cambridge Bio-science). Fura-2 free acid (2 μl) was added to 198 μl of calibration buffer in a glass bottom cuvette, created by removing the bottom of a plastic cuvette and mounting a glass coverslip on it. The cuvette was fitted with a polystyrene lid, then the calibration buffer was maintained at 37°C or 45°C by placing the cuvette in a micro-perfusion chamber containing water at the appropriate temperature. The temperature of the buffer was monitored throughout the measurement

by a thermocouple. Background fluorescence was measured using calibration buffer before the addition of the dye. The background intensities were then subtracted from the fluorescence intensities at 350 and 380 nm and the fluorescence ratio at 350/380 nm, corresponding to a particular concentration of free calcium, was calculated. However, as the binding affinity of EGTA and calcium varies with temperature, pH and ionic strength, it is necessary to correct the dissociation constant of calcium and EGTA to the relevant conditions.

The dissociation constant (K_d) for EGTA and calcium is defined as:

$$\frac{[\text{Free } Ca^{2+}]\,[\text{All forms of EGTA not bound to calcium}]}{[Ca \cdot \text{EGTA complex}]} \qquad (1)$$

At pH > 4, three forms of metal-free EGTA are present. They are

$[EGTA]^{4-}$, $[EGTA{\cdot}H]^{3-}$, $[EGTA{\cdot}H_2]^{2-}$,

where $[EGTA{\cdot}H]^{3-} = [EGTA]^{4-} \times 10^{(pK1-pH)}$

$[EGTA{\cdot}H_2]^{2-} = [EGTA]^{4-} \times 10^{(pK2-pH)}$

Substitution of the above expression into equation (1) and then simplication gives the following equation (Tsien and Pozzan, 1989):

$$K_d = \frac{1 + 10^{(pK_1 - pH)} + 10^{(pK_2 + pK_1 - 2pH)}}{K_{ca}}$$

$$K_d = 1 + 10^{(pK_1 - pH)} + 10^{(pK_2 + pK_1 - 2pH)} \times (K_{ca}^{-1}) \qquad (2)$$

Where $K_{ca} = \frac{[Ca \cdot EGTA]^{2-}}{[Ca^{2+}][EGTA]^{4-}}$, and K_1 and K_2 are the association constants for EGTA and H^+

Since both K_1, K_2 and K_{ca} are affected by temperature, ionic strength and pH, they must be converted to the correct value before applying to equation (2)

The conversion is according to those described by Harrison and Bers (1989)

The correction of association constants for temperature is given by :

$$\log K' = \log K + \frac{\Delta H(\frac{1}{T} - \frac{1}{T'})}{2.303R} \qquad (3)$$

Where K = association constant at 20°C

K' = new association constant after temperature correction

T = 20°C (293K)

T' = temperature of the experimental condition (in Kelvin)

ΔH for K_1 and K_2 = -5.8 Kcal/mol

ΔH for K_{ca} = -8.1 Kcal/mol

At 20°C, 0.1 M ionic strength ,

$pK_1 = 9.58$, $pK_2 = 8.96$, $K_{ca} = 10^{10.97}$, R = 1.9872 $KcalK^{-1}mol^{-1}$

Substituting all the values into equation (3), the corrected K_1, K_2 and K_{Cal} values at 37°C and 45°C are as follows:

	pK_1	pK_2	K_{ca}
37°C	9.34	8.72	$10^{10.64}$
45°C	9.24	8.62	$10^{10.5}$

Since pH is altered as temperature changes, the pH of the calibration buffer was determined by a Unicam combustion pH electrode which gave a reading up to 3 decimal places.

Substituting both pH (Table 5.3) and the corrected pK_1, pK_2 and pK_{ca} values into equation (2) to calculate the dissociation constants for calcium and EGTA at both 37 and 45°C, the corrected K_d for Ca-EGTA at 0.1 M ionic strength is given by:

Temperature (°C)	Corrected K_d (nM)
37	126
45	203

$$\text{Now, free calcium concentration in the buffer} = K_d \cdot \frac{[Ca \cdot EGTA]}{[EGTA]} \qquad (4)$$

The [Ca·EGTA] and [EGTA] were given by the manufacturer, so the free calcium concentration at a particular temperature and pH could be calculated by using equation (4).

The corrected free calcium concentrations together with the fluorescence ratios obtained at 350 and 380 nm were then fitted using a least squares program in order to determine the $K_{1/2}$. This $K_{1/2}$ value was then used to calculate the K_d of fura-2 for calcium (Groden *et al.*, 1991) as follows:

$$K_d = \frac{K_{1/2}}{\frac{Sf_2}{Sb_2}} \qquad (5)$$

where $K_{1/2}=[Ca^{2+}]_i$ for which $R = (R_{min} + R_{max})/2$

Sb_2= fluorescence intensity obtained at 380 nm at zero calcium concentration

Sf_2= fluorescence intensity obtained at 380 nm at saturating calcium concentration

The K_d of fura-2 and calcium were then used to convert the ratio obtained from the experiments to the free $[Ca^{2+}]_i$ according to the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d(\frac{R - R_{min}}{R_{max} - R})(\frac{Sf_2}{Sb_2}) \quad (6)$$

where R = Ratio obtained at 350/380 nm

R_{max} = Ratio obtained at saturating calcium

R_{min} = Ratio obtained at zero calcium

5.2.2 Determination of cytosolic free calcium during heating

(i) Culture conditions

WRK-1 cells from a 25 cm^2 flask were harvested, resuspended in growth medium and counted. The suspension was further diluted by growth medium to give 80000 cells/ml. The diluted cell suspension (0.1 ml) was then added to a number of wells in 6-well plates with a 10-mm diameter coverslip in each well. Cells were maintained at 37°C in an air/CO_2 (19:1, v/v) atmosphere. Cells were used between 18-24 h later.

(ii) Stock solutions of calcium indicators

On receipt of fura-2/AM or the free acid, the powder was dissolved in DMSO, then mixed for 10 min at room temperature, to give a stock solution of 1 mM. Dye solution (20 µl) was then dispensed into microfuge tubes together with 10 µl of 10 % (w/v) pluronic acid in DMSO, which is included to give a better dye loading. Tubes were stored at -20°C for up to 3 months and were thawed only once before use.

(iii) Dye loading

Fura-2/AM stock solution was thawed in an enclosed warm-up box containing dry silica gel to prevent condensation. The thawed dye (30 µl) was then mixed with 1.97 ml

of serum-free, phenol-red free EMEM containing 20 mM Hepes, pH 7.4 (medium A) to give a 10 μM fura-2/AM dye solution. The diluted dye solution was then mixed on a vortex mixer for 10 min. The coverslip with attached cells was transferred to a well of a 24-well plate and 200 μl of the dye solution was added to it. The plate was then incubated in a 30°C incubator for 1.5 h.

(iv) Cytosolic free calcium measurements during heating

Following the loading of cells with fura-2/AM, the coverslip was washed three times with medium A or the appropriate solution that would be used in the measurement. Then it was transferred to the microscope perfusion chamber containing 1 ml of medium A or calcium-containing buffer (138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose and 20 mM Hepes, pH 7.4) or calcium-free buffer (138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 5.5 mM glucose and 20 mM Hepes, pH 7.4) at 37°C. A background reading was taken in the same field as the cell that would be used. Then a cell was focused and the ratio was taken at 37°C. The temperature was then increased to 45°C using a combination of the temperature controller and by perfusing hot medium (50°C) through the system. Using this technique, it took about 2-3 min to reach 45°C. Measurements were taken intermittently for 8 s in every 200 s interval, because continous monitoring was found to accelerate the rate of photobleaching.

5.2.3 Determination of cell survival

The effect of calcium on hyperthermic cell death was studied by heating the cells in EMEM, calcium-containing buffer (1.8 mM Ca^{2+}) or calcium-free buffer. Furthermore, the effect of a calcium channel blocker on cell death was studied.

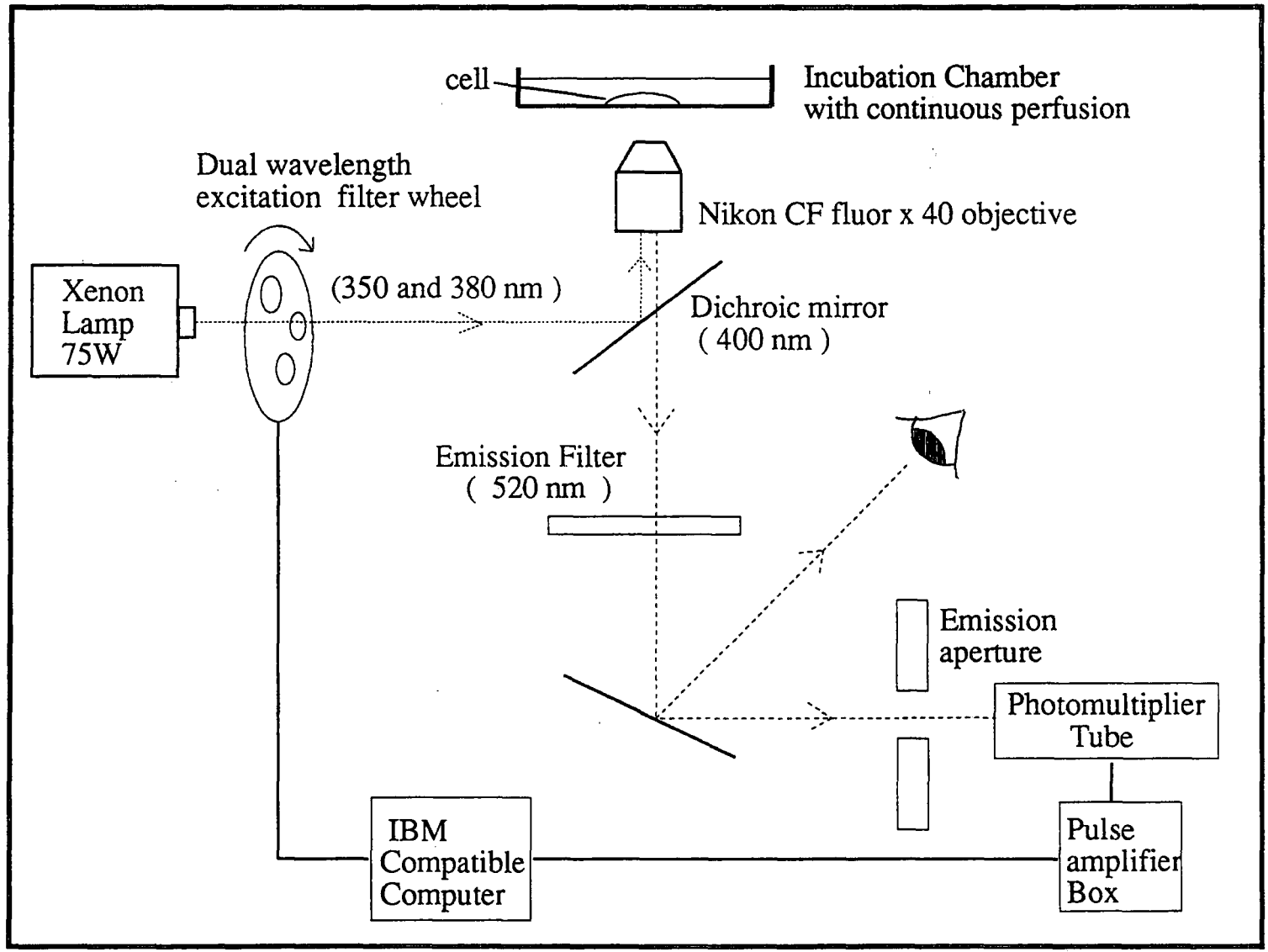
(i) Effect of extracellular calcium concentration on hyperthermic cell death

WRK-1 cells were harvested and incubated as described in section 3.2.2(ii). On the day of heating (day 3), the medium was replaced either by 1 ml of EMEM, calcium-containing buffer or calcium-free buffer, together with 24 mM sodium bicarbonate and 20 mM Hepes, pH 7.4. The plates were then returned to the 37°C incubator, containing an atmosphere of air/CO_2 (19:1, v/v), for 20 min. After 20 min, plates were sealed then equilibrated under the surface of a water bath for 15 min at 37°C. The plates were then transferred and submerged in a 46.5°C water bath for 4 min before moving to a 45°C bath. Plates were removed from this latter water bath every 7 min (including the 4 min lag period) for up to 42 min. Plates were cleaned with 70% (v/v) alcohol and unsealed. Then 10 µl of 181.8 mM calcium chloride was added to the wells containing calcium-free buffer. Sera were added to all of the wells to give a final concentration of 2% (v/v) rat serum and 5% (v/v) FBS. Plates were then returned to culture in the 37°C incubator and, after 4 h the medium was replaced by fresh growth medium and with one medium change on day 5. Cell viability was assessed by the MTT assay on day 6.

(ii) Effect of calcium channel blocker on hyperthermic cell death

Cells were harvested and seeded as described in section 3.2.2(ii). On day 3, the medium was replaced by either EMEM medium or EMEM containing 50 µM nifedipine. In both cases, 24 mM sodium bicarbonate and 20 mM Hepes, pH 7.4 were included. Plates were returned to the incubator at 37°C, containing an atmosphere of air/CO_2 (19:1, v/v), for 20 min. Plates were then sealed and then equilibrated at 37°C in a water bath for 15 min and heated as described in section 5.2.3(i). Plates were then cleaned with 70% (v/v) alcohol and unsealed. Sera were added to all of the wells to give a final concentration of 2% (v/v) rat serum and 5% (v/v) FBS. Furthermore, 1 µl of 50 mM nifedipine was added to the wells containing EMEM alone, to give a final concentration

of 50 μM. Plates were then returned to culture at 37°C and the medium was replaced with growth medium after 4 h and with one medium change on day 5. Cell viability was assessed by the MTT assay on day 6.


cell
Incubation Chamber
with continuous perfusion
Dual wavelength
excitation filter wheel
Nikon CF fluor x 40 objective
Xenon
Lamp
75W
(350 and 380 nm)
Dichroic mirror
(400 nm)
Emission Filter
(520 nm)
Emission
aperture
Photomultiplier
Tube
IBM
Compatible
Computer
Pulse
amplifier
Box

5.3 Results

The effect of heat on the cytosolic free calcium concentration ($[Ca^{2+}]_i$) was determined by an epifluorescent microscopic technique (sections 5.2.2(iv)). This system enabled the determination of $[Ca^{2+}]_i$ at the single cell level and allowed continuous perfusion of appropriate buffers, so the artifacts due to dye leakage out of the cells into the medium were avoided.

In order to correlate free calcium concentration with the ratio obtained from the fluorescent signal following excitation at wavelengths 350 and 380 nm, calibration is necessary. *In vitro* calibration was performed using fura-2 free acid together with buffers containing different concentrations of free calcium (in nM to μM range). This required a high buffering chelator and EGTA suits this purpose. However, as the dissociation constant of EGTA and calcium is affected by pH, ionic strength and temperature, failure to consider these factors will result in incorrect estimation of free calcium concentration in the buffer. The Ca-EGTA dissociation constant has been determined experimentally (Bers, 1982). Alternatively, the dissociation constant can be calculated by using individual stoichiometric association constants of EGTA for protons and Ca^{2+} (Harrison and Bers, 1989). Since the association constants of EGTA for protons and Ca^{2+} are modified by changes in temperature, pH and ionic strength, these constants must be adjusted for conditions similar to those used experimentally prior to the determination of the dissociation constant of Ca-EGTA ($K_{dCa\text{-}EGTA}$). It was found that increasing the temperature from 37°C to 45°C resulted in a decrease in pH value (Table 5.3). The $K_{dCa\text{-}EGTA}$ was then calculated by using the converted association constants of EGTA for protons and Ca^{2+}. It was found that, as the temperature increased from 37 to 45°C, the dissociation constant of Ca-EGTA increased by about 61% (Figure 5.4).

After the K_d values of Ca-EGTA were corrected for temperature, they were then

used for calculating the free calcium concentrations. The ratios obtained at 350/380 nm excitation wavelength were plotted against free calcium concentrations (in log scale) and the data points were analysed by a least squares analysis program to give the best fit curves at 37 and 45°C (Figure 5.5). The Hill coefficients of these curves were 1 at both temperatures, suggesting that fura-2 and calcium had a binding stoichiometry of 1:1. The $K_{1/2}$ value obtained from the curve was 623.8 nM at 37°C and 1238.8 nM at 45°C. These $K_{1/2}$ values were then used to calculate the dissociation constant of the fura-2/Ca^{2+} complex by substituting these values into equation (5) as described in section 5.2.1(iv). The calculated dissociation constants of fura-2/Ca^{2+} complex were 151 nM and 209 nM at 37 and 45°C, respectively.

Fura-2/AM, the hydrophobic acetoxy methylester form of the dye, can cross the cell membrane very easily. Once inside the cells, the acetoxymethyl groups are cleaved by esterases to give a highly charged fura-2, so it is trapped in the cytosol. If the cytosolic esterase activity is low, such that the ester bonds are not cleaved rapidly, the fura-2/AM can cross the membranes of intracellular organelles. Also, the dye can enter into the organelles via endocytosis and pinocytsis. The fura-2 so sequestered is insensitive to changes in $[Ca^{2+}]_i$ in the cytosol (Steinberg *et al.*, 1987). The problem of dye sequestration can be eliminated by reducing the temperature of dye loading. For example endocytosis and compartmentalization can be slowed down by a reduction in the loading temperature e.g. from 37°C to 32°C (Poenie *et al.*, 1986) or at 15°C (Malgaroli *et al.*, 1987). However, dye loading at low temperature followed by transferring of WRK-1 cells to 45°C meant that a larger temperature jump would be experienced by the cells, and this might produce artefacts in hyperthermic studies on the cells. Thus a loading temperature close to the normal growth temperature was sought. It was found that loading at 30°C for 1.5 h achieved an even distribution of the dye, i.e. prevented dye sequestration, as well as providing a high enough fluorescent signal to be detected. Thus this loading condition was used. The dispersing agent pluronic acid was

also included in the dye solution to prevent the incorporation of the dye into endocytic vesicles (Tsien, 1989).

Phenol red, which is present in most growth media, has been reported to give a highly fluorescent signal (Moore *et al.*, 1990). Thus in this study, phenol red-free EMEM medium was used instead. Serum was also omitted from the dye solution and the experimental medium to prevent the possibility of calcium mobilisation triggered by hormones or growth factors that are present in serum.

Once the appropriate loading condition was established and a suitable dye-loading medium had been chosen, determination of $[Ca^{2+}]_i$ was performed. To ensure that this loading system can detect rapid changes in $[Ca^{2+}]_i$, 1 μM vasopressin was added to the cells by perfusion. Vasopressin at 1 μM concentration has been reported to release Ca^{2+} from intracellular stores of WRK-1 cells (Mouillac *et al.*, 1989). Upon addition of this hormone (through perfusion) the ratio increased from 0.8 to about 4 i.e. $[Ca^{2+}]_i$ increased from 54±4.8 nM to 506±56.5 nM (8 determinations). Typical examples of the increase in $[Ca^{2+}]_i$ (under 1.6 mM extracellular calcium concentration or in calcium-free condition) upon vasopressin stimulation are shown in Figure 5.6. An increase in $[Ca^{2+}]_i$ was detected within 10 sec from the point when perfusion started and returned to basal level after about 100 sec. This increase in $[Ca^{2+}]_i$ was detected in either EMEM (Figure 5.6a) or in calcium-free buffer (Figure 5.6b). Results imply that rapid changes in $[Ca^{2+}]_i$ can be detected readily by this fluorescence system.

The effect of hyperthermia on $[Ca^{2+}]_i$ was then investigated in WRK-1 cells. Heating was performed in EMEM (1.6 mM extracellular calcium), calcium-containing buffer (1.8 mM extracellular calcium), calcium-free buffer (including 0.5 mM EGTA) and EMEM medium containing calcium channel blockers (nifedipine or La^{3+}).

When WRK-1 cells were heated at 45°C in EMEM medium, an increase in $[Ca^{2+}]_i$ was observed in most of the cells measured, and typical examples of the heat-induced increase in $[Ca^{2+}]_i$ are shown in Figure 5.7. Out of 50 cells analysed, 76% showed an elevated $[Ca^{2+}]_i$ within the heating period used (up to 30 min). No change in $[Ca^{2+}]_i$ was observed in control cells incubated at 37°C for up to 30 min (data not shown). However, when heating to 45°C was carried out in a simple calcium-containing buffer (containing 1.8 mM $CaCl_2$), only 25% of the cells showed an increase in $[Ca^{2+}]_i$ level, out of 24 cells analysed (Figure 5.8). To determine $[Ca^{2+}]_i$ under these heating conditions, fluorescent ratios obtained from these cells were converted to free calcium concentration using equation (6) described in section 5.2.1(iv). The mean $[Ca^{2+}]_i$ and standard error of the mean were plotted against duration of heating at 45°C (Figure 5.9). In both cases, the increase in $[Ca^{2+}]_i$ was dependent upon duration of heating. For cells heated in calcium-containing buffer, the mean $[Ca^{2+}]_i$ reached 250 nM after 1000 sec. Similarly, cells heated in EMEM medium resulted in an increase in mean $[Ca^{2+}]_i$ to the same level after 1000 sec and $[Ca^{2+}]_i$ continue to increase as the heating time increased, achieving a mean $[Ca^{2+}]_i$ of 800 nM after 1600 sec. On the other hand, there was insufficient data to determine $[Ca^{2+}]_i$ for heating times longer than 1000 sec in calcium-containing buffer. Whether $[Ca^{2+}]_i$ increased continuously until the intracellular and extracellular calcium concentration reached equilibrium, as a result of an increase in membrane permeability as suggested by Stevenson *et al.* (1987), is unknown since the fluorescence intensity of the cells became very low on prolonged heating. As shown in Figure 5.7 and 5.8, the lag time before an elevation in $[Ca^{2+}]_i$ was observed varied from one cell to another. The lag time required for cells to show an increase in $[Ca^{2+}]_i$ is shown in Figure 5.10. It was observed that $[Ca^{2+}]_i$ in some cells started to rise as early as 50 sec at 45°C, whereas some did not show any changes until very late in the measurement.

In order to compare the rate of increase in $[Ca^{2+}]_i$ upon heating in EMEM and calcium-containing buffer, the time at which an increase in $[Ca^{2+}]_i$ was observed was

normalised to 0 sec and $[Ca^{2+}]_i$ obtained under both conditions was compared at every 200 sec thereafter (Figure 5.11). It can be deduced from the graph that the initial rate of increase in $[Ca^{2+}]_i$ in both conditions was similar, whereas the rate of increase in $[Ca^{2+}]_i$ started to slow down after 600 sec in cells heated in calcium-containing buffer.

The results so far suggest that an increase in $[Ca^{2+}]_i$ was observed in many cases when cells were heated in calcium-containing media, although this occurred more frequently in EMEM than in calcium-containing buffer. In order to determine whether this increase was due to calcium released from internal stores or to calcium influx from the extracellular medium, experiments were performed in calcium-free buffer (Figure 5.12). The heat-induced increase in $[Ca^{2+}]_i$ was completely abolished under calcium-free conditions (19 determinations).

Hence the data suggest that (1) elevation of $[Ca^{2+}]_i$ upon heating was due primarily to calcium influx from the extracellular medium, (2) EMEM had a more profound effect in promoting a heat-induced increase in $[Ca^{2+}]_i$, so it was employed in the rest of this study to investigate the possible pathway for calcium entry.

Calcium influx through voltage-gated calcium channels has been successfully blocked by inorganic channel blockers such as Co^{2+}, La^{3+} and Cd^{2+} as well as by organic channel blockers such as nifedipine, verapamil and diltiazem (Hallett *et al.*, 1990). In this study, the effects of La^{3+} and nifedipine on the heat-induced increase in $[Ca^{2+}]_i$ were studied (Figure 5.13). It was found that the resting $[Ca^{2+}]_i$ value was not affected by the presence of 50 μM La^{3+} (Figure 5.13a) or 50 μM nifedipine (Figure 5.13b), nor did they abolish the heat-induced increase in $[Ca^{2+}]_i$ (Figure 5.14). Since the lag period before an increase in $[Ca^{2+}]_i$ was observed varied in different cells, the time at which the increase in $[Ca^{2+}]_i$ began was normalised to 0 sec and $[Ca^{2+}]_i$ in heated cells was compared every 200 sec thereafter (Figure 5.15). It was found that the rate of

increase in $[Ca^{2+}]_i$ in the presence of either channel blocker was insignificantly different from those heated in EMEM (control). These results suggested that the heat-induced increase in $[Ca^{2+}]_i$ was not due to an influx of calcium through the voltage-gated calcium channel. This conclusion was further supported by exposure of cells to a high concentration (50 mM) of K^+ (Figure 5.16). Medium containing this concentration of K^+ causes depolarisation of the cell membrane and opens voltage-gated calcium channels to allow calcium influx (Ozawa and Sand, 1986), but this treatment did not show any change in $[Ca^{2+}]_i$, suggesting that voltage-gated calcium channels were absent in these cells.

The Na^+/Ca^{2+} exchanger is present in both excitable and non-excitable cells, and it has been suggested to play a role in controlling calcium homeostasis (Carafoli, 1987). The action of this exchanger can be reversed depending on the magnitude and direction of the Na^+ electrochemical gradient, resulting in net movement of Ca^{2+} into or out of the cells (Carafoli, 1987; Reeves, 1990). The effect of heat on the Na^+/Ca^{2+} exchanger has been investigated in A-431 cells upon heating at 45°C (Kiang *et al.*, 1992). These authors suggested that the heat-induced increase in $[Ca^{2+}]_i$ was due to a reversal of the normal operation of this exchanger during heating, thus causing Ca^{2+} influx. Evidence leading to this conclusion included: (1) The increase in $[Ca^{2+}]_i$ during heating was dependent upon the extracellular calcium concentration, higher levels of extracellular calcium concentrations resulting in a higher level of heat-induced $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ was correlated with an increase in Ca^{2+} influx when assayed by $^{45}Ca^{2+}$. (2) The heat-induced rise in $[Ca^{2+}]_i$ was dependent on extracellular sodium concentration and a higher level of heat-induced $[Ca^{2+}]_i$ rise was observed as the extracellular Na^+ concentration increased. Removal of the sodium gradient by loading the cells with Na^+, by employing a Na^+-K^+-ATPase blocker such as ouabain, caused an increase in resting $[Ca^{2+}]_i$, but heating under these conditions only caused a slight increase in $[Ca^{2+}]_i$, suggesting that the heat-induced increase in $[Ca^{2+}]_i$ was related to the Na^+ gradient

across the plasma membrane. (3) The heat-induced increase in $[Ca^{2+}]_i$ was blocked by 5 μM amiloride or 1 μM 5'(N,N'-dimethyl)amiloride (an amiloride analogue which is a slightly more potent inhibitor of Na^+/Ca^{2+}exchanger), which were presumably acting by exerting their effects on the Na^+/Ca^{2+}exchanger, though the effect of these inhibitors on other Na^+ channels and the Na^+/H^+ exchanger were not excluded.

When WRK-1 cells were heated in the presence of 5 μM amiloride (in EMEM), the heat-induced increase in $[Ca^{2+}]_i$ was not abolished, though it occurred in a smaller proportion of heated cells, and the increase in $[Ca^{2+}]$ required a longer heating time (Figure 5.17). At present, there is insufficient data to make a precise decision on the role of the Na^+/Ca^{2+} exchanger in the heat-induced increase in $[Ca^{2+}]_i$. It could be possible that 5 μM amiloride is insufficient to inhibit the Na^+/Ca^{2+} exchanger as it has been found that 1 mM amiloride is required to inhibit this exchanger in epithelial cells (Kaczorowski *et al.*, 1985). Furthermore, it has been reported that although amiloride is an inhibitor of the Na^+/Ca^{2+} exchanger, it is a more potent inhibitor of voltage-gated calcium channels and the Na^+/H^+ exchanger (Kleyman and Cragoe, 1988). Thus when using amiloride in investigating the effect of heat on Na^+/Ca^{2+} exchanger, the effect of this inhibitor on other systems must be taken into account. It is clear that further studies such as using different concentrations of amiloride and/or using more potent and selective inhibitors (such as amiloride analogues bearing substituents on the 5-amino and terminal guanidino nitrogen atoms) of Na^+/Ca^{2+} exchanger must be carried out to clarify the role of Na^+/Ca^{2+} exchanger in heat-induced $[Ca^{2+}]_i$ elevation.

To test whether the heat-induced increase in $[Ca^{2+}]_i$ played a role in hyperthermic cell death, the $[Ca^{2+}]_i$ during heating was modulated by using different media. For example when heating was performed in EMEM, 76% of the cells had an elevated $[Ca^{2+}]_i$ level. By heating in 1.8 mM calcium-containing buffer, only 25% showed a rise

in $[Ca^{2+}]_i$. Furthermore, using calcium-free buffer, the $[Ca^{2+}]_i$ remained unchanged (maintained at about 40-50 nM).

The killing curves obtained from these conditions are shown in Figure 5.18. The data points on the second phase (linear portion) of the curves were fitted with regression lines. The survival parameters, D_q, D_0, LD_{50} and LD_{90} were calculated from the regression lines of the curves and are shown in Table 5.4. D_q measures the width of the shoulder region and Jung (1986) has pointed out that it may be the time-dependency of the conversion from non-lethal to lethal damage. D_0 measures the time required to reduce the survival on the exponential part of the curve to 37% of an initial value. The survival parameters of cells heated in EMEM or in calcium-containing buffer were insignificantly different from each other. On the other hand, both D_q and D_0 values were reduced when cells were heated in calcium-free buffer compared with cells heated in EMEM, showing an increase in heat sensitisation under this condition.

The effect of nifedipine on the survival of WRK-1 cells following exposure to hyperthermia was also investigated. The experiment was complicated by the fact that cells often detach from wells during heating and must be left for 4 h to reattach before the medium can be changed. Thus, nifedipine cannot be removed immediately after heating, and must remain in contact with the cells for a further 4 h. To correct for this, one group of cells was exposed to nifedipine during heating and throughout the 4 h post-heating (reattachment) period, while a second group was exposed to nifedipine only during the 4 h post-heating period. Survival parameters D_0, D_q, LD_{50} and LD_{90} in these two groups were compared with a third group of cells, heated in EMEM only (Figure 5.18b and Table 5.4). The survival parameters in the two nifedipine-treated groups were similar, except for the D_0 value, which was significantly higher ($p<0.05$) when nifedipine was added post-heating. This implies that the presence of nifedipine during and after heating was more effective in killing cells than its presence during the post-

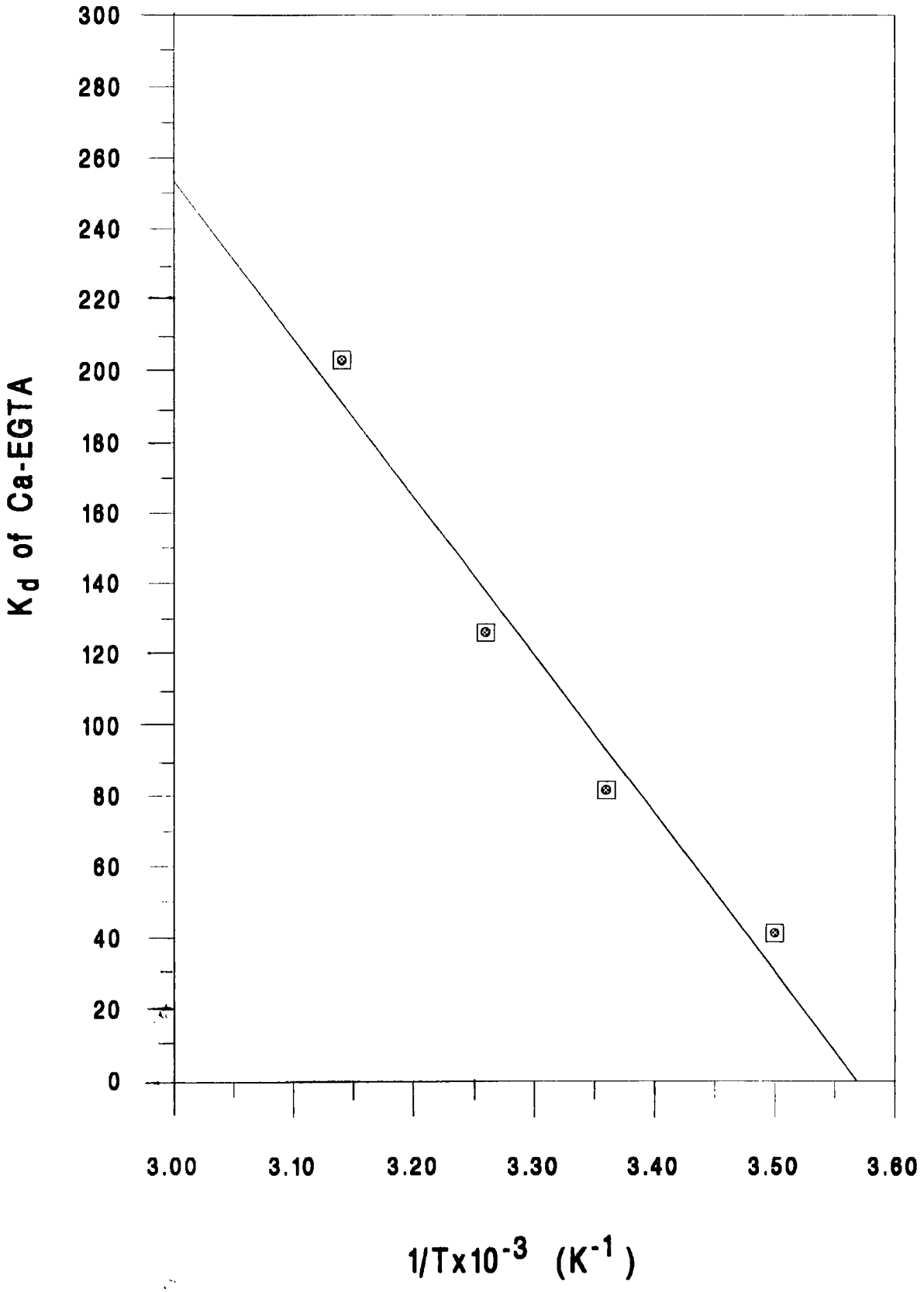
heating period only. However, the presence of nifedipine in both cases resulted in a significant difference ($p<0.05$) in survival parameters D_0, D_q and LD_{50} compared with cells heated in EMEM alone (Table 5.4). Thus, the presence of nifedipine (either during heating and the post-heating period or during the 4 h post-heating period only) increased cell death compared with cells heated in EMEM only.

Table 5.3. Effect of temperature on the pH of calcium-EGTA buffers

Since the pH of the buffers was affected by the temperature, the pH of the buffer at 37 and 45°C was determined using an Unicam combustion pH electrode which gave a reading up to three decimal places. Measurements were repeated three times.

Temperature (°C)	pH of buffer
37	7.166±0.002
45	7.032±0.015

Values represent mean ± SEM from three determinations.


300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
K_d of Ca-EGTA
3.00
3.10
3.20
3.30
3.40
3.50
3.60
$1/T \times 10^{-3}$ (K^{-1})

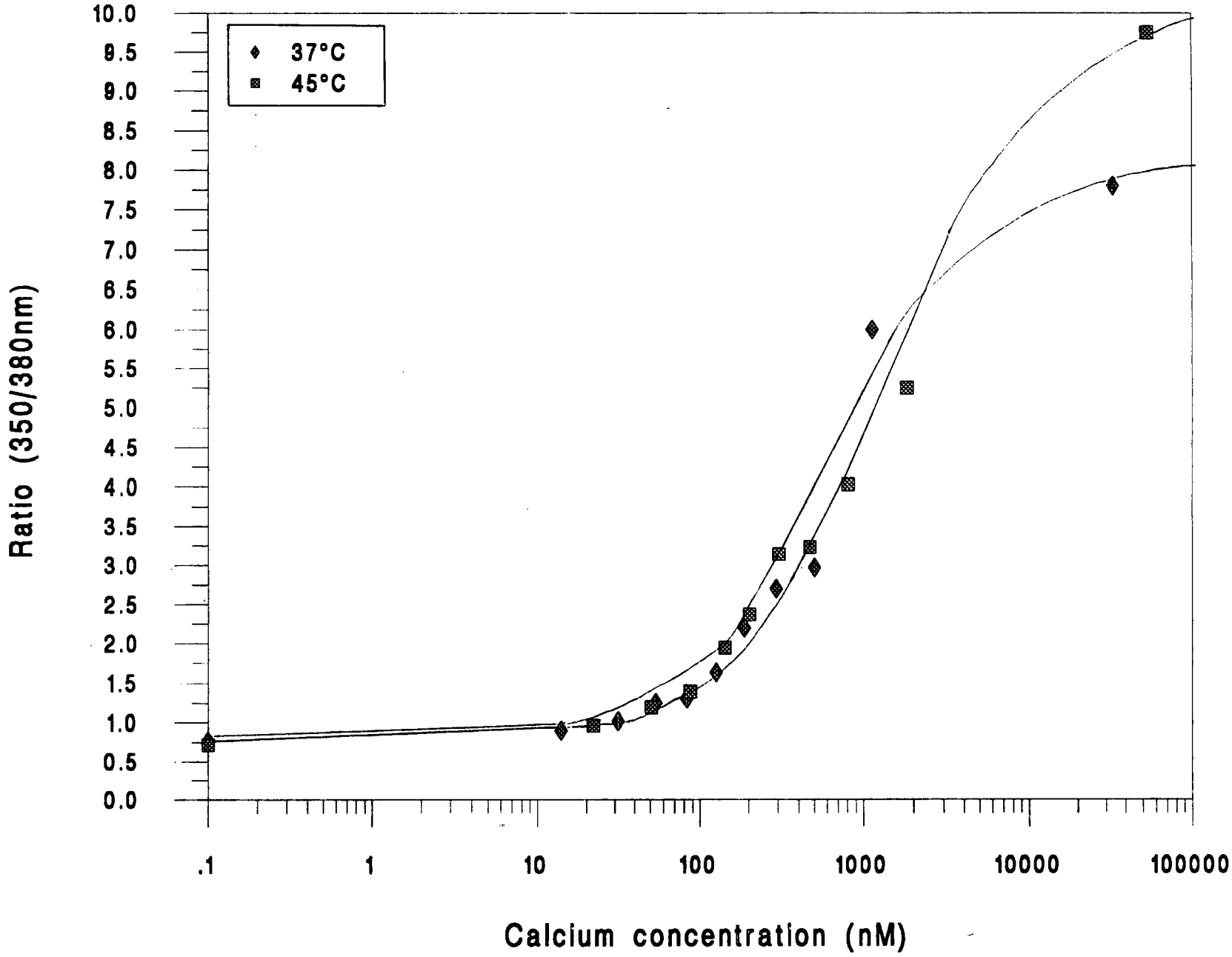

37°C
45°C
Ratio (350/380nm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
.1
1
10
100
1000
10000
100000
Calcium concentration (nM)

Figure 5.6a. Changes in $[Ca^{2+}]_i$ upon vasopressin stimulation in EMEM

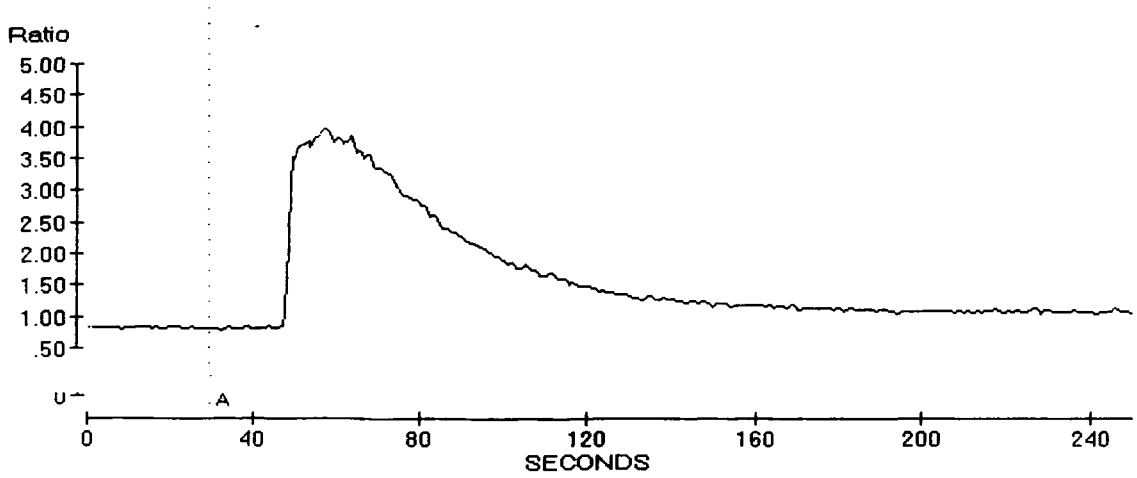
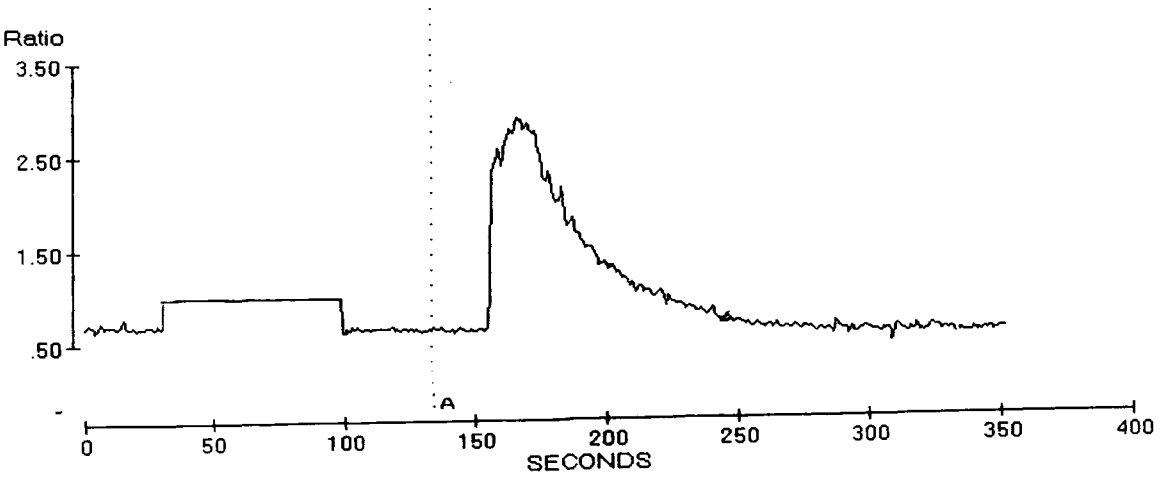


Figure 5.6b. Changes in $[Ca^{2+}]_i$ upon vasopressin stimulation in calcium-free buffer



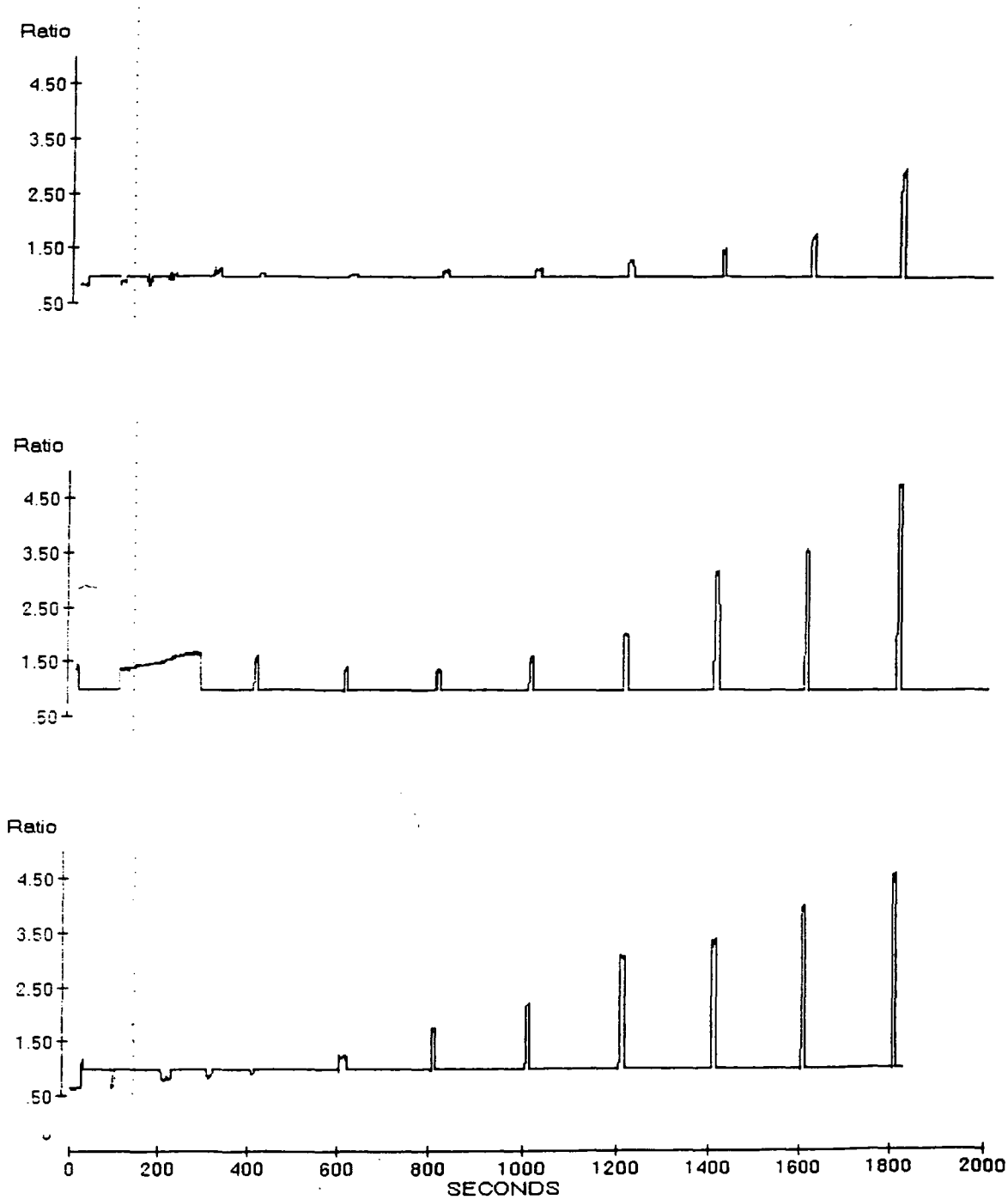

Ratio
4.50
3.50
2.50
1.50
.50
Ratio
4.50
3.50
2.50
1.50
.50
Ratio
4.50
3.50
2.50
1.50
.50
0
200
400
600
800
1000
1200
1400
1600
1800
2000
SECONDS

Figure 5.8a.

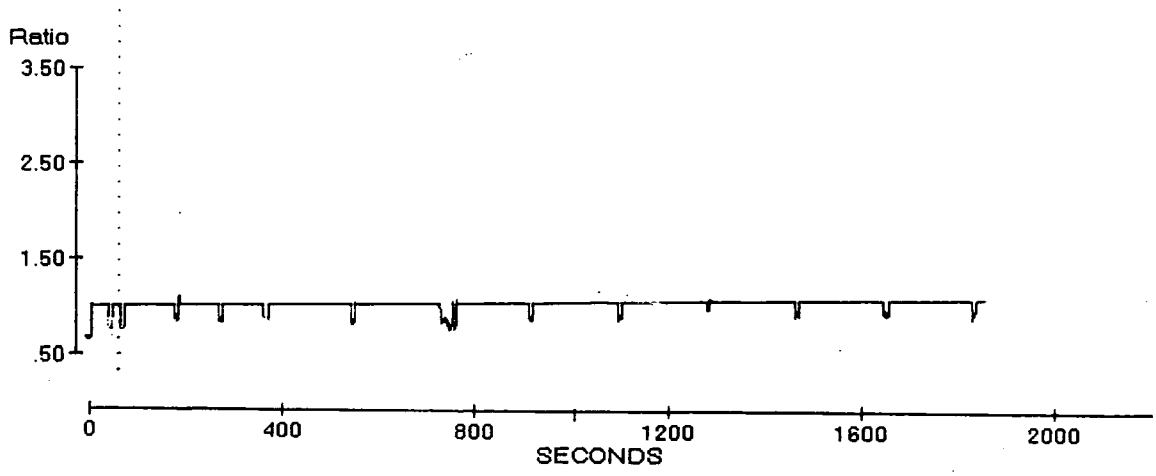
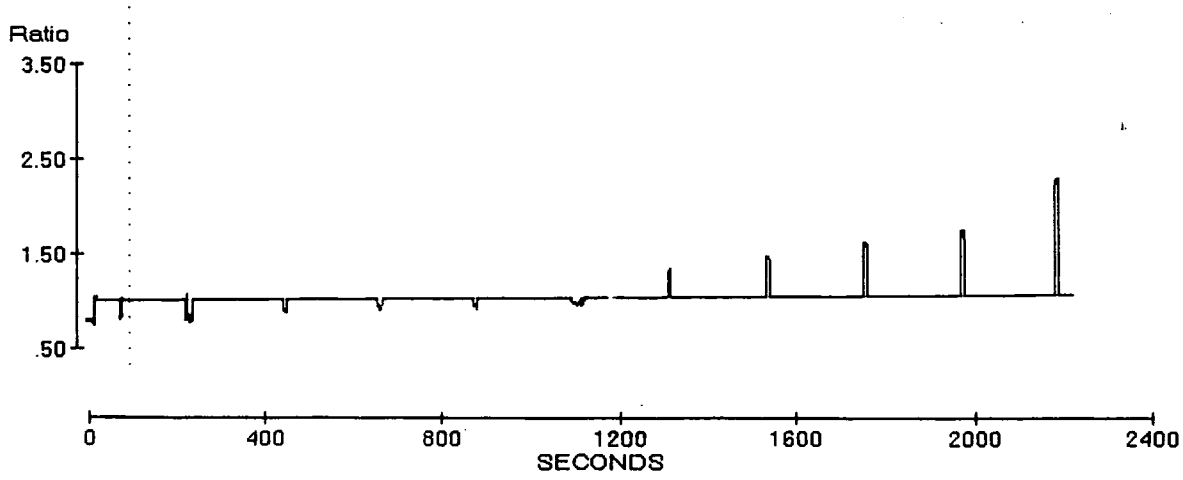


Figure 5.8b.



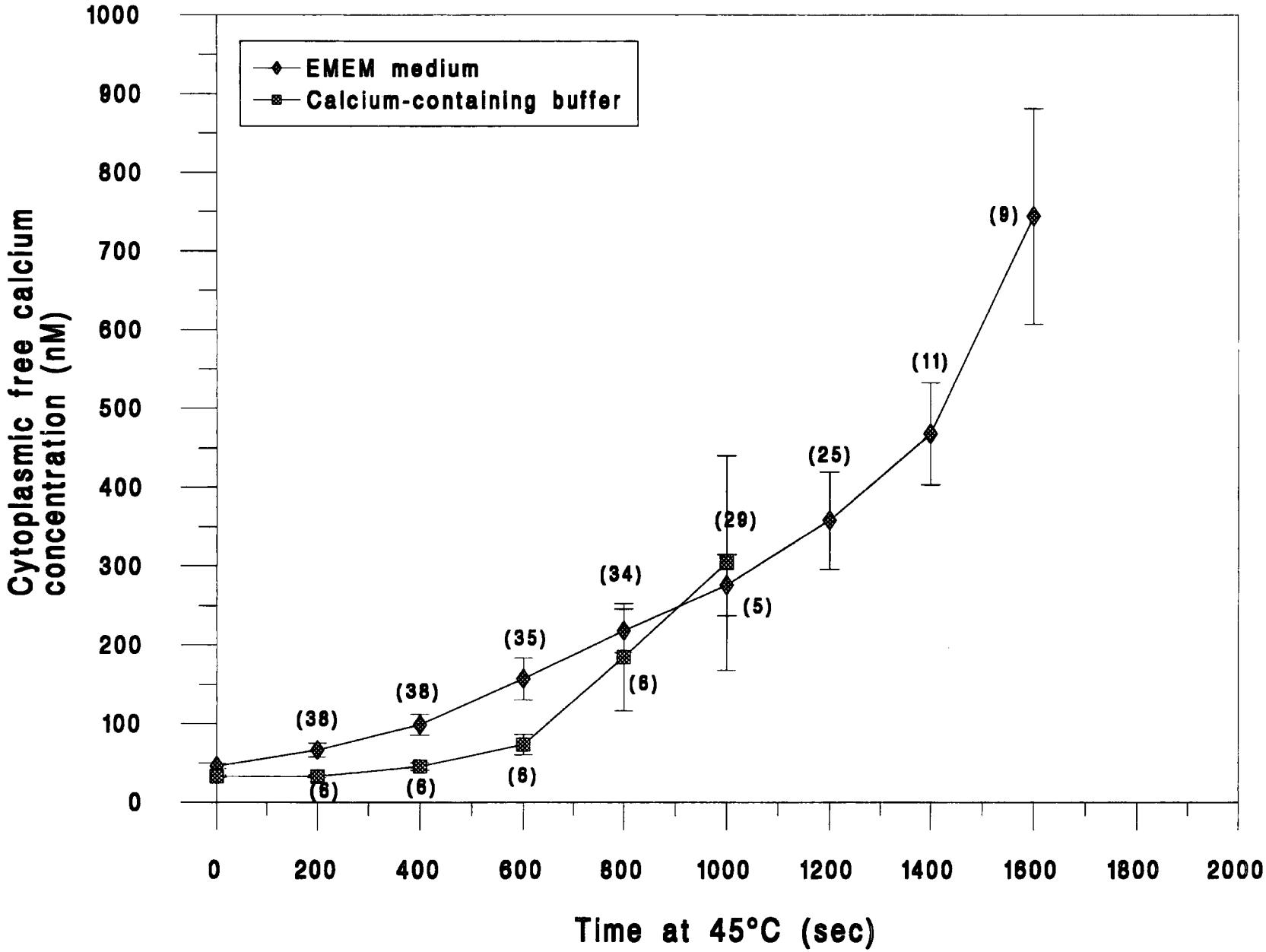

EMEM medium
Calcium-containing buffer
Cytoplasmic free calcium concentration (nM)
Time at 45°C (sec)
0
100
200
300
400
500
600
700
800
900
1000
0
200
400
600
800
1000
1200
1400
1600
1800
2000
(38)
(38)
(35)
(34)
(29)
(25)
(11)
(9)
(6)
(6)
(6)
(6)
(5)

Figure 5.10a. The lag time at 45°C before an increase in $[Ca^{2+}]_i$ was observed when heated in EMEM medium

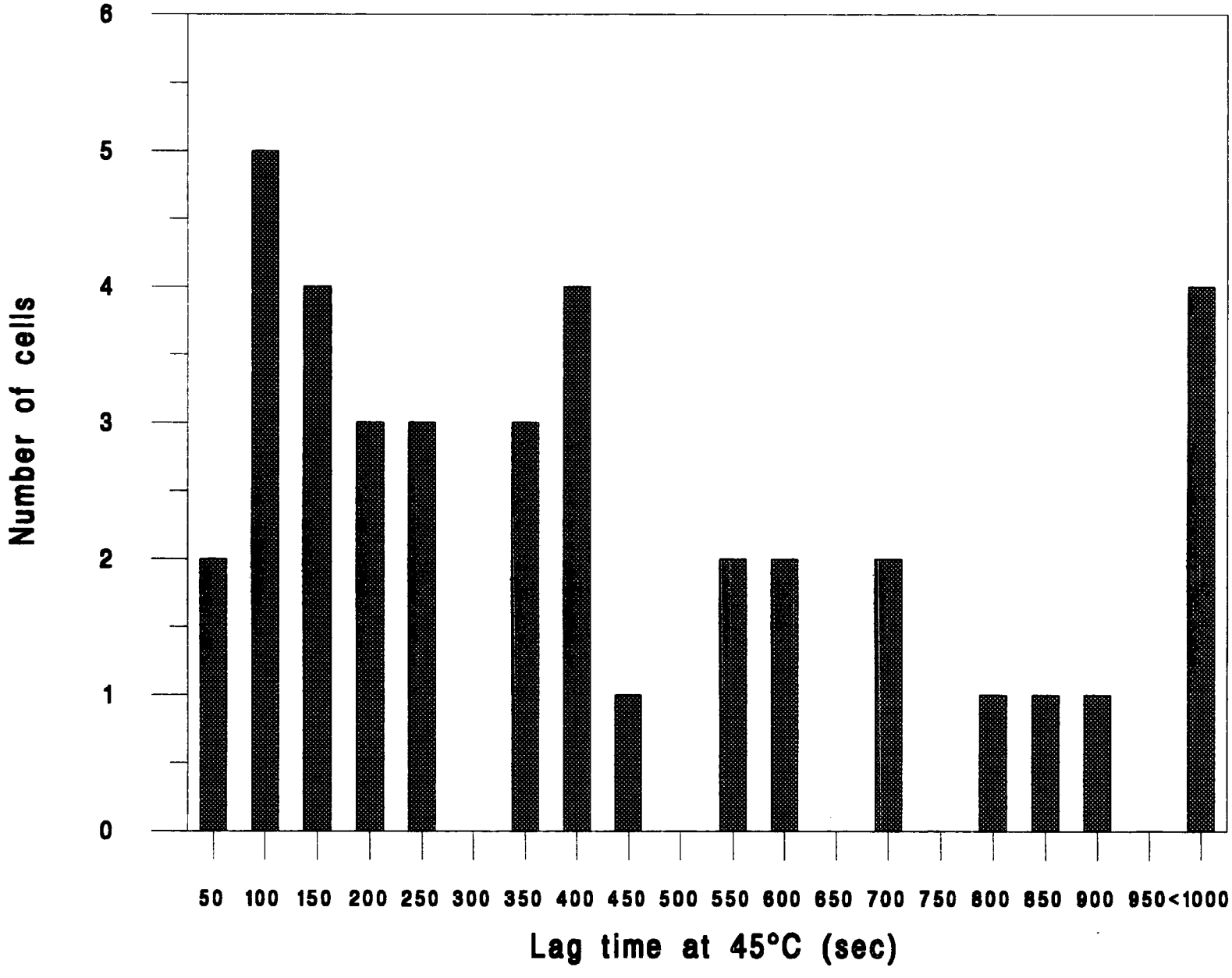
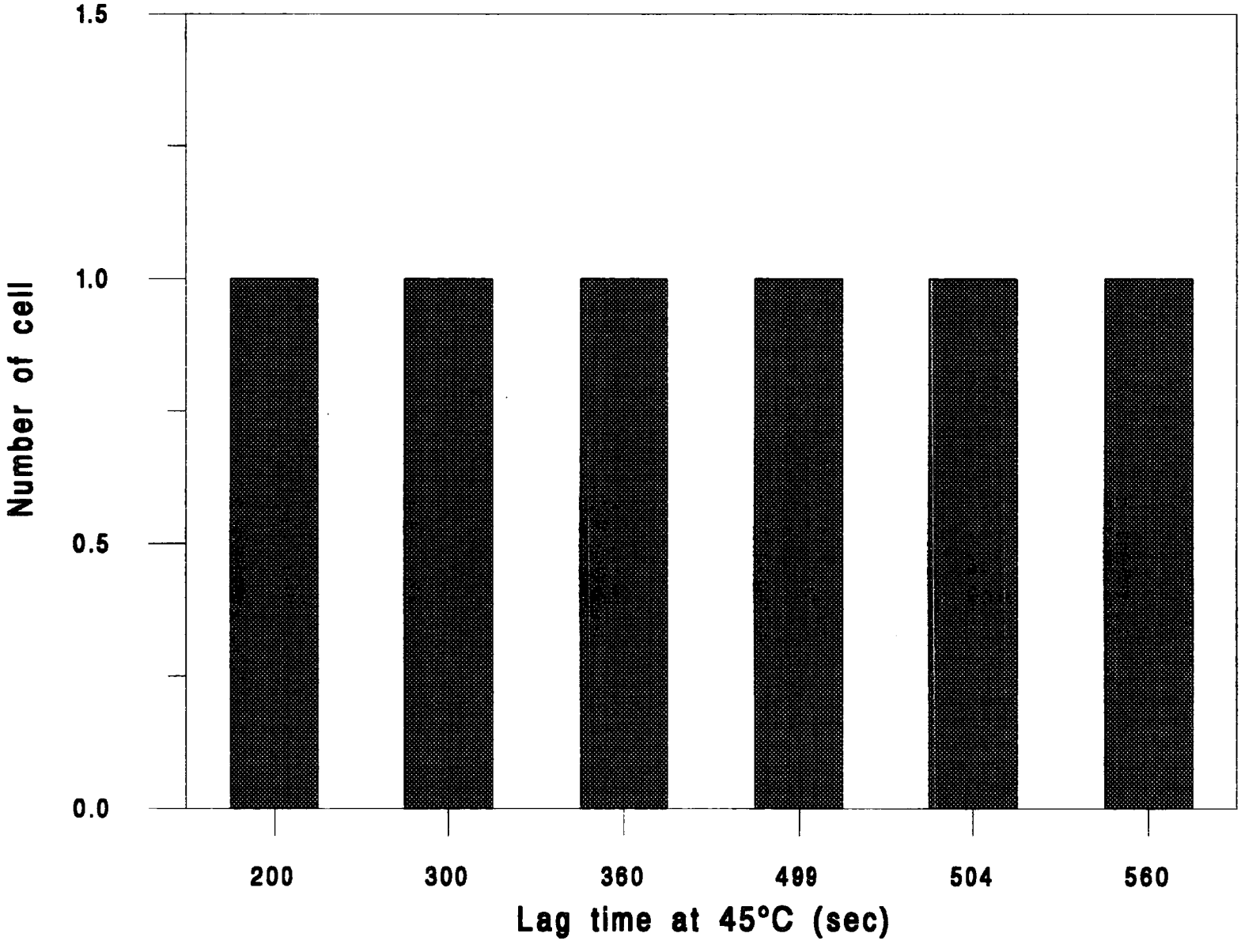
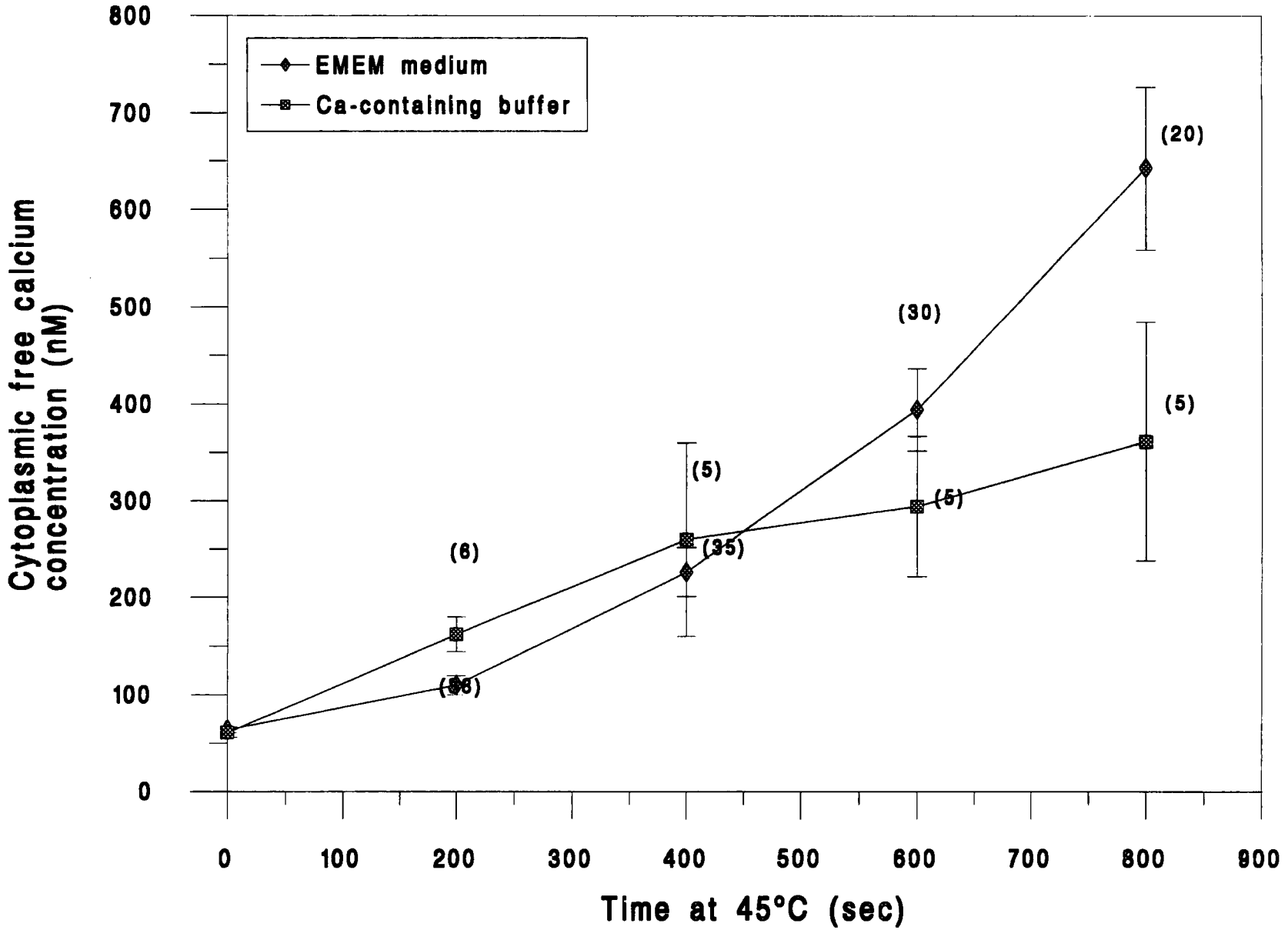
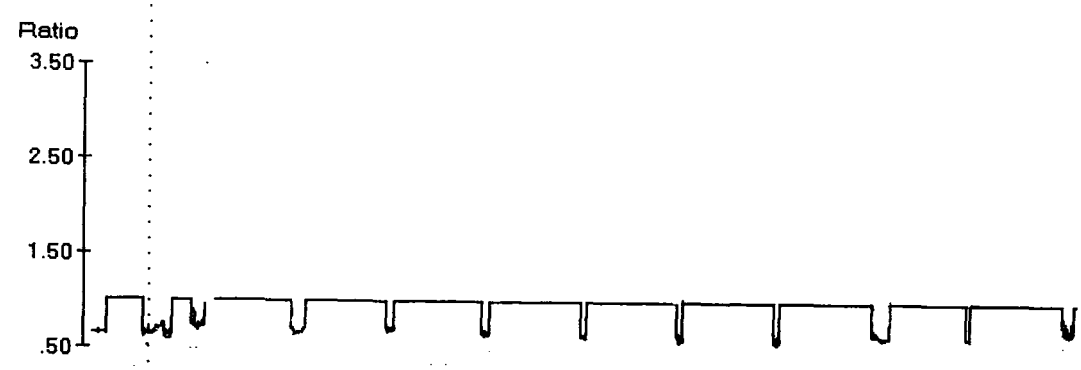


Figure 5.10b. The lag time at 45°C before an increase in $[Ca^{2+}]_i$ was observed when heated in calcium-containing buffer




EMEM medium
Ca-containing buffer
Cytoplasmic free calcium concentration (nM)
Time at 45°C (sec)
0
100
200
300
400
500
600
700
800
900
(6)
(5)
(35)
(30)
(5)
(20)
(5)


Ratio
3.50
2.50
1.50
.50

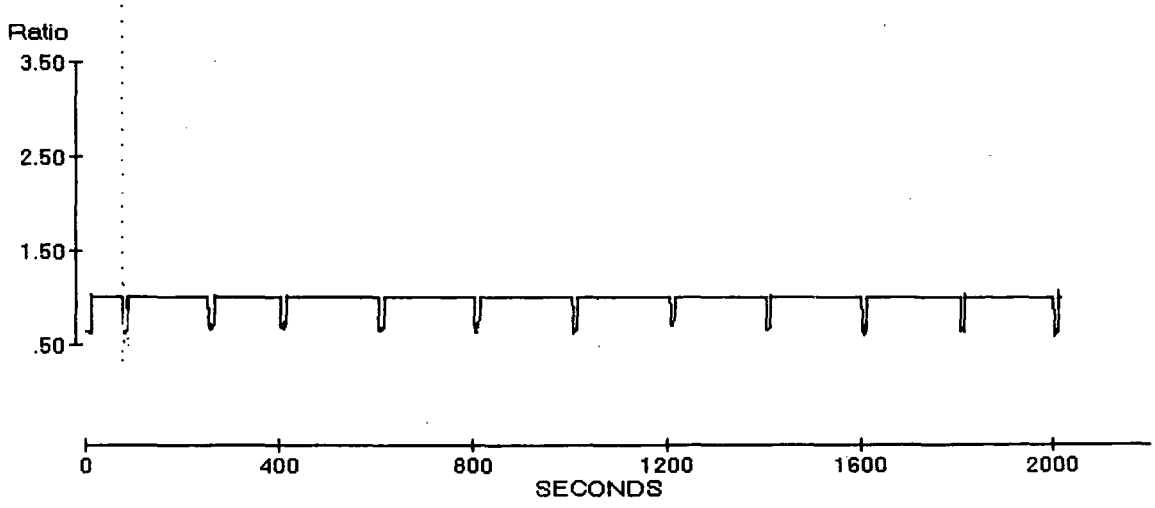

Ratio
3.50
2.50
1.50
.50
0
400
800
1200
1600
2000
SECONDS

Figure 5.13a. Effect of heat on $[Ca^{2+}]_i$ in the presence of 50 µM La^{3+}

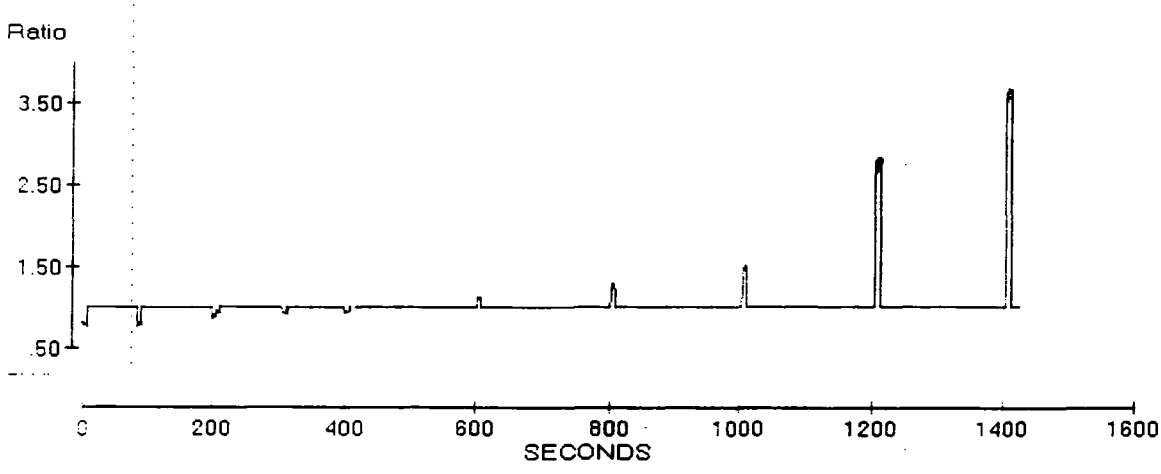
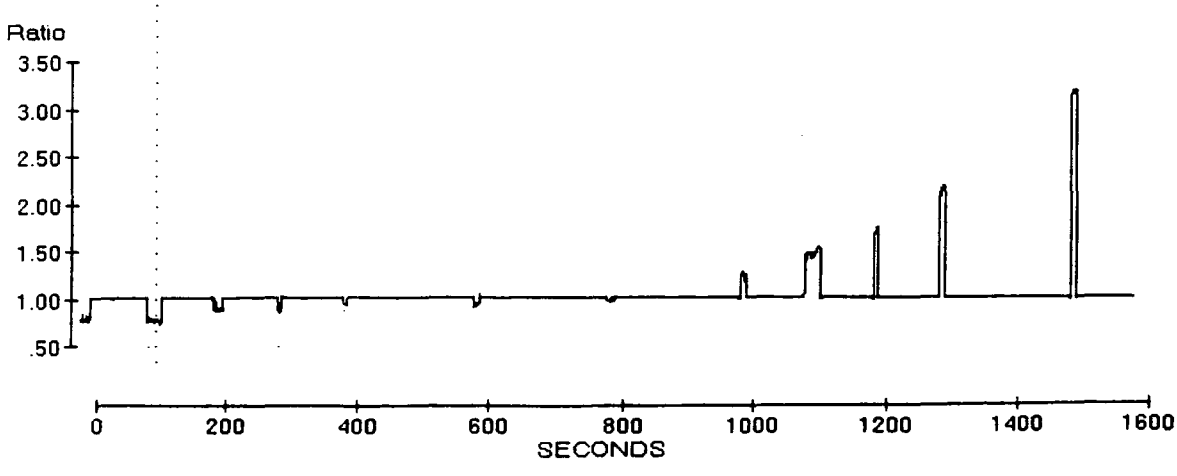
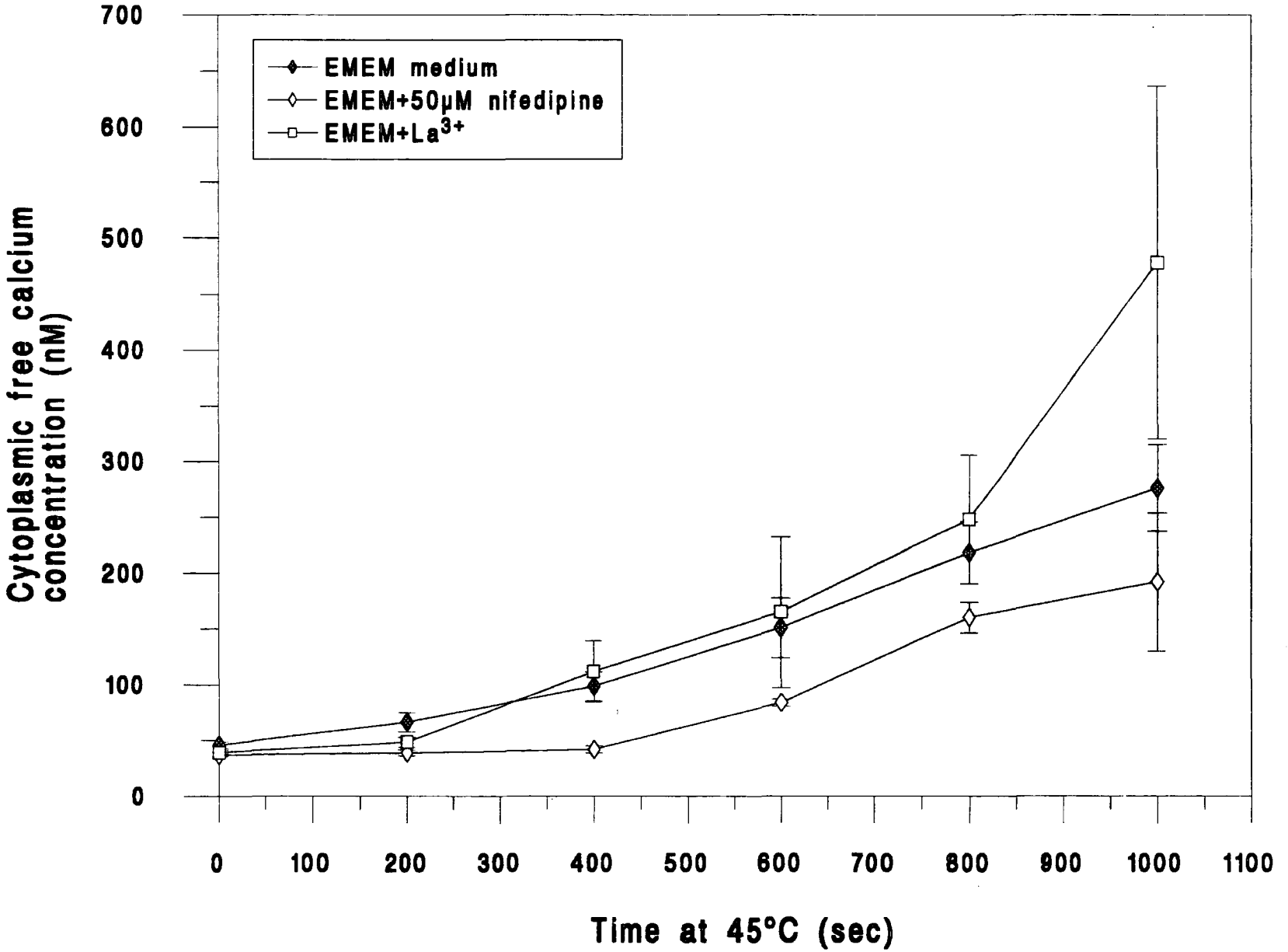
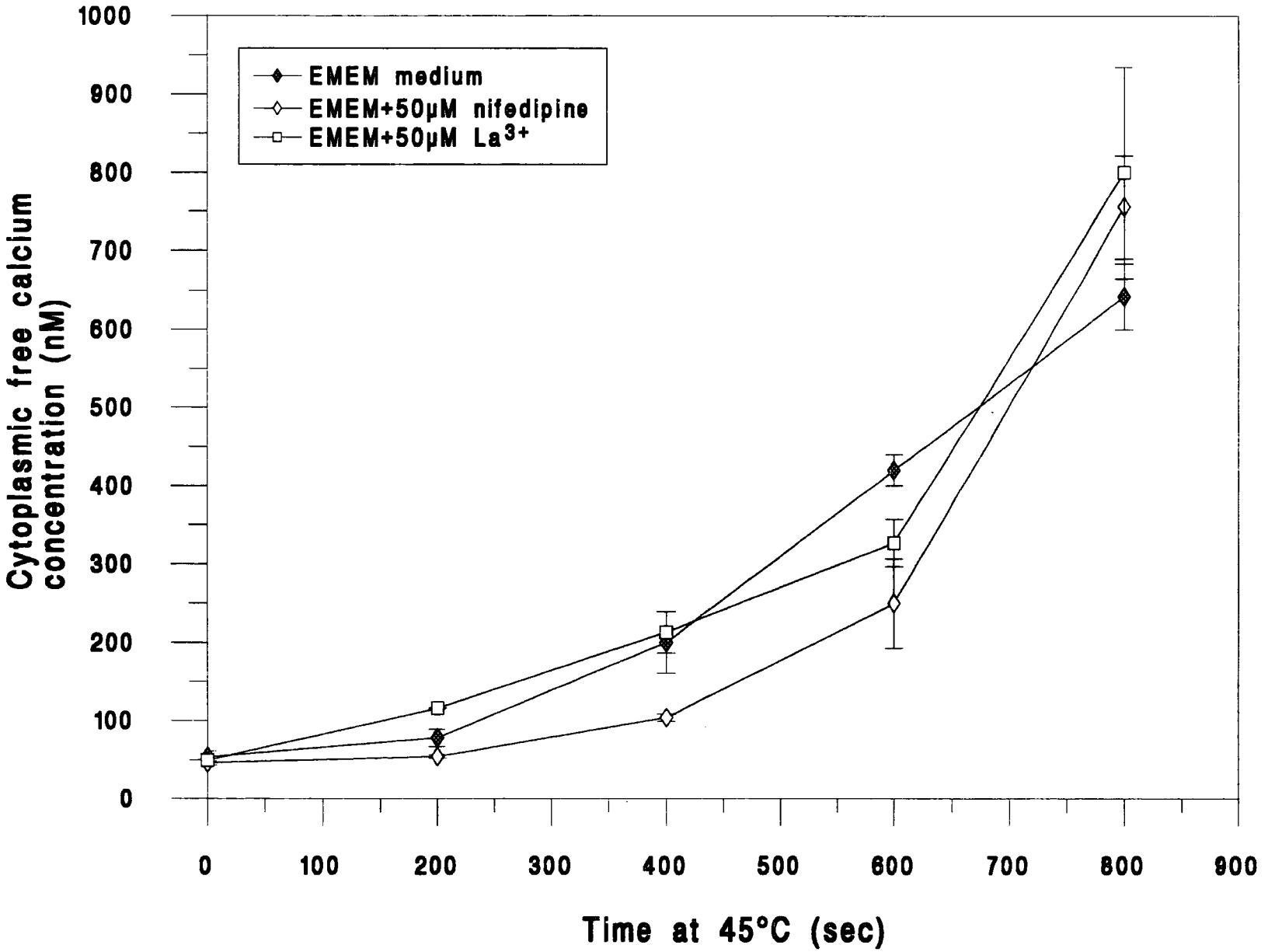
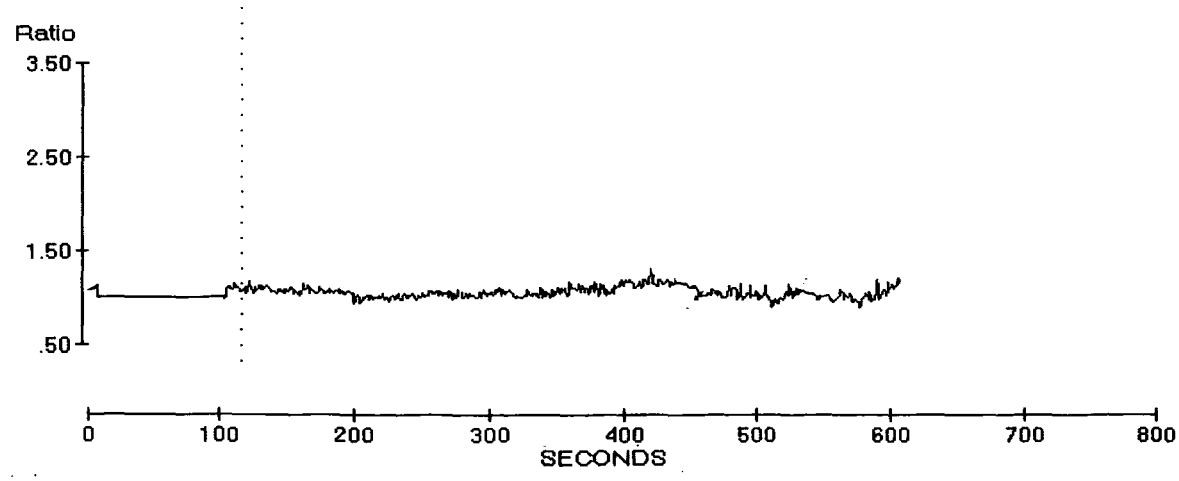


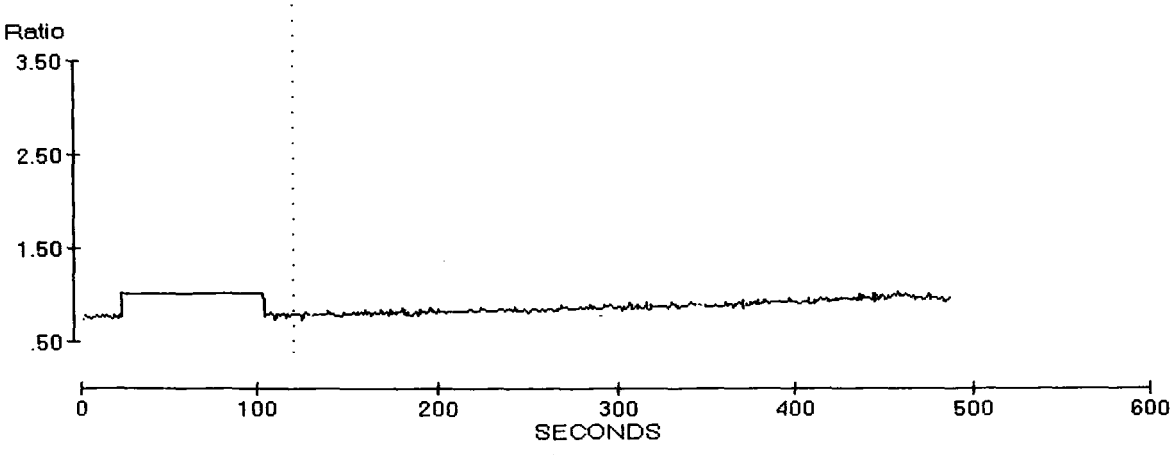
Figure 5.13b. Effect of heat on $[Ca^{2+}]_i$ in the presence of 50 µM nifedipine

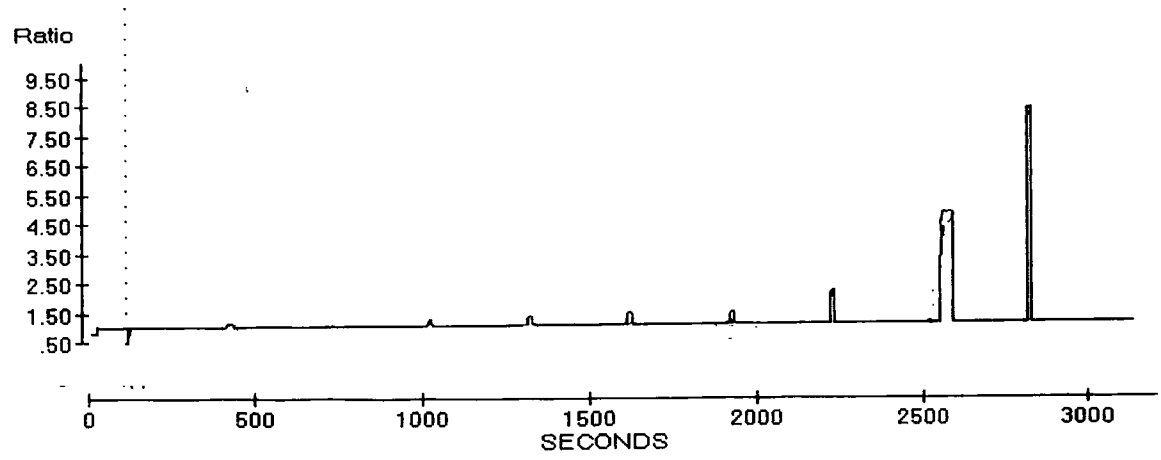



EMEM medium
EMEM+50μM nifedipine
EMEM+La3+
Cytoplasmic free calcium concentration (nM)
700
600
500
400
300
200
100
0
0
100
200
300
400
500
600
700
800
900
1000
1100
Time at 45°C (sec)


EMEM medium
EMEM+50μM nifedipine
EMEM+50μM La3+
Cytoplasmic free calcium concentration (nM)
1000
900
800
700
600
500
400
300
200
100
0
0
100
200
300
400
500
600
700
800
900
Time at 45°C (sec)


Ratio
3.50
2.50
1.50
.50
0
100
200
300
400
500
600
700
800
SECONDS


Ratio
3.50
2.50
1.50
.50
0
100
200
300
400
500
600
SECONDS


Ratio
9.50
8.50
7.50
6.50
5.50
4.50
3.50
2.50
1.50
.50
0
500
1000
1500
2000
2500
3000
SECONDS

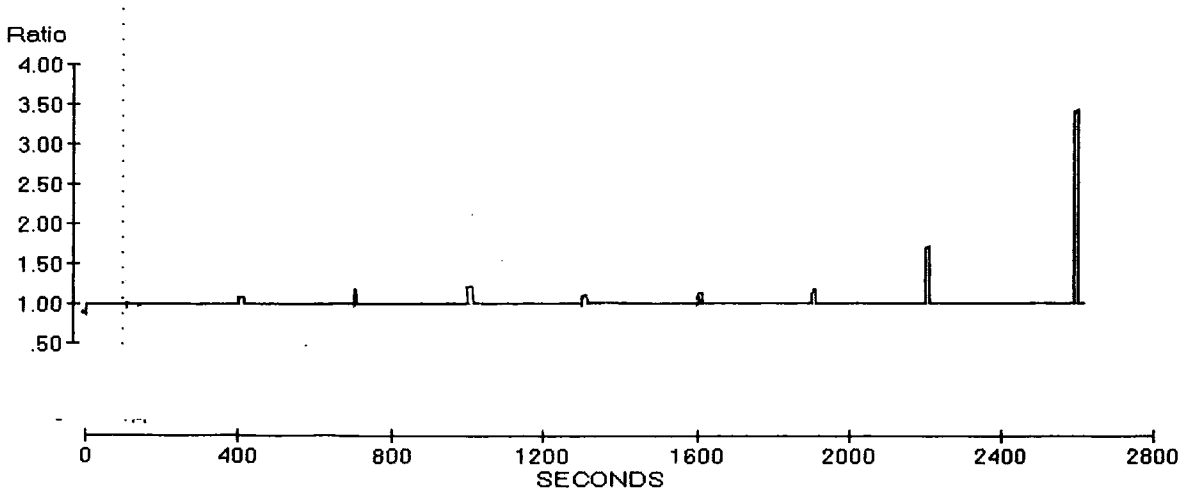

Ratio
4.00
3.50
3.00
2.50
2.00
1.50
1.00
.50
0
400
800
1200
1600
2000
2400
2800
SECONDS

Figure 5.18a. Effect of extracellular calcium concentration on hyperthermic cell death

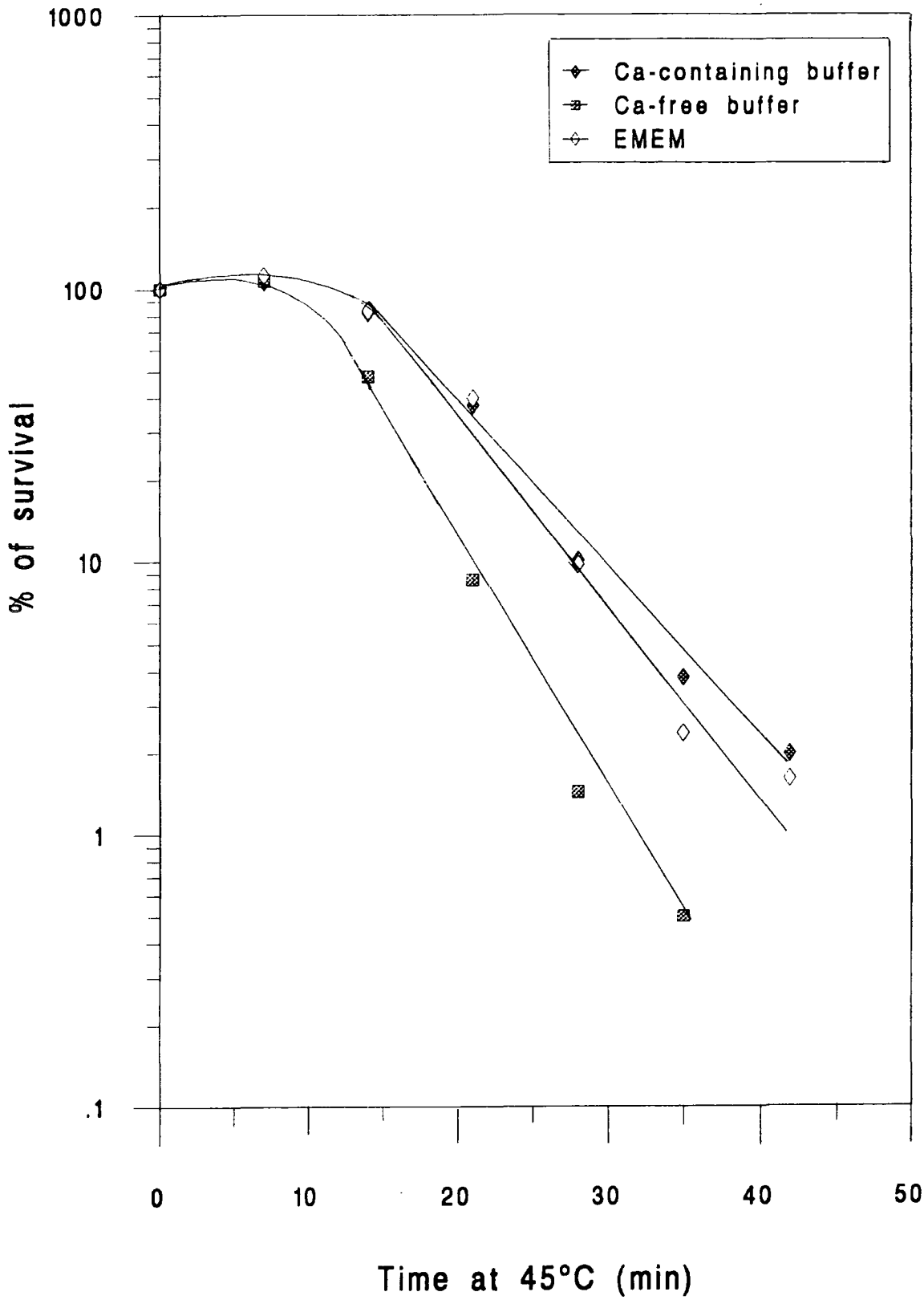


Figure 5.18b. Effect of nifedipine on hyperthermic death

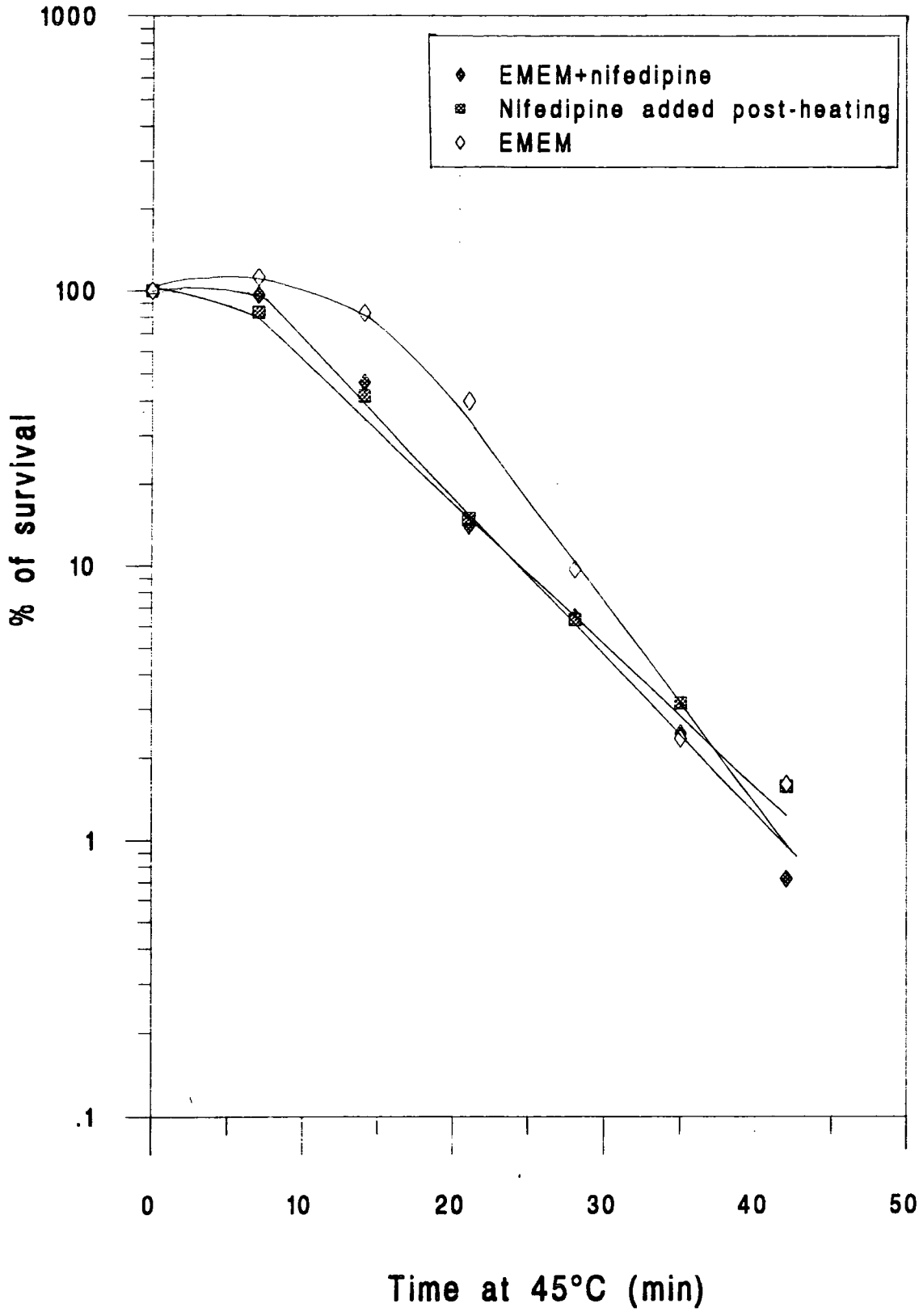


Table 5.4. Comparison of the survival parameters calculated from Figure 5.18

The survival parameters were calculated from the linear part of the killing curve from Figure 5.18.

Parameters (min)	EMEM w/o serum	EMEM with nifedipine	Nifedipine added post heating	Calcium containing buffer	Calcium free buffer
D_q	14.1±0.69	6.46±1.16*	4.56±1.20*	13.6±0.90	9.99±1.14*
D_0	13.6±0.29	17.5±0.32*	20.0±0.35*†	13.9±0.35	8.79±0.32*
LD_{50}	18.6±0.65	11.8±1.17*	10.7±1.20*	17.9±0.90	12.7±1.15*
LD_{90}	28.0±0.63	24.0±1.12	24.5±1.15	27.6±0.89	18.8±1.11*

Data in the table represent the estimate±SEM calculated from the regression lines (the 4 min lag period was included in the data shown)

* found to be significantly different ($p<0.05$) from the control value (EMEM was chosen as the control condition) by using Student's t-test analysis.

† found to be significantly different ($p<0.05$) when the survival parameters in the presence of nifedipine during heating and when it was added post-heating were compared by Student's t-test.

5.4 Discussion

Disruption of calcium homeostasis has been suggested to play an important role in several types of cell death (Schanne *et al.*, 1979; Trump *et al.*, 1980; Orrenius *et al.*, 1989). However, its role in hyperthermic cell death is controversial. Some workers found that heat cell death was neither sensitised nor protected when HT-29 cells were heated in extracellular calcium concentration between 0-15 mM at 44°C for up to 2 h (Mikkelsen *et al.*, 1991a). On the other hand, when Reuber H35 rat hepatoma cells were heated at 44°C, heat cell death was decreased in the presence of 0.03 mM extracellular calcium, but enhanced in the presence of 7.5 mM calcium, compared with cells heated in the presence of 1.5 mM extracellular calcium (Wiegant *et al.*, 1984). However, the relationship between $[Ca^{2+}]_i$ and hyperthermic cell death was obscure since $[Ca^{2+}]_i$ was not determined in these studies. In addition, the $[Ca^{2+}]_i$ within a cell population may vary from one cell to the other, so it is important to determine $[Ca^{2+}]_i$ at the single cell level. In the present study, $[Ca^{2+}]_i$ was determined at the single cell level by fluorescence microscopy. By employing different extracellular calcium concentrations and calcium channel blockers, the $[Ca^{2+}]_i$ during heating was manipulated, and the relationship between $[Ca^{2+}]_i$ and hyperthermic cell death was explored.

In the present study, changes in $[Ca^{2+}]_i$ were monitored in fura-2 loaded WRK-1 cells by using fluorescence microscopy. Sequestration of calcium-binding dyes into different organelles has been observed in several cell lines, and this can produce errors in estimation of $[Ca^{2+}]_i$ (Almers and Neher, 1985). For example, sequestered fura-2 has been found in endocytic vesicles of Balb C 3T3 cells (Di Virgilio *et al.*, 1990), in lysosomes of human skin fibroblasts (Malgaroli *et al.*, 1987), in mitochondria of endothelial cells (Steinberg *et al.*, 1987) and in the secretory granules of mast cells (Almers and Neher, 1985). Thus, an appropriate loading condition was determined to prevent this dye sequestration problem. It was found that loading at 30°C for 1.5 h gave

an even distribution of fura-2, so this loading procedure was adopted in the present study.

Calibration was performed at 37 and 45°C using fura-2 free acid and calibration buffers containing different concentrations of free calcium. Prior to the construction of the calibration curves, the dissociation constants of calcium-EGTA were corrected for temperature and pH. These values were then used to determine the free calcium concentrations. The K_d values for the Ca^{2+}/fura-2 complex obtained from the calibration curve were 151 nM and 210 nM at 37 and 45°C respectively. These values lie in the range of the K_d values reported by Grynkiewicz *et al.* (1985) and were comparable to those reported by Kantengwa *et al.* (1990) which were 191 nM and 234 nM at 37 and 45°C respectively.

In order to investigate the effectiveness of WRK-1 cells to buffer the elevated $[Ca^{2+}]_i$ upon agonist stimulation, cells were exposed to vasopressin, which is known to mobilise calcium from intracellular stores in these cells (Mouillac *et al.*, 1989). Upon stimulation, the $[Ca^{2+}]_i$ increased from 54±4.8 nM to 506±56.5 nM within 10 sec (8 determinations). A lag period was expected since vasopressin was added to the cells through superfusion. The elevation was transient since the $[Ca^{2+}]_i$ value returned to the basal level after 100 sec. A similar vasopressin-induced increase in $[Ca^{2+}]_i$ has been observed previously in WRK-1 cells, when $[Ca^{2+}]_i$ increased from 172±7 nM up to 1180 ±52 nM and returned to basal level after 30 sec (Mouillac *et al.*, 1989). The result suggested that WRK-1 cells have effective systems for agonist-induced elevation of $[Ca^{2+}]_i$ and subsequent removal of the excess cytosolic free calcium, at least under hormonal challenge.

The effect of hyperthermia on $[Ca^{2+}]_i$ has been investigated in several cell lines by using fluorescent indicators such as indo-1, fura-2 and quin2. Three different techniques

have been used together with the dyes. They are flow cytometry, spectrofluorimetry and fluorescent microscopy. By using a flow cytometry technique to measure the $[Ca^{2+}]_i$ in indo-1 loaded mouse NIH3T3 cells and C3H10T1/2 cells upon heating, Vidair *et al.* (1990) found that heat caused an increase in $[Ca^{2+}]_i$ in both cell types. However, it must be noted that the measurement was done at room temperature immediately following heating, so the increase in $[Ca^{2+}]_i$ may not be fully representative of the heat-induced increase in $[Ca^{2+}]_i$. Using spectrofluorimetry to determine $[Ca^{2+}]_i$ upon heating, an increase in $[Ca^{2+}]_i$ was observed in human epidermoid A-431 cells (Kiang *et al.*, 1992), CHO HA-1 cells (Stevenson *et al.*, 1986), mouse fibroblast 3T3 cells (Stege *et al.*,1993a) and Drosophila salivary gland (Drummond *et al.*, 1988); but not in HeLa cells (Stege *et al.*, 1993b), Ehrlich ascites tumour cells (EAT) (Stege *et al.*, 1993b), murine lymphoma cells (Stege *et al.*, 1993b) or human monocytic U-937 cells (Kantengwa *et al.*, 1990). The discrepancy in these studies may reflect intrinsic differences in the cell types employed. For example, using 6 different cell types including EAT cells, HeLaS3 cells and 3T3 fibroblasts, Wierenga *et al.* (1994) found that only 3T3 fibroblasts showed an increase in $[Ca^{2+}]_i$ upon heating for 1 h at 44°C, although this heat dose killed more than 95% of all 6 cell types. Another possible explanation for heat-induced increase in $[Ca^{2+}]_i$ in some cases but not others may be involved dye leakage out of the cells (Malgaroli *et al.*, 1987). This problem becomes significant at higher temperature (Wierenga and Konings, 1989). The artifact due to dye leakage is more pronounced when spectrofluorimetry is used, since the leaked dye will be maintained in the fluorimeter cuvette. If the extracellular medium contains a high concentration of calcium, the leaked dye will contribute some fluorescence signal and will result in an increase in fluorescence ratio. This increase in ratio will be interpreted as an increase in $[Ca^{2+}]_i$ upon heating which may not be the case. When dye leakage was prevented by using an anion transport inhibitor, probenecid, it was found that heat did not cause any alteration in $[Ca^{2+}]_i$ level in EAT cells (Stege *et al.*, 1993b), HeLa cells (Stege *et al.*, 1993b) and human monocytic U-937 cells (Kantengwa *et al.*, 1990).

By using fluorescence miscroscopy, the signal contributed by the secreted dye as described for spectrofluorimetry is eliminated because of continuous superfusion during measurement. In addition, the fluorescence microscopy technique enables $[Ca^{2+}]_i$ to be determined at the single cell level. This is another advantage over spectrofluorimetry, which only measures the average $[Ca^{2+}]_i$ in a cell population since this average value could reflect (a) a uniform change in all cells or (b) the sum of different changes in $[Ca^{2+}]_i$ in different cells. Fluorescence microscopy techniques have been used to measure $[Ca^{2+}]_i$ upon heating human colon HT-29 cells (Mikkelsen *et al.*, 1991a), mouse mammary tumour MMT060562 cells (Furukawa *et al.*, 1992) and mouse mammary carcinoma FM3A cells (Kondo *et al.*, 1993). It was found that heat caused an elevation in $[Ca^{2+}]_i$ in HT-29 (Mikkelsen *et al.*, 1991a) and FM3A cells (Kondo *et al.*, 1993) when they were heated at 44°C for 1 h, but did not cause a significant increase $[Ca^{2+}]_i$ when MMT060562 cells were heated at 44°C for 20 min (Furukawa *et al.*, 1992).

In the present study, the effect of heat on $[Ca^{2+}]_i$ in WRK-1 cells was investigated using fluorescence miscroscopy. When heated in EMEM, 76% of the cells showed an increase in $[Ca^{2+}]_i$ within 30 min heating at 45°C. The increase was observed as early as 50 sec at 45°C in some cells but some did not show any changes until 700 sec at 45°C, a heat dose which killed more than 50% of these cells. For those cells that showed a heat-induced increase in $[Ca^{2+}]_i$, the increase was found to occur in 2 phases. The first phase was a gradual increase in $[Ca^{2+}]_i$, which could proceed for several hundred seconds. The second phase was a rapid increase in $[Ca^{2+}]_i$ and occurred once the $[Ca^{2+}]_i$ achieved approximately 110 nM. This 2-phase increase in $[Ca^{2+}]_i$ during heating could be explained if, once $[Ca^{2+}]_i$ of a cell reached a threshold value (about 100 nM), heated cells were no longer able to control the $[Ca^{2+}]_i$ elevation. An increase in $[Ca^{2+}]_i$ was observed within 2 min when CHO HA-1 cells were heated at 45°C (Stevenson *et al.*, 1986), and in Drosophila salivary gland as soon as the temperature reached 35°C

(Drummond *et al.*, 1988). On the other hand, an increase in $[Ca^{2+}]_i$ was not observed until HT-29 cells were heated for more than 30 min at 44°C (Mikkelsen *et al.*, 1991a). In the present study, the varying time course of the heat-induced increase in $[Ca^{2+}]_i$ observed in different WRK-1 cells may be due to cells at different stages of the cell cycle were measured (as asynchronous cell populations were used). Since the thermosensitivity of the cell varies at different stages of the cell cycle (Hahn, 1982), the effect of heat on $[Ca^{2+}]_i$ may vary as well.

When cells were heated in calcium-containing buffer, only 25% of the cells showed a rise in $[Ca^{2+}]_i$. The discrepancy obtained between cells heated in EMEM or calcium-containing buffer could be due to effects exerted by some of the constituents present in EMEM, but this possibility was not investigated further.

So far, the results suggested that heat caused an elevation in $[Ca^{2+}]_i$ in some cases. This increase in $[Ca^{2+}]_i$ could be due to (1) calcium influx, (2) calcium release from intracellular pool, or (3) both influx and intracellular calcium redistribution. In order to investigate the possible mechanism(s) of $[Ca^{2+}]_i$ elevation upon heating, calcium-free buffer was used as the extracellular medium. Heat-induced elevation of $[Ca^{2+}]_i$ was abolished in calcium-free buffer, which suggested that calcium influx was the primary cause of the rise in $[Ca^{2+}]_i$. This result was in accordance with the findings in human epidermoid A-431 cells (Kiang *et al.*, 1992) and mouse mammary carcinoma cells (Furukawa *et al.*, 1992), whereas redistribution of the intracellular calcium during heating was reported as an initial event of the heat-induced increase in $[Ca^{2+}]_i$ in CHO HA-1 cells (Stevenson *et al.*, 1986), Drosophila salivary gland (Drummond *et al.*, 1988) and mouse mammary carcinoma FM3A cells (Kondo *et al.*, 1993).

In order to investigate the role of voltage-gated calcium channels in the heat-induced rise in $[Ca^{2+}]_i$, the voltage-gated channel blockers nifedipine and La^{3+} were

used. When WRK-1 cells were heated in the presence of La^{3+}, 75% of the cells (out of 8 determinations) showed a heat-induced increase in $[Ca^{2+}]_i$. When cells were heated in the presence of nifedipine, 35% (out of 14 determinations) showed an elevated $[Ca^{2+}]_i$ level. Although the lag time before an increase in $[Ca^{2+}]_i$ was observed under these conditions varied from one cell to another, the average $[Ca^{2+}]_i$ value and its rate of change from these 2 conditions were insignificantly different compared with $[Ca^{2+}]_i$ values obtained from cells heated in EMEM. These results imply that the voltage-gated calcium channel was not involved in the heat-induced rise in $[Ca^{2+}]_i$. This conclusion was further supported by using 50 mM K^+ in extracellular medium. High extracellular K^+ concentration has been shown to depolarise the plasma membrane potential, opening voltage-gated calcium channels and allowing calcium influx (Ozawa and Sand, 1986). Perfusing 50 mM K^+-containing buffer through the chamber did not alter the $[Ca^{2+}]_i$, suggesting that voltage-gated calcium channels were absent in these cells.

The Na^+/Ca^{2+} exchanger could be involved in the heat-induced rise in $[Ca^{2+}]_i$. It is an antiport and its action is reversible depending on the extracellular sodium concentration. In its normal state, it is responsible for pumping calcium out and allowing sodium to enter the cell. The energy for this exchanger is derived from the sodium concentration gradient which is established by the action of Na^+-K^+-ATPase and the Na^+/H^+ antiporter. This exchanger may be inhibited upon heating (resulting from the thermal denaturation of the protein) or reversed in action, such that it allows an increase in $[Ca^{2+}]_i$. Previously, it has been reported that the heat-induced increase in $[Ca^{2+}]_i$ in A-431 cells was due to the reverse action of this exchanger (Kiang *et al.*, 1992). Evidence for this conclusion included: (1) the heat-induced increase in $[Ca^{2+}]_i$ depended upon extracellular calcium concentration, and was inhibited when cells were heated in calcium-free medium. Using $^{45}Ca^{2+}$ to measure Ca^{2+} fluxes, it was found that $^{45}Ca^{2+}$ influx correlated well with the heat-induced rise in $[Ca^{2+}]_i$. (2) the heat-induced increase in $[Ca^{2+}]_i$ depended upon the extracellular sodium concentration. When the sodium

gradient across the plasma membrane was removed by overloading the cells with Na^+, through ouabain inhibition of Na^+-K^+-ATPase, the basal $[Ca^{2+}]_i$ value was higher than in untreated cells and the heat-induced increase in $[Ca^{2+}]_i$ was inhibited. This result suggested that the heat-induced rise in $[Ca^{2+}]_i$ was related to the Na^+ gradient across the plasma membrane. Since sodium channels also play a role in intracellular sodium homeostasis, the authors used a sodium channel agonist and antagonist to test out the contribution of these channels in the heat-induced $[Ca^{2+}]_i$ elevation. It was found that blocking these channels with tetrodotoxin (Na^+ channel antagonist) abolished the heat-induced increase in $[Ca^{2+}]_i$. On the other hand, treatment with veratridine (Na^+ channel agonist) resulted in a higher $[Ca^{2+}]_i$ upon heating compared with those cells heated in the absence of this agonist. These results suggested that Na^+ influx through tetrodotoxin-sensitive Na^+ channels plays a crucial role in the heat-induced $[Ca^{2+}]_i$ elevation. (3) It was found that the heat-induced rise in $[Ca^{2+}]_i$ was blocked by 5 μM amiloride or 1 μM 5'-(N,N-dimethyl)amiloride, presumably through the inhibition of the Na^+/Ca^{2+} exchanger, though the possibility that amiloride exerted its effect on other Na^+ channels such as the Na^+/H^+ exchanger cannot be excluded (Kiang *et al.*, 1992).

In this study, it was found that the heat-induced increase in $[Ca^{2+}]_i$ in WRK-1 cells was not blocked by 5 μM amiloride, suggesting that reversal of the Na^+/Ca^{2+} exchanger was unlikely to be responsible for the change in $[Ca^{2+}]_i$. However, further studies are needed before final conclusions on the role of the Na^+/Ca^{2+} exchanger in heat-induced $[Ca^{2+}]_i$ elevation are drawn. This is due to several reasons: (1) It is possible that the concentration of amiloride used in this study was not sufficient to inhibit the Na^+/Ca^{2+} exchanger in WRK-1 cells (if this cell type possesses this exchanger). It has been reported that a high concentration of amiloride (mM range) is required to inhibit the Na^+/Ca^{2+} exchanger in epithelial cells (Kaczorowski *et al.*, 1985). (2) Amiloride is not a potent inhibitor of the Na^+/Ca^{2+} exchanger, instead, it is a more potent inhibitor of the Na^+/H^+ exchanger and other types of Na^+-channel and voltage-gated calcium

channels (Besterman *et al.*, 1985; Kleyman and Cragoe, 1988). (3) Having an excitation spectrum similar to fura-2, amiloride may affect the fluorescence signal of fura-2 (Kleyman and Cragoe, 1990). Thus a comprehensive investigation of the effect of heat on Na^+/Ca^{2+} exchnager requires the use of different concentrations of amiloride as well as its analogues, such as those bearing a substituent on the 5-amino and terminal guanidino nitrogen atoms, which have been shown to be specific inhibitors of the Na^+/Ca^{2+} exchanger.

In summary, the results suggested that the heat-induced increase in $[Ca^{2+}]_i$ was due to calcium influx from the extracellular medium by a mechanism that did not involve voltage-gated calcium channels. However, the study only characterised the initial rise in $[Ca^{2+}]_i$ upon heating, and the subsequent increase in $[Ca^{2+}]_i$ during prolonged heating may result from calcium-induced calcium release, $InsP_3$-dependent calcium release (as a secondary event following the $[Ca^{2+}]_i$ rise) and/or reduction in calcium efflux. In addition, accumulation of $[Ca^{2+}]_i$ upon heat treatment in EMEM or calcium-containing buffer could result from several mechanisms, including an increase in membrane permeability (Stevenson *et al.*, 1987), reduced calcium efflux (Vidair *et al.*, 1990), inhibition of calcium uptake into calcisomes or ER (Mikkelsen and Stedman, 1990) and/or inhibition of Ca^{2+}-ATPase resulting from thermal denaturation (Anghileri *et al.*, 1984). Further studies involving utilisation of $^{45}Ca^{2+}$ to measure the calcium fluxes, employing inhibitors such as TMB-8 and heparin to block intracellular calcium mobilisation must be carried out before any further conclusions are drawn. Whether $[Ca^{2+}]_i$ continues to rise in heated cells until intracellular and extracellular calcium concentration achieve equilibrium is unknown.

Several investigators have reported changes in $[Ca^{2+}]_i$ in the recovery period following heating. When $[Ca^{2+}]_i$ was monitored in A-431 cells after pretreatment at 45°C for 20 min, it was found that the calcium level returned to the unheated value by 70

min post-heating (Kiang *et al.*, 1992). When $[Ca^{2+}]_i$ was determined in C3H10T1/2 cells at 3.5 h after heating at 45°C for up to 54 min, it was found that the $[Ca^{2+}]_i$ value in cells heated for 32 or 46 min was similar to unheated cells, whereas the value in cells heated for 54 min was 60% higher than the unheated control. When $[Ca^{2+}]_i$ was measured in these cells at 24 h post-heating, it was found that the value was similar to unheated cells, irrespective of the heating duration (Vidair *et al.*, 1990). Furthermore, Wang *et al.* (1991) found that the only factor that affected the $[Ca^{2+}]_i$ value, in both heated and unheated NIH3T3 cells, was the extracellular calcium concentration in which the cells were suspended during measurement and post-heating, suggesting that calcium homeostasis remained intact following heating at 45°C for 40 min, a heat dose that killed more than 95% of these cells. On the other hand, Mikkelsen *et al.* (1991a) found a correlation between $[Ca^{2+}]_i$ and hyperthermic cell death of HT-29 cells. Those cells having a $[Ca^{2+}]_i$ value greater than 200 nM at 4-6 h after heating at 44°C for 1 h were the clonogenically dead cells. However, the observed high $[Ca^{2+}]_i$ in this study may simply be a post-mortem effect.

Changes in $[Ca^{2+}]_i$ during and after heating have been investigated by several laboratories. By varying the extracellular calcium concentration, $[Ca^{2+}]_i$ during and after heating can be manipulated. For example, on heating C3H10T1/2 cells at 45°C for 30 min in buffer containing 0.03 mM extracellular calcium concentration, the $[Ca^{2+}]_i$ value was 200 nM which was lower than that in cells incubated at 37°C in medium containing 2 mM extracellular calcium. When cells were heated at 45°C for 30 min in medium containing 15 mM extracellular calcium, the $[Ca^{2+}]_i$ value achieved 1000 nM (Vidair *et al.*, 1990). In both conditions, cells were neither sensitised nor protected from heat cell death. A similar finding was observed in CHL V79 cells heated in different extracellular calcium concentrations (Malhotra *et al.*, 1987).

In the present study, it was found that when WRK-1 cells were heated in EMEM,

76% of the cells showed an elevated $[Ca^{2+}]_i$, whereas only 25% of the cells showed a rise in $[Ca^{2+}]_i$ when heated in the simple calcium-containing buffer. When comparing the survival parameters, D_q and D_0 values obtained from both heating conditions, it was found that they were insignificantly different. On the other hand, the D_q, D_0, LD_{50} and LD_{90} values obtained from cells heated in calcium-free medium were significantly lower than the values obtained from cells heated in EMEM or calcium-containing buffer, suggesting that hyperthermic cell death was sensitised in calcium-free conditions. Given that the $[Ca^{2+}]_i$ level remained at about 40-50 nM during heating in calcium-free conditions, it is possible that an increase in $[Ca^{2+}]_i$ may have a protective role in heat cell death. The sensitisation of cell death was likely to occur during the heating period rather than in the 4 h recovery period, as sera and calcium were added back to the solution immediately after heating. A similar finding was observed in hepatocytes (Malhotra *et al.*, 1986). The protective effect of calcium on cell death might be due to:

(1) decreased membrane fluidity upon heating as a result of the increase in $[Ca^{2+}]_i$. Several laboratories agree that increasing fluidity enhances cell death (Yatvin, 1977; Lepock, 1982) and an increase in $[Ca^{2+}]_i$ could perhaps decrease membrane fluidity upon heating. Ca^{2+} decreases the membrane fluidity through 2 distinct mechanisms. It can be due to the binding of Ca^{2+} to the phospholipid headgroups (Jacobson and Papahadjopoulos, 1975). This mechanism only accounts for a minor pathway in which Ca^{2+} exerts its effect on membrane fluidity and is reversible by addition of EGTA (Jacobson and Papahadjopoulos, 1975). On the other hand, a decrease in membrane fluidity can be due to a metabolic alteration of the lipid composition owing to the action of calcium-dependent enzymes and this is an irreversible mechanism (Rasmussen *et al.*, 1979). When the mechanism of Ca^{2+} in the alteration of membrane lipid fluidity was investigated in plasma membranes isolated from hepatocytes, it was found that addition of Ca^{2+} decreased the arachidonic acid content and reduced the double bond index of the fatty acids (Storch and Schachter, 1985). The authors found that this change in lipid composition resulted in the reduction in lipid fluidity and was likely due to the action of

calcium-dependent PLA_2. Activation of this enzyme would cleave the fatty acyl chain in the *sn*-2 position, especially arachidonic acid, and thus reduce the double bond index. Thus, it is possible that the heat-induced increase in $[Ca^{2+}]_i$ would reduce the membrane fluidity via one of the 2 mechanisms mentioned above, thus protecting the cells from hyperthermic cell death.

(2) Inhibition by Ca^{2+} (Cheng, 1989) of the thermal inactivation of Ca^{2+}-ATPase in the sarcoplasmic reticulum (Cheng *et al.*, 1987). The heat-induced increase in $[Ca^{2+}]_i$ level may protect Ca^{2+}-pumping ATPase proteins from thermal damage, so the Ca^{2+}-ATPase can act to reduce the elevated $[Ca^{2+}]_i$ back to the basal level. Hence this feedback mechanism of calcium may play an important role in the responses of cells during or after hyperthermic treatment by protecting proteins that may be important in calcium homeostasis from thermal denaturation. It is clear that further studies are necessary to clarify the role of calcium on other cellular proteins upon heating.

In this study, the presence of nifedipine, at a concentration which did not block the heat-induced rise in $[Ca^{2+}]_i$, resulted in a lower D_q value compared with the D_q value obtained from cells heated in EMEM. On the other hand, the presence of nifedipine resulted in a higher D_0 value compared with those cells heated in EMEM. In addition, when cells were heated in the presence of nifedipine (+ 4 h post-heating) or when nifedipine was present during the 4 h post-heating period only, a significantly lower ($p< 0.05$) LD_{50} value was observed compared with cells heated in EMEM only. Thus, when cells were heated in the presence of nifedipine or when it was included during the 4 h post-heating period, an increase in cytotoxicity was found. However, the mechanism of nifedipine in the increase in cytotoxicity of WRK-1 cells is unknown. It is possible that the presence of nifedipine alters the distribution of cytosolic free Ca^{2+} (Church and Zsotér, 1980) and alters the activities of calcium-dependent enzymes in particular organelles. The effect of calcium channel blockers on hyperthermic cell death has been investigated in HT-29 cells (Mikkelsen *et al.*, 1991a) and CHO 10B4 cells (Coss *et al.*,

1989). Mikkelsen and co-workers (1991a) found that using verapamil at a concentration ranging from 0-20 μM or nifedipine at 25 μM, hyperthermic cell death of HT29 cells upon heating at 44°C for up to 2 h was neither sensitised nor protected. However, when CHO 10B4 cells were heated at 44°C for up to 80 min in the presence of 50-75 μM verapamil or 100-250 μM diltiazem, cell death was enhanced compared with heat alone, whereas no effect of verapamil or diltiazem was observed when cells were incubated at 37°C (Coss *et al.*, 1989). Similarly, heating mouse mammary carcinoma FM3A cells in the presence of 100 μM verapamil or diltiazem at 44°C for 1 h delayed cell growth induced by heat (Kondo *et al.*, 1994). The discrepancies in the literature could be due to the difference in thermosensitivity of different cells and the concentration of the calcium channel blockers used. However, out of three studies described above, only Kondo and co-workers (1994) measured $[Ca^{2+}]_i$ upon heating in the presence of either verapamil or diltiazem. Surprisingly, heating in the presence of either agent caused a further increase in $[Ca^{2+}]_i$ compared with heat treatment alone. Hence, this result implied that the potentiation of cell death by these agents was unrelated to their antagonistic effect on calcium influx through voltage-gated calcium channels in the plasma membrane (its normal action), rather it exerted its effect via a mechanism that may be related to the disruption of other aspects of calcium homeostasis. As pointed out by these workers, verapamil has been reported to bind to calmodulin, thus affecting calmodulin-activated processes and perhaps reducing the ability of ER or mitochondria to control the heat-induced increase in $[Ca^{2+}]_i$ (Kondo *et al.*, 1994).

In summary, the results suggest that the heat-induced elevation in $[Ca^{2+}]_i$ did not play a critical role in enhancing hyperthermic cell death. Instead, it may have a protective role in heat cell death. The $[Ca^{2+}]_i$ determined in this study was the average of a single cell. Given the complex spatiotemporal distribution of cytosolic free Ca^{2+} and the presence of calcium-dependent enzymes in different organelles, further study by using digitised imaging microscopy to measure calcium changes at different subcellular

regions, as well as the redistribution of this cation within a particular organelle, is necessary before the relationship of the heat-induced increase in $[Ca^{2+}]_i$ to hyperthermic cell death is clarified.

Chapter 6

General Discussion

The major rationale for exploring the use of hyperthermia in cancer treatment is that treatment of cancer by other techniques such as chemotherapy and radiotherapy often produces only remission rather than a cure in the majority of patients. Relapse occurs either due to failure of control of local disease or because of metastases and any new method which could increase either local or systemic control would be of interest. Both local and systemic hyperthermia have now been clearly demonstrated to produce regressions in patients in which conventional methods have failed.

The suggestion that there may be differential thermal sensitivity between tumour and normal tissues has also stimulated interest in hyperthermia as a cancer treatment. Early studies by Cavaliere and co-workers (1967) suggested that tumour cells were intrinsically more sensitive to elevated temperature than normal cells. However, more recent experimental data indicate that there is very little inherent difference in intrinsic heat sensitivity between transformed cells and their normal cell counterpart (Symonds *et al.*, 1981). Instead, the differential thermal sensitivity between tumour and normal cells *in vivo* appears to be due to inadequate vascular supply in tumours, leading to a reduction in cooling ability during hyperthermia (Field, 1987). Furthermore, the impaired blood supply in tumour tissue causes deprivation of nutrients and oxygen, leading to anaerobic respiration and a consequent decrease in pH in the tumour. This decrease in tumour tissue pH is believed to enhance sensitivity of tumour cells to hyperthermia (Hahn, 1982; Field, 1987). Other results indicate that heat could be used to complement radiotherapy since cells in the S-phase of the cell cycle are resistant to x-rays but more sensitive to heat (Westra and Dewey, 1971). In addition, an inefficient

microcirculation leads to areas of hypoxia, which are more resistant to radiation and are thought to be the cause of some radiation therapy failures, but this can be overcome by hyperthermic treatment. Heat also increases the cellular sensitivities to some chemotherapeutic agents including nitrosoureas and cisplatin (Hahn, 1979).

The current interest in the use of hyperthermia as a potential modality for cancer treatment has led to studies of the heat response of cells because detailed knowledge of the dynamics of heat-induced reproductive cell death could lead to the development of methods to increase the effectiveness of hyperthermia in the clinical treatment of cancer.

Although extensive studies of the mechanism(s) of hyperthermic cell death have been performed in tissue culture, the identification of the primary cellular target(s) of heat damage has proved to be very difficult. This is mainly due to the all-pervasive effect of heat, making it difficult to distinguish between primary, secondary and tertiary targets (Jung, 1986; Bowler, 1987). However, despite the uncertainty of the target(s) of hyperthermic cell death, biological membranes have been suspected for a long time to be an important, and perhaps primary site of hyperthermic cell damage (Bowler *et al.*, 1979; Hahn, 1982; Bowler, 1987; Konings, 1988). Evidence supporting the view that the plasma membrane is a target of heat damage has been described in Chapter 1 and can be grouped into 4 categories: (1) heat affects the morphology of the membrane, causing bleb formation; (2) some compounds, such as local anaesthestics or alcohols which interact with the plasma membrane and decrease membrane order, act synergistically with heat to increase cell killing. On the other hand, agents such as D_2O and glycerol that are known to stabilise either proteins or membranes, protect cells from heat cell death; (3) alteration of fatty acyl composition of membrane lipids (e.g. by dietary supplementation with unsaturated fatty acid) alters the thermal sensitivities of the cells; (4) heat impairs plasma membrane functions.

Many plasma membrane functions are found to be affected by heat. For example, Na^+-dependent amino acid transport in rat thymocytes was inhibited when cells were exposed to 37-43°C (Lin *et al.*, 1978). On the other hand, glucose uptake was stimulated when CHO cells were exposed to 45°C (Vidair and Dewey, 1993). Heat has also been found to affect ion fluxes, though the results are equivocal. For example, when LM fibroblasts were heated at 44°C, the intracellular K^+ content decreased in a time-dependent manner, but this effect was reversed by 16 h (Ruifrok *et al.*, 1985). However, no effect on intracellular levels of K^+, Na^+ and Mg^{2+} was found when CHO cells were exposed to 45°C for 30 min (Vidair and Dewey, 1986).

Many altered plasma membrane functions induced by hyperthermia are presumably mediated in part, if not entirely, by proteins embedded in the plasma membrane. An earlier model of heat-induced cell lethality, based upon Eyring's Transition State Theory, suggested heat-induced protein denaturation as a critical lethal process in cells exposed to elevated temperature (Westra and Dewey, 1971). Furthermore, Lepock *et al.* (1983) have shown that hyperthermic cell death of CHL V79 cells correlated with the transition in membrane proteins rather than membrane lipid transition. By measuring both intrinsic protein fluorescence and energy transfer from a protein fluorophore to trans-paranaric acid, in order to probe the changes in protein structure, it was found that both mitochondrial and plasma membrane proteins underwent irreversible transitions above 40°C. The results led the authors to propose that the alteration in the structure of the proteins above 40°C could cause many of the observed changes in the plasma membrane and the irreversible protein transition may be involved in hyperthermic cell killing (Lepock *et al.*, 1983).

However, the fluorescent intensity measured by these authors was an average of all of the membrane proteins and it is likely that different proteins in the same membrane have differential sensitivity to heat. In fact, this differential heat sensitivity of membrane

proteins has been observed in CHO HA-1 cells (Stevenson *et al.*, 1983) and HTC cells (Ladha, 1990). When CHO HA-1 cells were exposed to 45°C, the binding of insulin to its receptor was inhibited in a time- and temperature-dependent manner. On the other hand, the concanavalin A binding glycoproteins of CHO HA-1 cells are heat resistant, even at 50°C (Stevenson *et al.*, 1983). When the activities of 4 different enzymes in plasma membranes isolated from HTC cells were determined, it was found that adenylyl cyclase, Na^+-K^+-ATPase and Mg^{2+}-ATPase were not substantially affected by heating the membranes at 43-45°C, but alkaline phosphodiesterase I was activated by the same treatment (Ladha, 1990). The results obtained by Ladha (1990) show that heat does not necessary lead to enzyme inactivation but can also result in enzyme activation in some instances.

Thermal activation of membrane proteins, in particular of enzymes such as adenylyl cyclase and phosphoinositide-specific phospholipase C (PI-PLC) which are involved in second messenger production, could have catastrophic effects on cell metabolism. The phosphoinositide signalling pathway is composed of 3 different components, including a hormone receptor, a G protein and an effector protein (PI-PLC). In contrast, growth factor signalling involves a receptor that is directly coupled to PI-PLC and activation of PI-PLC requires phosphorylation of tyrosine residues by the intrinsic tyrosine kinase activity of the receptor. Any heat-induced change in the activities of any of these components could lead to an impairment of cell metabolism, which might lead to a profound cellular injury.

Several lines of evidence suggest that receptor-linked signal transduction pathways are affected by heat. These include (1) binding of EGF to its receptor in Rat-1 fibroblasts was inhibited when these cells were exposed to 45°C for 30 min, as a result of a reduction of binding affinity (Magun and Fennie, 1981); (2) binding of insulin to its receptor in CHO HA-1 cells was inhibited by exposing these cells to 43-45°C due to a

decrease in receptor number (Calderwood and Hahn, 1983); (3) a transient increase in diacylglycerol level (DAG) was observed in heated CHO HA-1 cells (Calderwood *et al.*, 1987) whereas a sustained increase in diacylglycerol level was observed during the 2 h post-heating period (at 37°C) following 10 min heating at 45°C (Stevenson *et al.*, 1986); (4) increases in inositol monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) were observed in several mammalian cell lines upon heat treatment (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993; Kiang and McClain, 1993); (5) heat also leads to an increase in cytosolic free calcium ($[Ca^{2+}]_i$) and the increase in $[Ca^{2+}]_i$ may be correlated with an increase in $InsP_3$ levels in CHO HA-1 cells (Stevenson *et al.*, 1986); (6) heat altered the distribution of protein kinase C (PKC) between cytosolic and particulate fractions in P388 ascites cells. In addition, a decrease in PKC activity in heated P388 cells was found. However, phosphorylation of 3 proteins having molecular weight of 14, 25 and 33 kD increased in P388 cells following heating at 45°C for 1 h (Bagi and Hidvégi, 1990); (7) using several cultured cells such as Swiss 3T3 cells, chicken embryo fibroblasts and neuroblastoma N_2A cells, it was found that protein tyrosine phosphorylation increased in heated cells (Maher and Pasquale, 1989). Although the exact mechanisms of heat shock stimulation of protein tyrosine phosphorylation is unknown, it is likely that heat may activate tyrosine kinases by altering the conformation of the growth factor receptor or may alter the structure of the carboxyl terminus of the tyrosine kinases, leading to an increase in tyrosine kinase activity (Maher and Pasquale, 1989). (8) heat has also been shown to activate S6 protein kinase (Jurivich *et al.*, 1991) which is a serine/threonine kinase and activation of this enzyme is the primary response to mitogenic stimuli (Chen and Blenis, 1990).

Ca^{2+} is somewhat of a paradoxical second messenger to employ for cell signalling because, whilst it is clear that Ca^{2+} is capable of stimulating a variety of cellular processes, it is also toxic (Schanne *et al.*, 1979; Trump *et al.*, 1980; Farber, 1981; Orrenius *et al.*, 1989; Trump and Berezesky, 1992). Due to this reason, Ca^{2+} carries out

its signalling role by elevation of $[Ca^{2+}]_i$ in very short bursts. Deleterious effects of prolonged Ca^{2+} elevation are known to be related to cell death (Trump *et al.*, 1980; Farber, 1981; Orrenius *et al.*, 1988; Trump and Berezesky, 1992). Elevation of $[Ca^{2+}]_i$ may directly affect activities of Ca^{2+}-dependent enzymes or may act indirectly, by activation of calmodulin (CaM), thus activating CaM-dependent enzymes. Cells have access to 2 major sources of Ca^{2+}, an infinite external supply and much more finite internal sources. Ca^{2+} entry from the external medium is controlled by voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+} channels or second messenger-operated Ca^{2+} channels, whilst release of Ca^{2+} from internal stores, principally the endoplasmic reticulum (ER) is controlled by the second messenger Ins(1,4,5)P_3 (Berridge and Irvine, 1984). Ins(1,4,5)P_3 is generated along with 1,2-DAG from the hydrolysis of a relatively minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), by the action of PI-PLC (Berridge, 1984; Berridge, 1987). 1,2-DAG remains in the membrane lipid matrix and activates PKC, which in turn phosphorylates specific protein substrates (Nishizuka, 1984; Ryu *et al.*, 1990; Liscovitch, 1992; Nishizuka, 1992). On the other hand, Ins(1,4,5)P_3 can be dephosphorylated sequentially to free inositol which is then used for the synthesis of inositol lipids or it is phosphorylated to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 3-kinase, whose activity is regulated by Ca^{2+}/CaM-dependent kinase (Irvine *et al.*, 1986). Ins(1,3,4,5)P_4 then dephosphorylates to free inositol sequentially and is used for the synthesis of inositol lipids. Ins(1,3,4,5)P_4 has also been suggested to play a role in controlling Ca^{2+} influx (Berridge and Irvine, 1984; Boynton *et al.*, 1990; Irvine, 1990).

In order to make a comparison of the effect of heat on intracellular signalling, especially the phosphoinositide pathway, it is first worth mentioning 2 proposed mechanisms of heat-induced activation of the phosphoinositide signalling system based on results observed in CHO HA-1 cells (Stevenson *et al.*, 1986; Calderwood *et al.*, 1987; Stevenson *et al.*, 1987; Calderwood and Stevenson, 1989; Calderwood *et al.*, 1987;

Calderwood and Stevenson, 1993; Calderwood *et al.*, 1993) and in human epidermoid A-431 cells (Kiang *et al.*, 1991; Kiang *et al.*, 1992; Kiang and McClain, 1993).

In CHO HA-1 cells, heat caused an increase in $InsP_1$, $InsP_2$ and $InsP_3$ but not $InsP_4$ levels above 40°C (Calderwood *et al.*, 1988; Calderwood and Stevenson, 1993), and the magnitude of the increase depended on temperature and duration of heating. The changes in $InsP_3$ depended on the $[Ca^{2+}]_i$ value, since the heat-induced increase in $InsP_3$ was amplified by heating in the presence of ionomycin, but abolished by pretreatment of cells in calcium-free (EGTA-containing) buffer for 1 to 3 h before heating at 43°C (Calderwood and Stevenson, 1993). Furthermore, heat-induced $InsP_3$ formation in digitonin-permeabilised CHO HA-1 cells was stimulated by GTPγS (a non-hydrolysable analogue of GTP), but not by guanosine-5'-o-(2-thiodiphosphate) (Calderwood *et al.*, 1993). This effect of GTPγS was concentration-dependent and occurred at lower concentrations in heated, permeabilised cells ($>10^{-7}$ M GTPγS) compared to unheated, permeabilised cells ($>10^{-6}$ M GTPγS). Pretreatment of intact CHO HA-1 cells with pertussis toxin (PTX) for 3 h did not abolish the heat-induced increase in $InsP_3$, although it eliminated thrombin stimulation of $InsP_3$ levels. These observations led Calderwood *et al.* (1993) to suggest that heat may activate a PTX-insensitive G protein (perhaps G_q) which could activate PI-PLC.

A heat-induced increase in $[Ca^{2+}]_i$ was also observed in CHO HA-1 cells heated at 45°C, and this increase was rapid (within 2 min) and occurred in very low extracellular free calcium concentration (less than 1 μM), suggesting that heat led to calcium release from internal stores (Stevenson *et al.*, 1986). The formation of $InsP_3$ preceded the heat-induced increase in $[Ca^{2+}]_i$, suggesting that there may be a correlation between these two events (Stevenson *et al.*, 1986). However, whether the increase in $[Ca^{2+}]_i$ was caused by $InsP_3$-induced calcium release from $InsP_3$ sensitive stores or by other mechanisms was not clear (Stevenson *et al.*, 1986). On the other hand, Ca^{2+} influx, as analysed by $^{45}Ca^{2+}$

fluxes, was not detected until cells were heated at 45°C for 30 min (Calderwood *et al.*, 1988).

Activation of PI-PLCβ1 enzyme, and hydrolysis of inositol lipids can also lead to the formation of 1,2-DAG (Berridge, 1984). Heating [^{3}H]arachidonic acid-labelled CHO HA-1 cells at 45°C resulted in a transient increase in ^{3}H-DAG level (unspecified isomer), reaching maximum levels (40% increase) at about 2 min (Calderwood *et al.*, 1987). However, a more pronounced increase in ^{3}H-DAG level was observed during the 2 h recovery period at 37°C following treatment at 45°C for 10 min, and this increase in ^{3}H-DAG was accompanied by an increase in ^{3}H-PA level (Stevenson *et al.*, 1986). Formation of PA has been found to be correlated with the increase in $^{45}Ca^{2+}$ influx in CHO HA-1 cells and may contribute to the increase in $[Ca^{2+}]_i$ on prolonged heating (Stevenson *et al.*, 1986; Calderwood *et al.*, 1988). Although an increase in $[Ca^{2+}]_i$ was observed upon heating, no correlation was found between the heat-induced increase in $[Ca^{2+}]_i$ and hyperthermic killing of CHO HA-1 cells (Stevenson *et al.*, 1987). Instead, Calderwood and co-workers found that rapid exponential cell killing commences when plasma membrane permeability to Ca^{2+} reaches a maximum (Stevenson *et al.*, 1987).

The above observations have led Calderwood and coworkers to propose a model for the heat-induced increase in $InsP_3$ and $[Ca^{2+}]_i$ and this model is shown in Figure 6.1. Briefly, they suggest that heat may provide the activation energy required to stimulate the exchange of GDP with GTP on the G_q subclass of G proteins. As a consequence, structural changes occur in G_q, and the heterotrimer dissociates, giving rise to the α-subunit and βγ-complex. The α-subunit then activates PI-PLCβ1 and causes the hydrolysis of inositol lipids (Calderwood *et al.*, 1993) to release $InsP_3$ and 1,2-DAG.

The effect of heat on the phosphoinositide signalling pathway has also been investigated in A-431 cells (Kiang and McClain, 1993). A heat-induced increase in

$[Ca^{2+}]_i$ was found and this was dependent upon the temperature and the duration of heating. This heat-induced increase in $[Ca^{2+}]_i$ was suggested to be due to calcium influx from the extracellular medium as a result of the reversed mode of action of Na^+/Ca^{2+} exchanger or calcium influx through verapamil-sensitive calcium channel (as a result of a heat-induced increase in cAMP level). Supportive evidence for the involvement of the Na^+/Ca^{2+} exchanger in the heat-induced increase in $[Ca^{2+}]_i$ came from the studies using: (1) amiloride, an inhibitor of the Na^+/H^+ exchanger and (to some extent) the Na^+/Ca^{2+} exchanger, which inhibited the heat-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner, (2) a sodium channel antagonist (tetradotoxin) and a sodium channel agonist (veratridine), which resulted in the abolition of the heat-induced increase in $[Ca^{2+}]_i$. (3) heat in the presence and absence of extracellular calcium, which showed that the presence of extracellular calcium was necessary for the heat-induced increase in $[Ca^{2+}]_i$ (Kiang *et al.*, 1992).

Heating A-431 cells also led to an increase in cAMP levels which was proposed to lead to calcium influx through verapamil-sensitive calcium channels, possibly through the phosphorylation of the calcium channel by PKA, thus contributing to the heat-induced increase in $[Ca^{2+}]_i$. Supportive evidence for this proposed role of cAMP in Ca^{2+} influx was obtained from observations that: (1) Incubation 8-BrcAMP increased $[Ca^{2+}]_i$ compared with resting $[Ca^{2+}]_i$ in untreated cells. (2) The increase in $[Ca^{2+}]_i$ induced by 8-BrcAMP was inhibited if 1 mM La^{3+} or verapamil was also present during the incubation.

Heat caused an increase in $InsP_3$ levels in A-431 cells above 40°C, and the magnitude of the increase was temperature and heating time-dependent (Kiang and McClain, 1993). Cells heated for 20 min at 45°C showed a slight increase in $Ins(1,3,4)P_3$, a 2.4-fold increase in $Ins(1,4,5)P_3$ and a 3.6-fold decrease in $Ins(1,3,4,5)P_4$ isomers when the inositol phosphate extract was analysed by HPLC (Kiang and

McClain, 1993). The heat-induced increase in $[Ca^{2+}]_i$ (due to calcium influx) was a prerequisite for the increase in $InsP_3$ since heating in the absence of extracellular calcium completely abolished this phenomenon. In addition, when A-431 cells were heated at 45°C in calcium-containing growth medium in the presence of 1 mM amiloride (a concentration that was shown to completely block the heat-induced increase in $[Ca^{2+}]_i$, possibly by inhibiting the Na^+/Ca^{2+} exchanger), no change in $InsP_1$, $InsP_2$ and $InsP_3$ levels was observed. Furthermore, these workers suggested that the heat-induced increase in $InsP_3$ level required a G protein, likely to be a PTX-sensitive one. This view was supported by 3 observations: (1) Heating A-431 cell homogenate in the presence of 10 μM Gpp(NH)p at 45°C for 20 min resulted in an increase in $InsP_3$ level which was comparable to the $InsP_3$ levels obtained in intact cells heated for the same length of time. (2) Pretreatment of cells with PTX before heating did not lead to an increase in $InsP_3$ upon heating at 45°C. (3) When cells were treated with 0.1-1 μM U-73122 at 37°C for 20 min (a compound that inhibits GTPγS-stimulated $InsP_3$ formation in GH_3 cells), the heat-induced increase in $InsP_3$ was inhibited when cells were heated in the presence of U-73122, and the magnitude of inhibition was concentration-dependent, complete inhibition occurring at 10 μM U-73122. Collectively, these observations suggest that a PTX-sensitive G protein and a PLC mediated process are involved in the heat-induced increase in $InsP_3$ in A-431 cells (Kiang and McClain, 1993).

Heat caused an influx in calcium which preceded and was indispensable for the heat-induced increase in the $Ins(1,4,5)P_3$ level in A-431 cells. This increase in $Ins(1,4,5)P_3$ did not seem to play a major role in mobilising Ca^{2+} from internal stores upon heating, since blocking the heat-induced increase in $Ins(1,4,5)P_3$ (by pretreatment of A-431 cells with PTX) only slightly affected the heat-induced increase in $[Ca^{2+}]_i$ (Kiang *et al.*, 1992) However, experiments suggested that Ca^{2+} released from internal stores during heating did contribute to the heat-induced increase in $[Ca^{2+}]_i$. When cells were heated at 45°C for 20 min in the presence of 100 μM TMB-8, a known blocker of

calcium mobilisation from internal stores, the magnitude of increase in $[Ca^{2+}]_i$ was only half of that obtained from cells heated in the absence of TMB-8 (Kiang *et al.*, 1992). Furthermore, TMB-8 did not change the heat-induced increase in $^{45}Ca^{2+}$ influx. The above observations led to the proposal of a model for the events leading to the heat-induced increase in $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ in A-431 cells (Kiang and McClain, 1993), and this is shown in Figure 6.2. Briefly, it was postulated that heat causes an influx of Ca^{2+} from the extracellular medium, partly by reversal of the Na^+/Ca^{2+} exchanger and partly by cAMP-dependent activation of calcium channels. This increase in $[Ca^{2+}]_i$ then activates calcium-dependent PI-PLC, leading to the hydrolysis of inositol lipids and the formation of $Ins(1,4,5)P_3$ leading to release of Ca^{2+} from internal stores. The significance of the heat-induced increase in $[Ca^{2+}]_i$ in hyperthermic cell death remained unclear. These authors postulated that the heat-induced increase in $InsP_3$ level in A-431 cells may be one of the reconstructive mechanism that allows cells to recover from heat-induced dysfunction (Kiang and McClain, 1993).

The major differences between the mechanism proposed by Calderwood and co-workers and those proposed by Kiang and co-workers are (1) Calderwood and co-workers found that the heat-induced increase in $InsP_3$ in CHO HA-1 cells preceded the increase in $[Ca^{2+}]_i$ whereas Kiang and co-workers found that heat induced an increase in $[Ca^{2+}]_i$ before the formation of $InsP_3$ was observed. (2) The heat-induced influx in extracellular Ca^{2+} in the model proposed by Calderwood *et al.* was proposed to be due to accumulation in the membrane of PA (a metabolite of DAG) which is likely to act as calcium ionophore, and was a late event. On the other hand, Ca^{2+} influx in A-431 cells was observed as early as 2 min at 45°C and was proposed to occur via the reversed action of Na^+/Ca^{2+} exchanger as well as verapamil-sensitive calcium channels. (3) In both models, a G protein was proposed to be involved in the heat-induced increase in $InsP_3$ formation. However, Calderwood and co-workers suggested that the G protein was PTX-insensitive, whereas Kiang and co-workers suggested that it was PTX-sensitive.

The aim of the present study was to investigate the effect of hyperthermia on the phosphoinositide signalling system, with particular emphasis on heat-induced changes in (1) 1,2-DAG levels in both CHO-K1 cells and WRK-1 cells during acute and chronic exposure to 45°C; (2) 1,2-DAG levels (and other neutral lipids) in the post-heating period in both cell types; (3) $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_{4/5/6}$ levels in WRK-1 cells. In addition, HPLC was employed to investigate which $InsP_3$ isomer(s) was/were affected. (4) the response of WRK-1 cells to vasopressin during and after hyperthermia. (5) $[Ca^{2+}]_i$, determined at the single cell level in WRK-1 cells in the presence and absence of extracellular calcium. Furthermore, the effect of voltage-gated Ca^{2+} channel blockers on the heat-induced increase in $[Ca^{2+}]_i$ was investigated. Finally, the role of $[Ca^{2+}]_i$ in hyperthermic cell death was investigated.

Before these investigations took place, it was necessary to determine the growth rate of Chinese hamster ovary K1 cells (CHO-K1 cells) and rat mammary tumour WRK-1 cells so as to facilitate the planning of future experiments. In Chapter 2, cell sizing, doubling time and plating efficiency of these cells are reported. It was found that the doubling time for CHO-K1 cells and WRK-1 cells were 15.7 h and 21.6 h, respectively. The plating efficiency for CHO-K1 cells and WRK-1 cells were 94% and 63%, respectively. Different inoculated cell numbers resulted in similar percentage of plating efficiency.

In the preliminary study it was found that WRK-1 cells tend to migrate together so the clonogenic assay, which is the most commonly used method in the determination of thermal cell survival, could not be used. Alternatively, the MTT colorimetric assay, which relies on the ability of the metabolically viable cells to reduce yellow 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to blue formazan product was used (Mosmann, 1983; Denizot and Lang, 1986; Kingston *et al.*, 1989). Thus, the thermal sensitivity of WRK-1 cells was determined by the MTT assay,

whereas the clonogenic assay was used for the determination of the thermal sensitivity of CHO-K1 cells. By using clonogenic or MTT assays, a relationship between heating duration and cell death could be determined.

Having determined the thermal sensitivities of these cell types, the effect of heat on monoacylglycerol (MAG), 1,2-DAG and triacylglycerol (TAG) was investigated in CHO-K1 cells and WRK-1 cells. In the present studies, [2-^{3}H]glycerol or [^{3}H]arachidonic acid were used to label the lipids pool of WRK-1 cells and CHO-K1 cells, respectively. However, it must be noted that radioactive labelling methods may not reflect the changes in 1,2-DAG mass, especially during short-term labelling (Farese *et al.*, 1988). To minimise this problem, long-term labelling methods were employed in both cell types, whereas a short term labelling method was only used when the effect of heat on small, metabolically-active lipid pools was investigated. Several workers have determined the mass of 1,2-DAG directly by using the DAG kinase method, which depends on the conversion of 1,2-DAG (from sample of interest) to ^{32}P-PA when incubated in buffer containing DAG kinase (from bacterial sources) and [γ-^{32}P]ATP (Preiss *et al.*, 1986). The ^{32}P-PA thus formed can be separated by one-dimensional TLC and the original 1,2-DAG mass is calculated by comparing the ^{32}P-PA formed from known amounts of 1,2-DAG standard (e.g. diolein) (Preiss *et al.*, 1986). However, the drawback of the DAG kinase method is that it cannot distinguish between 1,2-DAG and the ether-linked analogue of 1,2-DAG. On the other hand, the method employing [2-^{3}H]glycerol as a precursor enabled 1,2-DAG to be distinguished from its ether-linked analogue, as the latter is not labelled with [2-^{3}H]glycerol (Farese and Cooper, 1990). It also enabled the determination of levels of MAG and TAG which might be metabolites of 1,2-DAG. Provided that the labelling is long enough for the lipid pool to reach isotopic equilibrium labelling, changes in radioactivity of 1,2-DAG reflect changes in 1,2-DAG mass (Farese *et al.*, 1988).

Following establishment of the techniques for labelling cells and the separation of neutral lipid classes, the effect of heat on 1,2-DAG, MAG and TAG levels was then investigated in CHO-K1 cells and WRK-1 cells. CHO-K1 cells were labelled for 24 h with [^{3}H]arachidonic acid and were then heated at 45°C for up to 10 min, a heat dose that killed about 50% of these cells. This treatment resulted in a decrease in ^{3}H-1,2-DAG levels, whereas no effect was observed in ^{3}H-TAG or in ^{3}H-MAG levels. When CHO-K1 cells were exposed to 45°C for up to 45 min, a chronic heat dose that killed greater than 99% of these cells, an increase in ^{3}H-TAG levels was observed at 30 min and 45 min, and a decrease in ^{3}H-1,2-DAG level was observed throughout the 45 min heating period (except at 30 min). Exposure of WRK-1 cells that had been labelled for 4 days with [2-^{3}H]glycerol to 45°C for 12.5 min (a heat dose that resulted in 20 % of metabolically dead cells) resulted in a decrease in ^{3}H-1,2-DAG level after 3.5 min and this remained lower than control level for up to 12.5 min. On the other hand, an increase in ^{3}H-TAG level was observed in WRK-1 cells from 4.5 min to 12.5 min at 45°C. On prolonged heating (for up to 45 min) a decrease in ^{3}H-1,2-DAG level was observed for up to 30 min heating. However at 45 min, ^{3}H-1,2-DAG levels in both heated and unheated cells were similar. An increase in ^{3}H-TAG levels was observed in these cells for up to 30 min at 45°C, but levels then declined to the control levels by 45 min heating. Neither acute nor chronic heating at 45°C showed a difference in ^{3}H-MAG levels in both cell types compared with unheated cells.

Results obtained in the present study from both cell types are in contrast to the results reported by Calderwood *et al.* (1987). These workers found that exposure of CHO HA-1 cells to 45°C led to a transient increase in DAG levels, reaching a maximum by 2-3 min (140% of control level) which returned rapidly to the control level by 4 min at 45°C. However, it was not mentioned in their study whether 1,2-DAG or 1,3-DAG were analysed separately or were combined together for analysis. Since only DAG bearing the *sn*-1,2 configuration is an activator of PKC, the measurement of 1,2-DAG,

in particular, is important. Furthermore, these authors only studied the effect of heat on DAG levels for up to 15 min at 45°C, a heat dose that did not result in any clonogenic cell death in CHO HA-1 cells (Stevenson *et al.*, 1986; Calderwoood *et al.*, 1987). The present study provides a more detailed investigation, by monitoring changes in levels of 1,2-DAG, MAG and TAG levels following both chronic and acute exposure to 45°C in 2 tumour cell lines.

In the present study, results from experiments using the DAG kinase inhibitor dioctanoylethylene glycol (diC_8EG) excluded the possibility that the heat-induced decrease in the 1,2-DAG level observed in CHO-K1 cells and WRK-1 cells was due to a higher turnover rate of 1,2-DAG to PA as a result of a heat-induced increase in DAG kinase activity. Long-term [2-^{3}H]glycerol labelled WRK-1 cells were incubated in EMEM medium in the presence of 100 μM diC_8EG at 37°C for 30 min before heating at 45°C in the same medium. This diC_8EG concentration and incubation period has been shown to block 70-100% conversion of 1,2-DAG to PA in platelets upon thrombin stimulation (Bishop *et al.*, 1986). When WRK-1 cells were heated at 45°C, a heat-induced decrease in ^{3}H-1,2-DAG levels was observed after 12.5 min heating, compared to cells at 37°C, and this was not affected by pretreatment of the cells with diC_8EG. Similarly, WRK-1 cells heated at 45°C for up to 45 min showed a decrease in ^{3}H-1,2-DAG levels, compared to unheated controls, and the magnitude of this effect was not altered by the presence of diC_8EG. In contrast, ^{3}H-TAG levels were elevated in WRK-1 cells at 37°C, and the presence of diC_8EG tended to increase this heat-induced elevation in TAG under these conditions, possibly by preserving more 1,2-DAG precursor to TAG.

Provided that cells are labelled with radioactive precursor for long enough to allow the lipid pool to label to near isotopic equilibrium, changes in radioactivity may reflect changes in 1,2-DAG mass (Farese *et al.*, 1988). However, if only a small, metabolically

active pool responds to heat treatment, then long term labelling studies may prevent the changes in 1,2-DAG being detected against a high background of the ^{3}H-DAG content (Farese and Cooper, 1990). To probe this possibility further, the experiment was repeated but this time both cell types were labelled for 2 h only with radioactive precursors, in order to label the metabolically active pools. On exposure of short term labelled CHO-K1 cells to 45°C for up to 45 min, a decrease in ^{3}H-1,2-DAG was observed for heating times longer than 10 min, whereas an increase in ^{3}H-TAG was observed at 4 min and 30 min heating. Similarly, exposure of short-term labelled WRK-1 cells to 45°C for up to 42.5 min resulted in a decrease in ^{3}H-1,2-DAG level throughout the heating period, except at 32.5 min. On the other hand, an increase in ^{3}H-TAG was observed in heated cells throughout the 42.5 min heating period, except at 12.5 min. Thus, the possibility that heat stimulated turnover of a rapidly-labelled metabolically active pool of 1,2-DAG was excluded.

Exposure of long term labelled CHO-K1 cells (24 h labelling with [^{3}H]arachidonic acid) or WRK-1 cells (4-days labelling with [2-^{3}H]glycerol) to 45°C for 10 min, followed by 2 h recovery at 37°C resulted in a heat-induced decrease in ^{3}H-1,2-DAG levels during the recovery period, whereas an increase in the levels of ^{3}H-TAG was observed in both cell types. The results obtained in this study are in contrast to those observed in CHO HA-1 cells (Stevenson *et al.*, 1986). Following a 2 h labelling with [^{3}H]arachidonic acid, CHO HA-1 cells were exposed to 45°C for 10 min, then the ^{3}H-DAG level (unspecified isomer) was determined during 2 h recovery period at 37°C. It was found that ^{3}H-DAG continued to increase during the 2 h post-heating period, reaching 160% of the control level. These authors also found that the time course of the increase in ^{3}H-DAG levels paralleled an increase in ^{3}H-PA level which is likely to be a metabolite of ^{3}H-DAG (Stevenson *et al.*, 1986).

The increase in ^{3}H-TAG and decrease in ^{3}H-1,2-DAG levels observed in heated

cells of both cell types in this study may reflect either (1) an enhanced *de novo* synthesis of TAG via 1,2-DAG or (2) hydrolysis of pre-labelled phospholipid followed by rapid translocation to ER of 1,2-DAG, and conversion to TAG in heated cells. The reported decrease in labelled 1,2-DAG could have been accentuated by the 'cold-chase' effect discussed in Chapter 3. Evidence for the translocation to the ER of 1,2-DAG, which is then metabolised to TAG, comes from studies using radioactive 1,2-DAG analogues (Florin-Christensen *et al.*, 1993). By introduction of radiolabelled 1,2-DAG species into the plasma membrane of NIH 3T3 fibroblast by a liposome fusion technique, it was found that 1,2-DAG was converted to a mixture of PtdCho and TAG. Since the enzymes catalysing the conversion of 1,2-DAG to TAG and PtdCho are known to reside in the ER, these results suggest that there is a transport process conveying 1,2-DAG from the plasma membrane to the ER where it was then metabolised. It is known that 1,2-DAG can derive from: (1) *de novo* synthesis of PA and conversion to 1,2-DAG, which is an intermediate for the *de novo* synthesis of phosphatidylcholine and phosphatidylethanolamine in mammalian tissues (Farese *et al.*, 1987); (2) hydrolysis of phospholipids such as PtdIns and PtdCho (Exton, 1990). It would be interesting to test whether these two pathways are differentially affected by heat. An extension to this would be to analyse the fatty acyl composition of the 1,2-DAG subfractions following heating, to determine the phospholipid source of the 1,2-DAG.

The decrease in ^{3}H-1,2-DAG levels observed in this study in both cell types upon heat treatment was in good agreement with the decrease in PKC activity found in P388 cells following 45°C heating for 1 h (Bagi and Hidvégi, 1990). Since PKC is involved in many cellular functions such as cell growth, a heat-induced decrease in the 1,2-DAG level may decrease PKC activity, and this could perhaps be related to hyperthermic cell death. Supportive evidence that decrease in PKC activity may be involved in hyperthermic cell death came from a study using PKC inhibitors (Mikkelsen *et al.*, 1991b). It was found that presence of the PKC inhibitors, tamoxifen and H7, enhanced

hyperthermic cell death of HT-29 cells, CHL V79 cells and MCF-7 cells heated at 44.5°C. On the other hand, when these cells were heated in the presence of HA1004, a structural analogue of H7 with much lower affinity for PKC than H7, the hyperthermic cell death was not potentiated even when the concentration of HA1004 was 5-fold higher than H7 (Mikkelsen *et al.*, 1991b). Thus it would be interesting to relate the 1,2-DAG level, PKC isoenzyme activities (together with protein phosphorylation) and hyperthermic cell death in one cell type. However, it has been found that the activity of the group C PKC isoenzymes are unaffected by 1,2-DAG and Ca^{2+} levels, so it is possible that heat affects the activity of PKC through a mechanism that is unrelated to the 1,2-DAG content.

Analysis of the 1,2-DAG level upon heating is complicated by the fact that it can derive from (1) hydrolysis of phospholipids such as PtdCho or PtdIns(4,5)P_2, or (2) dephosphorylation of PA, produced by synthesis *de novo*. A more precise method to determine the effect of heat on the phosphoinositide signalling pathway is to label inositol lipids only, so that changes in inositol lipid levels or inositol phosphates that are generated by hydrolysis of inositol lipids can be determined. Consequently, myo[2-^{3}H]inositol was used to label inositol lipids, and levels of inositol phosphates in both heated and unheated cells were determined following separation by anion exchange chromatography or by HPLC, and the results were summarised in Chapter 4. Due to the lack of time, the effect of heat on inositol phosphates was only investigated in WRK-1 cells.

WRK-1 cells were labelled with myo[2-^{3}H]inositol for 4 days then heated at different temperatures. A temperature-dependent increase in ^{3}H-InsP_2 and ^{3}H-InsP_3 levels was observed when cells were heated at 39-45°C for 30 min in the presence of serum. The increase in ^{3}H-InsP_2 levels was detected at 42°C or higher temperatures, whereas the increase in ^{3}H-InsP_3 levels was not observed unless the cells were heated at

45°C. The $InsP_1$ and $InsP_{4/5/6}$ levels were similar between heated and unheated cells. A temperature-dependent increase in $InsP_3$ levels has been observed previously in several cell lines including A-431 cells, CHO HA-1 and HeLa cells. Exposure of A-431 cells to 45°C showed an increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels during the first 20 min, achieving a maximal level (190% of control level) by 20 min. The levels of $InsP_1$, $InsP_2$ and $InsP_3$ then declined to those of control level by 30 min heating (Kiang and McClain, 1993). On the other hand, exposing cells to 45°C for 30 min resulted in a 40% increase in the $InsP_3$ level in CHO HA-1 cells and a 70% increase in $InsP_3$ level in HeLa cells (Calderwood and Stevenson, 1993). In the present study, a maximum increase of 40% in $InsP_3$ level was observed in WRK-1 cells on heating at 45°C for 30 min. Thus the effect of heat on phosphoinositide signalling pathway may depend on cell types, and may be related to the thermal sensitivity of a particular cell line. In order to investigate if any particular isomer(s) is/are affected by heat, and to separate $InsP_4$, $InsP_5$ and $InsP_6$, cells were labelled to high specific radioactivity and heated at 45°C for 30 min (in the presence of serum). Inositol phosphate extracts were then separated by HPLC. Fractions corresponding to ^{3}H-Ins(1,3)P_2 and ^{3}H-Ins(?)P_2 showed a significant increase compared with unheated cells ($p<0.05$). The route that could lead to the accumulation of these 2 fractions has already been suggested in Chapter 4. In brief, formation of Ins(1,3)P_2 and Ins(?)P_2 could result from heat-induced hydrolysis of polyphosphoinositide, generating Ins(1,4,5)P_3. This isomer may then be phosphorylated to Ins(1,3,4,5)P_4, via the action of Ins(1,4,5)P_3 3-kinase. Ins(1,3,4,5)P_4 can then be dephosphorylated to Ins(1,3,4)P_3, which is then further dephosphorylated to Ins(1,3)P_2 or Ins(3,4)P_2, but the presence of 10 mM Li^+ would favour the formation of Ins(1,3)P_2 since Li^+ is an uncompetitive inhibitor of Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase (Barker *et al.*, 1992).

When WRK-1 cells were heated in serum-free EMEM medium at 45°C for up to 20 min, a transient increase in ^{3}H-$InsP_3$ was observed at approximately 11 min and this returned to the control level by 14 min. However, a sustained elevation of total ^{3}H-$InsP_2$

in WRK-1 cells was observed for up to 20 min at 45°C. Analysis of the individual $InsP_3$ isomers by HPLC suggested that there was an increase in the mean dpm count in all 3 isomers, compared to values in control cells, but the difference was statistically insignificant. The discrepancy of a significant difference found in the total ^3H-$InsP_3$ fraction of heated versus control cells, eluted from the ion exchange column, but an insignificant difference found in any single $InsP_3$ isomers or the total $InsP_3$ dpm count after HPLC separation could be explained by sample loss during recovery of the sample after freeze-drying. It is clear that further study is necessary to clarify the effect of heat on individual $InsP_3$ isomers as well as other inositol phosphate isomers. The dependency of $InsP_3$ levels on heating duration has been observed by other workers in A-431 cells, CHO HA-1 cells and HeLa cells. For example, heating A-431 cells at 45°C caused an increase in $InsP_3$, reaching a maximum level after 20 min (70% increase) while further heating caused a decline in $InsP_3$ level (Kiang and McClain, 1993). On the other hand, the $InsP_3$ levels in both CHO HA-1 cells and HeLa cells continued to increase during heating at 45°C for up to 30 min (Calderwood and Stevenson, 1993).

The transient increase in total ^3H-$InsP_3$ observed (at approximate 11 min) in WRK-1 cells heated at 45°C did not require extracellular Ca^{2+}, since both the resting ^3H-$InsP_3$ level and the magnitude of the increase in the $InsP_3$ levels in cells heated in either EMEM (containing 1.8 mM extracellular calcium) or in calcium-free buffer was similar. This observation is in contrast to the proposed mechanism (Figure 6.2) of the heat-induced increase in $InsP_3$ levels proposed in A-431 cells (Kiang and McClain, 1993), in which extracellular Ca^{2+} is suggested to be indispensible for $InsP_3$ formation. The existence of a calcium-independent PI-PLC has been observed in WRK-1 cells, and calcium-independent PI-PLC activation has been suggested as one of the routes of vasopressin-stimulated $InsP_3$ formation (Mouillac *et al.*, 1990). Thus it is possible that this enzyme is stimulated by heat, releasing $InsP_3$ from phosphoinositides in Ca^{2+}-

independent manner. It is clear that further investigation is necessary to clarify this possibility.

The possibility that heat led to a disruption of the phosphoinositide signalling system, such that cells could not respond to hormones following heating was tested. When WRK-1 cells were incubated at 37°C or heated at 45°C for 2 min before cells were challenged with vasopressin for up to 2 min at either 37°C or 45°C, respectively, the metabolism of inositol phosphates during vasopressin-stimulation at 45°C was altered. Increases in $InsP_3$ and $InsP_{4/5/6}$ levels accompanied by a decrease in $InsP_1$ and $InsP_2$ levels were observed in heated cells stimulated with vasopressin at 45°C for up to 2 min, compared with unheated cells treated with vasopressin. When cells were heated at 45°C for up to 40 min and then returned to 37°C for 2 min (to allow the medium to equilibrate at 37°C) before cells were challenged with vasopressin for 30 sec, it was found that levels of ^3H-$InsP_1$, ^3H-$InsP_2$, ^3H-$InsP_3$ and ^3H-$InsP_{4/5/6}$ in heated cells were similar to those in unheated cells following vasopressin stimulation. The experimental data show that the response of WRK-1 cells to vasopressin was unaffected by a heat dose sufficient to kill (ultimately) greater than 95% of these cells (determined by the MTT colorimetric assay). Other workers found that vasopressin and bradykinin access a common phosphoinositide pool, but there is a small fraction of hormone-sensitive lipid which responds only to bradykinin in WRK-1 cells (Monaco *et al.*, 1990). Thus, it is possible that WRK-1 cells respond differently to different hormones or growth factors following heating. The effect of heat on the $InsP_3$ level (and its metabolites) upon agonist stimulation has been investigated in A-431 cells and CHO HA-1 cells. When A-431 cells were incubated at 37°C or 46°C for 30 min, then EGF added for the last 5 min of incubation, the EGF-treatment caused an increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels, giving 35% and 45% in the $InsP_3$ level at 37°C and 46°C, respectively (Liu and Carpenter, 1992). On the other hand, an additive increase in $InsP_1$, $InsP_2$ and $InsP_3$ levels was found in CHO HA-1 cells when they were stimulated with thrombin at 43°C

(0.5-30 min) compared with thrombin stimulation at 37°C (Calderwood and Stevenson, 1993). It is clear that further investigation is necessary to determine the significance of the distortion of inositol phosphate metabolism in relation to heat cell death.

Effects of agents that have been shown to affect either G proteins or the PI-PLC of the phosphoinositide signalling pathway were also investigated. No significant difference in the total ^{3}H-$InsP_3$ levels were found between WRK-1 cells incubated in 5% (v/v) ethanol or 50 μM sodium orthovanadate compared with cells incubated in serum-free EMEM medium at 37°C for 30 min. However, a significant increase in total ^{3}H-$InsP_3$ levels was observed when cells were incubated either with AlF_4^- (5 mM NaF and 10 μM $AlCl_3$) at 37°C for 30 min or heated at 45°C for 30 min in EMEM medium alone, compared with cells incubated in EMEM for 30 min at 37°C. Incubation in the presence of serum at 37°C for 30 min also resulted in an increase in total ^{3}H-$InsP_3$ level, which was further increased by heating at 45°C for 30 min, compared with cells incubated in serum-free EMEM medium at 37°C for 30 min. However, the present study did not have sufficient data to make a precise decision on whether heat and AlF_4^- exerted effects on the same or different components of the phosphoinositide signalling pathway. Experiments using PLC inhibitors (e.g. neomycin), non-hydrolysable GTP analogues or a combination of AlF_4^- and heat treatment, to find out if they give a synergistic effect on inositol phosphate levels, may clarify this uncertainty.

The role of the increase in $[Ca^{2+}]_i$ in hyperthermic cell death remains equivocal. It is known that heat induces an increase in $[Ca^{2+}]_i$ in cell lines such as CHO HA-1 cells (Calderwood *et al.*, 1988), Drosophila salivary gland (Drummond *et al.*, 1988), human colon HT-29 cells (Mikkelsen *et al.*, 1991a), human epidermoid A-431 cells (Kiang *et al.*, 1992), mouse mammary FM3A cells (Kondo *et al.*, 1993) and NIH3T3 fibroblasts (Stege *et al.*, 1993b). However, no heat-induced increase in $[Ca^{2+}]_i$ was observed in HeLa S3 cells (Stege *et al.*, 1993a), L5178Y-S cells (Stege *et al.*, 1993b), L5178Y-R

cells (Stege *et al.*, 1993b) and mouse mammary MMT060562 cells (Furukawa *et al.*, 1992). These discrepancies may be due to differences in the temperature or duration of heating of the cell lines. For example, in HT-29 cells were heated for 1 h at 44°C (Mikkelsen *et al.*, 1991a) whereas mouse mammary MMT060562 cells were heated at 44°C for 30 min only (Furukawa *et al.*, 1992). Alternatively, Stege and co-workers (1993a,b) suggested that the heat-induced increase in $[Ca^{2+}]_i$ was solely cell type-dependent. Furthermore, the $[Ca^{2+}]_i$ reported in most of the above studies was measured by spectrofluorimetry, a technique that measures the changes in a cell population rather than at the single cell level, and it has been reported that dye leakage from cells can present a more serious problem at higher temperature when using this technique (Wierenga and Konings, 1989). Clearly, if dye leakage into a calcium-containing medium occurs during spectrofluorimetry, an increase in 350 nm/380 nm ratio would be recorded, and this could be incorrectly interpreted as an increase in $[Ca^{2+}]_i$. However, by using fluorescence microscopy, the dye leakage problem can be eliminated. In the study performed by Mikkelsen and co-workers (1991a), the level of $[Ca^{2+}]_i$ upon heating was measured at single cell level in fura-2 loaded HT-29 cells by fluorescence microscopy. No linear correlation was found between cells having an increase in $[Ca^{2+}]_i$ during heating and cell death. It was found that 80% of cells showed an increase in $[Ca^{2+}]_i$ following heat treatment at 44°C for 1 h but more than 40% of cells survived this treatment. Instead, a correlation between elevated $[Ca^{2+}]_i$ and cell death was observed during the post-heating period. These authors found that those cells with a heat-induced elevation in $[Ca^{2+}]_i > 200$ nM that persisted for 4-6 h following heating correlated with the number of dead cells (Mikkelsen *et al.*, 1991a). Similarly, no correlation between the heat-induced increase in $[Ca^{2+}]_i$ and cell death was observed in 6 different cell lines by Wierenge *et al.* (1994). It was found that a heat treatment (44°C for 1 h) that killed more than 90% of cells did not lead to changes in $[Ca^{2+}]_i$ in at least 4 cell lines.

To further address the relationship between $[Ca^{2+}]_i$ and hyperthermic cell death,

heat-induced changes in $[Ca^{2+}]_i$ were measured in fura-2 loaded WRK-1 cells at the single cell level by fluorescence microscopy. The results are presented in Chapter 5. It is clear from these results that heat led to an increase in $[Ca^{2+}]_i$ when calcium was present in the extracellular medium. However the proportion of the cell population that showed an increase in $[Ca^{2+}]_i$ depended on the medium used. The most pronounced effect on the increase in $[Ca^{2+}]_i$ was observed when cells were heated at 45°C in EMEM, when 76% of cells showed an increase in $[Ca^{2+}]_i$ compared with cells heated in calcium-containing buffer, in which 25% showed an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ upon heating did not result from Ca^{2+} influx through voltage-gated calcium channels, as the effect was not blocked by voltage-gated calcium channel blockers (La^{3+} or nifedipine). In addition, perfusing 50 mM K^+-containing buffer (which is sufficient to cause membrane depolarisation and Ca^{2+} influx via any voltage-gated channels) did not show any alteration in $[Ca^{2+}]_i$, implying that voltage-gated calcium channels are absent in WRK-1 cells. Furthermore, the $[Ca^{2+}]_i$ value was not elevated when cells were heated in calcium-free buffer, suggesting that extracellular calcium is the major (sole?) source of the heat-induced elevation in $[Ca^{2+}]_i$. This result is comparable to that reported by Kiang *et al.* (1992) who found that the heat-induced increase in $[Ca^{2+}]_i$ in A-431 cells was due to Ca^{2+} influx from the extracellular medium.

A significant increase in $[Ca^{2+}]_i$ was observed upon heat treatment of WRK-1 cells in EMEM medium at 45°C for ≥ 600 sec, compared to unheated cells. This increase in $[Ca^{2+}]_i$ displayed a time course similar to that of the heat-induced elevation of total ^3H-$InsP_3$ level, which appeared to be Ca^{2+}-independent event in WRK-1 cells. Thus, it is possible that heat induced an increase in $InsP_3$ levels which then mobilised Ca^{2+} from an internal store. However, if this were true, it would be expected that the heat-induced increase in $[Ca^{2+}]_i$ would be observed even in the absence of extracellular free Ca^{2+} and this was not the case, as WRK-1 cells heated at 45°C for up to 30 min in calcium-free buffer did not show a heat-induced increase in $[Ca^{2+}]_i$. In essence, therefore, the results

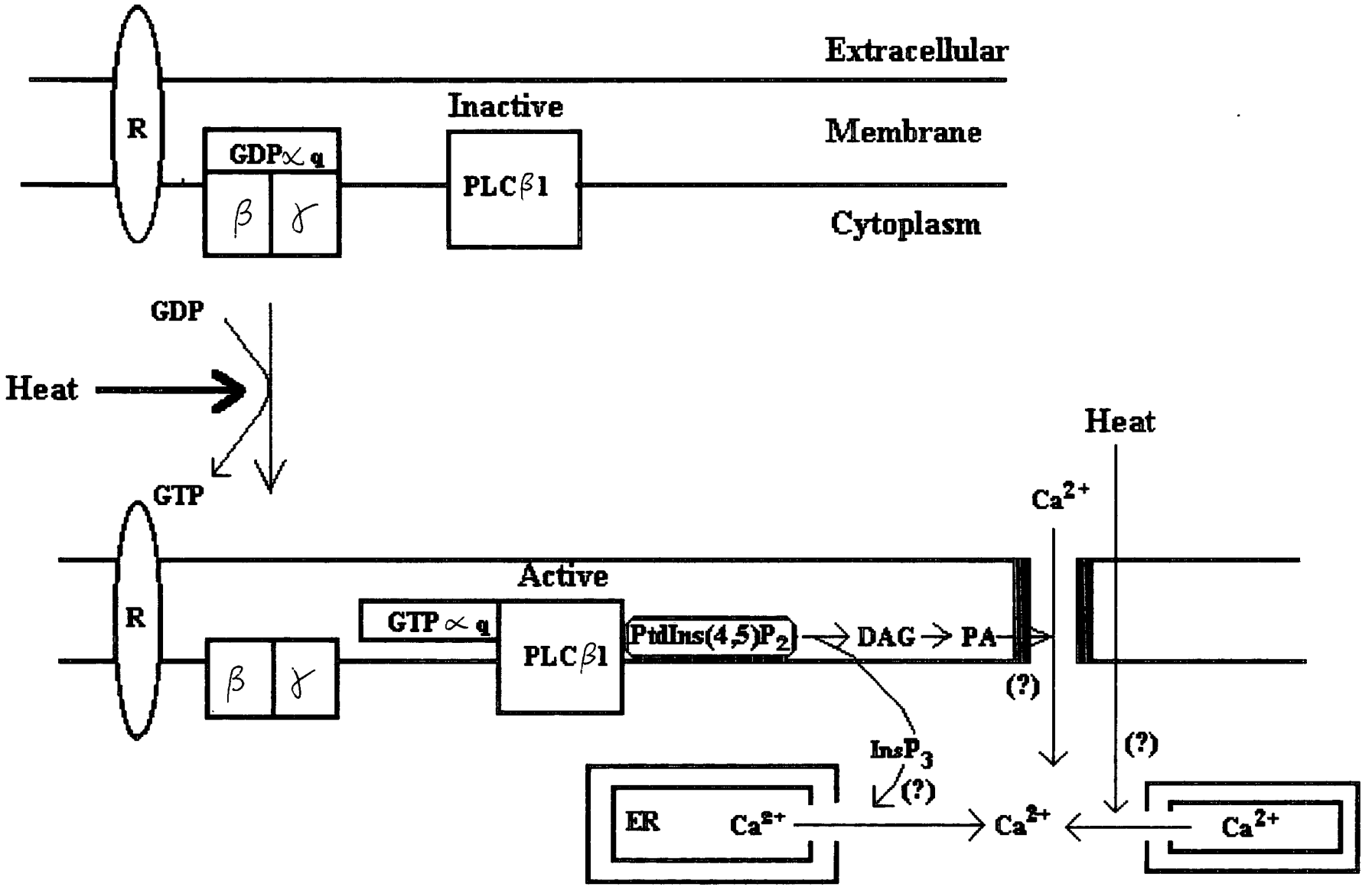
show that heat induced an elevation in $[Ca^{2+}]_i$, which derived mainly (solely?) from extracellular sources, as well as an elevation in $InsP_3$ levels, which occurred by a Ca^{2+}-independent mechanism. This is consistent with the report of a Ca^{2+}-independent PI-PLC activity in WRK-1 cells (Mouillac *et al.*, 1990), so heat-induced activation of this enzyme (perhaps via activation of a G protein) could elevate $InsP_3$ levels, even in the absence of changes in $[Ca^{2+}]_i$.

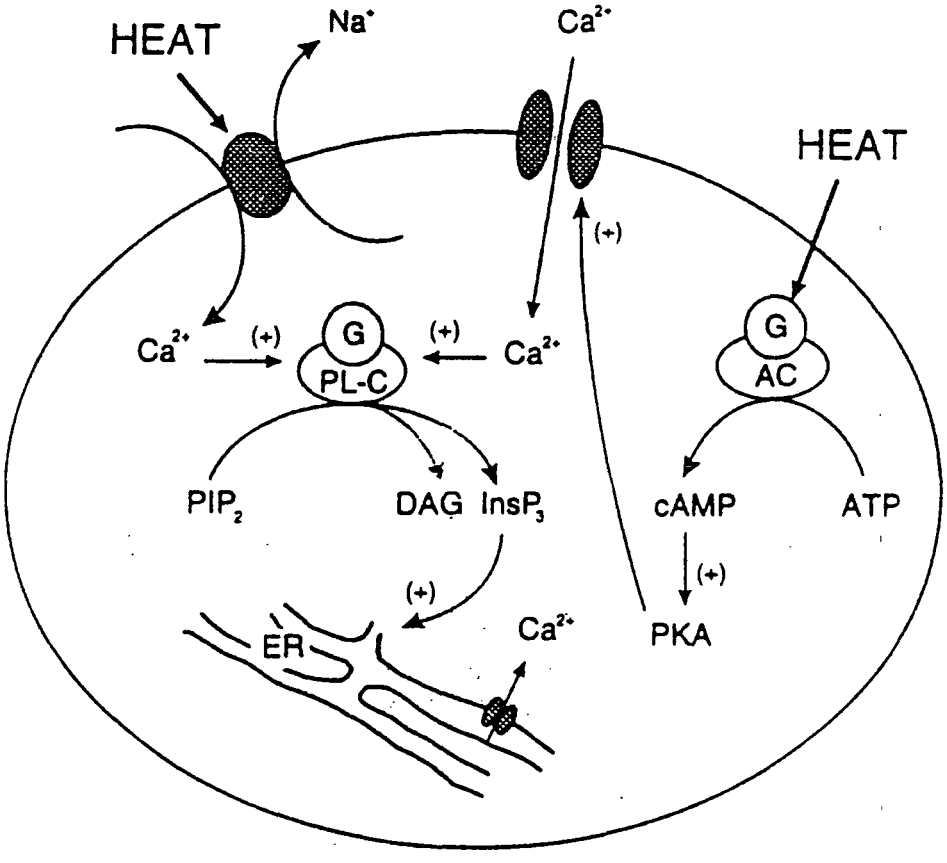
The heat-induced increase in $[Ca^{2+}]_i$ in WRK-1 cells may play a protective role against hyperthermic cell death. When heated in calcium-free buffer, to prevent elevation of $[Ca^{2+}]_i$, hyperthermic cell death of WRK-1 cells was enhanced compared with cells heated in EMEM medium or in calcium-containing buffer (1.8 mM $CaCl_2$). This result was compatible with that observed in hepatocytes in which it was found that cell death was potentiated in calcium-free buffer (Malhotra *et al.*, 1986). This effect may be due to the induction of heat shock protein (HSP) as a result of a heat-induced increase in $[Ca^{2+}]_i$ (Price and Calderwood, 1991; Kiang and McClain, 1992; Kiang *et al.*, 1994). Induction of HSP synthesis may play a protective role against cell death (Landry *et al.*, 1988; Li *et al.*, 1991). In addition, the heat-induced increase in $[Ca^{2+}]_i$ may protect Ca^{2+}-ATPase from thermal denaturation (Cheng, 1989), thus maintaining Ca^{2+} homeostasis in the cells. Furthermore, it is possible that Ca^{2+} exerts an effect on cytoskeletal organisation through the action of CaM-dependent enzymes and this may protect against cell death. Supportive evidence for this notion comes from a study using a CaM antagonist. Heating H_{35} cells at 43°C for 1 h in the presence of the CaM antagonist, trifluoperazine (TFP), prevented the destruction of stress fibres and rounding up of these cells (Wiegant *et al.*, 1985). On the other hand, when N_2A cells were heated in the presence of TFP, the heat-induced aggregation of vimentin around the nucleus and loss of microtubular networks was prevented. However, cell death was enhanced in both cell types when heated in the presence of TFP, so it was suggested that there was a relationship between heat cell death and the alteration of cytoskeletal organisation

(Wiegant *et al.*, 1985). A similar result was observed in mouse tumour clone C MTC cells (Evans and Tomasovic, 1989). Furthermore, in the present study, cell death was enhanced when cells were heated in the presence of nifedipine or when nifedipine was added during the 4 h post-heating period. The mechanism of the cytotoxic effect of this nifedipine treatment is not clear but certainly it was not due to different $[Ca^{2+}]_i$ values in cells heated in this condition compared with cells heated in EMEM. However, it is possible that nifedipine increased Ca^{2+} efflux during subsequent incubation at 37°C for 4 h (Church and Zsotér, 1980).

The decrease in 1,2-DAG levels (rather than increase) in heat-treated WRK-1 cells may be due to thermal activation of DAG acyltransferase, causing accumulation of TAG.

The results obtained from 2 tumour cell lines — CHO-K1 cells and WRK-1 cells in this study suggest that physical stress such as heat can be converted to chemical messengers through the phosphoinositide signalling pathway. The exact role of such changes in relation to hyperthermic cell death remains to be clarified, as the effects are potentially antagonistic. For example, it is possible that the decrease in 1,2-DAG level (and perhaps decrease in PKC activity) in heated cells may have a role in hyperthermic cell death because it has been shown that antagonism of PKC by inhibitors sensitises cells to heat (Mikkelsen *et al.*, 1991b). However, the transient increase in $InsP_3$ and the sustained increase in $[Ca^{2+}]_i$ may play a protective role, possibly via the induction of HSP synthesis, against cell death (Landry *et al.*, 1988; Price and Calderwood, 1991; Kiang and McClain, 1992).


Extracellular
Membrane
Cytoplasm
R
GDP∝q
β
γ
Inactive
PLCβ1
GDP
Heat
GTP
Heat
Ca2+
Active
R
GTP∝q
β
γ
PLCβ1
PtdIns(4,5)P2
DAG
PA
(?)
InsP3
(?)
(?)
ER
Ca2+
Ca2+
Ca2+


HEAT
Na+
Ca2+
HEAT
(+)
Ca2+
(+)
G
PL-C
(+)
Ca2+
G
AC
PIP2
DAG
InsP3
cAMP
ATP
(+)
(+)
ER
Ca2+
PKA

References

Abdel-Latif, A.A., Akhtar, R.A. and Hawthorne, J.N. (1977) Acetylcholine increases the breakdown of trisphosphoinositide of rabbit iris muscle prelabelled with [^{32}P] phosphate. Biochem. J. 162, 61-73.

Almers, W. and Neher, E. (1985) The calcium signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. FEBS Lett. 192, 13-18.

Anghileri, L.J., Crone, M-C., Marchal, C. and Robert, J. (1983) Comparative enhancement of hyperthermia lethality on tumor cells by procaine and lanthanum. Neoplasma 30, 547-549.

Anghileri, L.J., Crone-Escanyé, M-C Marchal, C., and Robert, J. (1984) Plasma membrane changes during hyperthermia: probable role of ionic modifications in tumor cell death. Proc. Hyperthermic Oncol, 4th international symposium., 49-52.

Anghileri, L.J., Marchal, C., Crone-Escanyé, M-C and Robert, J. (1985a) Effects of extracellular calcium on calcium transport during hyperthermia of tumor cells. Eur. J. Cancer. Clin. Oncol. 21, 981-984.

Anghileri, L.J., Crone-Escanyé, M-C and Robert, J. (1985b) Calcium, calcium ATPase and cyclic AMP changes in Ehrlich ascites cells submitted to hyperthermia. Arch. Geschwuisttorsch 55, 171-175.

Arslan, P., Di Virgillo, F., Beltrame, M., Tsien, R.Y. and Pozzan, T. (1985) Cytosolic Ca^{2+} homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free calcium. J. Biol. Chem. 260, 2719-2727.

Aubin, J.E. (1979) Autofluorescence of viable cultured mammalian cells. J. Histochem. Cytochem. 27, 36-43.

Augert, G., Blackmore, P.F. and Exton, J.H. (1989) Changes in the concentration and fatty acid composition of phosphoinositides induced by hormones in hepatocytes. J. Biol. Chem. 264, 2574-2580.

Back, J.F., Oakenfull, D. and Smith, M.B. (1979) Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry 18, 5191-5196.

Bagi, G. and Hidvégi, E.J. (1990) Protein phosphorylation and kinase activities in tumour cells after hyperthermia. Int. J. Radiat. Biol. 58, 633-650.

Balla, T., Guillemette, G., Baukal, A.J. and Catt, K.J. (1987) Formation of inositol 1,3,4,6-tetrakisphosphate during angiotensin II action in bovine adrenal glomerulosa cells. Biochem. Biophys. Res. Commun. 148, 199-205.

Banochback, M.W., Geison, R.L. and O'Brien, J.F. (1974) Use of [1-^{14}C]acetic anhydride to quantitate diglycerides: a new analytical procedure. Anal. Biochem. 59, 617-627.

Barker, C.J., Morris, A.J., Kirk, C.J. and Michell, R.H. (1988) Inositol tetrakisphosphates in WRK-1 cells. Biochem. Soc. Trans. 16, 984-985.

Barker, C.J., Wong, N.S., Maccallum, S.M., Hunt, P.A., Michell, R.H. and Kirk, C.J. (1992) The interrelationships of the inositol phosphates formed in vasopressin-stimulated WRK-1 rat mammary tumour cells. Biochem. J. 286, 469-474.

Barnes, D. and Sato, G. (1980) Method for growth of cultured cells in serum-free medium. Anal. Biochem. 102, 255-270.

Bass, H., Coakley, W.T., Moore, J.L. and Tilley, D. (1982) Hyperthermia-induced changes in the morphology of CHO-K1 and their refractile inclusions. J. Thermal Biol. 7, 231-242.

Bauer , K.D. and Henle, K.J. (1979) Arrhenius analysis of heat survival curves from normal and thermotolerance CHO cells. Radiat. Res. 78, 251-263.

Becker, P.L. and Fay, F.S. (1987) Photobleaching of fura-2 and its effect on determination of calcium concentrations. Am. J. Physiol. 253, C613-C618.

Bennett, C.F. and Crooke, S.T. (1987) Purification and characterisation of a phosphoinositide-specific phospholipase C from guinea pig uterus. J. Biol. Chem. 262, 13789-13797.

Berridge, M.J. and Fain, J.N. (1979) Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. Biochem. J. 178, 59-69.

Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220, 345-360.

Berridge, M.J. and Irvine, R.F. (1984) Inositol tetrakisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315-332.

Berridge, M.J. (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann. Rev. Biochem. 56, 159-193.

Berridge, M.J. and Irvine, R.F. (1989) Inositol phosphates and cell signalling. Nature 341, 197-205.

Berridge, M.J., Downes, C.P. and Hanley, M.R. (1989) Neural and developmental actions of lithium: a unifying hypothesis. Cell 59, 411-419.

Berridge, M.J. (1993) Inositol trisphosphate and calcium signalling. Nature 361, 315-325.

Bers, D.M. (1982) A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. Am. J. Physiol. 242, C404-C408.

Besterman, J.M., May, W.S., Jr., LeVine, H., Cragoe, E.J., Jr. and Cuatrecasas, P. (1985) Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. An amiloride analog selectively inhibits Na^+/H^+ exchange. J. Biol. Chem. 260, 1155-1159.

Bhuyan, B.K., Day, K.J., Edgerton, C.E. and Ogunbase, O. (1977) Sensitivity of different cell lines and of different phases in the cell cycle to hyperthermia. Cancer Res. 37, 3780-3784.

Bhuyan, B.K. (1979) Kinetics of cell kill by hyperthermia. Cancer Res. 39, 2277-2284.

Billah, M.M. and Anthes, J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. 269, 281-291.

Bird, G.stJ., Rossier, M.F., Hughes, A.R., Shears, S.B., Armstrong, D.L. and Putney, J.W., Jr. (1991) Activation of Ca^{2+} entry into acinar cells by a non-phosphorylable inositol trisphosphate. Nature 352, 162-165.

Bishop, W.R., Ganong, B.R. and Bell, R.M. (1986) Attenuation of *sn*-1,2-diacylglycerol second messengers by diacylglycerol kinase. J. Biol. Chem. 261, 6993-7000.

Blackmore, P.F., Bocckino, S.B., Waynick, L.E. and Exton, J.H. (1985) Role of a guanine nucleotide-binding regulatory protein in the hydrolysis of hepatocyte phosphatidylinositol 4,5-bisphosphate by calcium-mobilizing hormones and the control of cell calcium. J. Biol. Chem. 260, 14477-14483.

Blaker, G.J., Birch, J.R. and Pirt, S.J. (1971) The glucose, insulin and glutamine requirements of suspension cultures of HeLa cells in defined culture medium. J. Cell Sci. 9, 529-537.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J Biochem. Physiol. 37, 911-917.

Bocckino, S.B., Blackmore, P.F. and Exton, J.H. (1985) Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine and angiotensin II. J. Biol. Chem. 260, 14201-14207.

Bocckino, S.B., Wilson, P.B. and Exton, J.H. (1987) Ca^{2+}-mobilising hormones elicit phosphatidylethanol accumulation via phospholipase-D activation. FEBS Lett. 225, 201-204.

Bolander, F. (1989) Calcium, calmodulin, and phospholipids. In: Molecular Endocrinology. Ed. Bolander, F., Academic Press, pp147-167.

Borrelli, M.J., Wong, R.S.L. and Dewey, W.C. (1986) A direct correlation between hyperthermia-induced membrane blebbing and survival in synchronous G_1 CHO cells. J. Cell. Physiol. 126, 181-190.

Bowler, K., Duncan, C.J., Gladwell, R.T. and Davison, T.F. (1979) Cellular heat injury. Comp. Biochem. Physiol. 45A, 441-450.

Bowler, K. (1987) Cellular heat injury: are membranes involved? In: Temperature and Animal Cells, SEB symposium XLI, eds. Bowler, K. and Fuller, B.J., Cambridge: The Company of Biologist Ltd, pp157-185.

Boynton, A.L., Dean, N.M. and Hill, T.D. (1990) Inositol 1,3,4,5-tetrakisphosphate and regulation of intracellular calcium. Biochem. Pharmacol. 40, 1933-1939.

Brock, T.D. (1985) Life at high temperatures. Science 230, 132-138.

Burgess, G.M., Mckinney, J.S., Fabiato, A., Leslie, B.A. and Putney, J.W., Jr. (1983) Calcium pools in saponin-permeabilized guinea pig hepatocytes. J. Biol Chem. 258, 15336-15345.

Burgess, G.M., Bird, G. St. J, Obie, J.F. and Putney, J.W., Jr. (1991) The mechansim for synergism between phospholipase C- and adenylyl cyclase-linked hormones in liver. J. Biol. Chem. 266, 4772-4781.

Burns, D.J. and Bell, R.M. (1992) Lipid regulation of protein kinase C. In: Protein Kinase C. Current concepts and future perspectives, eds. Lester, D.S. and Epand, R.M., Ellis Horwood Ltd., pp 25-40.

Burrow, M.T. (1910) The cultivation of tissues of the chick-embryo outside the body. J. Am. Med. Ass. 55, 2056-2058.

Calderwood, S.K. and Hahn, G.M. (1983) Thermal sensitivity and resistance of insulin-receptor binding. Biochim. Biophys. Acta 756, 1-8.

Calderwoood, S.K., Bump, E.A., Stevenson, M.A., Kersen, I.V. and Hahn, G.M. (1985) Investigation of adenylate energy charge, phosphorylation potential, and ATP concentration in cells stressed with starvation and heat. J. Cell. Physiol. 124, 261-268.

Calderwood, S.K., Stevenson, M.A. and Hahn, G.M. (1987) Heat stress stimulates inositol trisphosphate release and phosphorylation of phosphoinositides in CHO and Balb C3T3 cells. J. Cell. Physiol. 130, 369-376.

Calderwood, S.K., Stevenson, M.A. and Hahn, G.M. (1988) Effects of heat on cell calcium and inositol lipid metabolism. Radiat. Res. 113, 414-425.

Calderwood, S.K., Bornstein, B., Farnum, E.K. and Stevenson, M.A. (1989) Heat shock stimulates the release of arachidonic acid and the synthesis of prostaglandins and leukotriene B_4 in mammalian cells. J. Cell. Physiol. 141, 325-333.

Calderwood, S.K. and Stevenson, M.A. (1989) Role of phosphoinositides in the response of mammalian cells to heat shock. In: Cell Calcium Metabolism. Ed. Fiskum, G., Plenum Press, pp 499-505.

Calderwood, S.K. and Stevenson, M.A. (1993) Inducers of the heat shock response stimulate PLC and PLA_2 activity in mammalian cells. J. Cell. Physiol. 155, 248-256.

Calderwood, S.K., Stevenson, M.A. and Price, B.D. (1993) Activation of PLC by heat shock requires GTP analogs and is resistant to pertussis toxin. J. Cell. Physiol. 156, 153-159.

Camps, M., Hou, C., Sidiropoulos, D., Stock, J.B., Jakobs, K.H. and Gierschik, P. (1992) Stimulation of phospholipase C by guanine-nucleotide-binding proteins $\beta\gamma$ subunits. Eur. J. Biochem. 206, 821-831.

Carafoli, E. (1987) Intracellular calcium homeostasis. Ann. Rev. Biochem. 56, 395-433.

Carbone, E. and Lux, H.D. (1984) A low voltage-activated, fully inactivating Ca-channel in vertebrate sensory neurons. Nature 310, 501-502.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. and Mitchell, J.B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetic assay: assessment of chemosensitivity testing. Cancer Res. 47, 936-942.

Castagna, M., Takai, Y. Kaibuchi, K,Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847-7851.

Catt, K.J., Hunyady, L. and Balla, T (1991) Second messengers derived from inositol lipids. J. Bioenergetics and Biomembranes 23, 7-27.

Cavaliere, R., Ciocatto, E.C., Giovanella, B.C., Heidelberger, C., Johnson, R.O., Margottini, M., Moricca, G. and Rossi-Fanelli, A. (1967) Selective heat sensitivity of cancer cells. Cancer 20, 1351-1381.

Changya, L., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1989) Inositol 1,3,4,5-tetrakisphosphate and inositol 1,4,5-tisphosphate act by different mechanisms when controlling calcium in mouse lacrimal acinar cells. FEBS Lett. 251, 43-48.

Chen, S.S. (1974) Studies of radiocalcium efflux in single barnacle muscle fibres: effects of procaine and external divalent cations. J. Physiol. 238, 313-328.

Chen, R.H. and Blenis, J. (1990) Identification of xenopus S6 protein kinase homologs ($pp90^{rsk}$) in somatic cells: phosphorylation and activation during initiation of cell proliferation. Molecular Cell Biol. 10, 3204-3215.

Cheng, K-H., Hui, S.W. and Lepock, J.R. (1987) Protection of the membrane calcium adenosine trisphosphatase by cholesterol from thermal inactivation. Cancer Res. 47, 1255-1262.

Cheng, K-H. (1989) Role of calcium in the thermal inactivation of calcium transport proteins. Cancer Res. 49, 7029-7030.

Cheung, W.Y. (1980) Calmodulin plays a pivotal role in cellular regulation. Science 207, 19-27.

Christiansen, E.N. and Kvamme, E. (1969) Effects of thermal treatment on mitochondria of brain, liver and ascites cells. Acta Physiol. Scand. 76, 472-484.

Chu, G.L. and Dewey, W.C. (1988) The role of low intracellular or extracellular pH in sensitization to hyperthemia. Radiat. Res. 114, 154-167.

Church, J. and Zsotér, T.T. (1980) Calcium antagonistic drugs. Mechanism of action. Can. J. Physiol. Pharmacol. 58, 254-264.

Cobbold, P.H. and Rink, T.J. (1987) Fluorescence and bioluminescence measurements of cytosolic free calcium. Biochem. J. 248, 313-328.

Cockcroft, S. and Thomas, G.M.H. (1992) Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem. J. 288, 1-14.

Connolley, T.M., Wilson, D.B., Bross, T.E. and Majerus, P.W. (1986) Isolation and characterisation of the inositol cyclic phosphate products of phosphoinositide cleavage by PLC. J. Biol. Chem. 261, 122-126.

Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987) The metabolism of tris- and tetrakisphosphates of inositol by 5-phosphomonoesterase and 3-kinase enzymes. J. Biol. Chem. 262, 2146-2149.

Coss, R.A., Dewey, W.C. and Bambury, J.R. (1979) Effect of hyperthermia (41.5ºC) on Chinese hamster ovary cells analyzed in mitosis. Cancer Res. 39, 1911-1918.

Coss, R.A., Dewey, W.C. and Bamburg, J.R. (1982) Effects of hyperthermia on dividing Chinese hamster ovary cells and on microtubules *in vitro*. Cancer Res. 42, 1059-1071.

Coss, R.A., Felgar, R.E., Stokes, D.G. and Smith, N.N. (1989) Effects of verapamil and diltiazem on hyperthermic cell death in CHO cells. Int. J. Hyperthermia 5, 383-387.

Cossins, A.R., Bowler, K. and Prosser, C.L. (1981) Homeoviscous adaptation and its effect upon membrane-bound proteins. J. Thermal Biol. 6, 183-187.

Cossins, A.R. and Raynard, R.S. (1987) Adaptative responses of animal cell membranes to temperature. In: Temperature and Animal Cells, SEB symposium XLI. Eds. Bowler, K. and Fuller, B.J., Cambridge: The Company of Biologist Ltd., pp 95-112.

Creba, J.A., Downes, P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilising hormones. Biochem. J. 212, 733-747.

Cress, A.E. and Gerner, E.W. (1980) Cholesterol levels inversely reflect the thermal sensitivity of mammalian cells in culture. Nature 283, 677-679.

Cress, A.E., Culver, P.S., Moon, T.E. and Gerner, E.W. (1982) Correlation between amounts of cellular membrane components and sensitivity to hyperthermia in a variety of mammalian cell lines in culture. Cancer Res. 42, 1716-1721.

Crompton, M., Capano, M. and Carafoli, E. (1976) The sodium-induced efflux of calcium from heart mitochondria. A possible mechanism for the regulation of mitochondrial calcium. Eur. J. Biochem. 69, 453-462.

Cubitt, A.B., Zhang, B. and Gershengorn, M.C. (1990) Analysis by base exchange of thyrotropin-releasing hormone responsive and unresponsive inositol lipid pools in rat pituitary tumor cells. J. Biol. Chem. 265, 9707-9714.

Dawson, R.M.C. and Clarke, N. (1972) D-*myo*inositol 1:2-cyclic phosphate 2-phosphohydrolase. Biochem. J. 127, 113-118.

Dean, N.M. and Beaven, M.A. (1989) Methods for analysis of inositol phosphates. Anal. Biochem. 183, 199-209.

Dekker, L.V. and Parker, P.J. (1994) Protein kinase C — a question of specificity. Trends Biochem. Sci. 19, 73-77.

Demel, R.A., Van Kessel, W.S.M. and Van Deenen, L.L.M. (1972) The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. Biochim. Biophys. Acta 266, 26-40.

Denizot, F. and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271-277.

DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P. and Kaczmarek, L.K. (1985) Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. Nature 313, 313-326.

Dewey, W.C., Westra, A., Miller, H.H. and Nagasawa, H. (1971) Heat-induced lethality and chromosomal damage in synchronized Chinese hamster cells treated with 5-bromodeoxyuridine. Int. J. Radiat. Biol. 20, 505-520.

Dice, F., Chiang H-L, Terlecky, S.R. and Olson, T.S. (1991) A role for the 73 kD heat shock cognate protein in lysosomal pathway of intracellular protein degradation. In: Heat Shock. Eds. Maresca, B. and Lindquist, S, Springer-Verlag, pp181-192.

Dickson, J.A. and Oswald, B.E. (1976) The sensitivity of a malignant cell line to hyperthermia (42°C) at low intracellular pH. Br. J. Cancer 34, 262-271.

Dickson, J.A. and Calderwood, S.K. (1983) Thermosensitivity of neoplastic tissue in vitro. In: Hyperthermia in Cancer Therapy. Eds. Storm, F.K. and Hall, G.K., Boston, MA, pp63-140.

Di Virgilio, F., Steinberg, T.H. and Silverstein, S.C. (1990) Inhibition of fura-2 sequestration and secretion with organic anion transport blockers. Cell Calciuim 11, 57-62.

Downes, C.P. (1989) The cellular functions of myo-inositol. Biochem. Soc. Trans. 17, 259-268.

Downes, C.P. and MacPhee, C.H. (1990) Myo-inositol metabolites as cellular signals. Eur. J. Biol. Chem. 195, 1-18.

Drummond, A.H. (1985) Bidirectional control of cytosolic free calcium by thyrotropin-releasing hormone in pituitary cells. Nature 315, 752-755.

Drummond, I.A.S., Livingston, D. and Steinhardt, R.A. (1988) Heat shock protein synthesis and cytoskeletal rearrangements occur independently of intracellular free calcium increases in drosophila cells and tissues. Radiat. Res. 113, 402-413.

Dynlacht, J.R. and Fox, M.H. (1992a) The effect of 45°C hyperthermia on the membrance fluidity of cells of several lines. Radiat. Res. 130, 55-60.

Dynlacht, J.R. and Fox, M.H. (1992b) Heat-induced changes in the membrane fluidity of Chinese hamster ovary cells measured by flow cytometry. Radiat. Res. 130, 48-54.

Dynlacht, J.R., Hyun, W.C. and Dewey, W.C. (1993) Changes in intracellular free calcium during hyperthermia: effects of local anaesthetics and induction of thermotolerance. Cytometry 14, 223-229.

Eagle, H. (1955) The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. Biol. Chem. 214, 839-852.

Eagle, H. (1973) The effect of environment pH on the growth of normal and malignant cells. J. Cell. Physiol. 82, 1-8.

Evans, D.P. and Tomasovic, S.P. (1989) The effect of calmodulin antagonists on hyperthermic cell killing and the development of thermotolerance. Int. J. Hyperthermia 5, 563-578.

Exton, J. H. (1990) Signalling through phosphatidylcholine breakdown. J. Biol. Chem. 265, 1-4.

Exton, J.H., Taylor, S.J., Augert, G. and Bocckino, S.B. (1991) Cell signalling through phospholipid breakdown. Molecular and Cellular Biochem. 104, 81-86.

Exton, J.H. (1994) Phosphoinositide phospholipases and G-protein in hormone action. Ann. Rev. Physiol. 56, 349-369.

Farber, J.L. (1981) The role of calcium in cell death. Life Sci. 29, 1289-1295.

Farese, R.V., Konda, T.S., Davis, J.S., Standaert, M.L., Pollet, R.J. and Cooper, D.R. (1987) Insulin rapidly increases diacylglycerol by activating *de novo* phosphatidic acid synthesis. Science 236, 586-589.

Farese, R.V., Cooper, D.R., Konda, T.S., Nair, G., Standaert, M.L, Davis, J.S. and Pollet, R.J. (1988) Mechanisms whereby insulin increases diacylglycerol in BC3H-1 myocytes. Biochem. J. 256, 175-184.

Farese, R.V. and Cooper, D.R. (1990) Lipid-related second massengers. In: Peptide Hormone Action. A Practical Approach. Eds. Siddle, K. and Hutton, J.C., IRL Press, pp185-222.

Field, S.B. (1987) Hyperthermia in the treatment of cancer. Phys. Med. Biol. 32, 789-811.

Fisher, H.W., Puck, T.T. and Sato, G. (1958) Molecular growth requirements of single mammalian cells: the action of fetuin in promoting cell attachment of glass. Proc. Natl. Acad. Sci. (USA) 44, 4-10.

Fisher, G.A., Li, G.C. and Hahn, G.M. (1982) Modification of the thermal response by D_2O. 1 Cell survival and the temperature shift. Radiat. Res. 92, 530-540.

Florin-Christensen, J., Florin-Christensen, M., Delfino, J.M., Stegmann, T. and Rasmussen, H. (1992) Metabolic fate of plasma membrane diacylglycerols in NIH 3T3 fibroblasts. J. Biol. Chem. 267, 14783-14789.

Florin-Christensen, J., Florin-Christensen, M., Delfino, J.M. and Rasmussen, H. (1993) New patterns of diacylglycerol metabolism in intact cells. Biochem. J. 289, 783-788.

Fredin, B.L., Seiffert, S.C. and Gelehrter, T.D. (1979) Dexamethasone-induced adhesion in hepatoma cells: The role of plasminogen activator. Nature 277, 312-313.

French, P.J., Bunce, C.M., Stephens, L.R., Lord, J.M., McConnell, F.M., Brown, G., Creba, J.A. and Michell, R.H. (1991) Changes in the levels of inositol lipids and phosphates during the differentiation of HL60 promyelocytic cells towards neutrophils or monocytes. Proc. R. Soc. London B 245, 193-201.

Friend, C., Patuleia, M.C. and Nelson, J.B. (1966) Antibiotic effect of tylosin on a mycoplasma contaminant in a tissue culture leukaemia cell line. Proc. Soc. Exp. Biol. Med. 121, 1009-1010.

Furukawa, M., Enomoto, K-I, Kato, H., Ishida, T. and Maeno, T. (1992) Effects of hyperthermia on intracellular calcium concentration and responses of cancerous mammary cells in culture. Cell Biochem. and Function 10, 225-232.

Gallo-Payet, N. and Payet, M.D. (1989) Excitation-secretion coupling: involvement of potassium channels in ACTH-stimulated rat adrenocortical cells. J. Endocrinol. 120, 409-421.

Ganong, B.R., Loomis, C.R., Hannum, Y.A. and Bell, R.M., (1986) Specificity and mechanism of PKC activation by *sn*-1,2-diacylglycerol. Proc. Natl. Acad. Sci. (USA) 83, 1184-1188.

Garcia-Martin, E., Gonzales-Cabanillas, S. and Gutierrez-Merino, C. (1990) Modulation of calcium fluxes across synaptosomal plasma membrane by local anaesthetics. J. Neurochem. 55, 370-378.

Gawler, D.J., Potter B.V.L, Gigg R. and Nahorski S.R. (1991) Interactions between inositol tris- and tetrakis-phosphates effect on intracellular calcium mobilisation in SH-SY5Y cells. Biochem. J. 276, 163-167.

Gerner, E.W., Boone, R., Connor, W.G., Hicks, J.A. and Boone, M.L.M. (1976) A transient thermotolerant survival response produced by single thermal doses in HeLa cells. Cancer Res. 36, 1035-1040.

Gerner, E.W., Cress, A.E., Stickney, D.G., Holmes, D.K. and Culver, P.S. (1980) Factors reglating membrane permeability alter themal resistance. Ann. NY Acad. Sci. 335, 215-233.

Gerweck, L.E. (1977) Modification of cell lethality at elevated temperatures: the pH effect. Radiat. Res. 70, 224-235.

Gerweck, L.E. and Richards, B. (1981) Influence of pH on the thermal sensitivity of cultured human glioblastoma cells. Cancer Res. 41, 845-849.

Gerweck, L.E., Dahlberg, W.K., Epstein, L.F. and Shimms, D.S. (1984) Influence of nutrient and energy deprivation on cellular response to single and fractionated heat treatments. Radiat. Res. 99, 573-581.

Giovanella, B.C., Stehlin, J.S., Jr. and Morgan, A.C. (1976) Selective lethal effect of supranormal temperatures on human neoplastic cells. Cancer Res. 36, 3944-3950.

Glass, J.R., DeWitt, R.G. and Cress, A.E. (1985) Rapid loss of stress fibres in Chinese hamster ovary cells after hyperthermia. Cancer Res. 45, 258-262.

Goldman, R.D., Goldman, A.E., Green, K.J., Jones, J.C.R., Jones, S.M. and yang, H-Y (1986) Intermediate filament networks: organisation and possible functions of a diverse group of cytoskeletal elements. In: The Cytoskeleton: Cell Function and Organisation. Eds. Lloyd, C.W., Hyams, J.S.H. and Warn, R.M., Cambridge: The Company of Biologist Ltd., pp69-98.

Gonzalez-Mendez, R., Minton, K.W. and Hahn, G.M. (1982) Lack of correlation between membrane lipid composition and thermotolerance in Chinese hamster ovary cells. Biochim. Biophys. Acta 692, 168-170.

Gospodarowicz, D. and Moran, J. (1974) Growth factors in mammalian cell culture. Ann. Rev. Biochem. 45, 531-558.

Green, L.M., Reade, J.L. and Woare, C.F. (1984) Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J. Immunol. Methods 70, 257-268.

Greene, D.A. and Lattimer, S.A. (1986) Protein kinase C against acutely normalize decreased ouabain-inhibitable respiration in diabetic rabbit nerve: implications for Na^+ K^+-ATPase regulation and diabetic complications. Diabetes 35, 242-245.

Groden, D.L., Guan, Z. and Stokes, B.T. (1991) Determination of fura-2 dissociation constants following adjustment of the apparent Ca-EGTA association constant for temperature and ionic strength. Cell Calcium 12, 279-287.

Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440-3450.

Guffy, M.M., Rosenberger, J.A., Simon, I. and Burns, C.P. (1982) Effects of cellular fatty acid alteration on hyperthermic sensitivity in cultured L1210 murine leukaemia cells. Cancer Res. 42, 3625-3630.

Guilbert, L.J. and Iscove, N.N. (1976) Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures. Nature 263, 594-595.

Guillon, G., Mouillac, B. and Balestre M-N (1986a) Activation of polyphosphoinositide phospholipase C by fluoride in WRK1 cell membranes. FEBS Lett. 204, 183-188.

Guillon, G., Balestre, M-N, Mouillac, B. and Devilliers, G. (1986b) Activation of membrane phospholipase C by vasopressin. FEBS Lett. 196, 155-159.

Hahn, G.M. (1974) Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment. Cancer Res. 34, 3117-3123.

Hahn, G.M. and Strande, D.P. (1976) Cytotoxic effects of hyperthermia and adriamycin on Chinese hamster cells. J. Natl. Cancer Inst. 57, 1063-1067.

Hahn, G.M. Steinberg, D. and Fisher, G. (1978) Protection by deuterium oxide against hyperthermic inactivation of Chinese hamster cells. Radiat. Res. 74, 476-477.

Hahn, G.M. (1979) Potential for therapy with drugs and hyperthermia. Cancer Res. 39, 2264-2268.

Hahn, G.M. (1982) Hyperthermia and cancer. Plenum Press. N.Y.

Hahn, G.M. and Li, G.C. (1982) Interactions of hyperthermia and drugs: treatments and probes. Natl. Cancer Inst. Monogr. 61, 317-323.

Hallett, M.B., Dormer, R. L. and Campbell, A.K. (1990) Cytoplasmic free calcium: measurement and manipulation in living cells. In: Peptide Hormone Action. A Practical Approach. Eds. Siddle, K. and Hutton, J.C., IRL Press, pp115-150.

Ham, R.G. (1962) Clonal growth of diploid Chinese hamster cells in synthetic medium supplemented with purified protein fractions. Exp. Cell Res. 28, 489-500.

Ham, R.G. and McKeehan, W.L. (1978) Development of improved media and culture conditions for clonal growth of normal diploid cells. In Vitro 14, 11-22.

Hammond, S.L., Ham, R.G. and Stampfer, M.R. (1984) Serum-free growth of human mammary epithelial cells:rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. (USA) 81, 5435-5439.

Harisiadis, L., Hall, E.J., Kraljevic, U. and Borek, C. (1975) Hyperthermia: biological studies at the cellular level. Radiol. 117, 447-452.

Harrison, S.M. and Bers, D.M. (1989) Correction of proton and calcium association constants of EGTA for temperature and ionic strength. Am. J. Physiol. 256, C1250-C1256.

Haveman, J. and Hahn, G.M. (1981) The role of energy in hyperthermia-induced mammalian cell inactivation: a study of the effects of glucose starvation and an uncoupler of oxidative phosphorylation. J. Cell. Physiol. 107, 237-241.

Heine, U., Severak, L., Kondratick, J. and Bonar, R.A. (1971) The behaviour of HeLa-S3 cells under the influence of supranormal temperatures. J. Ultrastruct. Res. 34 375-396.

Henle, K.J. and Leeper, D.B. (1979) Effects of hyperthermia (45°C) on macromolecular synthesis in Chinese hamster ovary cells. Cancer Res. 39, 2665-2674.

Henle, K.J. (1981) Interaction of mono- and polyhydroxy alcohols with hyperthermia in CHO cells. Radiat. Res. 88, 392-402.

Henle, K.J. and Warters, R.L. (1982) Heat protection by glycerol *in vitro*. Cancer Res. 42, 2171-2176.

Hepler, J.R. and Gilman, A.G. (1992) G protenis. Trends Biochem. Sci. 17, 383-387.

Herchuelz, A., Sener, A. and Malaisse, W. (1980) Fluxes in rat pancreatic-islets-calcium extrusion by sodium calcium countertransport. J. Membr. Biol. 57, 1-12.

Higgins, P.J., Darzynkiewicz, Z. and Melamed, M.R. (1983) Secretion of albumin and alpha-foetoprotein by DMSO-stimulated hepatocellular carcinoma cells. Br. J. Cancer 48, 485-493.

Hightower, L.E. (1991) Heat shock, stress proteins, chaperones and proteotoxicity. Cell 66, 191-197.

Hoek, J.B., Thomas, A.P., Rubin, R. and Rubin, E. (1987) Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. J. Biol. Chem. 262, 682-691.

Hofer, K.G., Brizzard, B. and Hofer, M.G. (1979) Effect of lysosome modification on the heat potentiation of radiation damage and direct heat death of BP-8 sacroma cells. Eur. J. Cancer 15, 1449-1457.

Hokin, M.R. and Hokin, L.E. (1953) Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 203, 967-977.

Holmsen, H., Male, R., Rongved, S., Langeland, N. and Lillehang, J. (1989) Platelet-derived-growth-factor-stimulated heterogeneous phosphoinositide metabolism and phosphate uptake in C3H fibroblasts. Biochem. J. 260, 589-592.

Horwich, A.L. Hartl, F-U and Cheng, M.Y. (1991) Role of HSP60 in folding/assembly of mitochondrial proteins. In: Heat Shock. Eds Maresca, B. and Lindquist, S., Springer-Verlag, pp165-174.

Huang, K-P (1989) The mechanisms of protein kinase C activation. Trends Neurosci. 12, 425-432.

Hunyady, L., Bankal, A.J., Guillémette, G., Balla, T. and Catt, K.J. (1988) Metabolism of inositol-1,3,4,6-tetrakisphosphate to inositol pentakisphosphate in adrenal glomerulosa cells. Biochem. Biophys. Res. Commun. 157, 1247-1252.

Irvine, R.F. (1986) Calcium transients: mobilization of intracellular calcium. Br. Medical Bulletin 42, 369-374.

Irvine, R.F., Letcher, A.J., Heslop, J.P. and Berridge, M.J. (1986) The inositol tris/tetrakisphosphate pathway — demonstration of Ins(1,4,5)P_3 3-kinase activity in animal tissues. Nature 320, 631-634.

Irvine, R.F. and Moore, R.M. (1987) Inositol 1,3,4,5-tetrakisphosphate-induced activation of sea urchin eggs requires the presence of inositol tetrakisphosphate. Biochem. Biophys. Res. Commun. 164, 284-290.

Irvine, R.F. (1990) 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates — a possible mechanism. FEBS Lett. 263, 1, 5-9.

Irvine, R.F. (1992) Is inositol tetrakisphosphate the second messenger that controls calcium entry into cells? In: Advances in Second Messenger and Phosphoprotein Research vol. 26. Ed. Putney, J.W., Jr., New York: Raven Press Ltd., pp161-185.

Irvine, R.F. and Culler, P.J. (1993) Will the real IP_4 receptor please stand up? Current Opin. in Cell Biol. 3, 540-543.

Isaacks, R.E. and Harkness, D.R. (1980) Erythrocyte organic phosphates and haemoglobin function in birds, reptiles and fishes. Am. Zool. 20, 115-129.

Iscove, N.N. and Melchers, F. (1978) Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharride-reactive B lymphocytes. J. Exp. Med. 147, 923-933.

Ivorra, I., Gigg, R., Irvine, R.F. and Parker, I. (1991) Inositol 1,3,4,6-tetrakisphosphate mobilizes calcium in *Xenopus* oocytes with high potency. Biochem. J. 273, 317-321.

Jabbar, S.A.B., Twentyman, P.R. and Watson, J.V. (1989) The MTT assay underestimates the growth inhibitory effects of interferons. Br. J. Cancer. 60, 523-528.

Jacobson, K. and Papahadjopoulos, D. (1975) Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH and concentration of bivalent cations. Biochemistry 14, 152-161.

Johnson, H.A. (1974) On the thermodynamics of cell injury. Some insights into the molecular mechanisms. Am. J. Pathol. 75, 13-26.

Jung, H. (1986) A generalised concept for cell killing by heat. Radiat. Res. 106, 56-72.

Jurivich, D.A., Chung, J. and Blenis, J. (1991) Heat shock induces two distinct S6 protein kinase activities in quiescent mammalian fibroblasts. J. Cell. Physiol. 148, 252-259.

Kabara, J.J. and Chen, J.S. (1976) Microdetermination of lipid classes after thin-layer chromatography. Anal. Chem. 48, 814-817.

Kaczorowski, G.J., Barros, F., Dethmers, J.K., Trumble, M.J. and Crogoe, E.J., Jr. (1985) Inhibition of Na^+/Ca^{2+} exchange in pituitary plasma membrane vesicles by analogues of amiloride. Biochemistry 24, 1394-1403.

Kampinga, H.H., Turkel-Uygur, N., Roti Roti, J.L. and Konings, A.W.T. (1989) The relationship of increased nuclear protein content induced by hyperthermia to killing of HeLa S3 cells. Radiat. Res. 117, 511-522.

Kantengwa, S., Capponi, A.M., Bonventre, J.V. and Polla, B.S. (1990) Calcium and the heat-shock response in the human monocytic line U-937. Am. J. Physiol. 259, C77-C83.

Kao, F.T. and Puck, T.T. (1967) Genetics of somatic mammalian cells. IV Properties of Chinese hamster cell mutants with respect to the requirements for proline. Genetics 55, 513-524.

Kapiszewska, M. and Hopwood, L.E. (1986) Changes in bleb formation following hyperthermia treatment of Chinese hamster ovary cells. Radiat. Res. 105, 405-412.

Kapiszewska, M. and Hopwood, L.E. (1988) Mechanisms of membrane damage for CHO cells heated in suspension. J. Cancer Res. Clin. Oncol. 114, 23-29.

Kapp, D.S. and Hahn, G.M. (1979) Thermosensitization by sulfhydryl compounds of exponentially growing Chinese hamster cells. Cancer Res. 39, 4630-4635.

Kiang, J.G., McKinney, L.C. and Gallin, E.K. (1990) Heat induces intracellular acidification in human A-431 cells: role of Na^+-H^+ exchange and metabolism. Am. J. Physiol. 259, C727-737.

Kiang, J.G., Wu, Y.Y. and Lin, M.C. (1991) Heat treatment induces an increase in intracellular cyclic AMP content in human epidermoid A-431 cells. Biochem. J. 276, 683-689.

Kiang, J.G., Koeing, M.L. and Smallridge, R.C. (1992) Heat shock increases cytosolic free calcium concentration via Na^+-Ca^{2+} exchange in human epidermoid A431 cells. Am. J. Physiol. 263, C30-C38.

Kiang, J.G. and McClain, D.E. (1992) Hsp-72 induction caused by heat shock depends on an increase in $[Ca^{2+}]_i$ in human A-431 cells. Molecular Cell Biol. 3, 249a.

Kiang, J.G. and McClain, D.E. (1993) Effect of heat shock, $[Ca^{2+}]_i$ and cAMP on inositol trisphosphate in human epidermoid A-431 cells. Am. J. Physiol. 264, C1561-C1569.

Kiang, J.C., Carr, F.E., Burns, M.R. and McClain, D.E. (1994) HSP-72 synthesis is promoted by increase in $[Ca^{2+}]_i$ or activation of G proteins but not pH_i or cAMP. Am. J. Physiol. 267, C104-C114.

Kidwell, W.R., Monaco, M.E., Wicha, M.S. and Smith, G.S. (1978) Unsaturated fatty acid requirements for growth and survival of a rat mammary tumor cell line. Cancer Res. 38, 4091-4100.

Kim, J.H. (1988) Modification of thermal effects: chemical modifiers. In: Hyperthermia and Oncology. I Thermal Effects on Cells and Tissues. Eds. Urano, M. and Douple, E., VSP Press, pp99-119.

Kingston, C.A., Ladha, S., Manning, R. and Bowler, K. (1989) Hyperthermic cell death of HTC cell in culture: a comparison of assays. Biochem. Soc. Trans. 17, 531-532.

Kirk, C.J. (1990) Recent developments in our understanding of the inositol lipid signalling system. In: Hormone Perception and Signal Transduction in Animals and Plants, SEB symposium XLIV. Eds. Roberts, J., Kirk, C.J. and Venis, M., Cambridge: The Company of Biologist Ltd., pp173-180.

Kirk, C.J., Maccallum, S.H., Michell, R.H. and Barker, C.J. (1990a) Inositol phosphates in receptor-mediated cell signalling: metabolic origins and interrelationships. Biotech. and Applied Biochem. 12, 489-495.

Kirk, C.J., Morris, A.J. and Shears, S.B. (1990b) Inositol phosphate second messengers. In: Peptide Hormone Action. A Practical Approach. Eds. Siddle, K. and Hutton, J. C, IRL Press, pp151-184.

Kleyman, T.R. and Cragoe, E.J. (1988) Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. 105, 1-21.

Kleyman, T.R. and Cragoe, E.J. (1990) Cation transport series: the amiloride series. Method in Enzymol. 191, 739-755.

Kondo, T., Kano, E., Habara, Y. and Kanno, T. (1993) Enhancement of cell killing and increase in cytosolic calcium concentration by combined treatments with hyperthermia and TMB-8 in mouse mammary carcinoma FM3A cells. Cell Calcium 14, 621-629.

Kondo, T., Habara, Y., Kanno, T. and Kano, E. (1994) Thermosensitization and modification of cytosolic calcium concentration by verapamil and diltiazem in mouse mammary carcinoma cells. Int. J. Radiat. Oncol. Biol. Phys. 29, 511-517.

Konings, A.W.T. and Ruifrok, A.C.C. (1985) Role of membrane lipids and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells. Radiat. Res. 102, 86-98.

Konings, A.W.T. (1988) Membrane as targets for hyperthermic cell killing. In: Recent Results in Cancer Research, Springer-Verlag, pp9-21.

Koréh, K. and Monaco, M.E. (1986) The relationship of hormone-sensitive and hormone-insensitive phosphatidylinositol to phosphatidylinositol 4,5-bisphosphate in the WRK-1 cells. J. Biol. Chem. 261, 88-91.

Kwant, W.O. and Seeman, P. (1969) The displacement of membrane calcium by a local anaesthetic (chlorpromazine). Biochim. Biophys. Acta 193, 338-349.

Labarca, R., Janowsky, A., Patel, J. and Paul, S.M. (1984) Phorbol esters inhibit agonist-induced [^{3}H]inositol-1-phosphate accumulation in rat hippocampal slices. Biochem. Biophys. Res. Commun. 123, 703-709.

Ladha, S. (1990) The effect of changes in plasma membrane lipid composition on the heat sensitivity of hepatoma tissue culture cells and selected plasma membrane enzymes, Ph.D. Thesis, Durham University.

Ladha, S., Kingston, C.A., Manning, R.M. and Bowler, K. (1993) Effect of dietary modulation of membrane lipid composition on the thermostability of HTC cells and of a membrane enzyme. Int. J. Hyperthermia 9, 503-515.

Lamarche, S., Chrétien, P. and Landry, J. (1985) Inhibition of the heat shock response and synthesis of glucose-regulated proteins in Ca^{2+}-deprived rat hepatoma cells. Bichem. Biophys. Res. Commun. 131, 868-876.

Landry, J., Crête, P., Lamarche, S. and Chrétien, P. (1988) Activation of Ca^{2+}-dependent processes during heat shock: role in cell thermoresistance. Radiat. Res. 113, 426-436.

Lapetina, E.G. (1990) Eicosanoid formation and regulation of phospholipase A_2. In: Biology of Cellular Transducing Signals. Ed. Vanderhoek, J.Y., Plenum Press, pp275-280.

Lassing, I. and Lindberg, U. (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 314, 472-474.

Laszlo, A. and Li, G.C. (1985) Heat resistant variants of Chinese hamster fibroblasts altered in expression of heat shock potein. Proc. Natl. Acad. Sci. (USA) 82, 8029-8033.

Laszlo, A. (1992) The effects of hyperthermia on mammalian cell structure and function. Cell Prolif. 25, 59-87.

Laval, R. and Michel, S. (1982) Enhancement of hyperthermia-induced cytotoxicity upon ATP deprivation. Cancer Lett. 15, 61-65.

LeCavalier, D. and Mackillop, W.J. (1985) The effect of hyperthermia on glucose transport in normal and thermotolerance Chinese hamster ovary cells. Cancer Lett. 29, 223-231

Lee, C.O., Taylor, A. and Windhager, E.C. (1980) Cytosolic calcium ion activity in epithelial cells of *Necturus* kidney. Nature 287, 859-861.

Lee, D.C. and Chapman, D. (1987) The effect of temperature on biological membranes and their models. In: Temperature and Animal Cells, SEB symposium XLI, eds. Bowler, K. and Fuller, B.J., Cambridge: The Company of Biologist Ltd., pp95-112.

Lee, C.H. and Hokin, L.E. (1989) Inositol 1,2-cyclic 4,5-trisphosphate is an order of magnitude less potent than inositol 1,4,5-trisphosphate in mobilising intracellular stores of calcium in mouse pancreatic acinar cells. Biochem. Biophys. Res. Commun. 159, 561-565.

Lee, Y.J., Hou, Z-Z, Curetty, L. and Corry, P.M. (1992) Expression, synthesis, and phosphorylation of HSP28 family during development and decay of thermotolerance in CHO plateau-phase cells. J. Cell. Physiol. 150, 441-446.

Leith, J.L., Miller, R.C., Gerner, E.W. and Boone, M.L.M. (1977) Hyperthermic potentiation: biological aspects and application to radiation therapy. Cancer 39, 766-779.

Lepock. J.R. (1982) Involvement of membranes in cellular responses to hyperthermia. Radiat. Res. 92, 433-438.

Lepock, J.R., Cheng, K-H, Al-Qysi, H. and Kruuv, J. (1983) Thermotropic lipid and protein transitions in Chinese hamster lung cell membranes: relationship to hyperthermic cell killing. Can. J. Biochem. Cell Biol. 61, 421-427.

Lepock, J.R., Frey, H.E., Heynen, M.P., Nishio, J., Waters, B., Ritchie, K.P. and Kruuv, J. (1990) Increased thermostability of thermotolerant CHL V79 cells as determined by differential scanning calorimetry. J. Cell. Physiol. 142, 628-634.

Levine, E.M. and Robbins, E.B. (1970) Differential temperature sensitivity of normal and cancer cells in culture. J. Cell. Physiol. 76, 373-380.

Li, G.C. and Hahn, G.M. (1978) Ethanol induced tolerance to heat and adriamycin. Nature 274, 699-701.

Li, G.C., Petersen, N.S. and Mitchell, H.K. (1982) Induced thermal tolerance and heat shock protein synthesis in Chinese hamster ovary cells. Int. J. Radiat. Oncol. Biol. Phys. 8, 63-67.

Li, G.C. and Hahn, G.M. (1987) Influence of temperature on the development and decay of thermotolerance and heat shock proteins. Radiat. Res. 112, 517-524.

Li, G.C., Liu, R., Li, L., Mak, J.Y. and Lee, M.W. (1991) Stable expression of human HSP 70 gene in rodent cells confers thermal resistance. In: Heat Shock. Eds. Maresca, B. and Lindquist, S., Springer-Verlag, pp257-267.

Lin, P-S, Kwock, L., Hefter, K. and Wallach, D.F.H. (1978) Modification of rat thymocyte membrane properties by hyperthermia and ionizing radiation. Int. J. Radiat. Biol. 33, 371-382.

Lin, P-S, Hefter, K. and Ho, K-C (1984) Modification of membrane function, protein synthesis, and heat killing effect on cultured Chinese hamster cells by glycerol and D_2O. Cancer Res. 44, 5776-5784.

Lindquist, S. and Craig, E.A. (1988) The heat shock proteins. Ann. Rev. Genetics 22, 631-677.

Liscovitch, M. (1992) Crosstalk among multiple signal-activated phospholipases. Trends Biochem. Sci. 17, 394-399.

Liu, S.M. and Carpenter, G. (1992) Activity of the epidermal-growth factor receptor and phospholipase C-γ1 in heat-stressed fibroblasts and A-431 cells. Biochem. J. 286, 541-547.

Llinas, R., Sugimori, M., Lin, J-W and Cherksey, B. (1989) Blocking and isolation of a calcium channel from neurons in mammals and cephalopods ultilizing a toxin fraction (FTX) from funnel-web spider poison. Proc. Natl. Acad. Sci. (USA) 86, 1689-1693.

Lovelock, J.E. (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochim. Biophys. Acta 11, 28-36.

Maccallum, S.H., Barker, C.J., Hunt, P.A., Wong, N.S., Kirk, C.J. and Michell, R.H. (1989) The use of cells doubly labelled with [^{14}C]inositol and [^{3}H]inositol to search for a hormone-sensitive inositol lipid pool with atypically rapid metabolic turnover. J. Endocrinol. 2, 379-389.

Madden, T.D., Chapman, D. and Quinn, P.J. (1974) Cholesterol modulates activity of calcium-dependent ATPase of the sarcoplasmic reticulum. Nature 279, 538-541.

Magun, B.E. (1981) Inhibition and recovery of macromolecular synthesis, membrane transport and lysosomal function following exposure of cultured cells to hyperthermia. Radiat. Res. 87, 657-669.

Magun, B.E. and Fennie, C.W. (1981) Effects of hyperthermia on binding, internalisation, and degradation of epidermal growth factor. Radiat. Res. 86, 133-146.

Maher, P.A. and Pasquale, E.B. (1989) Heat shock induces protein tyrosine phosphorylation in cultured cells. J. Cell Biol. 108, 2029-2035.

Majerus, P.W. (1992) Inositol phosphate biochemistry. Ann. Rev. Biochem. 61, 225-250.

Malgaroli, A., Milani, D., Meldolesi, J. and Pozzan, T. (1987) Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. J. Cell Biol. 105, 2145-2155.

Malhotra, A., Kruuv, J. and Lepock, J.R. (1986) Sensitization of rat hepatocytes to hyperthermia by calcium. J. Cell. Physiol. 128, 279-284.

Malhotra, A., Heynen, M.L.P. and Lepock, J.R. (1987) Role of extracellular calcium in the hyperthermic killing of CHL V79 cells. Radiat. Res. 112, 478-489.

Martin, J.L., Pumford, N.R., LaRosa, A.C., Martin, B.M., Gonzaga, H.M.S., Beavan, M.A. and Pohl, L.R. (1991) A metabolite of halothane covalently binds to an endoplasmic reticulum protein that is highly homologous to phosphatidylinositol-specific PLC-α but has no activity. Biochem. Biophys. Res. Commun. 178, 679-685.

McCleskey, E.W., Fox, A.P., Feldman, D.H., Cruz, L.J., Olivera, B.M., Tsien, R.W. and Yoshikami, D. (1987) ω-conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc. Natl. Acad. Sci. (USA) 84, 4327-4331.

McLean, J.S., Frame, M.C., Freshney, R.I., Vaughan, P.F.T. and Mackie, A.E. (1986) Phenotypic modification of human glioma and non-small lung carcinoma by glucocorticoids and other agents. Anticancer Res. 6, 1101-1106.

Means, A.R., Tash, J. S. and Chafouleas, J.G. (1982) Physiological implications of the presence, distribution, and regulation of calmodulin in eukaryotic cell. Physiol. Rev. 62, 1-39.

Mehdi, S.Q., Recktenwald, D.J., Smith, L.M., Li, G.C., Armour, E.P. and Hahn, G.M. (1984) Effect of hyperthermia on murine cell surface histocompatibility antigens. Cancer Res. 44, 3394-3397.

Meldolesi, J. and Pozzan, T. (1987) Pathways of calcium influx at the plasma membrane: voltage-, receptor-, and second messenger-operated channels. Exp. Cell Res. 171, 271-283.

Menniti, F.S., Oliver, K.G., Nogimori, K., Obie, J.F., Shears, S.B. and Putney, J.W., Jr. (1990) Origins of myo-inositol tetrakisphosphate in agonist-stimulated rat pancreatoma cells. Stimulation by bombesin of myo-inositol 1,3,4,5,6-pentakisphosphate breakdown to myo-inositol 3,4,5,6-tetrakisphosphate. J. Biol. Chem. 265, 11167-11176.

Menniti, F.S., Miller, R.N., Putney, J.W., Jr. and Shears, S.B. (1993a) Turnover of inositol polyphosphate pyrophosphates in pancreatoma cells. J. Biol. Chem. 268, 3850-3856.

Menniti, F.S., Oliver, K.G., Putney, J.W., Jr. and Shears, S.B. (1993b) Inositol phosphates and cell signalling: new views of $InsP_5$ and $InsP_6$. Trends Biochem. Sci. 18, 53-56.

Meryman, H.T. (1974) Freezing injury and its prevention in living cells. Ann. Rev. Biophys. Bioeng. 3, 341-363.

Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) The stimulation of inositol lipid metabolism that accompanies calcium mobilisation in stimulated cells: defined characteristics and unanswered questions. Phil. Trans. R. Soc. London B 296, 123-137.

Mikkelsen, R.B. and Stedman, T. (1990) Cytotoxic hyperthermia and calcium homeostasis: the effect of heat on Ca^{2+} uptake by nonmitochondrial intracellular Ca^{2+} stores. Radiat. Res. 123, 82-86.

Mikkelsen, R.B., Reinlib, L., Donowitz, M. and Zahniser, D. (1991a) Hyperthermia effects on cytosolic $[Ca^{2+}]_i$: analysis at the single cell level by digitized imaging microscopy and cell survival. Cancer Res. 51, 359-364.

Mikkelsen, R.B., Stedman, T., Schmidt-Ullrich, R. and Lin, P.S. (1991b) Effects of tamoxifen and PKC inhibitors on hyperthermic cell killing. Int. J. Radiat. Oncol. Biol. Phys. 20, 1039-1045.

Monaco, M.E. (1982) The phosphatidylinositol cycle in WRK-1 cells. J. Biol. Chem. 257, 2137-2139.

Monaco, M.E. and Lippman, M.E. (1982) A new model system for studying the phosphatidylinositol cycle. J. Cell. Physiol. 112, 148-153.

Monaco, M.E. and Woods, D. (1983) Characterization of the hormone-sensitive phosphatidylinositol pool in WRK-1 cells. J. Biol. Chem. 258, 15125-15129.

Monaco, M.E. (1987a) Inositol metabolism in WRK-1 cells. Relationship of hormone-sensitive to insensitive pools of phosphoinositides. J. Biol. Chem. 262, 13001-13006.

Monaco, M.E. (1987b) Calcium and the phosphoinositide cycle in WRK-1 cells. J. Biol. Chem. 262, 147-151.

Monaco, M.E., Attinasi, M. and Koréh, K. (1990) Effect of dual agonists on phosphoinositide pools in WRK-1 cells. Biochem, J. 269, 633-637.

Monaco, M.E. and Gershengorn, M.C. (1992) Subcellular organization of receptor-mediated phosphoinositide turnover. Endocrine Rev. 23, 707-718.

Moore, E.D.W., Becker, P.L., Fogarty, K.E., Williams, D.A. and Fay, F.S. (1990) Ca^{2+} imaging in single living cells: theoretical and practical issues. Cell Calcium 11, 157-179.

Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. Nature 330, 653-655.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55-63.

Mouillac, B., Balestre, M-N and Guillon, G. (1989) Transient inositol (1,4,5) trisphosphate accumulation under vasopressin stimulation in WRK 1 cells: correlation with intracellular calcium mobilization. Biochem. Biophys. Res. Commun. 159, 953-960.

Mouillac, B., Balestre, M-N and Guillon, G. (1990) Positive feedback regulation of PLC by vasopressin-induced calcium mobilization in WRK 1 cells. Cellular signalling 2, 497-507.

Mulcahy, R.T., Gould, M.N., Hidvergi, E., Elson, C.E. and Yatvin, M.B. (1981) Hyperthermia and surface morphology of P388 ascites tumour cells: effects of membrane modifications. Int. J. Radiat. Biol. 39, 95-106.

Nahorski, S.R., Ragen, C.I. and Challiss, R.A.J. (1991) Lithium and the phosphoinositide cycle: an example of incompetitive inhibition and its pharmacological consequences. Trends Pharmacol. Sci. 12, 297-303.

Nicholls, D.G. (1986) Intracellular calcium homeostasis. Br. Med. Bulletin 42, 353-358.

Nicotera, P., Hartzell, P., Davis, G. and Orrenius, S. (1986) The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic calcium is mediated by the activation of a non-lysosomal proteolytic system. FEBS Lett. 209, 139-144.

Nicotera, P., Thor, H. and Orrenius, S. (1989) Cytosolic-free Ca^{2+} and cell killing in hepatoma 1c1c7 cells exposed to chemical anoxia. FASEB J. 3, 59-64.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308, 693-698.

Nishizuka, Y. (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science 258, 607-614.

Oliver, K.G., Putney, J.W., Jr., Obie, J.F. and Shears, S.B. (1992) The interconversion of inositol (1,3,4,5,6)-pentakisphosphate and inositol tetrakisphosphates in AR4-2J cells. J. Biol. Chem. 267, 21526-21534.

Orrenius, S., McConkey, D.J., Jones, D.P. and Nicotera, P. (1988) Ca^{2+}-activated mechanisms in toxicity and programmed cell death. ISI Altas of Science-Pharmacol. 2, 319-324.

Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. (1989) Role of Ca^{2+} in toxic cell killing. Trends Pharmacol. Sci. 10, 281-285.

Overgaard, K. and Overgaard, J. (1972) Investigations on the possibility of thermic tumour therapy. I. Short-wave treatment of a transplanted isologous mouse mammary carcinoma. Eur. J. Cancer 8, 65-78.

Overgaard, J. (1976a) Ultrastructure of a murine mammary carcinoma exposed to hyperthermia *in vivo*. Cancer Res. 36, 983-995.

Overgaard, J. (1976b) Influence of extracellular pH on the viability and morphology of tumour cells exposed to hyperthermia. J. Natl. Cancer Inst. 56, 1243-1250.

Ozawa, S. and Sand, O. (1986) Electrophysiology of excitable endocrine cells. Physiol. Rev. 66, 887-952.

Paris, S. and Pouysségur, J. (1987) Further evidence for a phospholipase C-coupled G protein in hamster fibroblasts. J. Biol. Chem. 262, 1970-1976.

Park, D.J., Min, H.K. and Rhee, S.G. (1992) Inhibition of CD-3 linked phospholipase-C by phorbol ester and by cAMP is associated with decreased phosphotyrosine and increased phosphoserine contents of PLC-gamma-1. J. Biol. Chem. 267, 1496-1501.

Pearlman, D. (1979) Use of antibiotics in cell culture media. In: Cell Culture. Basic methods in enzymology. Ed.. Jakoby, W. and Pastan, I.H., pp110-116.

Pittet, D., Schlegel, W., Lew, D.P., Monod, A. and Mayr, G.W. (1989) Mass changes in inositol tetrakis and pentakisphosphate isomers induced by chemotactic peptide stimulation in HL-60 cells. J. Biol. Chem. 264, 18489-18493.

Plumb, J.A., Milroy, R. and Kaye, S.B. (1989) Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res. 49, 4435-4440.

Poenie, M., Alderton, J., Steinhardt, R. and Tsien, R. (1986) Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. Science 233, 886-889.

Portilla, D., Mordhorst, M., Bertrand, . and Morrison, A.R. (1988) Protein kinase C modulates phospholipase C and increase arachidonic acid release in bradykinin stimulated MDCK cells. Biochem. Biophys. Res. Commun. 153, 454-462.

Price, B.D. and Calderwood, S.K. (1991) Ca^{2+} is essential for multistep activation of the heat shock factor in permeabilised cells. Molecular Cell Biol. 11, 3365-3368.

Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Neidel, J.E. and Bell, R.M. (1986) Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes and ras-and sis-transformed normal rat kidney cells. J. Biol. Chem. 261, 8597-8600.

Puck, T.T., Marcus, P.I. and Cieciura, S.J. (1955) Clonal growth of mammalian cells *in vitro*. J. Exp. Med. 103, 273-284.

Puck, T.T., Cieciura, S.J. and Robinson, A. (1958) Genetics of somatic mammalian cells. III Long term cultivation of euploid cells from human and animal subjects. J. Exp. Med. 108, 945-956.

Putney, J.W., Jr. (1986) Identification of cellular activation mechanisms associated with salivary secretion. Ann. Rev. Physiol. 48, 75-88.

Raaphorst, G.P., Romano, S.L., Mitchell, J.B., Bedford, J.S. and Dewey, W.C. (1979) Intrinsic differences in heat and/or x-ray sensitivity of seven mammalian cell lines cultured and treated under identical conditions. Cancer Res. 39, 396-401.

Raaphorst, G.P., Vadasz, J.A., Azzam, E.I., Sargent, M.D., Borsa, J. and Einspenner, M. (1985) Comparison of heat and/or radiation sensitivity and membrane composition of seven x ray-transformed C3H 10T1/2 cell lines and normal C3H 10T1/2 cells. Cancer Res. 45, 5452-5456.

Rasmussen, H. Calyberger, C. and Gustin, M.C. (1979) In: Secretory mechanisms, SEB symposium XXXIII, eds. Hopkins, C.R. and Duncan, C.J., Cambridge: The Company of Biologist Ltd, pp161-197.

Read, R.A., Fox, M.H. and Bedford, J.S. (1984) The cell cycle dependence of thermotolerance. Radiat. Res. 98, 491-505.

Reeves, J.P. (1990) Sodium calcium exchange. In: Intracellular Calcium Regulation. Ed. Bronner, F., Wiley-Liss, pp305-347.

Renard, D., Poggioli, J., Berthon, B. and Claret, M. (1987) How far dose phospholipase C activity depend on the cell calcium concentration? Biochem. J. 243, 391-398.

Rhee, S.G., Suh, P-G, Ryu, S-H and Lee, S.Y. (1989) Studies of inositol phospholipid-specific phospholipase C. Science 244, 546-550.

Rhee, S.G. and Choi, K.D. (1992a) Multiple forms of phopholipase C isozymes and their activation mechanisms. In: Adv. in Second Messenger and Phosphoprotein Res. 26, pp35-61.

Rhee, S.G. and Choi, K.D. (1992b) Regulation of inositol phospholipid-specific phospholipase C isozymes. J. Biol. Chem 267, 12393-12396.

Rhodes, D., Prpíc, V., Exton, J.H. and Blackmore, P.F. (1983) Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in hepatocytes by vasopressin. J. Biol. Chem. 258, 2770-2773.

Rice, G.C., Gray, J.W. and Dewey, W.C. (1985) FACS analysis of a hyperthermia-induced alteration in Hoechst 33342 permeability and direct measurement of its relationship to cell survival. J. Cell. Physiol. 122, 387-396.

Ringer, S. (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. 4, 29-42.

Robins, H.I., Dennis, W.H. and Vorpahl, J.W. (1982) Systemic lidocaine enhancement of tumour regression after hyperthermia. Natl.Cancer. Inst. Monogr. 61, 243-245.

Roe, M.W., Lemasters, J.J. and Herman, B. (1990) Assessment of fura-2 for measurements of cytosolic free calcium. Cell Calcium 11, 63-73.

Rosenberg, B., Kemeny, G., Switzer, R.C. and Hamilton, T.C. (1971) Quantitative evidence for protein denaturation as the cause of thermal death. Nature 232, 471-473.

Ross, W.M. and Watmough, D.J. (1986) Clinical and scientific aspects. In Hyperthermia, eds. Watmough, D.J. and Ross, W.M., Blackie and Son Ltd., pp1-13.

Ross, T.S. and Majerus, P.W. (1991) Inositol-1,2-cyclic-phosphate 2-inositol-phosphohydrolase substrate. Specificity and regulation of activity by phospholipids, metal ion chelators and inositol 2-phosphate. J. Biol. Chem. 266, 851-856.

Rossomanudo, A., Wu, J., Weber, M. J. and Sturgill, T.W. (1992) The phorbol ester-dependent activator of the mitogen-activated protein kinase $p42^{mapk}$ is a kinase with specificity for the threonine and tyrosine regulatory sites. Proc. Natl. Acad. Sci. (USA) 89, 5221-5225.

Roti Roti, J.L. and Winward, R.T. (1978) The effects of hyperthermia on the protein-to-DNA ratio of isolated HeLa chromatin. Radiat. Res. 74, 159-169.

Roti Roti, J.L. (1982) Heat-induced cell death and radiosensitization: Molecular mechanisms. Natl. Cancer Inst. Monogr. 61, 3-10.

Roti Roti, J.L. and Laszlo, A. (1988) The effects of hyperthermia on cellular macromolecules. In: Hyperthermia and Oncology. I. Thermal effects on cells and tissues. Eds. Urano M. and Douple E, VSP Press, pp13-56.

Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determnation of phospholipids by phosphorus analysis of spots. Lipids 5, 494-496.

Roux, W. (1885) Beitrage der entwicklungsmechanik des embryo. Z. Biol. 21, 411-526.

Rudland, P.S., Twiston-Davis, A. and Warburton, M.J. (1982) Prostaglandin-induced differentiation or DMSO-induced differentiation: reduction of the neoplastic potential rat mammary stem-cell line. J. Natl. Cancer Inst. 69, 1083-1093.

Ruifrok, A.C.C., Kanon, B. and Konings, A.W.T. (1985) Correlation of colony forming ability of mammalian cells with potassium content after hyperthermia under different experimental conditions. Radiat. Res. 103, 452-454.

Ryu, S.H., Kim, Uh-H, Wahl, M.I., Brown, A.B., Carpenter, G., Huang, K-P and Rhee, S.G. (1990) Feedback regulation of phospholipase C-β by protein kinase C. J. Biol. Chem. 265, 17941-17945.

Sabine, J.R. (1983) Membrane homeostasis: is there an optimal level of membrane cholesterol? Bioscience Reports 3, 337-344.

Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. (1979) Calcium dependence of toxic cell death. Science 206, 700-702.

Schmitt, K., Daeubener, W., Bitter-Suermann, D. and Hadding, U. (1988) A safe and efficient method for elimination of cell culture mycoplasma using ciprofloxacin. J. Immunol. Methods 109, 17-26.

Shears, S.B. (1992) Metabolism of inositol phosphates. In: Advances in Second Messenger and Phosphoprotein Research. vol 26. Ed. Putney, J.W., Jr., Raven Press Ltd., pp63-92.

Siesjö, B.K. and Wieloch, T. (1985) Cerebral metabolism in ischaemia: neurochemical basis for therapy. Br. J. Anaesth. 57, 47-62.

Siesjö, B.K. (1989) Calcium and cell death. Magnesium 8, 223-237.

Simpson, C.M.F., Batty, I.H. and Hawthorne, J.N. (1992) Physiological responses to receptor activation: phosphoinositide turnover. In: Neurochemistry. A Practical Approach. Eds. Turner, A.J. and Bachelard, H.S., IRL Press, pp193-223.

Sinensky, M. (1974) Homeostasis adaptation — a homeostasis process that regulates the viscosity of membrane lipids in *Escherichia Coli*. Proc. Natl. Acad. Sci. (USA) 71, 522-525.

Singer, S.J. and Nicholson, G.L. (1972) The fluid mosaic model of the structure of cell membrane. Science 175, 720-731.

Slater, T.F., Sawyer, B. and Sträuli, U. (1963) Studies on succinate-tetrazolium reductase systems III. Points of coupling of four different tetrazolium salts. Biochim. Biophys. Acta 77, 383-393.

Slusser, H., Hopwood, L.E. and Kapiszawska, M. (1982) Inhibition of membrane transport by hyperthermia. Natl. Inst. Cancer Monogr. 61, 85-87.

Smith, M.W., Phelps, P.C. and Trump, B.F. (1991) Cytosolic Ca^{2+} deregulation and blebbing after $HgCl_2$ injury to cultured rabbit proximal tubule cells as determined by digital imaging microscopy. Proc. Natl. Acad. Sci. (USA) 88, 4926-4930.

Smith, B.J. and Yaffe, M.P. (1991) Uncoupling thermotolerance from the induction of heat shock proteins. Proc. Natl. Acad. Sci. USA 88, 11091-11094.

Smith, P.M. (1992) $Ins(1,3,4,5)P_4$ promotes sustained activation of the Ca^{2+}-dependent Cl^- current in isolated mouse lacrimal cells. Biochem. J. 283, 27-30.

Somylo, A.P., Bond, M. and Somylo, A.V. (1985) Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*. Nature 314, 622-625.

Spiro, I.J., Sapareto, S.A., Raaphorst, G.P. and Dewey, W.C. (1982) The influence of chronic and acute heat conditioning on the development of thermal tolerance. Int. J. Radiat. Oncol. Biol. Phys. 8, 53-58.

Stabel, S. and Parker, P.J. (1991) Protein kinase C. Pharma. Ther. 51, 71-95.

Stanbridge, E.J., Tischfield, J.A. and Schnsider, E.L. (1975) Appearance of hypoxanthine guanine phosphoribosyl-transferase activity as a consequence of mycoplasma contamination. Nature 256, 329-331.

States, B., Harris, D., Hummeler, K. and Segal, S. (1978) Amino acid disorders in tissue culture cells: effect of mycoplasma and antibiotics on cystene metabolism. Monogr. Human Genetics 9, 131-134.

Stege, G.J.J., Wierenga, P.K., Konings, A.W.T. and Kampinga, H.H. (1993a) Synergistic action of calcium-ionophores and hyperthermia is best interpreted as thermal enhancement of calcium toxicity. J. Cell. Physiol. 155, 452-460.

Stege, G.J.J., Wierenga, P.K., Kampinga, H.H. and Konings, A.W.T. (1993b) Hyperthermia, intracellular free calcium and calcium ionophores. Int. J. Radiat. Biol. 64, 459-468.

Steinberg, S.F., Bilezikian, J.P. and Al-Awqati, Q. (1987) Fura-2 fluorescence is localized to mitochondria in endothelial cells. Am. J. Physiol. 253, C744-C747.

Stephens, L., Hawkins, P.T., Carter, N., Chahwala, S.B., Morris, A.J., Whetton, A. D. and Downes, C.P. (1988) L-myo-inositol 1,4,5,6-tetrakisphosphate is present in both mammalian and avain cells. Biochem. J. 249, 271-282.

Stephens, L.R. and Irvine, R.F. (1990) Stepwise phosphorylation of myo-inositol leading to myo-inositol hexakisphosphate in Dicytostelium. Nature 340, 580-583.

Stephens, L.R and Downes, C.P. (1990) Product-precursor relationships amongst inositol polyphosphates. Biochem. J. 265, 435-452.

Stephens, L.R., Hawkins, P.T., Stanley, A.F., Moore, T., Poyner, D.R., Morris, P.J., Hanley, M.R., Kay, R.R. and Irvine, R.F. (1991) *Myo*-inositol pentakisphosphates. Biochem. J. 275, 485-499.

Stephens, L., Radenberg, T., Thiel, U., Vogel, G. Khoo, K-H, Dell, A., Jackson, T.R., Hawkins, P.T. and Mayr, G.W. (1993) The detection, purification, structural characterization and metabolism of diphosphoinositol pentakisphosphate(s) and bisdiphosphoinositol tetrakisphosphate(s). J. Biol. Chem. 268, 4009-4015.

Stevenson, A.P., Galey, W.R. and Tobey, R.A. (1983) Hyperthermia-induced increase in potassium transport in Chinese hamster cells. J. Cell. Physiol. 115, 75-86.

Stevenson, M.A., Calderwood, S.K and Hahn, G.M. (1986) Rapid increases in inositol trisphosphate and intracellular calcium after heat shock. Biochem. Biophys. Res. Commun. 137, 826-833.

Stevenson, M.A., Calderwood, S.K and Hahn, G.M. (1987) Effect of hyperthermia (45°C) on calcium flux in Chinese hamster ovary HA-1 fibroblasts and its potential role in cytotoxicity and heat resistance. Cancer Res. 47, 3712-3717.

Storch, J. and Schachter, D. (1985) Calcium alters the acyl chain composition and lipid fluidity of rat hepatocyte plasma membranes *in vitro*. Biochim. Biophys. Acta 812, 473-484.

Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Release of calcium from an non-mitochondrial intracellular store in pancreatic acinar cell by inositol-1,4,5-tetrakisphosphate. Nature 306, 67-69.

Stubbs, C.D. (1983) Membrane fluidity: structure and dynamics of membrane lipids. In: Essays in Biochemistry vol.19. Eds. Campell, P.N. and Marchall, R.D., Academic Press, pp.1-39.

Suzuki, N. and Yoshioka, T. (1987) Differential blocking action of synthetic ω-conotoxin on components of Ca^{2+} current in clonal GH_3 cells. Neurosci. Lett.. 75, 235-240.

Sylvia, V., Curtin , G., Norman, J., Stec, J. and Busbee, D. (1988) Activation of a low specific activity form of DNA polymerase α by inositol 1,4-bisphosphate. Cell 54, 651-658.

Symonds, R.P., Wheldon, T.E., Clarke, B. and Bailey, G. (1981) A comparison of the response to hyperthermia of murine haemopoietic stem cells (CUF-S) and L1210 leukaemia cells: enhanced killing of leukaemia cells in presence of normal marrow cells. Br. J. Cancer 44, 682-691.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. 91, 1218-1224.

Takuwa, Y., Takwwa, N. and Rasmussen, H. (1986) Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositide in bovine tracheal smooth muscle measurements of the mass of polyphosphoinositides, 1,2-diacylglycerol, and phosphatidic acid. J. Biol. Chem. 261, 14670-14675.

Taylor, C.W., Merritt, J.E., Putney, J.W.Jr. and Rubin, R.P. (1986) A guanine nucleotide-dependent regulatory protein couples substance P receptors to PLC in rat parotid gland. Biochem. Biophys. Res. Commun. 136, 362-368.

Taylor, C.W. (1990) The role of G proteins in transmembrane signalling. Biochem. J. 272, 1-13.

Thomas, G.P., Welch, W.J. , Mathews, M.B. and Feramisco, J.R. (1982) Molecular and cellular effects of heat shock and related treatments of mammalian tissue culture cells. Cold Spring Harbor Symposium on Quatitative Biol. 46, 985-996.

Tomasovic, S.P., Turner, G.N. and Dewey, W.G. (1978) Effect of hyperthermia on nonhistone protein isolated with DNA. Radiat. Res. 73, 535-552.

Trump, B.F., Berezesky, I.K., Laiho, K.U., Osornio, A.R., Mergner, W.J. and Smith, M.W. (1980) The role of calcium in cell injury. Scanning Electron Microscope 2, 437-462.

Trump, B.F. and Berezesky, I.K. (1992) The role of cytosolic Ca^{2+} in cell injury, necrosis and apoptosis. Current Opin. in Cell Biol. 4, 227-232.

Truter, M.R. (1976) Chemistry of the calcium ionophores. In: Calcium in Biological Systems. SEM Symposia XXX, ed. Duncan, C.J., Cambridge University Press, pp19-40.

Tsien, R.Y. and Rink, T.J. (1980) Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. Biochim. Biophys. Acta 599, 623-638.

Tsien, R.Y.(1989) Fluorescent probes of cell signalling. Ann. Rev. Neurosci. 12, 227-253.

Tsien, R. and Pozzan, T. (1989) Measurement of cytosolic free calcium with quin2. Methods in Enzymol. 172, 230-261.

Tsien, R.W. (1990) Calcium channels, stores and oscillations. Ann. Rev. Cell Biol. 6, 715-760.

Vallejo, M., Jackson, T., Lightman, S. and Hanley, M.R. (1987) Occurrence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. Nature 330, 656-658.

Van Bergen en Henegouwen, P.M.P., Jordi, W.J.R.M., Van Donger, G., Ramaskers, F.C.S., Amesz, H. and Linneman, W.A.M. (1985) Studies on a possible relationship between alterations in the cytoskeleton and induction of heat shock protein synthesis in mammalians cells. Int. J. Hyperthermia 1, 69-83.

Van Bergen en Henegouwen, P.M.P. and Linnemans, W.A.M. (1987) Heat shock gene expression and cytoskeletal alterations in mouse neuroblastoma cells. Exp. Cell Res. 171, 367-375.

Vernon, C. (1992) Hyperthermia in cancer growth regulation. Biotherapy 4, 307-315.

Vidair, C.A. and Dewey, W.C. (1986) Evalution of a role for intracellular Na^+, K^+, Ca^{2+} and Mg^{2+} in hyperthermic cell killing. Radiat. Res. 105, 187-200.

Vidair, C.A., Wang, Z. and Dewey, W.C. (1990) Non-involvement of the heat-induced increase in the concentration of intracellular free Ca^{2+} in killing by heat and induction of thermotolerance. Radiat. Res. 124, 156-164.

Vidair, C.A. and Dewey, W.C. (1993) Plasma membrane activities retained after lethal heat shock. Int. J. Radiat. Biol. 63, 589-596.

Volpe, P., Krause, K-H, Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. and Lew, D.P. (1988) 'Calciosome', a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive store of non-muscle cells? Proc. Natl. Acad. Sci. (USA) 85, 1091-1095.

Von Hoff, D.D., Forseth, B.J., Huong, M., Buckok, J.B. and Lanthan, B. (1986) Improved plating efficiencies for human tumours cloned in capillary tubes versus petri dishes. Cancer Res. 46, 4012-4017.

Wang, Z., Vidair, C.A. and Dewey, W.C. (1991) Maintenance of intracellular free calcium homeostasis following lethal heat shock. Radiat. Res. 128, 104-107.

Warters, R.L. and Henle, K.J. (1982) DNA degradation in CHO cells after exposure to hyperthermia. Cancer Res. 42, 4427-4432.

Warters, R.L., Brizgys, L.M. and Axtell-BartLett., J. (1985) DNA damage production in CHO cells at elevated temperatures. J. Cell. Physiol. 124, 481-486.

Welch, W.J. and Suhan, J.P. (1985) Morphological study of mammalian stress response: characterisation of changes in cytoplasmic organelles, cytoskeleton and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J. Cell Biol. 101, 1198-1211.

Westra, A. and Dewey, W.C. (1971) Variation in sensitivity to heat shock during the cell-cycle of Chinese hamster cells *in vitro*. Int. J. Radiat. Biol. 19, 467-477.

Wiegant, F., Kavelaars, A., Blok, F. and Linnemans, W. (1984) Effects of extracellular Ca^{2+} concentrations upon hyperthermia induced cell death. Hyperthermic Oncol. 1, 3-7.

Wiegant, F.A.C., Tuyl, M. and Linnemans, W.A.M. (1985) Calmodulin inhibitors potentiate hyperthermic cell killing. Int. J. Hyperthermia 1, 157-169.

Wierenga, P.K. and Konings, A.W.T. (1989) Absence of a relationship between $[Ca^{2+}]_i$ and heat-induced cell killing. Method Surveys Biochem. and Analysis 19, 475-478.

Wierenga, P.K., Stege, G.J.J., Kampinga, H.H. and Konings, A.W.T. (1994) Intracellular free calcium concentrations in cell suspensions during hyperthermia. Eur. J. Cell Biol. 63, 68-76.

Wilson, B.B. (1978) Cellular transport mechanism. Ann. Rev. Biochem. 47, 933-965.

Wong, N.S., Barker, C.J., Shears, S.B., Kirk, C.J. and Michell, R.H. (1988) Inositol 1:2(cyclic) 4,5-trisphosphate is not a major product of inositol phospholipid metabolism in vasopressin-stimulated WRK1 cells. Biochem. J. 252, 1-5.

Wong, N.S., Barker, C.J., Morris, A.J., Craxton, A., Kirk, C.J. and Michell, R.H. (1992) The inositol phosphates in WRK1 rat mammary tumour cells. Biochem. J. 286, 459-468.

Wreggett, K.A., Howe, L.R., Moore, J.P. and Irvine, R.F. (1987) Extraction and recovery of inositol phosphates from tissues. Biochem. J. 245, 933-934.

Yatvin, M.B. (1977) The influence of membrane lipid composition and procaine on hyperthermic death of cells. Int. J. Radiat. Biol. 32, 513-521.