

Gap junctional intercellular communication: methodologies for measurement and application to potential inhibitors of communication.

~~MEASUREMENT OF INTERCELLULAR COMMUNICATION  
ACROSS GAP JUNCTIONS IN CULTURED V79 CELLS~~

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SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

APRIL 1993

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## Abstract

As an adaptation to multicellular life, organisms have evolved aqueous channels which allow passive transport of small molecules between contiguous cells. These channels are called gap junctions. Junctional communication exercises homeostatic control on cellular physiology and takes part in regulating cell growth and differentiation. In adult organisms the blockage of junctional communication is implicated in the process of carcinogenesis. In embryo's, blockage results in abnormal development.

In this investigation metabolic cooperation was used to investigate junctional communication. A modification of an existing method was developed whereby 6-thioguanine (6-tg) resistant and sensitive strains of V79 cells were plated in a 1:1 ratio in multiwell plates. The co-cultures were treated with 6-tg. Metabolism of 6-tg in the sensitive cells and subsequent transfer to resistant cells led to inhibition of DNA synthesis in both cell types as assayed by <sup>3</sup>H-thymidine incorporation. DNA synthesis in segregated resistant cells is not affected by 6-tg because these cells cannot metabolize 6-tg due to an enzyme deficiency. The inhibition of DNA synthesis in resistant cells in co-culture demonstrates junctional communication. As a positive control junctional communication in the co-culture was inhibited by exposure to a known communication inhibitor, the phorbol ester TPA.

Using this method the effects of compounds on junctional communication were investigated. Calcium ionophore A23187 and DDT did not inhibit junctional communication whereas paracetamol appeared to inhibit junctional communication. To elucidate the mechanism of action of paracetamol BHA, aspirin and indomethacin were tested without showing an effect on junctional communication. Nordihydroguaiaretic acid also appeared to inhibit communication similarly to paracetamol. Using dye transfer as a comparative measure

of cell communication it was found that these compounds did not inhibit junctional communication but prevented the incorporation of the 6-tg metabolite into the resistant cells. Consequently hydroxyurea, a known inhibitor of DNA synthesis was tested and shown to mimic the effects of these compounds. The implications of these findings to junctional research are discussed.

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## Acknowledgements

I would like to thank Professor Andre McLean and Doctor Simon Wolff for their supervision, encouragement and friendship which made this work possible and a pleasure to carry out.

I would also like to thank members of the laboratory of toxicology for their friendship and support, especially Miss Jaswinder Sethi whose technical assistance enhanced the presentation of this work. Many thanks to Doctor Peter Tatham (Dept. of Experimental Pathology) for advice on making micropipettes and to Doctor Colin Green (Dept. of Anatomy) for carrying out a gap junction protein immunostaining study on the V79 cells. I would also like to extend my gratitude to Doctor Margaret Fox (Christie Hospital & Holt Radium Institute, Manchester) for supplying the V79 cell lines.

The financial assistance of the Medical Research Council is gratefully acknowledged.

## Chapter 1. Introduction

### 1.1. Cell Communication

Cells in multicellular organisms communicate with one another to co-ordinate cellular functions and to maintain homeostatic control of the organism. Communication may occur between spatially separated cells through nerve impulses or hormone signals and between adjacent cells through direct contact. Communication with adjacent cells is facilitated by structures called gap junctions.

#### 1.1.1. Gap Junctional Intercellular Communication

Cell communication through direct contact was demonstrated visually by Kanno & Loewenstein (1964), who showed that fluorescein injected into a cell diffused into adjacent cells but not into the extracellular medium. The idea that membrane impermeant molecules diffuse from one cell to another through distinct channels or junctions was put forward by Loewenstein (1966). This is now supported by extensive work. The permeability of the junctional channel was investigated by injection of molecules from 300-127,000 Dalton molecular weight into one cell. Following their passage into adjacent cells revealed junctional transfer. It was found that molecules above a certain molecular weight could not traverse the junctional channel (Kanno & Loewenstein 1966). Ultra structural studies of the junctional channels showed that a gap existed between membranes of apposed cells at the site where junctional channels aggregate (Revel & Karnovsky 1967). This aggregated junctional structure has since been widely called the gap junction. The term nexus is also used to

describe this structure (Dewey & Bar 1962).

### 1.1.2. Gap Junction Ultrastructure

The existence of an electrical intercellular connection was described by Furshpan & Potter (1959). They used electro-physiological techniques to measure currents across the giant motor synapses of crayfish. They observed that "electrotonic currents readily flow across the junction" . This bidirectional flow could not be adequately accounted for the by chemical mechanisms of synaptic transmission. Later Robertson et al. (1963) presented electron micrographs of Mauthner cell synapses of gold fish brain showing membrane structures which differed from adjacent regions and exhibited "relatively rigid parallelism of the pair of membranes". Robertson (1963) further characterized these membrane structures revealing a characteristic subunit organization.

Revel & Karnovsky (1967) used electron dense lanthanum salts to stain cell preparations and obtained electron micrographs of hexagonally packed subunits which we now know as gap junctions. In the centre of the subunits was a region into which the electron dense stain penetrated. This central zone was 20-30 Å in diameter and revealed the existence of the putative central channel through which intercellular communication may take place. A schematic diagram of gap junctions is depicted in figure 1.1

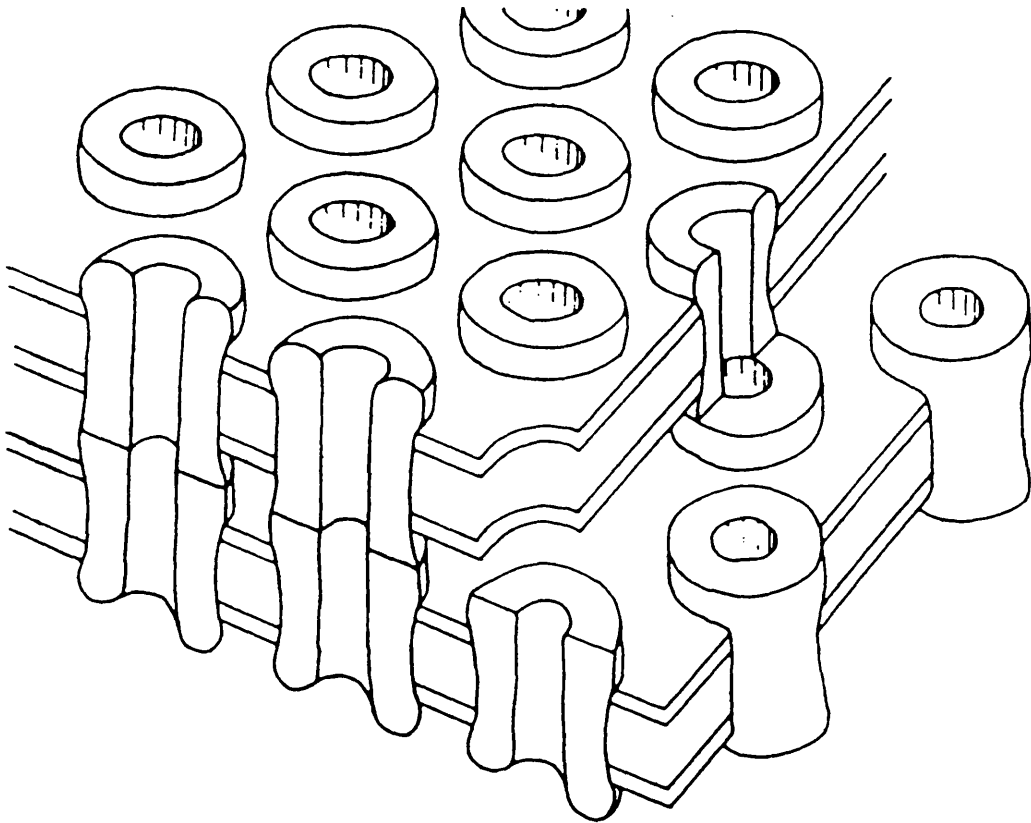
### 1.1.3. Purification And Isolation Of Gap Junctions

The study of gap junctional intercellular communication (GJIC) has been greatly facilitated by the availability of isolated and purified gap junctions. The process of isolation



**Figure 1.1**

A simple representation of gap junction structure



Adapted from Casper et al. (1977)

and purification was made possible by the fundamental observation that while most cell membrane proteins are soluble in detergents, gap junctions are not. The detergent treated residue which remains is enriched in gap junctions (Benedetti & Emmelot 1968; Evans & Gurd 1972; Goodenough & Stoeckenius 1972). Treatment of rat liver membranes with detergent leaves a residue which not only contains gap junctions but other proteins as well. Therefore, proteases were used to further purify the gap junctions. However, the partial degradation of gap junction proteins by this process led to confusion when protein composition from more than one laboratory was compared (Hertzberg et al. 1988). Since then, detergent-based methods have been developed for the isolation of gap junctions which avoid protease treatment. Gap junctions from mouse liver (Henderson et al. 1979) and rat liver (Hertzberg & Gilula 1979) have been prepared in this way. The insolubility of gap junctions in alkali has also enabled detergent free extraction to be carried out (Hertzberg 1984).

The low abundance of gap junctions and their insolubility has made their detailed characterization difficult. Gap junctions occupy 0.2% of the membrane area in V79 cells and 3% in liver (Yancey et al. 1982). Subsequently, the use of recombinant human gap junction cDNA over-expressed in insect cells in tissue culture has allowed gap junction proteins to be produced in milligram quantities. Their subsequent extraction using alkaline treatment and solubilization using detergents under high pH, high salt concentration and reducing agents has made further characterization possible (Stauffer et al. 1991).

#### 1.1.4. Gap Junction Organization In The Cell Membrane

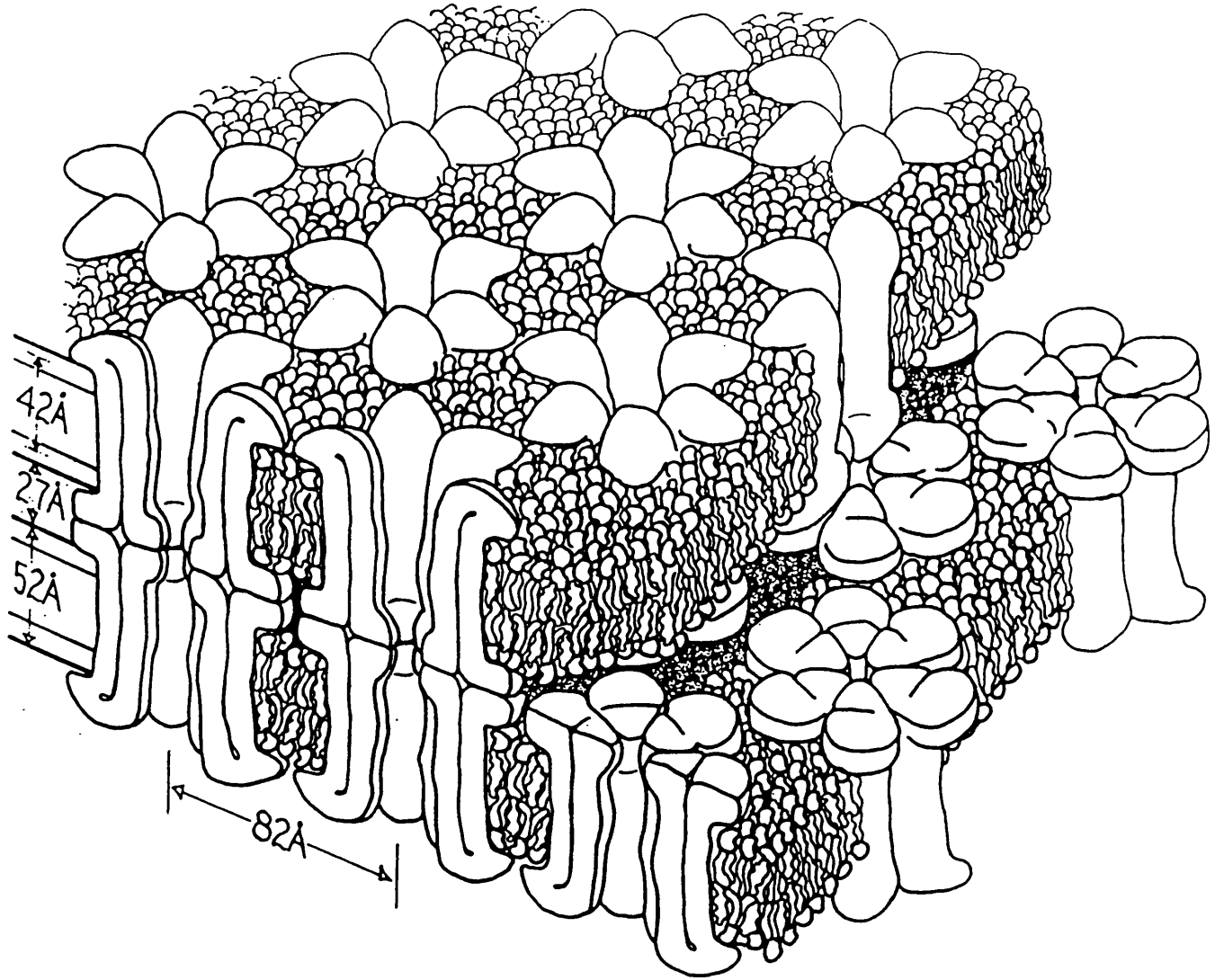
The structure of the gap junction in the plasma membrane was investigated by electron

microscopy by Benedetti & Emmelot (1968) who demonstrated that gap junctions were made up of two rows of 70 Å diameter globules. McNutt & Weinstein (1970) applied freeze-cleave techniques to electron microscopy to split plasma membranes into two lamellae. The two lamellae revealed pits on one face and particles on the other. The particles showed a 90-100 Å centre to centre distance which revealed the amount of packing of the junctional channels in the gap junctional plaques. Treatment of cells with hypertonic concentrations of disaccharides splits the junctions symmetrically through the middle of the gap revealing a half junction on each cell membrane (Goodenough & Gilula 1974). These half junctions have been called connexons (Goodenough 1975). The structure of connexons has been studied by low angle X-ray diffraction and a model of the connexon was proposed whereby the connexon consists of a hexameric protein. Two connexons are aligned on two apposed cell membranes to form a gap junction (Casper et al. 1977; Makowski et al. 1977). The average distance between the centres of connexons in gap junctions is 80-100 Å and the gap between apposed membranes (from which gap junctions derive their name) is 20-30 Å wide. The overall thickness of the gap junction is 150 Å (Casper et al. 1977). See figure 1.2.

#### 1.1.5. Junctional Channel Closure

Three dimensional maps of connexons have shown that the six subunits of the connexon are roughly rod shaped and inclined with respect to the gap junction pore axis. The connexon can take up alternative configurations. The different configurations may suggest a simple molecular mechanism by which gap junctional permeability is controlled (Unwin & Zampighi 1980). Movement of individual subunits by tilting and sliding along their lines of contact may close the junctional pore. On exposure to calcium the channel subunits become

Figure 1.2



Detailed diagram of gap junction structure  
Adapted from Makowski et al. (1977)

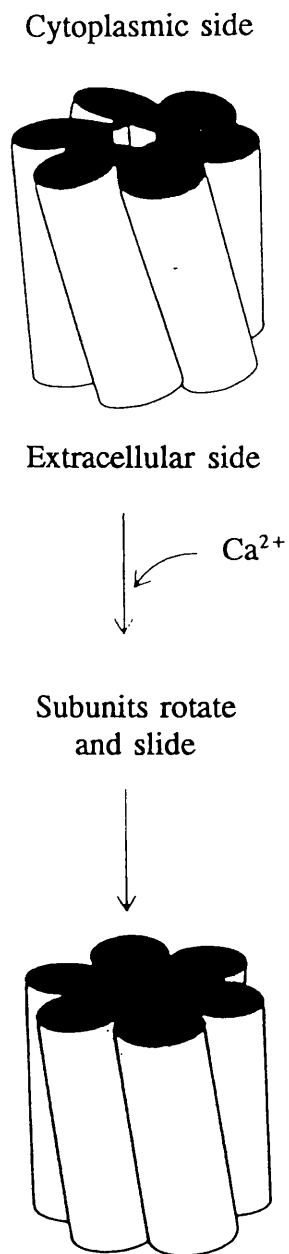
aligned nearly parallel to the channel pore axis at the extracellular end thereby restricting the opening. Subunits on either side of the channel tilt in opposite directions about the pore axis. The change in tilt of each subunit is only approximately  $7.5^\circ$  but the length of the protein means that the displacement at the cytoplasmic end is upto 18 Å. Although the cytoplasmic and the extracellular ends of the subunits move through the same tilt angle, the tilt axis is located towards the extracellular end. This means that the cytoplasmic ends of the subunits travel through a greater arc length. This movement as depicted in figure 1.3 would restrict the channel thus controlling the permeability of the junction (Unwin & Enis 1984).

## 1.2. Gap Junction Protein Biochemistry And Molecular Biology

Biochemical analysis of isolated gap junctions has been accomplished by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Two major bands are seen on electrophoretic gels of liver gap junctions with a molecular weight of 26-28 kDa and 47 kDa (Henderson et al. 1979; Nicholson et al. 1981; Traub et al. 1982). Could this mean that there is more than one major protein in the liver gap junction?

The 47 kDa protein was in fact a dimer of the lower molecular weight protein. The conditions under which the SDS-PAGE gels were run, caused the 26-28 kDa protein to dimerize thereby giving a false molecular weight of 47 kDa for the major liver gap junction protein (Dermietzel et al. 1990). Henderson et al. (1979) also noted a 21 kDa protein band which they attributed to a break down product of the larger protein. But because the two proteins have different immunological properties, they could be two distinct proteins (Traub et al. 1982). The levels of expression of the 21 kDa protein is also species specific. In mice it comprises 30-40% of the protein mass of isolated gap junctions but in rats it is less than

Figure 1.3



Transition of the junction from an open to a closed state  
Adapted from Unwin & Zampighi (1980).

7% (Dermietzel et al. 1990). Further adding to the diversity of junctional components is a 16 kDa protein isolated from mouse hepatic gap junctions. This protein is distinct from other gap junction proteins as shown by sequence analysis. The 16 kDa protein may contribute to the structure of the gap junction (Willecke et al. 1988). The 16 kDa protein is present in gap junctions in non stoichiometric amounts (Finbow & Meagher 1992). This means that it is unlikely that this protein is a component of the same multi-subunit complex because such structures usually have a fixed composition with respect to subunits.

### 1.2.1. Protein Sequence characterization

Cloning and sequencing of gap junction protein cDNA has shown that the 26-28 kDa liver junction protein has a true molecular mass of 32 kDa. The gap junction proteins have been proposed to be called connexins (Goodenough 1974). Hence the major liver gap junction protein is known as connexin 32. SDS-PAGE gels of heart gap junctions point to a major protein of 44-47 kDa. Sequencing data has shown that the heart gap junction protein has a true molecular weight of 43 kDa and is known as connexin 43. The 21 kDa protein has been shown to have a true molecular weight of 26 kDa and is designated connexin 26. Furthermore, connexins 30 and 38 have been discovered in xenopus embryo and connexin 46 in rat lens (Dermietzel et al. 1990). These proteins also show sequence homologies. A connexin of 70 kDa has been found in cardiac gap junctions (Harfst et al. 1990).

In the vertebrate lens a 70 kDa protein associated with gap junctions has also been found. This protein called MP70 has homology with cardiac and hepatic connexins but is immunologically distinct from connexin 46. Connexin 46 is also expressed in myocardium and kidney to some degree (Goodenough 1992).

### 1.2.2. Lipid Content Of Gap Junctions

Lipid analysis has shown that gap junctions extracted from mouse liver with the detergent Triton X-100 have greater levels of cholesterol relative to phospholipid. However, gap junctions purified using the detergent sarkosyl or with alkali were found to be depleted of sphingomyelin. Arachidonic acid and phosphatidyl-inositol have been found in relatively high levels with arachidonic acid constituting 10% of all fatty acids and phosphatidyl-inositol constituting 7% of all phospholipids. The significance of these findings is not known (Hertzberg et al. 1988).

### 1.2.3. Are Gap Junctions Communication Channels?

Proof that gap junctions are assembled from connexins comes from experiments carried out on a human hepatoma cell line (SKHep1). These hepatoma cells are unable to communicate via gap junctions as measured by transfer dye. They also have near zero junctional electrical conductance which is another measure of cell communication. Furthermore, immunocyto-chemistry and northern blot analysis could not detect connexins or connexin mRNA. When cDNA of connexin 32 from rat liver was stably transfected into the SKHep1 genome, the cells became dye coupled and exhibited electrical conductance. Antibodies to connexin 32 were able to show the presence of the protein (Eghbali et al. 1990).



#### 1.2.4. Genes Of Gap Junction Proteins

The connexins which make up junctional channels are coded by a family of genes. This family has been differentiated into two broad classes, groups I and II, depending on their relative homologies (Haefliger et al. 1992). Different connexin genes have been shown to be expressed in different tissues. But normal cells have not been found which only express one type of connexin. Co-expression of two or more connexins in most cell types seems probable. The different genes may be expressed in a tissue specific manner as indicated by mRNA analysis. The mouse connexin 37 is highly expressed in lung but to a lesser extent in brain, skin, spleen, liver, intestine and heart. Embryonic tissue expresses 2 to 5 fold greater levels of this connexin than the corresponding adult tissue (Willecke et al. 1991).

#### 1.2.5. Expression Of Different Connexins

Risek & Gilula (1991) studied the expression of three different types of connexins involved in communication in the uterus during rat pregnancy. They found that in the placenta and uterus connexins 43, 32 and 26 were expressed at different times during the gestation period, differentially and cell specifically. Connexin 43 and 26 were co-expressed. Connexins 32 and 26 were also co-expressed. They did not observe the co-expression of connexins 43 and 32. These studies show that genes which encode connexins can be switched on at different times. Different genes can be switched on together and different cells can express a specific combination of connexins. However, the physiological implications and the control mechanisms remain to be understood.

### 1.3. Methods For Assaying Gap Junctional Intercellular Communication

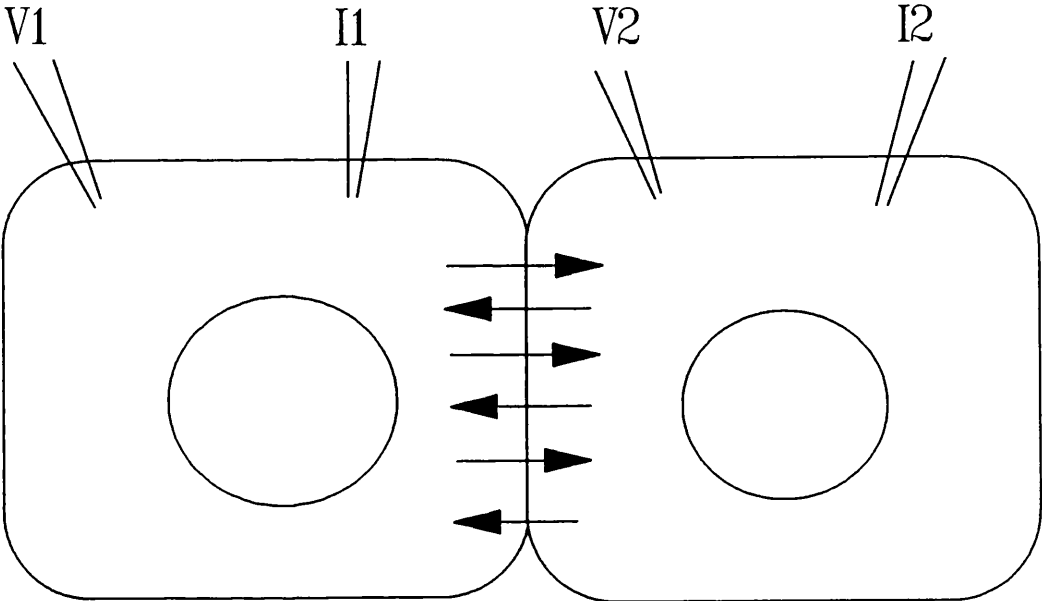
Several methods for assaying GJIC exist and the principle methods which have been successfully utilized are presented.

#### 1.3.1. Measurement Of Electrical Coupling

Electrical coupling measures the transfer of small ions between coupled cells. The simplest method used is to measure the coupling coefficient (the ratio  $V_2/V_1$ ) between a voltage (potential difference)  $V_1$  applied in one cell and the resulting voltage ( $V_2$ ) measured in a coupled cell. The coupling coefficient depends on the resistances of junctional and the non-junctional membranes. A change in the non-junctional membrane resistance can modify the coupling coefficient, even without change in gap junctions. This method only tells us whether ionic currents are able to pass from one cell to the next. However, it does not give an indication whether alterations in coupling are due to changes in the number of open channels or to modification in their elementary properties. Quantitative information about gap junctional permeability can be obtained by measuring electrical conductance.

In the double voltage clamp method two coupled cells are individually held at a particular voltage. A change in voltage in cell (1) induces a change in current in cell (2) because the voltage in cell (2) is held constant (Neyton & Trautmann 1986). The junctional conductance,  $G$ , is then given by the ratio between the changes in  $I_2$  and  $V_1$ . Figure 1.4 depicts the electrode arrangement in a pair of cells.

Figure 1.4



Voltage clamp electrode arrangement in a pair of cells

### 1.3.2. Microinjected Fluorescent Markers

Fluorescent markers are injected into cells with a micropipette using hydraulic or electrical (iontophoretic) pressure (Rose et al. 1977). Transfer of fluorescent tracers from injected cell into adjacent cells gives qualitative information about gap junction permeability. Fluorescein has been very popular as a fluorescent tracer (Loewenstein 1979). But recently a highly fluorescent and non-toxic dye, lucifer yellow CH has been extensively used. Lucifer yellow has a molecular mass of 457.3 Daltons and does not cross the plasma membrane and hence offers greater advantages for visualizing gap junctional dye transfer (Stewart 1981). Procion yellow is another dye which is retained by the cell membrane like lucifer yellow but has now been superseded by the latter dye. The quantum yield (ratio of photons emitted to photons absorbed) of procion yellow is about 500 times less than lucifer dyes (Stewart 1978). This means that a smaller volume of lucifer yellow dye needs to be injected to give equivalent fluorescence. With the benefit that cell volume is not greatly perturbed. Less dye injected also reduces cytotoxic effects. In figure 1.5 lucifer yellow was injected into one cell only. The dye then spread from that cell to the neighbouring cells showing junctional communication.

### 1.3.3. Scrape Loaded Fluorescent Tracers

Dyes can be introduced into cells by placing a membrane impermeant dye solution in a cell culture dish followed by scraping a line with an implement across the cultured cells. The scraping action damages cell membranes temporarily and allows dye to enter the cells after which the membranes seal again. The dye is then transferred from those cells to

Figure 1.5



10 μM

One cell injected with lucifer yellow

adjacent ones if gap junctions are permeable (El-Fouly et al. 1987). The advantage of this method is that expensive equipment for injecting dye into cells is not required.

The disadvantages of this method are that only cells which grow in monolayers can be assayed for junctional permeability. The rate of dye transfer from one cell to another cannot be measured. Subjectivity involved in counting fluorescent cells manually cannot be easily eliminated.

#### 1.3.4. Hydrophobic Fluorescein Tracers

Non polar esters of fluorescein such as diacetate, dipropionate or dibutyrate can diffuse into cells through the cell membrane due to their lipid solubility. In the cell they are hydrolysed by esterases to yield free fluorescein which is a polar molecule. This does not freely diffuse through the membrane. The loaded cells can then be co-cultured with non loaded cells and transfer through gap junctions can be monitored (Loewenstein 1979).

The measurement of dye transfer from one group of cells to another can be carried out using flow cytometry (fluorescence activated cell sorting). If a known number of cells containing fluorescent dye are co-cultured with dye free cells then after a period the cells can be removed and the distribution of dye in the culture can be determined. This provides an automated method for measuring dye transfer through gap junctions. If suitable fluorescent labels can be attached to specific metabolites then the transfer of these metabolites between cells can also be measured (Kavanagh et al. 1987;1988a).

### 1.3.5. Radioactive Tracers

Nucleotides can be radiolabelled in cells by incubating cell cultures in the presence of radiolabelled precursors. The labelled cells can then be co-cultured with non labelled cells and the transfer of nucleotides between cells can then be monitored by fixing the culture and carrying out autoradiography (Pitts & Simms 1977). Radioactive markers can also be injected into cells. Rieske et al. (1975) injected radioactively labelled fucose, glucosamine, glycine, leucine, orotic acid and uridine by means of microiontophoresis into neurons of the leech. They demonstrated by autoradiography the transfer of these metabolites from the injected cell to electrically coupled neighbours. This is a sensitive method but has the disadvantage that it is time consuming and relatively few cells can be monitored for gap junction permeability.

### 1.3.6. Metabolic Cooperation

This method is based on the observation that mutant cells deficient in an enzyme in the purine or pyrimidine salvage pathway are unable to incorporate exogenous radiolabelled bases or nucleosides into their nucleic acids. However, mutant cells do so when co-cultured with wild type cells. The metabolite absent in the mutant cell is passed from the wild type cell. The radioactively labelled metabolites act as markers for metabolic cooperation, highlighting transfer to adjacent mutant cells. The transfer is visualised by autoradiography. Contact between the mutant and wild type cells is required (Subak-Sharpe et al. 1966;1969). Metabolic cooperation between cells of the same phenotype can also be measured using radiolabelled nucleotides. This method has the advantage that mutant strains of cells are not required (Pitts & Simms 1977). A cell membrane permeable radiolabelled precursor is loaded

into some cells. The precursor is converted into an impermeable metabolite inside the cell and the precursor is washed out. The loaded cells are then co-cultured with unloaded cells and transfer of metabolite is assayed by autoradiography. This method has been successfully employed to show transfer of other nucleotides, proline, fucose, and choline phosphate (Pitts 1977).

A general introduction to nucleotide biochemistry here is a prerequisite for better understanding of other metabolic cooperation techniques.

#### 1.3.6.1. Nucleotide Biosynthesis And Incorporation.

Deoxyribonucleotides arise from the direct reduction of ribonucleotide diphosphates. Most of the ribonucleotides in DNA and RNA are supplied from pools which are very small with the exception of ATP. The cellular supply of DNA precursors is less than 1% of total requirement for S-phase DNA synthesis in mammalian cells. Intricate regulation of the synthetic pathway and efficient salvage of the available precursors provides the right amount and balance of nucleotides (Kornberg & Baker 1992).

#### 1.3.6.2. De Novo Pathway.

Ribose phosphate, certain amino acids,  $\text{CO}_2$  and  $\text{NH}_3$  are combined in successive reactions in bacterial and animal cells to form the nucleotides. Synthesis of nucleotides via this pathway takes place without passing through a stage or pool containing free bases or nucleosides (Kornberg & Baker 1992).



### 1.3.6.3. Salvage Pathway.

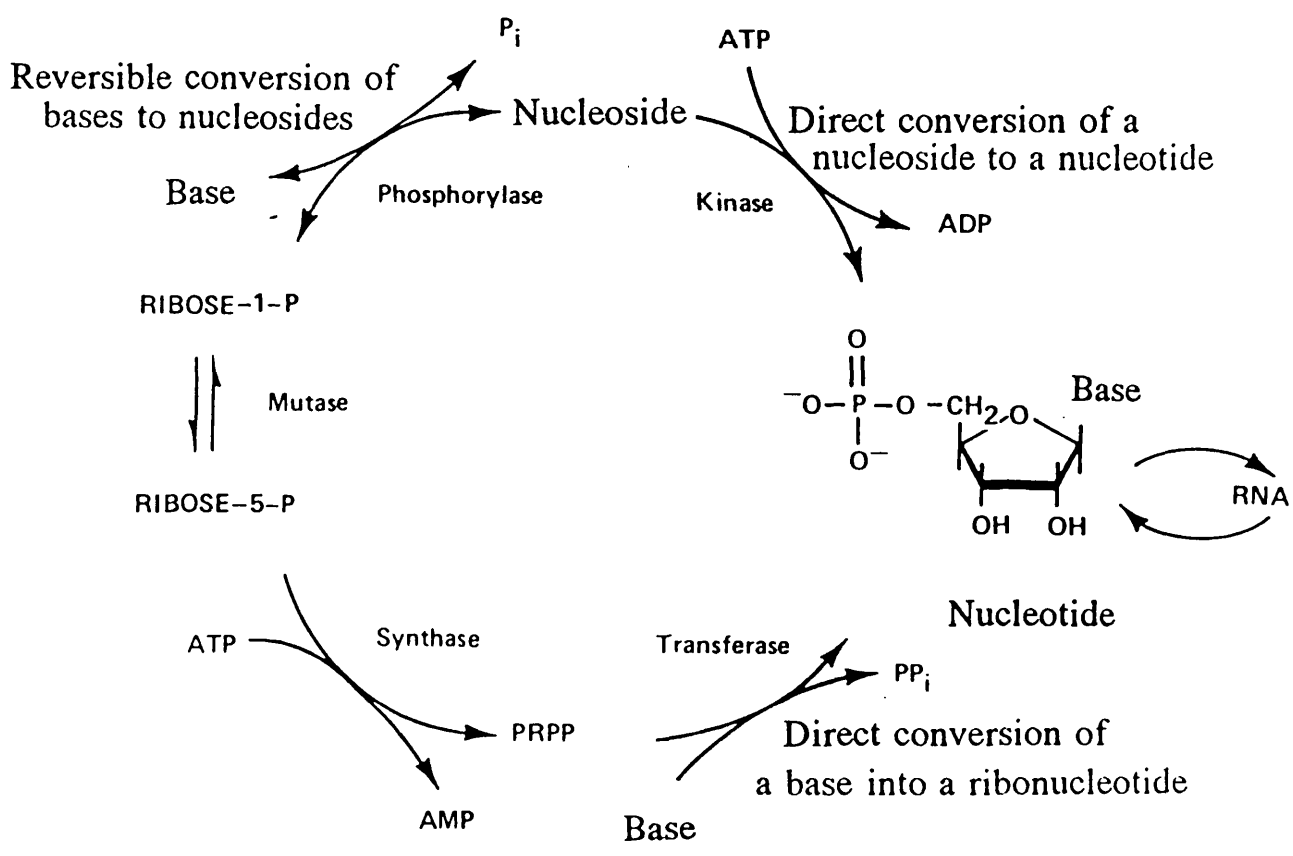
Hydrolytic degradation of nucleic acids and nucleotides makes available free bases which the cell can re-utilize to make nucleotides via the salvage reaction. This process is simpler and requires less energy than the de novo pathway.

In the salvage reaction of purine bases, the ribose phosphate group of phosphoribosyl-pyrophosphate is transferred to the purine to form the corresponding nucleoside.

There are two purine salvage enzymes with different specificities. Adenine phosphoribosyl transferase (APRT) catalyses the formation of adenylate from adenine and hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) catalyses the formation of inosinate from hypoxanthine and guanylate from guanine. Therefore, any exogenous purine base that is added to cell culture medium will be taken up and incorporated into the cellular DNA. Cells which are deficient in one of these enzymes will not be able to metabolize the corresponding exogenous base or base analogue. Consequently, base analogues which disrupt DNA replication by incorporation into nascent DNA will only kill wild type cells when added to culture medium. The mutant cells will survive.

The salvage pathway can also convert thymine to the nucleotide in two steps. 1) The thymine is converted to thymidine by thymidine phosphorylase. 2) Thymidine is phosphorylated by thymidine kinase to yield thymidylate. Exogenous radioactively labelled thymidine is already a deoxyribonucleoside and only requires phosphorylation via the salvage reaction before incorporation into DNA. Thymidine is not incorporated into RNA. Therefore, this labelling method is used to monitor DNA synthesis and treatments which affect it (Kornberg & Baker 1992; Stryer 1988). Figure 1.6 shows the diagrammatic representation of the salvage reactions.

Figure 1.6



Principal pathways for salvage of bases and nucleosides.

Adapted from Kornberg & Baker (1992)

#### 1.4. Using Salvage Reaction To Investigate Cell Physiology

Fujimoto et al. (1971) tried to select for cells lacking the salvage reaction enzyme HGPRT. They added 6-thioguanine (6-tg), (a base analogue toxic when metabolized and incorporated into DNA) to wild type and mutant cell co-cultures. In theory the mutant cells could not metabolize 6-tg. Wild type cells which possess HGPRT would metabolize 6-tg and die leaving behind mutant cells. But transfer of 6-tg metabolite via metabolic cooperation to the mutant cells killed them complicating the selection. Similarly, wild type cells in the co-culture were selected for by preventing de-novo nucleotide synthesis with aminopterin (see figure 1.7). Adding exogenous hypoxanthine would overcome this metabolic block via the salvage reaction in wild type cells. The mutant cells lack HGPRT and could not metabolize hypoxanthine and thus would die. Transfer of hypoxanthine metabolite from wild type to mutant cells rescued the mutant cells. Metabolic cooperation provided the means by which adjacent cells shared metabolites.

#### 1.5. Metabolic Cooperation Occurs Through Gap Junctions

The passage of metabolites through gap junctions has been confirmed by electrophysiological and metabolic cooperation experiments. Gilula et al. (1972) have shown that cell lines incapable of gap junctional communication are also incapable of metabolic cooperation. This circumstantial evidence has been substantiated by experiments which showed that isolated gap junctions incorporated into lipid bilayers exhibit similar properties to channels in intact hepatocytes (Spray et al. 1986).

### 1.5.1. Metabolic Cooperation As A Basis For Measuring Cell Communication

The phenomenon of metabolic cooperation has been used in several ways to assay junctional communication. It is known that mammalian cells of different species differ in their sensitivity to ouabain, a cardiac glycoside inhibitor of  $\text{Na}^+\text{-K}^+$  ATPase pump. Human cells are very sensitive by comparison with rodent cells. Corsaro & Migeon (1977) showed that in the presence of ouabain human skin fibroblasts became growth arrested but mouse 3T3 fibroblasts continued to proliferate. When the two types of cells were densely co-cultured enabling cell to cell contact, the human fibroblasts proliferated. The transfer of potassium from the mouse cells to the human cells allowed the latter to escape growth arrest. This  $\text{K}^+$  ion transfer took place through gap junctions as evidenced by the fact that human cells failed to grow in the presence of ouabain even though mouse cells were grown in the same culture dish sharing the same medium but spatially separated.

Therefore, measurement of intracellular potassium can form the basis for demonstrating junctional coupling. Ledbetter & Lubin (1979) used ouabain sensitive and resistant cells in co-culture and measured the total  $\text{K}^+$  in the cell in the culture in the presence of ouabain.  $^{86}\text{Rb}^+$  was used as a tracer for  $\text{K}^+$ . They found that the cells in co-culture contained more  $^{86}\text{Rb}^+$  when compared to the sum total of the  $^{86}\text{Rb}^+$  levels of the two types of cells grown separately in the presence of ouabain. Since the sensitive cells are unable to accumulate  $\text{K}^+$  due to ouabain inhibition of the  $\text{Na}^+\text{-K}^+$  ATPase pump, they hypothesised that the  $\text{K}^+$  accumulated in the sensitive cells must have been transferred through gap junctions from the resistant cells, thereby enhancing the total intracellular  $^{86}\text{Rb}^+$  in the culture. This cooperation for  $\text{K}^+$  required cell contact so that transfer could occur through gap junctions.

### 1.5.2. Modulation Of Metabolic Cooperation

Metabolic cooperation studies offer the advantage that a cell population is tested rather than individual cells. Assay systems using the toxic metabolite transfer method take a relatively longer time compared to dye transfer. Toxic nucleotide analogues depend on DNA synthesis to exert their toxic effects. Even if the nucleotide analogue acts without being incorporated into DNA the effects take several hours to manifest themselves. Communication inhibition will not be detected if it only lasts a short time and toxic metabolite transfer resumes. Therefore, this method screens for substances which modulate GJIC and sustain their gap junction inhibitory effects for several hours.

### 1.5.3. Use Of Toxic Nucleotide Analogue To Assay Junctional Communication

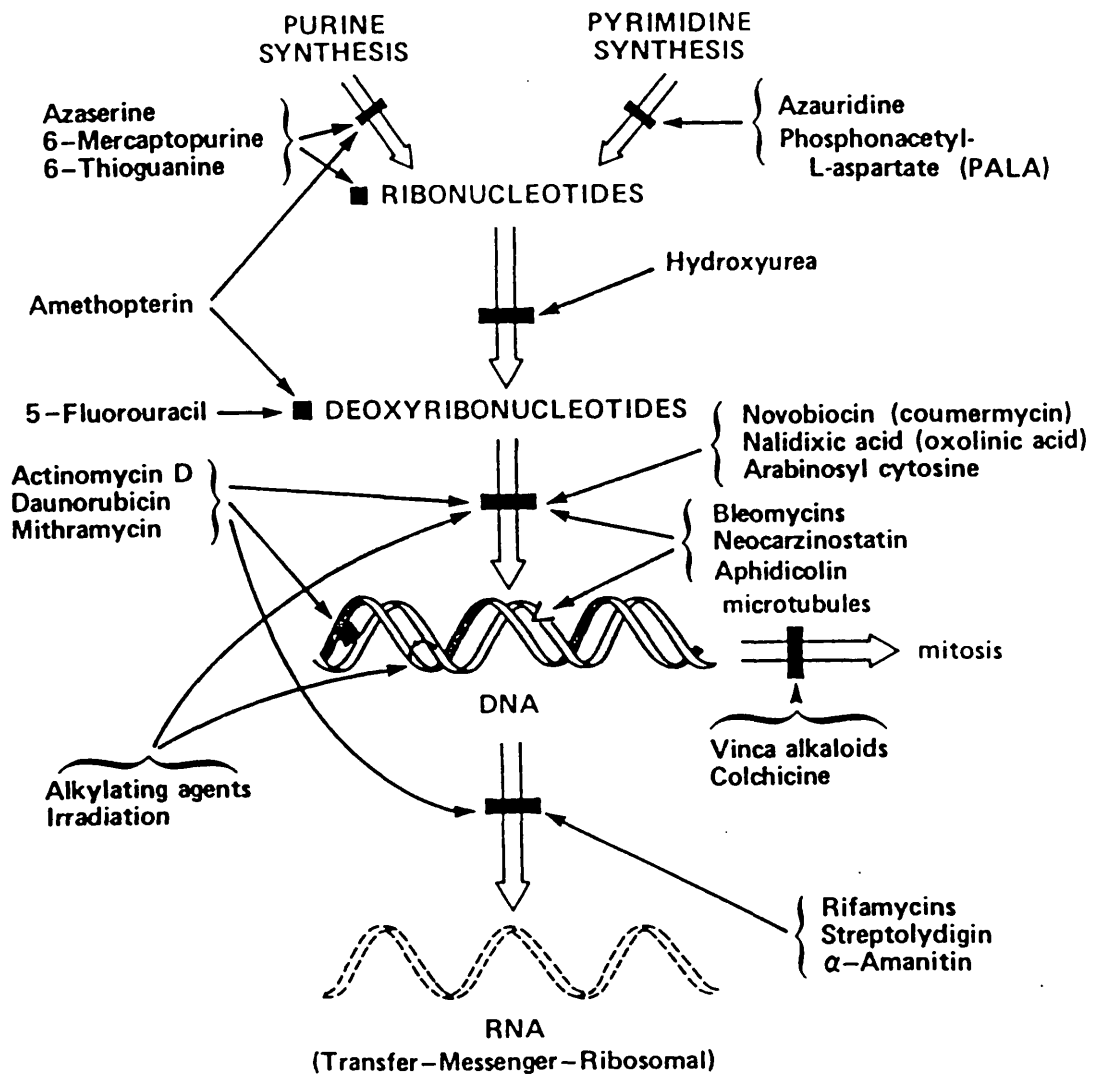
Another method which utilizes metabolic cooperation to measure the extent of junctional communication is that devised by Yotti et al. (1979). About 100 hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme deficient mutant cells unable to metabolize 6-thioguanine (6-tg) are co-cultured with about 800,000 wild type cells. The HGPRT enzyme in the wild type cells catalyses the transfer of the ribose phosphate part of 5-phosphoribosyl-1-pyrophosphate (PRPP) to 6-tg to form the 6-thioguanilate. The ribonucleotide is further reduced to the deoxyribonucleotide in a reaction catalyzed by ribonucleotide reductase. This metabolite inhibits DNA synthesis by incorporation into DNA and eventually kills the wild type cells. It also kills the mutant cells if it is transferred across gap junctions. Mutant cells which have not formed functional gap junctions with the wild type cells survive. The surviving mutant cells are then allowed to grow into visible colonies

which are stained and counted to provide measure of the level of gap junctional intercellular communication. This method allows assessment of compounds and treatment regimes to see if they modulate cell communication.

#### 1.5.3.1. How Does 6-tg Kill Cells?

The base analogue 6-tg has a sulphur atom at position six of the purine ring instead of oxygen. 6-Tg is accepted by DNA polymerases for pairing with the DNA template and is incorporated into DNA. Further chain growth is then prevented. The metabolite of 6-tg (6-thioguanilate) is also an inhibitor of 5-phosphoribosyl pyrophosphate (PRPP) amidotransferase, the first step in purine synthesis (Kornberg & Baker 1992). Figure 1.7 shows the points of action of 6-tg on DNA synthesis. Reichard (1988) points out that imbalance in nucleotide pools affects both DNA replication in S phase and DNA repair in resting lymphocytes resulting in cell death. In less extreme cases increasing the pool of one nucleotide caused point mutations and sister chromatid exchange in cultured cells. Therefore, addition of exogenous 6-tg could cause an imbalance in the relative nucleotide pools thereby contributing to the toxic effects without being metabolized. There is evidence to suggest that 6-tg can exert toxic effect on cells even without being incorporated into DNA. Non dividing hepatocytes and liver epithelial cells growth arrested by serum deprivation die in culture but their death was accelerated by 6-tg (Berman et al. 1985). This could explain why 6-tg at high concentrations is toxic to 6-tg resistant cells (see figure 3.6b).

Figure 1.7



Agents used to block pathways of DNA biosynthesis  
Adapted from Kornberg & Baker (1992)

## 1.6. Alternatives To Using Toxic Base Analogues For Measuring Metabolic Cooperation

Davidson et al. (1984) devised an alternative method. Normal human fibroblasts possess argininosuccinate synthetase and argininosuccinate lyase enzymes. They are therefore able to convert citrulline into arginine. Fibroblast cell lines are available which are deficient in either argininosuccinate synthetase or argininosuccinate lyase enzymes. The enzyme deficiency means that these two cell lines are unable individually to convert  $^{14}\text{C}$ -citrulline into  $^{14}\text{C}$ -arginine and incorporate it into protein. Co-culturing the two fibroblast cell lines restores  $^{14}\text{C}$ -citrulline incorporation to normal levels due to the intercellular transfer of argininosuccinate. This transfer occurs through gap junctions. Double labelling of the culture with  $^3\text{H}$ -leucine and  $^{14}\text{C}$ -citrulline and expressing the incorporation of labelled citrulline relative to leucine gives a measure of cell to cell communication. If a second label ( $^3\text{H}$ -leucine) incorporated independently of communication was not used then variations in cell number between different groups would make comparisons of level of communication difficult.

## 1.7. Regulation Of Gap Junctional Intercellular Communication

Gap junctions couple adjacent cells so that small molecules freely diffuse through this pathway. Molecules in the order of 1000 Dalton are able to traverse the non-arthropod gap junction but arthropod junctions allow the passage of up to 50% bigger molecules (Schwartzmann et al. 1981). Larger polypeptides, RNA and DNA cannot diffuse through the gap junction (Pitts & Simms 1977).

Gap junctional intercellular communication is labile and the strength of coupling is



governed by the number of channels in the membrane and also whether the channels are restricted or not. Over a short period of less than a minute, gap junction permeability may be modulated by opening and closing of existing channels in the membrane by a process termed gating. Over periods longer than a minute synthesis, assembly and degradation of gap junctions may exert control (Spray et al. 1988). Indeed, the main gap junction protein has been found to be present in lysosomes indicating possible degradation (Larson & Hai-Nan 1978). Studies of the kinetics of gap junction turnover give estimates of half-life ranging from 5 to 19 hours in mouse liver and rat liver respectively. Formation and growth of gap junctions to mature proportion in Novikoff hepatoma cells seems to occur quite rapidly but can take as long as 2 weeks in rodent luteal cells (Larsen & Risinger 1985).

#### 1.7.1. Role Of Calcium In Junctional Communication

Electrical coupling measurements by Loewenstein (1966) showed that elevation of internal calcium ion ( $\text{Ca}^{2+}$ ) concentration caused gap junction closure in insect salivary gland cells. Later, X-ray diffraction was employed to show that isolated liver gap junctions undergo a change in conformation upon exposure to  $\text{Ca}^{2+}$  causing junctional channel closure (Unwin & Ennis 1983). Gandolfi et al. (1990) found a direct relationship between increase in internal calcium of the mammalian lens and increase in inter-fibre electrical resistance.

#### 1.7.2. How Does pH Effect Junctional Coupling?

Treatments which reduce intracellular pH also reduce junctional coupling. Amphibian embryos exposed to  $\text{CO}_2$  which decreases cellular pH caused a reduction in cell electrical

coupling (Turin & Warner 1977). Duncan et al. (1988) also found this to occur in human lens epithelial cells.

### 1.7.3. Role Of Intermediates On Ion Effects On Gap Junctions

The role of  $\text{Ca}^{2+}$  and  $\text{H}^+$  in modulating GJIC has been established but the mechanism of action of these ions remains to be completely understood. Do these ions affect gap junction channels directly or through an intermediate? Ramon et al. (1988) perfused crayfish lateral axons with solutions of low pH (6.0) or high calcium concentration (0.1mM). They found that these treatments did not alter the electrical coupling of the axons. They concluded that a soluble intermediate was washed out by the perfusate. A possible candidate for the intermediate was calmodulin, a known calcium binding protein. When calcium activated calmodulin was perfused into the axons, the junctional resistance increased suggesting that calmodulin could indeed be the intermediary. The work of Gandolfi et al. (1990) confirms this finding. These workers found that calmodulin antagonists such as calmidazolium could prevent the electrical uncoupling induced by increased internal calcium in the rat lens. Spray et al. (1982) found that gap junctions were three to four orders of magnitude more sensitive to  $\text{H}^+$  than  $\text{Ca}^{2+}$ . According to these authors the concentrations of these ions in a healthy cell are similar. Therefore, physiological regulation of gap junctions is more likely to be achieved by  $\text{H}^+$  changes. Calcium ions may act to regulate junctional channels during pathological conditions.

#### 1.7.4. Voltage Sensitivity Of Gap Junctions

Some gap junctions are also sensitive to voltage. This was first demonstrated in crayfish synapse junctions but is more thoroughly characterized in amphibian embryos. Injecting current pulses into one cell of a pair causes coupling or uncoupling depending on the developmental beginnings of the cells (Spray & Bennett 1985). Two types of voltages are able to elicit gap junction gating. Voltages applied between cells and voltages applied between the cell cytoplasm and the extracellular fluid.

Comparison of gap junction protein sequences derived from cloned cDNA show that connexins do not share homology with the group of voltage sensitive ion channels which include the Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> channels (Verselis et al. 1991).

#### 1.7.5. Hormonal Control Of Gap Junctions

Gap junctions can also be regulated by hormones, growth factors and neurotransmitters (Trosko et al. 1990). Human chorionic gonadotropin (HCG) upon administration to hypophysectomized rats amplifies gap junction membrane in ovarian interstitial cells and cells of the theca interna. Adenocorticotropin (ACTH) induces gap junction formation upon application in ACTH-sensitive strains of Y-1 adrenal cortical tumour cells. These hormones increase intracellular cAMP (Larsen & Risinger 1985).

Platelet derived growth factor (PDGF) and epidermal growth factor (EGF) has been shown to inhibit junctional communication in NRK and Balb/C 3T3 cells. However, transforming growth factor beta (TGF Beta) acts to enhance junctional communication in Balb/C 3T3 cells but reduces communication in the NRK cell line (Maldonado et al. 1988).

### 1.7.6. Distinct Effects Of Different Growth Factors On Junctional Communication

Experiments carried out on human primary keratinocytes showed that both EGF and TGF beta inhibited junctional cell communication. EGF increased <sup>3</sup>H-thymidine incorporation and increased cellular proliferation in keratinocytes. TGF beta on the other hand decreased <sup>3</sup>H-thymidine incorporation in the culture and caused differentiation in some of the keratinocytes. The underlying mechanisms by which these factors inhibit cell communication may therefore be different (Madhukar et al. 1989).

Neyton & Trautmann (1986) found that acetylcholine could electrically uncouple rat lacrimal cells. The uncoupling by acetylcholine was slower when calcium was highly buffered using EGTA than when it was not. This suggests that more than one mechanism may be involved in mediating the effects of acetylcholine.

### 1.7.7. Membrane Effects On Junctional Communication

Meyer et al. (1990) has shown that in Novikoff hepatoma cells, supplementing culture media with cholesterol causes a five to six fold increase in gap junctional particle aggregation as observed by freeze fracture electron microscopy. Microinjected dye transfer showed that cholesterol supplementation caused increased junctional communication between cells. This was measured by looking at the time taken for dye to be transferred. Cholesterol supplementation even for one hour shortened dye transfer times significantly. This suggests that increased particle aggregation in gap junctions due to cholesterol is accompanied by increased junctional permeability. Inhibition of protein synthesis by cycloheximide blocked this increase in gap junction assembly but inhibiting RNA synthesis by actinomycin D did

not. Therefore, protein synthesis is required but not mRNA synthesis (transcription).

Addition of the protein component of low density lipoprotein (apolipoprotein B) to Novikoff cell culture medium also increases gap junction assembly and hence junctional coupling (Meyer et al. 1991). Apolipoprotein-B does not increase levels of gap junction proteins in the membrane but facilitates the assembly of junctions. Because gap junctions are located in the cell membrane treatments which alter the cell membrane can also alter junctional communication.

#### 1.7.8. Effect Of Phospholipid Metabolites On Junctional Communication

Treatment of rat lacrimal gland cells with arachidonic acid for 10-20 minutes reduced junctional communication as measured by electrical conductance. This effect seems to be mediated directly by arachidonic acid rather than through a metabolic product, since myristic and lauric acids which cannot be further metabolized into eicosanoids also inhibited junctional communication (Giaume et al. 1989).

#### 1.7.9. Effect Of cAMP On Junctional Communication

Introduction of cAMP into cells by using membrane permeable cAMP derivatives increases junctional communication as measured by junctional conductance. Similarly, treatment of cells with agents which increase cytoplasmic cAMP such as glucagon (hepatocytes) or isoproterenol (cardiac cells) also have the same effect. Inhibiting the catalytic subunit of cAMP dependent protein kinase (cAMP-dPK) with an intracellularly injected peptide inhibitor (Walsh inhibitor) prevents glucagon from affecting junctional

communication. This shows that protein phosphorylation is involved in regulating junctional communication (Saez et al. 1986; De Mello 1988).

#### 1.7.10. Role Of Protein Phosphorylation On Junctional Physiology

The main gap junction protein becomes phosphorylated at serine residues as revealed by p32 labelling. When cAMP is increased within the cell the proportion of gap junction protein labelled also increases. This suggests that phosphorylation of relevant proteins is the mediator of cAMP induced increase in junctional communication (Saez et al. 1986).

The gap junction protein is also phosphorylated by Protein Kinase C (PK C) and Calmodulin-Ca<sup>2+</sup>-dependent kinase II (CaM-Ca<sup>2+</sup>-dPK II). PK C phosphorylates at similar sites as cAMP-dPK but the site of phosphorylation by CaM-Ca<sup>2+</sup>-dPK II is different.

cAMP can act to decrease junctional communication in other cells and tissues.

(Teranishi et. al. 1982).

#### 1.8. Physiological Role Of Gap Junctional Intercellular Communication.

All solid tissues have cells that communicate with each other via gap junctions including nerve cells but the one exception is skeletal muscle (Warner 1988). Gap junctional communication has even been found between macrophages and in haemopoietic cells (Rosendaal et al. 1991). Junctional communication has been observed in a broad range of cell types in adult organisms from sponges to man. The widespread prevalence of junctional communication has led people to propose that this type of communication plays a fundamental role in coordinating cellular signalling.

### 1.8.1. Transfer Of Secondary Messengers Through Gap Junctions

A definitive experiment which shows the transmission of signals between cells through gap junctions was conducted by Lawrence et al. (1978). Mouse heart and rat ovarian granulosa cells were co-cultured and the granulosa cells were stimulated with follicle stimulating hormone (FSH). The granulosa cells responded by secreting plasminogen activator and the heart cells in contact with granulosa cells increased their beat frequency. Alternatively when the heart cells were stimulated with noradrenaline, they responded by increasing beat frequency and the granulosa cells in contact with them secreted plasminogen activator. Granulosa cells do respond directly to noradrenaline but this does not invalidate the experiment since they do not respond to the concentrations used in this experiment. Contact between the two cell types was essential. A likely candidate for the mediation of this intercellular communication is cAMP. Addition of cyclic nucleotide phosphodiesterase to the medium to eliminate any cAMP did not prevent this effect showing that signal transfer did not occur through the medium.

### 1.8.2. Coordination Of Tissue Cell Function Via Gap Junctions

Ciliated epithelial cells of the mammalian respiratory tract transport and expel mucus and debris from the airways of the lung. This function requires that many individual cilia act in unison. Mechanical stimulation of a ciliated epithelial cell in culture induces an acceleration of beating and increase in beat frequency of cilia in the stimulated cell and its contiguous neighbours. The nearby cells respond with a time lag which depended on the distance from the stimulated cell. Mechanical stimulation and IP<sub>3</sub> injection into one cell

produced an increase in intracellular  $\text{Ca}^{2+}$  which was propagated to other cells. A wave of increased beat frequency was also induced in surrounding cells. Blockage of gap junctions with the anaesthetic, halothane prevented the  $\text{Ca}^{2+}$  (wave) from being propagated. This suggests that the secondary messenger  $\text{IP}_3$ , communicated through gap junctions brings about this effect (Sanderson et al. 1990). This demonstrates that stimuli at the surface of one cell type can be transmitted via secondary messenger through gap junctions. Hence, gap junctional communication may play an important part in cell regulation, growth control and embryogenesis.

### 1.8.3. Gap Junctions Facilitate Sharing Of Important Metabolites

Glutathione (GSH) is a tripeptide molecule with a molecular weight of 307 Daltons. GSH is a constituent of all cells. Prolonged failure to maintain sufficient levels leads to cell death. Therefore GSH can be regarded as an essential constituent of cells (Kosower 1976). GSH is conjugated by GSH transferases to xenobiotic compounds for safe elimination of the latter from the body (Boyland & Chasseaud 1967). It has been shown that depletion of GSH in hepatocytes treated with paracetamol leads to cell damage (Hogberg & Kristoferson 1977). Also exposing cells to oxygen radicals leads to GSH depletion followed by protein thiol modification resulting in cellular damage. This damage due to generation of active oxygen is called oxidative stress. Oxidative stress has been reported as playing a part in the ageing process and cancer (Orrenius et al. 1988).

Kavanagh et al. (1988b) showed that when V79 cells depleted of GSH were co-cultured with normal V79 cells, the GSH levels equilibrated between the two cell lines in a density dependent way. When GSH depleted cells deficient in metabolic cooperation were



co-cultured with normal cells the GSH levels did not equilibrate. Subsequently, it was shown that cells depleted of GSH and unable to communicate were sensitive to damage caused by active oxygen even when co-cultured with normal cells. On the other hand, cells depleted of GSH but capable of metabolic cooperation were less susceptible to damage by oxygen radicals when co-cultured with normal cells. Therefore, this provides indirect evidence for the sharing of GSH by cells through gap junctions. Furthermore, blocking the synthesis of GSH in GSH depleted cells did not prevent contact mediated repletion of GSH from normal cells. Whereas, inhibition of metabolic cooperation with TPA prevented this contact mediated equilibration of GSH levels. This work provides evidence that GSH is transferred through gap junctions (Kavanagh et al. 1988a).

The vertebrate eye lens is another tissue in which gap junctions play a prominent role. Gap junctions in the lens interconnect the cells into a three dimensional syncytium and allow the transfer of ions, small metabolites, and water (Goodenough 1992). The correct maintenance of ions and metabolites in the lens by virtue of junctional communication keeps the crystallin proteins in the lens in solution. The aggregation of the crystallins leads to the formation of cataracts.

#### 1.8.4. Junctional Coupling Between Dividing Cells.

When a part of rat liver is surgically removed (partial hepatectomy), regeneration of liver tissue occurs. This regeneration occurs at a peak rate of one to two grams per 24 hours. Electrophysiological monitoring of gap junctional communication during this period of liver regeneration shows that the cells are junctionally coupled. With the knowledge that rapid cell division is taking place, it is conceivable that some of the measurements were taken on

mitotic cells. Junctional coupling was invariably found. This leads to the assumption that mitotic cells remain coupled (Loewenstein & Penn 1967). Other investigators have shown that mitotic chick embryo fibroblasts remain coupled to their interphase neighbours through cytoplasmic extensions even though the cell body has rounded up (O'lague et al. 1970). However, a more recent study by Stein et al. (1992) has shown by dye coupling in spontaneously immortalized rat granulosa cells that gap junctional communication is reduced in mitotic cells. This reduction in communication becomes apparent at the onset of prophase. The resumption of coupling occurs slowly, taking several hours after cell division. There is a difference in opinion as far as gap junctional communication in cell division is concerned.

#### 1.8.5. Effect Of Growth State On Junctional Communication

The degree of cell communication was found to vary with growth state as measured by dye transfer (Boreiko et al. 1989). Dye transfer diminished as cultures of mouse C3H/10T1/2 fibroblasts became confluent but as the culture thinned out (matured), extensive coupling was observed.

It has also been reported that senescent cells have diminished junctional communication ability (Xie et al. 1992). However this is not due to arrest of growth of the senescent cells in a particular phase of growth. These workers showed that although the senescent cells were growth arrested in G1/G0 phase of the cell cycle, younger cells experimentally growth arrested at G1/G0 by serum deprivation still exhibited greater junctional communication.

## 1.9. Gap Junctional Intercellular Communication In Development.

In the mouse embryo junctional communication becomes apparent at the 8 cell stage and all the cells are dye coupled at this stage. As the cells diverge in their differentiation they form compartments with respect to junctional linkage. Communication is generally limited within the compartments (Guthrie & Gilula 1989).

All embryonic tissue has also been found to be coupled through gap junctions and this seems to be a general feature of developmental physiology. Even cells of presumptive skeletal muscle make gap junctions but this ability is lost upon differentiation (Loewenstein 1979). Various workers have found that temporal and spatial variations in patterns of cell communication in embryos. Also in the embryo of the nematode *Caenorhabditis Elegans* dye transfer has been demonstrated at the two cell stage. In the later stages of development distinct dye coupling compartments appear. This demonstrates the changes that occur in cell communication during embryogenesis (Bossinger & Schierenberg 1992).

### 1.9.1. So What Role Does Gap Junctional Communication Play In Development?

When gap junctional coupling is inhibited in embryos it has certain consequences. Warner et al. (1984) took antibodies to the rat major gap junction protein and microinjected them into one cell of *Xenopus* embryo at the 8 cell stage. This inhibited dye transfer and electrical coupling between the progeny of the injected cell. The tadpoles which developed from these embryos had defects which included failure of eyes and the brain to form.

The development of the chick limb bud provides another example of how gap junctional communication may influence embryogenesis. The chick limb develops in a

anterior-posterior pattern. The limb pattern formation is influenced by mesenchymal tissue located at the posterior margin of the limb bud. This area is called the polarising region. It is thought that the polarising region produces a morphogen which diffuses across the limb and defines the limb pattern due to its local concentration. When polarising region tissue is grafted onto the anterior margin of the limb, duplication of digits occurs. Blockage of gap junctions with antibodies in the polarising cells and the anterior cells before grafting decreases digit duplication. This suggests that inhibiting junctional coupling blocks the transfer of the morphogen which leads to decrease in digit duplication (Allen et al. 1990).

Coelho & Kosher (1991) found that a gradient of gap junctional communication occurs along the anterior-posterior axis of the limb bud of a developing chick. They used a modified scrape loading/dye transfer assay to show that extensive communication occurs between posterior mesenchymal tissue and adjacent cells. The posterior mesenchymal tissue is adjacent to the zone of polarising activity. Mesenchymal tissue in the middle of the limb communicated much less and almost no communication was seen in the mesenchymal tissue at the anterior end of the limb bud.

### 1.9.2. Morphogen Diffuses Through Gap Junctions To Regulate Development

Further evidence for the developmental role played by gap junctions comes from the freshwater coelenterate Hydra. This organism has a discernable body plan. The head region produces a substance which inhibits other heads from forming. When a piece of tissue cut from the sub-apical region of the hydra was grafted on to other parts of the body, a second head usually did not form. This could be due to the inhibitor travelling through the cells via gap junctions and exerting its morphogenic effect. Only 11% of grafts formed second heads.

However, when hydra were loaded with anti gap junction antibodies by dimethylsulphoxide permeabilization, junctional intercellular coupling was abolished as revealed by dye transfer. Grafting of sub-apical regions from untreated hydra onto these communication inhibited hydra resulted in 22% of the hosts forming secondary heads. The blockage of junctional communication by the antibody could have prevented the inhibitory morphogen from reaching the graft (Fraser et al. 1987). Indeed if the head of the host hydra was removed, the graft formed a new head in 80% of the cases. This provides some evidence that some inhibitory substance diffuses from the head region to inhibit other heads from forming. A substance which exhibits the properties of this inhibitory morphogen has been partially purified and has a molecular mass of about 0.5 kDa. The size of this morphogen makes it plausible that it could pass through gap junctions (Guthrie & Gilula 1989).

#### 1.10. Communication Between Different Cell Types

The capacity of similar cells to communicate via gap junctions has been discussed but do cells from different organs, tissues and indeed different animals communicate with one another? To answer this question Michalke & Loewenstein (1971) co-cultured rabbit lens cells, SV40 virus transformed mouse 3T3 fibroblasts, hamster kidney BHK-21 fibroblasts, rat liver epithelial cells and other cell types. They found that all of these cell types were electrically coupled to each other to varying degrees, thereby demonstrating that heterologous cells can communicate with one another. However, the different degrees of coupling did demonstrate the fact that some communication specificity exists between cells of different origin. In another study metabolic transfer of radiolabelled uridine was used to show that a human mammary epithelial cell line (Hum E) derived from benign tumours exhibited

selective communication. Transfer of radiolabel was greatest to other Hum E cells compared to other cells. Similarly a rat liver cell line also showed communication specificity (Fentiman et al. 1976).

Pitts & Burk (1976) also showed that different cell lines communicate better among themselves using the radiolabelled metabolite transfer method. However, they found that with increase in time of co-culture, the communication between rat liver (BRL) and hamster fibroblast (BHK/C13) cells also increased. After one hour the level of communication in heterologous cultures in terms of radioactive marker transfer was less than 2% of the homologous cultures. After 18 hours this increased to about 50%. This shows that prolonged contact can lead to greater communication.

Gaunt & Subak-Sharpe (1979). also found selective communication between different cell lines using a metabolic cooperation assay. They also found that communication in heterologous cultures increased with time.

Mammalian (mouse 3T3 fibroblasts) and avian (chick embryo fibroblast) cells readily form low resistance gap junctions as revealed by electrical coupling. Mammalian and insect cells did not communicate even when in close apposition. Insect cells of different taxonomic orders also exhibited communication specificity. Although cells from within the same order did communicate (Epstein & Gilula 1977). Arthropod gap junctions have a different gap junctional structure to that of vertebrates and this could explain why vertebrate and arthropod cells do not communicate. The specificity of communication between different arthropod cells remains to be understood.

Cells in mixed cultures also segregate into domains of each cell type. These domains form compartments with respect to junctional communication. Coupling with cells across compartment boundaries is restricted. Dye coupling, electrical coupling and metabolic

cooperation measurements suggest that reduction in gap junction number rather than reduced channel size governs the level of communication across compartment boundaries (Pitts & Kam 1985). Homologous and heterologous junctions seem to be regulated differentially. Mehta & Loewenstein (1991) have found that in co-culture of normal and transformed 10T1/2 cells, retinoic acid decreases junctional communication. However, homologous communication among the normal or the transformed cells is increased.

#### 1.10.1. Expression Of Different Types Of Connexins Could Account For Communication Specificity

Different cells can and do express different connexins. This leads to the question of whether connexons (half junctions) of one cell, pair with connexons in another cell type expressing a different connexin (junction protein). Werner et al. (1989) microinjected connexin-43 mRNA into some *Xenopus* oocytes. The pairing of such oocytes with normal non mRNA injected oocytes produced functional gap junctions which exhibited properties contributed by both hemichannels. Connexin 43 channels are not voltage gated whereas oocyte channels are. The hybrid channels rectify.

#### 1.11. The Role Of Junctional Communication In Cellular Growth Control And Carcinogenesis.

The observation that embryonic tissue exhibits extensive gap junctional communication (Potter et al. 1966) and most cancer cells show aberrant junctional communication (Azarnia et al. 1972; Trosko et al. 1990) leads to the conclusion that gap junctional intercellular

communication plays some pivotal role in controlling cell growth and differentiation.

Gap junctional communication has been correlated with growth control and differentiation in non-excitable cells capable of proliferation. It has also been correlated with control of organ function such as heart or uterine muscle fibres, with ionic and nutrient dependence of non-proliferating excitable brain cells and with embryonic processes and wound healing (Trosko et al. 1990). Contact inhibition of growth is a property associated with normal cells (Levine et al. 1965). Tumour cells lack this contact inhibition (Borek & Sachs 1966). Gap junctional communication may serve to suppress the transformed phenotype by transferring regulatory molecules from normal to transformed cells. Co-culturing a small number of SV40 transformed swiss 3T3 cells with a large number of non infected cells causes the SV40 infected cells to assume normal morphology. But adding croton oil (which contains phorbol ester) and hence blocking junctional communication caused the infected cells to exhibit the transformed phenotype. This suggests that some substance transferred across gap junctions causes the suppression of the transformed phenotype. Therefore this experiment provides some evidence for the involvement of gap junctions in cellular growth control (Sivak & Van-Durren 1967).

#### 1.11.1. Junctional Communication In Tumour Cells Is Downgraded

Junctional communication studies on tumours were first carried out in solid tumours of liver, thyroid, and stomach. Communication was also investigated on azo-dye induced primary rat hepatoma, transplanted rat hepatoma, Novikoff's hepatoma and hamster thyroid tumours as well as human stomach carcinoma cells (Loewenstein 1979).

Electrophysiological measurements on these tumour cells revealed that they were



communication incompetent. As a comparison, it was found that normal tissues of the same origin under the same growth conditions were electrically coupled. However, not all tumour cells were found to be communication incompetent. Benign thyroid tumours such as human diffuse toxic goitre, nontoxic nodular goitre and human follicular adenoma were found to be predominantly coupled (Loewenstein 1979).

In the fruit fly *Drosophila*, cell proliferation terminates in a regular spatial pattern during the last stages of larval development. There are mutants however in which overgrowth of tissues occurs. Some of the hyperplastic mutants show a failure in gap junctional communication in the imaginal discs from which hyperplastic cell proliferation takes place (Bryant & Schmidt 1990). Also chemical and oncogene transformed Balb/C 3T3 cells were shown not to communicate with normal cells but selectively communicate with normal cells (Yamasaki et al. 1987). Mesnil & Yamasaki carried out dye transfer assay on transformed and non-transformed rat liver epithelial cells lines in culture. They found that all the cells lines communicated among themselves but cell lines showing a high degree of transformed phenotype did not communicate with normal cells. Cells of a line which showed the least degree of transformation, did communicate with their normal neighbours. The highly transformed and hence the most communication selective cells showed the most tumourigenicity when injected into immunodeficient nude mice. This selective communication may allow tumour cells to escape regulation and maintain the transformed phenotype (Mesnil & Yamasaki 1988). The ability of tumour cells to metastasize seems to be inversely related to their ability to communicate. Tumour cells which are unable to communicate among themselves are able to metastasize the most (Nicolson et al. 1988).

### 1.11.2. Density Dependence Of Cell Transformation

Observations in C3H/10T1/2 mouse embryo fibroblasts showed that, as the initial seeding density was increased in carcinogen-treated cultures, the frequency of transformation was found to decrease. At seeding densities above  $1 \times 10^5$  cells/60mm dish, transformed foci formation was reduced to zero. Therefore, increased cell contact reduces expression of transformation by carcinogens (Bertram 1990). Work on retinoids demonstrated their chemopreventive action which correlated with their ability to increase gap junctional intercellular communication (Hossain et al. 1989). However, it must also be stated that retinoic acid has been shown to inhibit junctional communication in a variety of cell lines (Pitts et al. 1986).

Further to the chemopreventive action of junctional communication it has been demonstrated that established neoplastically transformed cells can be growth inhibited when in close contact with C3H/10T1/2 cells. Agents which increased cellular cAMP levels prevented growth of neoplastic cells in co-culture with normal cells. But these drugs did not prevent the growth of neoplastic cells in the absence of normal cells. Therefore, contact with normal cells was required (Bertram et al. 1985) The growth arrest of these neoplastic cells was shown to correlate with the extent of gap junctional communication between the neoplastic and normal cells (Mehta et al. 1986). Cyclic AMP is known to increase gap junctional communication (Azarnia et al. 1981; Saez et al. 1986). UV irradiation of C3H/10T1/2 leads to the generation of cells whose transformed phenotype is suppressed by contact with normal cells. But blockage of junctional communication with TPA allows the expression of the transformed phenotype (Herschman et al. 1986).

### 1.11.3. Effect Of Oncogenes On Junctional Communication

Evidence which shows that cells transformed by expressed oncogenes lack junctional communication comes from work carried out on rat kidney, quail embryo, chick embryo and mouse 3T3 fibroblast cells. The cells are infected with a temperature sensitive mutant of the avian sarcoma virus. Whether these cells are transformed or not depends on the temperature at which the cells are grown. At the non-permissive temperature of 40.5°C the virus is unable to transform the cells and they are dye coupled. At the permissive temperature of 35°C, the cells become transformed and dye transfer becomes reduced (Atkinson et al. 1981; Azarnia et al. 1984). The product of the viral src (v-src) gene is a protein kinase (pp60v-src) which phosphorylates tyrosine residues. The pp60v-src has been located in the plasma membrane near to gap junctions (Atkinson et al. 1981).

Another protein kinase, is encoded by the cellular-src gene. This gene is highly conserved in evolution and present in all animal species. The product of this gene is pp60-cellular-src (pp60c-src) which is expressed in low levels in ordinary cells and is membrane bound. Over-expression of this gene in NIH 3T3 cells by recombinant techniques results in down regulation of junctional permeability. The higher the expression of this gene, the greater the down regulation of communication (Azarnia et al. 1988).

### 1.11.4. Oncogenes Alter Communication Specificity

Yamasaki et al. (1987) transfected Balb/C 3T3 cells with Harvey-ras oncogene which codes for a 21 kDa protein (p21). They found that the subsequently transformed cells were unable to transfer dye to neighbouring non-transformed cells. However, the transformed cells

were able to communicate among themselves. Either the transformed cell gap junctions differ from normal cell gap junctions and the transformed and non-transformed cells cannot form functional gap junctions. Or the transformed and normal cells have different recognition molecules and therefore do not recognize each other. Chemically transformed cells communicate only among themselves but treatment with dibutyl cAMP or retinoic acid induced them to communicate with their normal neighbours and to revert to their normal phenotype. Therefore, it seems likely that transformed and normal cells have different recognition molecules (Yamasaki et. al. 1987).

Transfection of NIH 3T3 cells with v-myc, v-src and v-ras oncogenes resulted in transformation of the cells. Culturing of a small number of these cells with mouse embryo fibroblasts (C3H10T1/2) resulted in the v-src and v-ras transformed cells overgrowing the normal cells and forming foci, the v-myc transfected cells did not. The v-src and v-ras transfected cells were shown to be communication incompetent with the normal cells. However, the v-myc transfected cells did show dye transfer with normal cells. Inhibition of this communication with phorbol ester enabled the v-myc transformants to escape growth control and form foci (Yamasaki 1988).

The evidence for the involvement of cell communication in tumourigenesis is mounting. Results of experiments carried out with oncogenes provides indirect but persuasive arguments in favour of a central role for gap junctional intercellular communication in malignant cell transformation and its expression.

#### 1.12. Changes In Gap Junctions In Myocardial Infarct Tissue.

According to Smith et al. (1991) ischemic heart disease is the most prevalent

cardiovascular disorder in western countries. Abnormal propagation of electrical impulses are frequently associated with myocardial infarction and ischemia. In normal myocardial tissue, gap junctions are organized in intercalated disks between adjoining cells and facilitate the transmission of electrical signals between the cells. Using antibody labelling and laser scanning confocal microscopy, Smith et al. (1991) were able to show that the gap junction organization in infarct tissue was disordered and gap junctional immunostaining was spread all over the myocyte surfaces. The importance of this observation is that disorderly arrangement of gap junctions in myocardium may alter the transmission of electrical impulses and hence contribute to arrhythmic beating of the heart. These arrhythmias are thought to be responsible for over half the deaths due to ischemic heart disease.

### 1.13. Aims And Objectives Of Project

The objective of this project was to understand the mechanism of how junctional communication is regulated and if and how junctional communication is involved in cell injury. The colony forming metabolic cooperation method for assaying junctional communication was modified. Using the modified method, it was planned to test various compounds for their effects on junctional communication, and to check the metabolic cooperation results using direct dye injection.

## Chapter 2. Materials And Methods

### 2.1. Tissue Culture

All experiments involving cells were conducted using Chinese hamster lung fibroblast (V79) wild type and 6-thioguanine (6-tg) resistant strains. These cells were obtained from Dr. M. Fox, Christie hospital & Holt Radium Institute, Manchester. The 6-tg resistant strain is deficient in the hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme and thus unable to phosphorylate 6-tg. Mutations in the HGPRT gene can be induced using chemical mutagens or X-radiation. A variety of different types of mutations are produced in the gene by these methods all resulting in the enzyme deficiency (Chaudhry & Fox 1990).

#### 2.1.1. Aseptic Technique

To maintain aseptic conditions all cultures were manipulated within a class II microbiological cabinet (M.D.H.) providing vertical downward flow of filtered air. Prior to use the cabinet was swabbed thoroughly with absolute alcohol to maintain a sterile environment. Routine maintenance of the cabinet was carried out to verify the integrity of the filter and to ensure that airflow was adequate by measuring with a flow meter.

A removable access hatch was used to maintain sterility whilst the cabinet was not in use. Consumable plastics were purchased ready sterilized by gamma irradiation. Pipette tips, media bottles and pasteur pipettes were sterilized by autoclaving. All solutions were filter sterilized using 0.2 $\mu$ M pore size filters (ICN Flow). Media and buffers were also purchased pre-sterilized.

### 2.1.2. Tissue Culture Materials

Materials used in the experiments requiring sterile tissue culture work were as follows:

<u>Materials</u>	<u>Description</u>	<u>Supplier</u>
Tissue culture flasks	75 cm <sup>2</sup> culture surface	Becton Dickinson U.K. (Falcon)
Culture dishes	35 mm diameter Easy grip type	"
Multi-well plates	24 well plates	"
Sterile pipettes	5, 10 and 25 ml plugged and individually wrapped	"
Centrifuge tubes	15 ml capacity clear polycarbonate conical tubes with 1ml graduations	"

Round bottom universal tubes	Sterile 30 ml capacity tubes with and 5 ml graduations	Gibco-Nunc
General purpose universals	Sterile 25 ml screen cap tubes with label and 5 ml graduations	Sterilin
Media Preparation ware	Duran autoclavable bottles (100,250 and 500 ml capacity)	Schott
Pasteur pipettes	Disposable glass pipettes	J.Poulten Ltd.
Disposable plastic Pipettes tips	Non sterile pipette tips for Gilson pipettes	Hughes & Hughes Ltd.
Cryogenic vials	Small 2 ml capacity Cryo-Vials	Whatman U.K. Ltd.



### 2.1.3. Subculturing Of Cells

The V79 cell line grows in a monolayer until the culture reaches confluency. The cells then start to die. It is necessary to subculture the cells before they reach confluency. Cell cultures were grown in Dulbecco's Modification of Eagles Minimal Medium (DMEM) (ICN Flow) with 20 mM HEPES and supplemented with 2mM Glutamine (Sigma), 10% foetal calf serum and 50 µg/ml Gentamycin (ICN Flow). Sterile plastic flasks with 75cm<sup>2</sup> surface area (Falcon) were used for cell culture. Medium was added at 20 ml per flask. The cells were subcultured twice weekly to maintain exponential growth. The monolayer was detached using 0.05% Trypsin + 0.02% EDTA in a special salt solution (ICN Flow). The detached cells were dispersed by repeated pipetting and expelling against the flask wall. The suspension was then pelleted in 15 mm centrifuge tubes at 900 rpm (110g) for 3 minutes. The pellet was resuspended in fresh medium and pelleted. This was done twice to remove all trypsin. The cells were then re-plated at a lower density of 2x10<sup>5</sup> cells per flask. The V79 6-tg resistant strain was cultured in medium supplemented with 1 µg/ml 6-tg to select out revertants. Cultures were incubated in a humidified CO<sub>2</sub> incubator (Haraeus) with 5% CO<sub>2</sub>, 95% air at 37°C with 98% relative humidity. Cell cultures were discarded once passage number had reached 40. New cells from original stock frozen down were thawed out.

### 2.1.4. Mycoplasma Testing

Infection with mycoplasma can change the characteristics of the culture. Mycoplasmas can incorporate <sup>3</sup>H-thymidine and hence affect the present study. Therefore, periodic

screening of cultures was important. Cultures were screened for mycoplasma by staining for extracellular DNA using fluorescent Hoestch 33258 stain (Sigma) in accordance with the method of Freshney (1987).

Presence of mycoplasma is detected by appearance of DNA stain in the extracellular media and on the cell surface. In a mycoplasma free culture only the cell nuclei are visible under fluorescent lighting conditions and the cell cytoplasm is not seen.

#### 2.1.5. Cryogenic Preservation Of Cultured Cell Lines

Cell lines were routinely frozen at  $-196^{\circ}\text{C}$  in plastic cryogenic vials which were submerged in liquid nitrogen. A cell suspension of  $1-2 \times 10^6$  cells/ml in new born calf serum containing 10% DMSO (v/v) was made according to Doyle et al. (1988). 2 ml of this suspension was then aliquoted into the cryogenic vials. The sealed vials were then packed in insulating material and placed in a  $-70^{\circ}\text{C}$  deep freeze overnight. This was done to cool the cells at a slow rate. The cryo-vials were then placed in liquid nitrogen cell store (Union Carbide 35HC).

Cells from the freeze store were recovered by rapid thawing at  $37^{\circ}\text{C}$ . The DMSO was removed from the cells by placing the suspension in a centrifuge tube and centrifuging at 900 rpm (110g) for 3 minutes to obtain a pellet. The cells were then resuspended in fresh medium to wash the cells and again pelleted. The resuspended cells were then plated in a flask to grow.

#### 2.1.6. Evaluation Of Cell Number And Viability

Cell numbers were counted using a haemocytometer. The cell suspension was diluted in trypan blue solution 0.4% (w/v in 0.15 M NaCl) and then applied to the haemocytometer. Non-viable cells are permeable to trypan blue and viable cells exclude this dye. Counting the stained cells and expressing them as a percentage of total cells gives a measure of cell viability. The cells in the 4 corner squares were counted. The total number of cells counted in a suspension diluted  $y$ -fold is given by  $n$ . Number of cells per square is  $n/4$ . The volume of the counting chamber is  $1 \times 10^{-4} \text{ cm}^3$ . Therefore, the number of cells per ml in the dilution is given by  $10^4 n/4$ . The cell number per ml in the original suspension is given by  $10^4 ny/4$ .

#### 2.1.7. Assessment Of Cytotoxicity (Dose Response Curves).

To test the cytotoxic effects of compounds on the V79 cell line, cells were plated at a density of 60,000 per well in 24 well plates. The surface area per well is  $1.13 \text{ cm}^2$ . Therefore, the number of cells per  $\text{cm}^2$  is approximately 53,000. The culture reaches confluency at about 440,000 cells per  $\text{cm}^2$ . After 24 hours the compound of interest was added at a range of concentrations in quadruplicate and incubated for 24 hours.

A quadruplicate control group was treated with solvents concurrently. After the exposure period the test compound was removed and tritiated thymidine incorporation was carried out for four hours. The incorporated radioactivity was then measured and a plot of concentration of compound against cell DNA synthesis was constructed to give a dose response curve.

## 2.2. General Methods.

### 2.2.1. Protein Estimation

Proteins were measured using the Pierce BCA Protein Assay Reagent kit. Proteins in aqueous solution at high pH reduce copper (II) to copper (I) which reacts with the BCA reagent to give a purple product. This has a strong absorbance at 562 nm and allows spectrophotometric quantitation. A range of protein standards were prepared using 2 mg/ml bovine serum albumin (BSA) stock. 0.1 ml of known protein standard or unknown protein sample was added to 2ml of working reagent in appropriately labelled tubes and incubated at 37°C for 30 minutes. After cooling to room temperature, the absorbance was measured at 562 nm blanked with a water reference. A plot of absorbance vs protein concentration was constructed for the protein standards and the absorbance of the unknown sample was compared with the standard curve to obtain the protein content of the sample.

### 2.2.2. Scintillation Counting

Radioactive samples were counted using a Packard Tri-Carb 460C scintillation counter (Packard Instruments Co. Pangbourne, U.K). A known volume of sample to be counted was mixed with 5 ml of Liquiscint scintillation fluid (National Diagnostics USA) in glass counting vials. The sample was counted until a stable value was reached. The background radiation count was measured by including a scintillation fluid blank in the samples to be counted first and the background value was automatically subtracted from the sample counts. The counts per minute (CPM) were automatically converted to disintegrations per minute (DPM) using

the quench curve parameter (QIP) for each sample and a standard quench curve. A standard quench curve is obtained by plotting the efficiency of counting against QIP value for each isotope under the conditions of counting used.

### 2.2.3. Measurement Of DNA Synthesis By Tritiated Thymidine Incorporation

DNA synthesis is a sensitive indicator of S-phase DNA replication and cell division. It was measured by adding exogenous tritiated thymidine to the cell culture medium and then measuring the amount of radioactivity incorporated into DNA by scintillation counting. The method used was a modification of the method used by Oreffo et al. (1985). After any treatment of cell cultures, the medium was removed and replaced by medium containing 0.1  $\mu\text{Ci/ml}$  tritiated thymidine in 500  $\mu\text{M}$  cold thymidine. Incubation was carried out for 4 hours during which incorporation is linear. The radioactive medium was then removed and the culture was washed twice with ice cold phosphate buffered saline (PBS). The cells were then detached using 0.5 ml of 0.25% trypsin (ICN Flow). The detached cells were placed in microcentrifuge tubes (Eppendorf) containing 0.4 ml of 20% TCA. Calf Thymus DNA (0.1 ml of 1mg/ml) was then added and the suspension was left to precipitate overnight. The precipitate was centrifuged at 12000 rpm (10,000g) for 15 minutes. The pellet was washed twice with ice cold 5% (v/v) TCA and once with ice cold absolute ethanol. The remaining pellet was dissolved in 1M NaOH and then added to scintillation fluid to be counted. The alkali was neutralized with equivalent amount of hydrochloric acid in the liquiscint. The amount of incorporated radioactivity of any treated group was compared to a control group. About 1% of counts are contributed by non-incorporated activity bound to cell material.

#### 2.2.4. Protein Synthesis Estimation By <sup>14</sup>C-Leucine Incorporation

Protein Synthesis was measured by incorporating radioactive leucine into cellular proteins to ascertain whether certain treatments inhibit protein synthesis. <sup>14</sup>C-leucine was incorporated into cells by adding 0.5  $\mu$ Ci/ml of radioactive leucine made up in 0.1 mM non-radioactive leucine in fresh medium and incubating for 30 minutes. The medium was removed and the cells were washed twice with PBS. The cells were then removed with 0.25% trypsin and precipitated in 5% TCA containing 10 mM leucine. The precipitate was pelleted by centrifugation at 12,000 rpm (10,000g) for 15 minutes and the pellet was washed with 5% TCA. The pellet was then dissolved in 1M NaOH and a known volume was counted for incorporated activity.

#### 2.2.5. MTT Assay For Cell Viability

The yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye is converted to a blue product in active mitochondria. This should only occur in live cells and can be used to measure cell viability. Stock solution of 5 mg/ml of MTT was made in PBS. This was diluted to give 1mg/ml MTT in serum free medium. The reagent was added to cells in culture and incubated for 2 hours. The reagent was then removed and 0.005 M HCl made up in isopropanol was added to dissolve the formazan product. The culture plate was agitated for a few minutes. The optical density of the blue formazan product was measured using a spectrophotometer at 560nm wavelength in a glass cuvette blanked with isopropanol.

### 2.2.6. Microinjection Of Cells.

Cells to be microinjected with fluorescent dye were plated on 35 mm tissue culture plates at a density of  $2 \times 10^6$  overnight. A 3% solution of Lucifer Yellow CH dye (Sigma) was made up in Phosphate Buffered Saline (PBS) and filtered using a  $0.2 \mu\text{M}$  syringe filter. The dye solution was then centrifuged for 15 minutes at 12000 rpm (10,000g). This was done to remove all debris which would frequently block the micropipette tip. The micropipettes were pulled from Clark 1.5 mm diameter glass capillaries with internal glass fibre (Clark Electromedical Instruments). The internal glass fibre facilitates backfilling of the micropipettes with dye. A Kopf vertical pipette puller was used to make the micropipettes. Micropipettes were backfilled using an Eppendorf pipette and Eppendorf microloader pipette tips. The loaded micropipette was positioned into close proximity of the cell to be injected using a Narashige MN-151 joystick micromanipulator and the cell was impaled using a Narashige MO-22 single axis hydraulic micromanipulator to provide smooth movement. Pressure from a large hypodermic syringe was applied via tubing to the micropipette to provide continuous flow of dye and inject the dye into the cell. Cells were microinjected for approximately 10 seconds after which the culture plate was washed with fresh medium to remove overspilled dye.

The microinjection apparatus was set up on a Nikon Diaphot-TMD inverted microscope with a epi-fluorescence attachment. Injected fluorescent dye was visualized by exposing the culture plate to UV light and the cells were photographed. Scotch Tungston slide film (ISO 650) was used for this purpose. The dye spread was then measured by counting the number of fluorescent cells on the slide/photograph. (See section 1.3.2.)

### 2.2.7. Statistical Analysis Of Cell Cooperation Experiments.

Comparative analysis and significance of results was determined using the Student t-test. The probability of getting a random result  $p \leq 0.05$  is considered to be significant. The tests were computed using a computer based statistical package (Statgraphics, CHILD Inst.).



Chapter 3. Characterization Of V79 Cells And Development Of A Method For Detecting Metabolic Cooperation.

3.1. Introduction

For the study of intercellular gap junctional communication the V79 chinese hamster lung fibroblast cell line was selected. This cell line is readily available and has been utilized in a variety of toxicological studies (Garle et al. 1987), mutagenicity studies (Diamond et al. 1982; Nakatsuka et al. 1990) and cell communication studies (Jongen et al. 1987; Kornburst et al. 1984). The use of this cell line in junctional communication assays has proved advantageous because V79 cells readily form gap junctions and mutant strains are available which lack the ability to metabolize 6-tg (Yotti et al. 1979).

3.2. Assessment Of Cell Communication By Metabolic Cooperation.

Measurement of cell communication by metabolic cooperation can be carried out in several ways. Radioactively labelled metabolites or precursors can be loaded into one group of cells and these cells are then co-cultured with non-labelled cells. The cells are then allowed to form functional gap junctions and transfer of labelled metabolite through gap junctions takes place. Transferred label is then measured by autoradiography. The drawback of this technique is that autoradiography is a lengthy process and relatively few cells can be assayed for junctional communication at any one time. The process of counting individual grains is also time consuming.

Yotti et al. (79) developed a method whereby two variants of the V79 cell line, one able and

the other unable to metabolize 6-tg to a nucleotide analogue are plated together. The nucleotide analogue prevents further DNA synthesis when incorporated into DNA. The two cell lines are both killed by production of the nucleotide analogue in the competent cells which is then passed through gap junctions to the deficient cells (see section 1.5.3.1). If junctional communication is inhibited, the cells unable to metabolize 6-tg survive and are allowed to grow into macroscopic colonies. These colonies are then stained and counted. This colony counting method for measuring metabolic cooperation was a distinct improvement over other methods. However, the whole process takes about two weeks to complete and the colonies formed exhibit a range of sizes. The large culture dishes used require relatively large amounts of expensive media. What was required was a method which measures metabolic cooperation and yield results relatively quickly. Hence, one of the objectives of this project was to develop a method for assaying junctional communication and test its efficiency using established methods for inhibiting junctional communication.

### 3.3. The Use Of Tetradecanoyl Phorbol Ester As An Inhibitor Of Junctional Communication

It was shown by Yotti et al. (79) and Murray & Fitzgerald (79) that TPA inhibits gap junctional communication. This compound has been used extensively as an inhibitor of gap junctional communication (Jongen et al. 1987). Indeed, TPA has become a standard compound for inhibition of junctional communication with which the communication inhibitory effects of other agents is compared (Zwijssen et al. 1990; Warngard et al. 1987). Any new method for assaying gap junctional communication would have to be tested using TPA to demonstrate inhibition of junctional communication. Therefore, TPA was selected

for the development of the new model system for measuring junctional communication.

#### 3.4. Materials

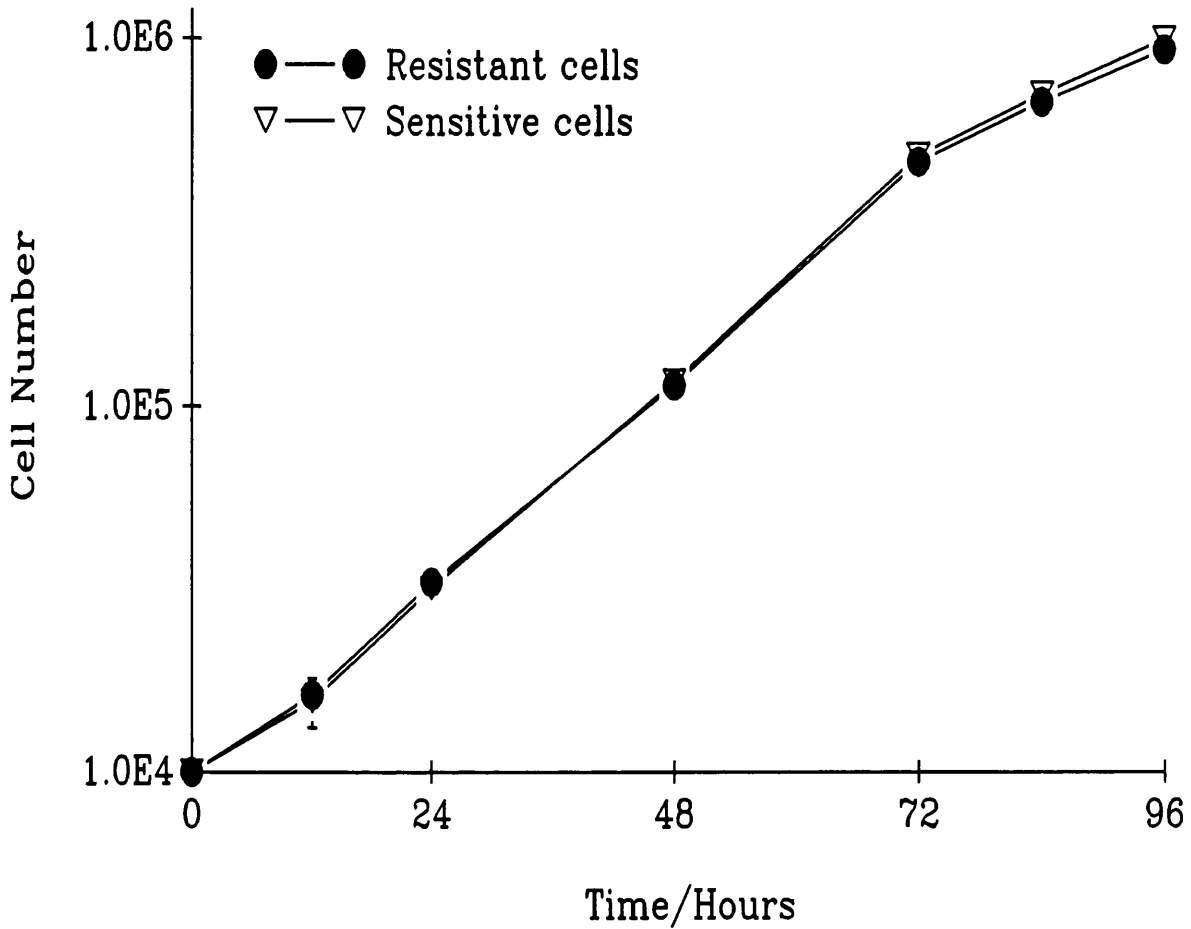
Tissue culture materials used were as described previously. 6-Thioguanine (6-tg) (Sigma) was dissolved in 0.5% sodium carbonate to give a 1mg/ml stock. 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Sigma) was dissolved in DMSO (99%) (Sigma) to give a 1mg/ml solution. <sup>3</sup>H-thymidine (Amersham) was made up into a 10  $\mu$ Ci/ml stock solution in 500  $\mu$ M cold thymidine (Sigma).

#### 3.5. Cell Characterization Methods

Before experiments could be conducted using these cells, basic parameters such as growth rates and responses to 6-tg of these cells had to be ascertained. The two V79 cell lines have a doubling time of 12-14 hours calculated from plot of cell number versus time as seen in figure 3.1. There is no initial lag phase apparent. This could be due to the cells having completed S-phase DNA synthesis before being plated. When the cultures become confluent at about 500,000 cells/well the cells become growth inhibited. This contact inhibition of growth is most apparent when <sup>3</sup>H-thymidine incorporation is used as a measure of cell division. Figure 3.2 shows that <sup>3</sup>H-thymidine incorporation falls sharply when the cultures become confluent.

Figure 3.1

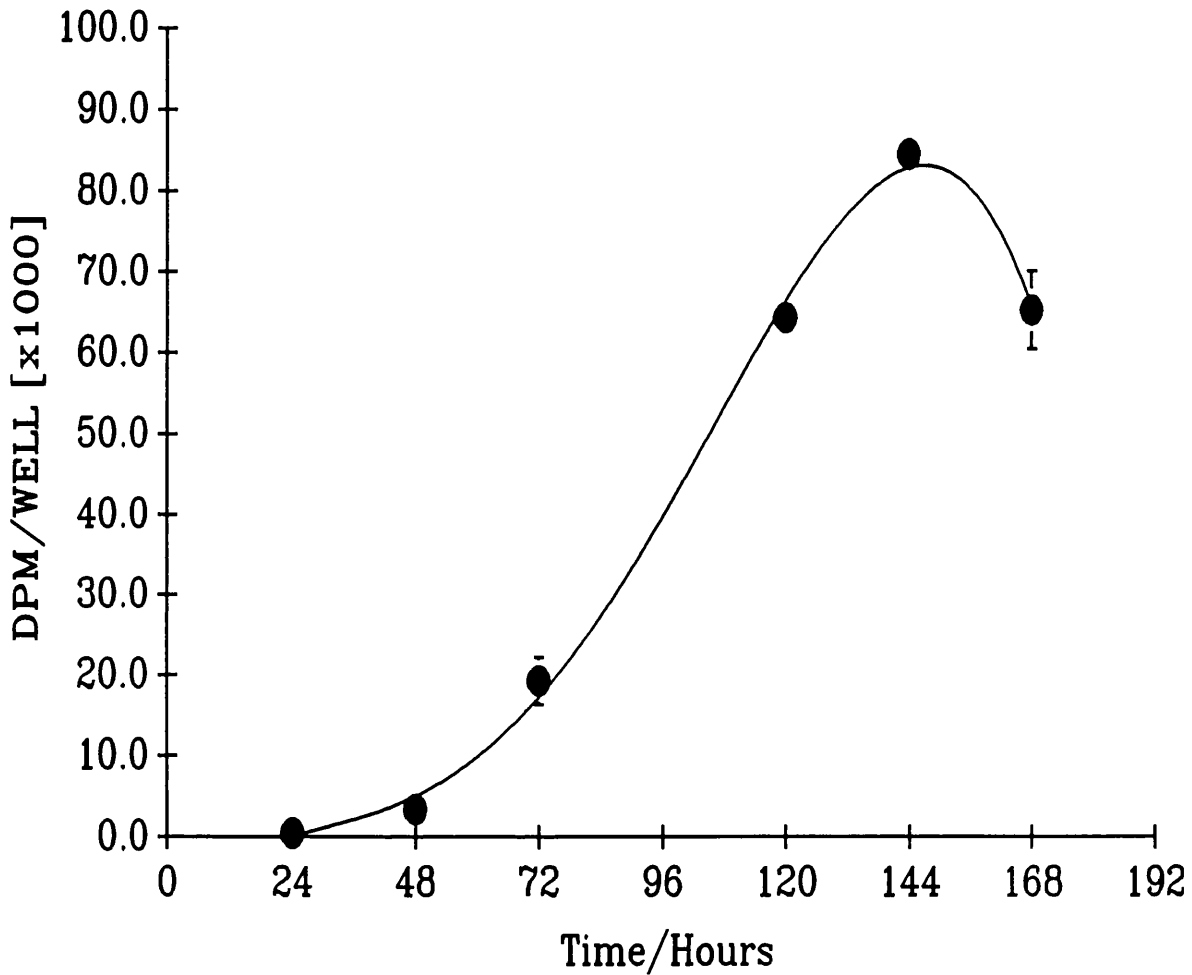
Growth curves for V79 cell lines  
Cell number vs Time



V79 6-tg resistant and sensitive cells were plated separately at a density of 10 K (1000) cells per well in 24 well plates. After every twelve hours, cells from both resistant and sensitive cultures were detached using trypsin and counted using a haemocytometer. Cells from four wells were counted per time point.

Figure 3.2

Growth curve of V79 wild type cells as measured by <sup>3</sup>H-thymidine incorporation



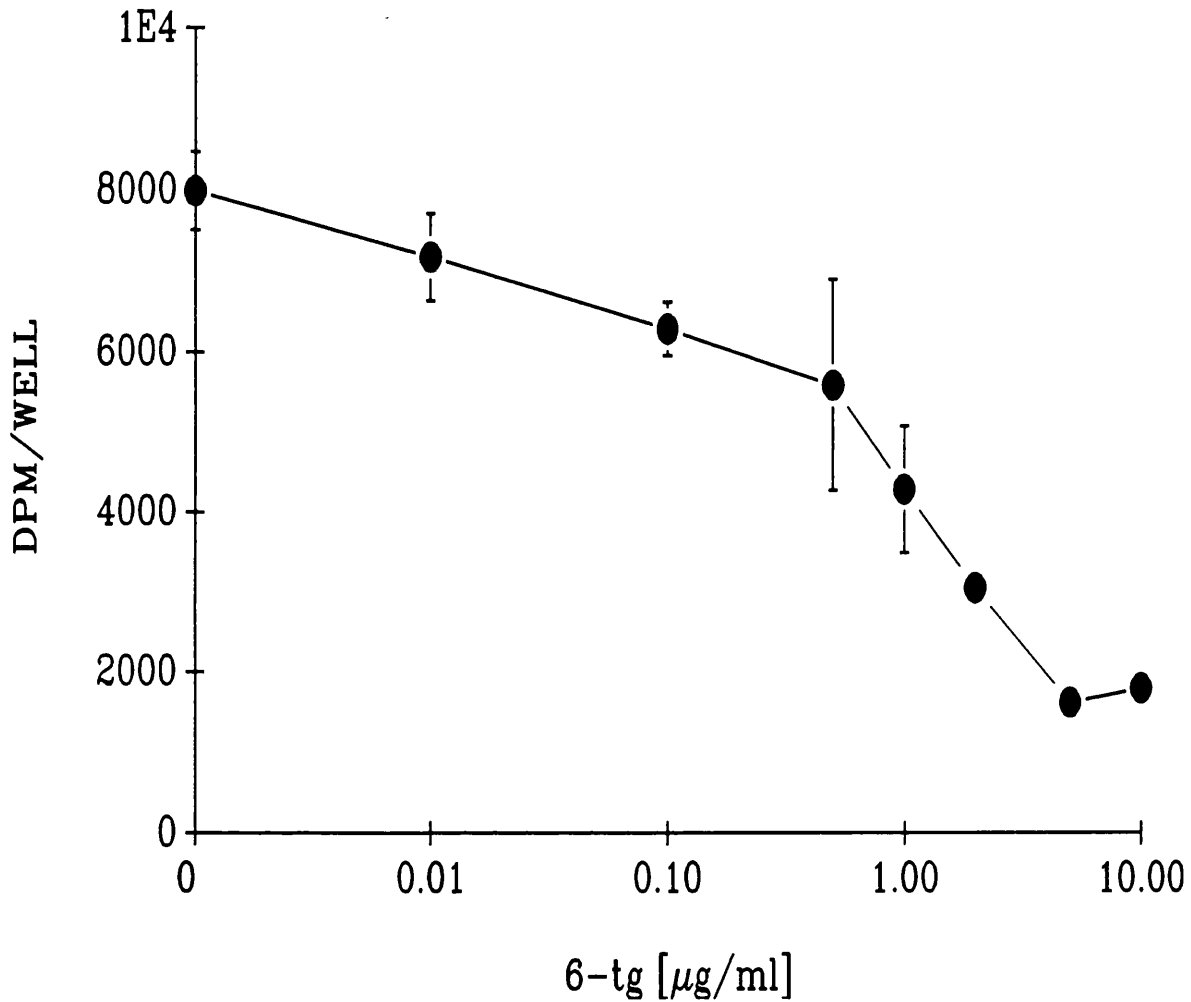
V79 wild type cells were plated at a density of 20 K per plate in 3.5 cm tissue culture plates. Every 24 hours a group of 3 plates was taken and tritiated thymidine incorporation was carried out for 4 hours. The incorporated radioactivity was then measured.

### 3.6. Effect Of 6-Tg On V79 Cells

The effect of 6-tg on these cell lines and the time required for this compound to act had to be elucidated. Both strains of the cells were treated with various concentrations of 6-tg for six hours to find an approximate concentration which is toxic to the wild type cell line but does not greatly affect the HGPRT<sup>-</sup> mutant (6-tg resistant) cell line. Figures 3.3a and 3.3b shows the effect of six hour exposure to 6-tg on wild type and 6-tg resistant cells respectively before incorporation of <sup>3</sup>H-thymidine. From this graph it is possible to see that a concentration of 1µg/ml of 6-tg is toxic to wild type cells but not to the mutant (resistant) cell line. The resistant cell line is unable to metabolize 6-tg (base analogue) and hence escapes the main toxicity of this compound which is due to incorporation into DNA. However, 6-tg can exert toxic effect on cells without being incorporated or metabolized (Berman et al. 1985) (see section 1.5.3.1). This could be one reason why 6-tg at high concentrations is toxic to the mutant cell line. The base analogue, 6-tg is also an inhibitor of 5-phosphoribosyl pyrophosphate (PRPP) amidotransferase and hence inhibits purine biosynthesis thereby exerting toxicity (Kornberg & Baker 1992). Another reason could be that exogenous 6-tg is causing an imbalance in the relative nucleotide pools. According to Reichard (1988) imbalance in nucleotide pools affects both DNA replication in S phase and DNA repair in resting lymphocytes resulting in cell death. In less extreme cases increasing the pool of one nucleotide caused point mutations and sister chromatid exchange in cultured cells.

Figure 3.3a

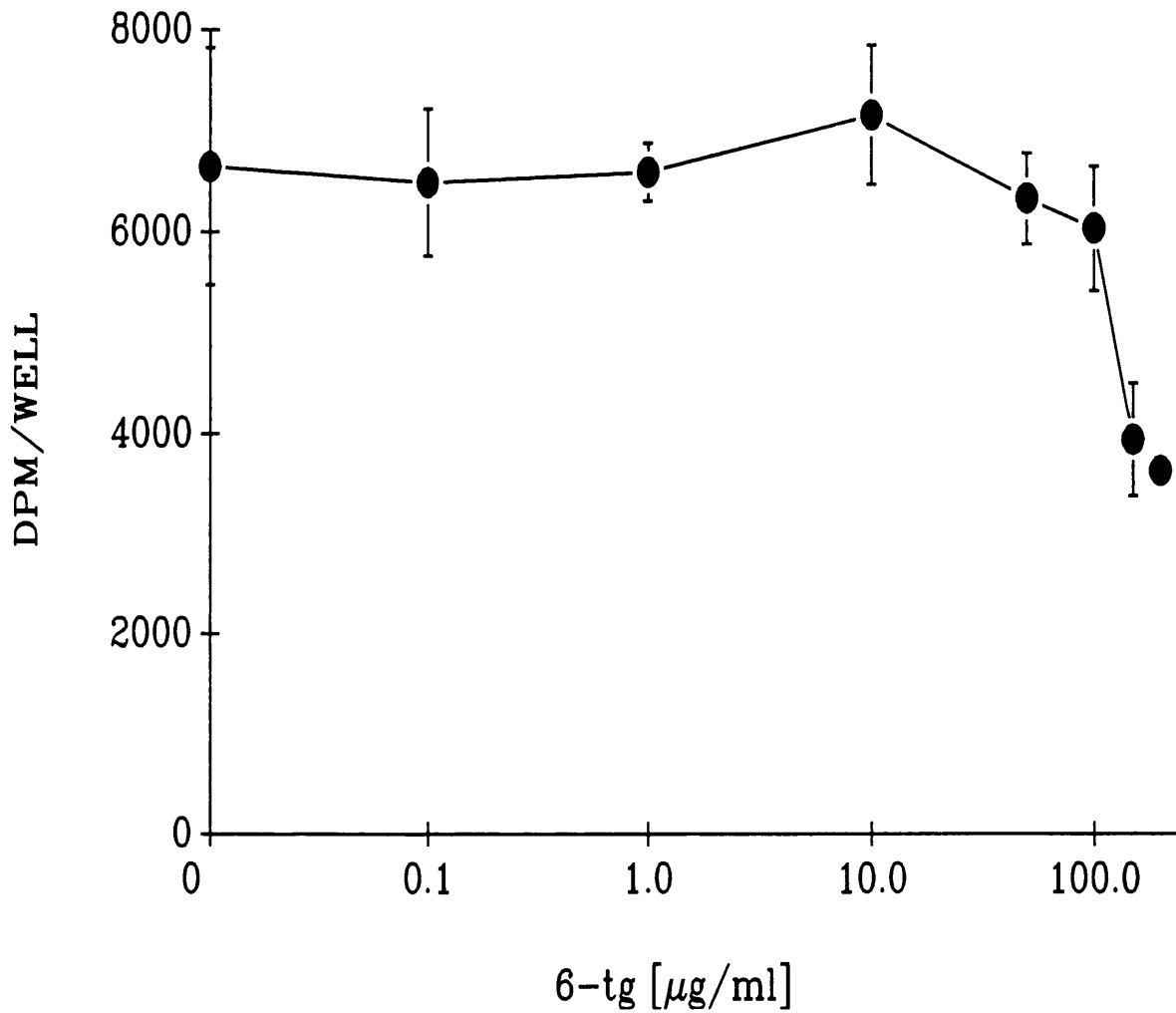
Effect of 6 hour 6-tg exposure on wild type cell growth and DNA synthesis



V79 wild type cells were plated at a density of 50 K per well in multiwell plates and left overnight. Medium containing various concentrations of 6-tg was added to groups of 3 wells for six hours. Tritiated thymidine without 6-tg was then incorporated for 4 hours and then the incorporated radioactivity was measured.

Figure 3.3b

Effect of 6 hour 6-tg exposure on resistant cell growth and DNA synthesis



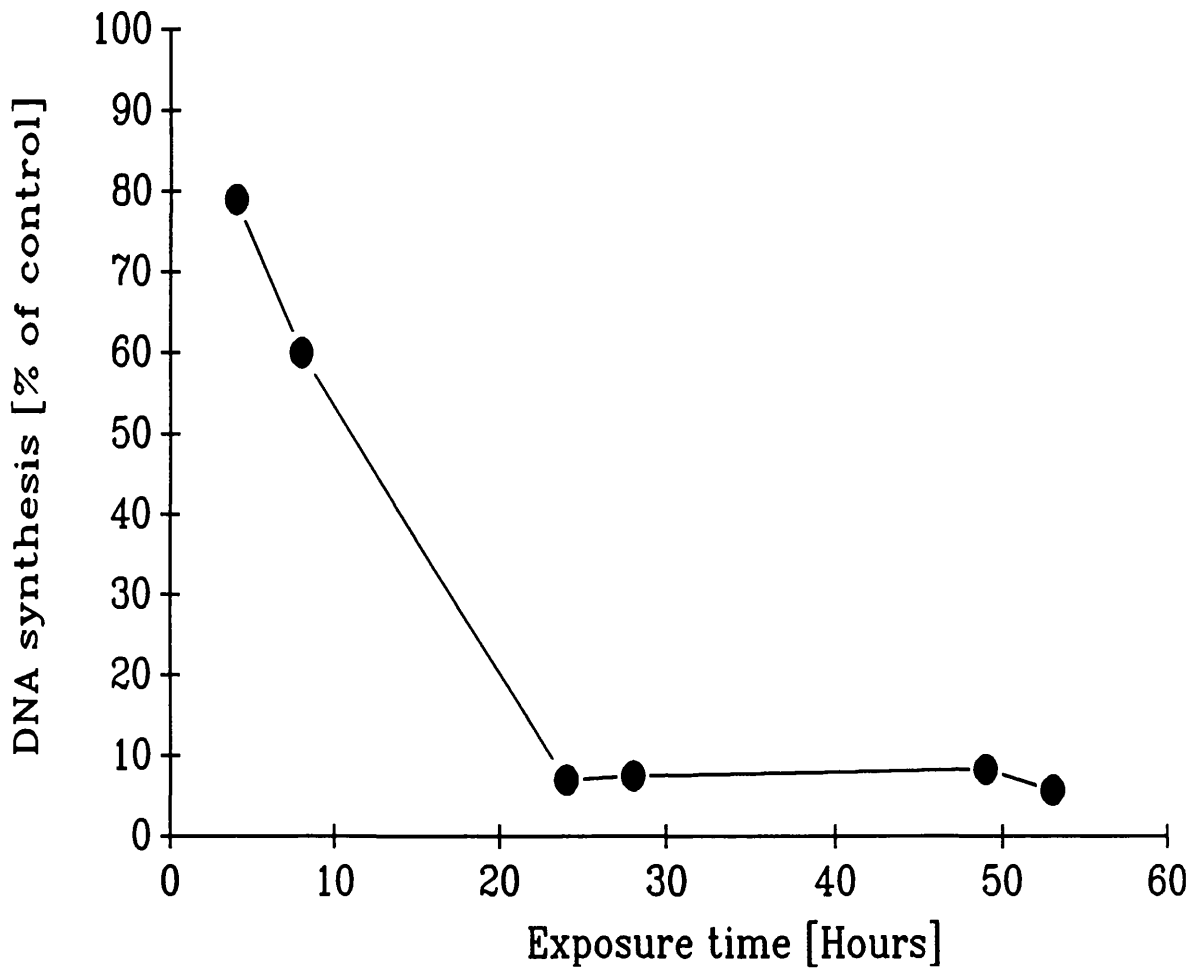
V79 6-tg resistant cells were plated at a density of 50 K per well in multiwell plates and left overnight. Medium containing different concentrations of 6-tg was added to groups of 3 wells for 6 hours. Tritiated thymidine was then incorporated for 4 hours and incorporated activity was measured.



The concentration of  $1\mu\text{g/ml}$  of 6-tg was then used to find a suitable time period of exposure to 6-tg which would reduce DNA synthesis to less than 10% in most wild type cells. In the next experiment, cell capacity to synthesis DNA was sampled at different time periods from four hours to over fifty hours after exposure to 6-tg. Figure 3.4 shows that 24 hour exposure to 6-tg is the best time period to use and reduces wild type cell DNA synthesis to under 10% of control cultures.

Figure 3.4

Time course of  $1\mu\text{g/ml}$  6-tg on wild type V79 cells



Wild type cells were plated at a density of 50 K and left overnight. They were then treated with  $1\mu\text{g/ml}$  6-tg. At intervals groups of 3 plates were taken and tritiated thymidine was incorporated for 4 hours. The incorporated activity was then measured.

### 3.7. Effect Of Short Exposure To 6-Tg

It is possible that treatment of the cultures with 6-tg for a shorter time period may accumulate sufficient 6-tg in the cells to inhibit DNA synthesis in the cells after a period of incubation. Figure 3.5 shows the effects on wild type cells of a short exposure of 6-tg followed by 24 hour incubation period to allow the 6-tg to act before carrying out <sup>3</sup>H-thymidine incorporation. A 90 minute exposure to 1 $\mu$ g/ml 6-tg followed by 24 hour incubation is sufficient reduce DNA synthesis to under 10% in most wild type cells. However, adding 6-tg for a short period and then removing it does not improve experimental design in any way. It adds an extra manipulation into the experiment and extra manipulations could introduce more errors into the results.

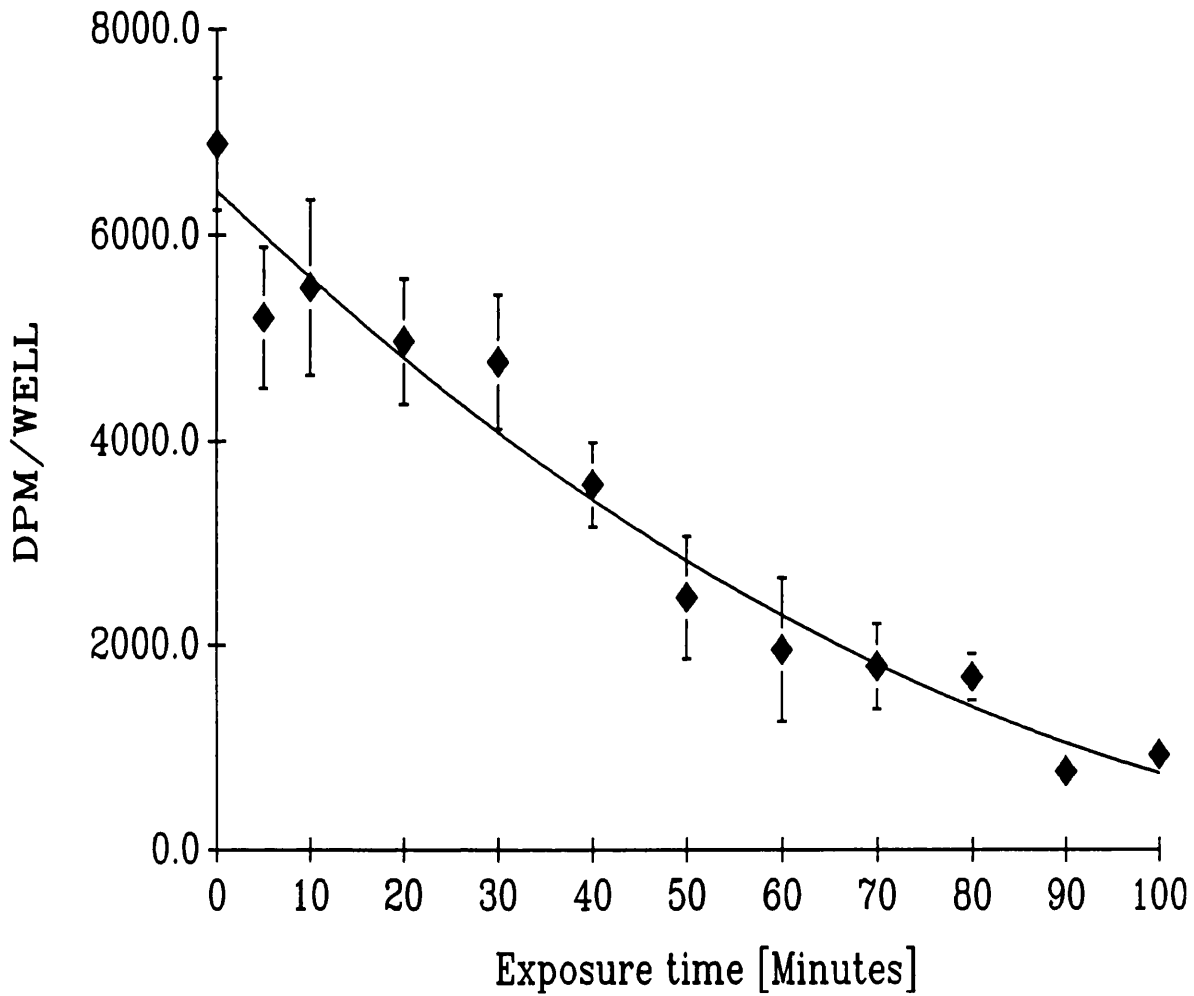
Having established a concentration of 6-tg to use and a reasonable period of exposure it was necessary to carry out a dose response curve again to further define the 6-tg toxicity. Figures 3.6a and 3.6b show the new dose responses of wild type and mutant cells to 6-tg respectively using the optimum 6-tg concentration and incubation period. Under these conditions 6-tg is one order of magnitude more toxic to the wild type cells.

### 3.8. Appearance Of Cells After 6-Tg Exposure

The V79 cells have an elongated shape when they are spreading over the culture surface. The membranes under light microscopy appear smooth. When 6-tg is added to wild type cells or co-culture the cells become more rounded and the membranes become crenulated. This occurs after about 10-12 hours after exposure to 6-tg. Some cells form blebs on the membrane and lift off from the culture surface.

Figure 3.5

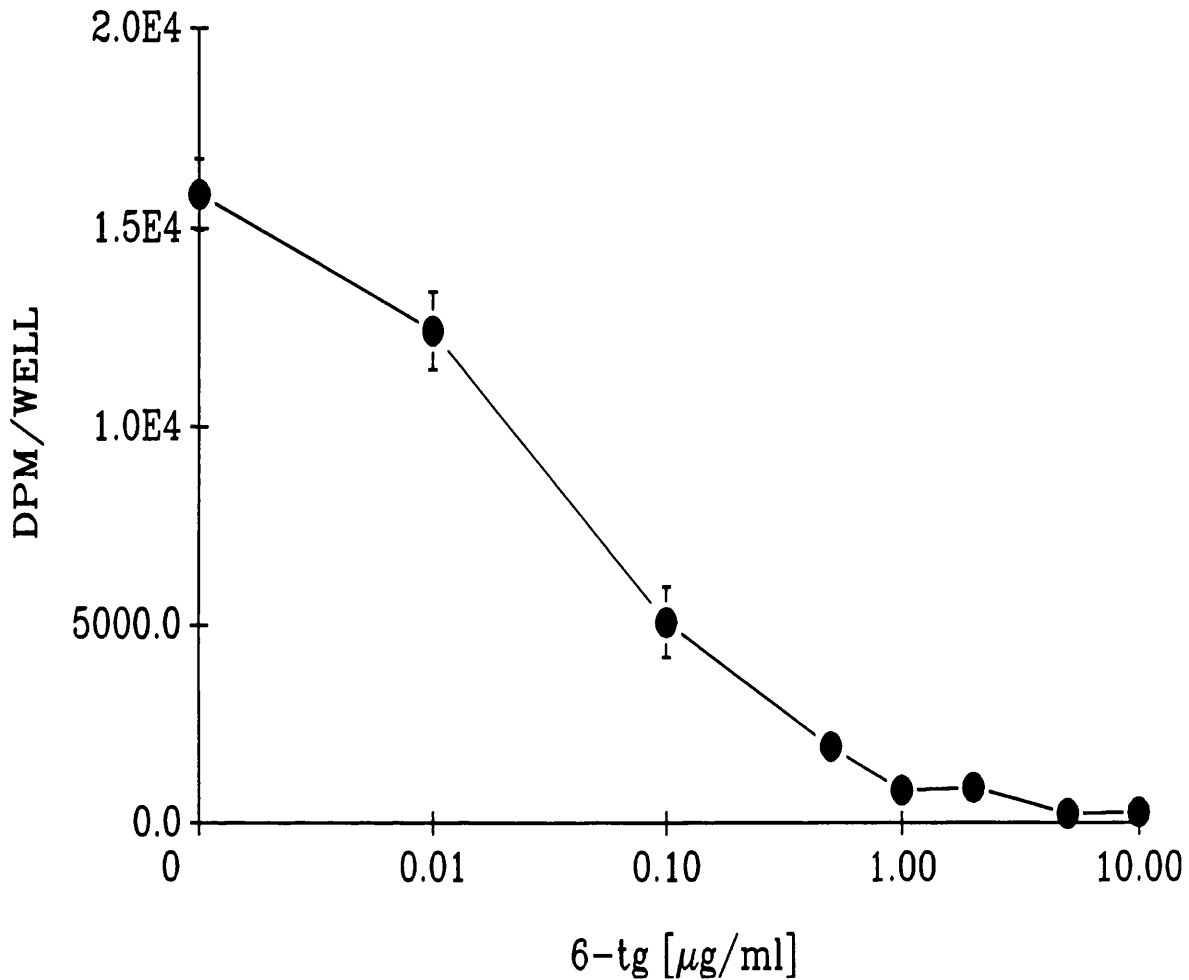
Effect of short exposure of 6-tg to wild type cells



Wild type cells were plated at a density of 50 K per well overnight. 6-Tg at  $1\mu\text{g/ml}$  was added for various time periods and then removed. The culture was then incubated in fresh medium until 24 hours had elapsed from the start of the experiment. Tritiated thymidine was then incorporated for 4 hours and then measured.

Figure 3.6a

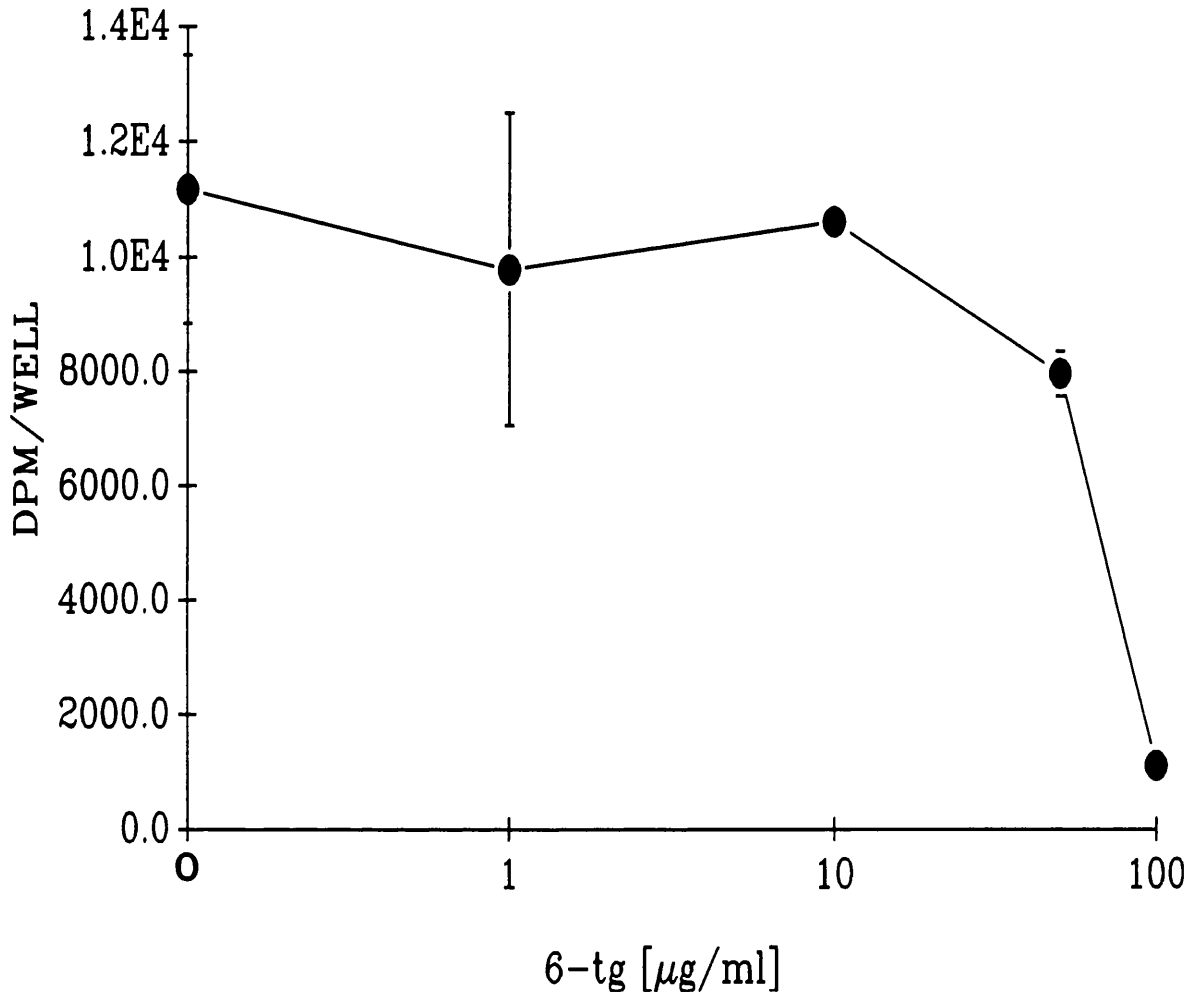
Effect of 24 hour 6-tg exposure on wild type cell growth and DNA synthesis



Wild type cells were plated at a density of 50 K per well overnight. 6-Tg at various concentrations was then added to the culture in triplicate and incubated for 24 hours. Fresh medium containing tritiated thymidine without 6-tg was then added. Tritiated thymidine incorporation was carried out for 4 hours and the incorporated activity was measured.

Figure 3.6b

Effect of 24 hour 6-tg exposure on resistant cell growth and DNA synthesis



V79 resistant cells were plated at a density of 50 K per well overnight. 6-tg at various concentrations was added to the culture in triplicate and incubated for 24 hours. Fresh medium containing tritiated thymidine without 6-tg was then added. Incorporated was carried out for 4 hours and the incorporated activity was measured.

### 3.9. Demonstration Of Communication Between Wild Type And Mutant Cells.

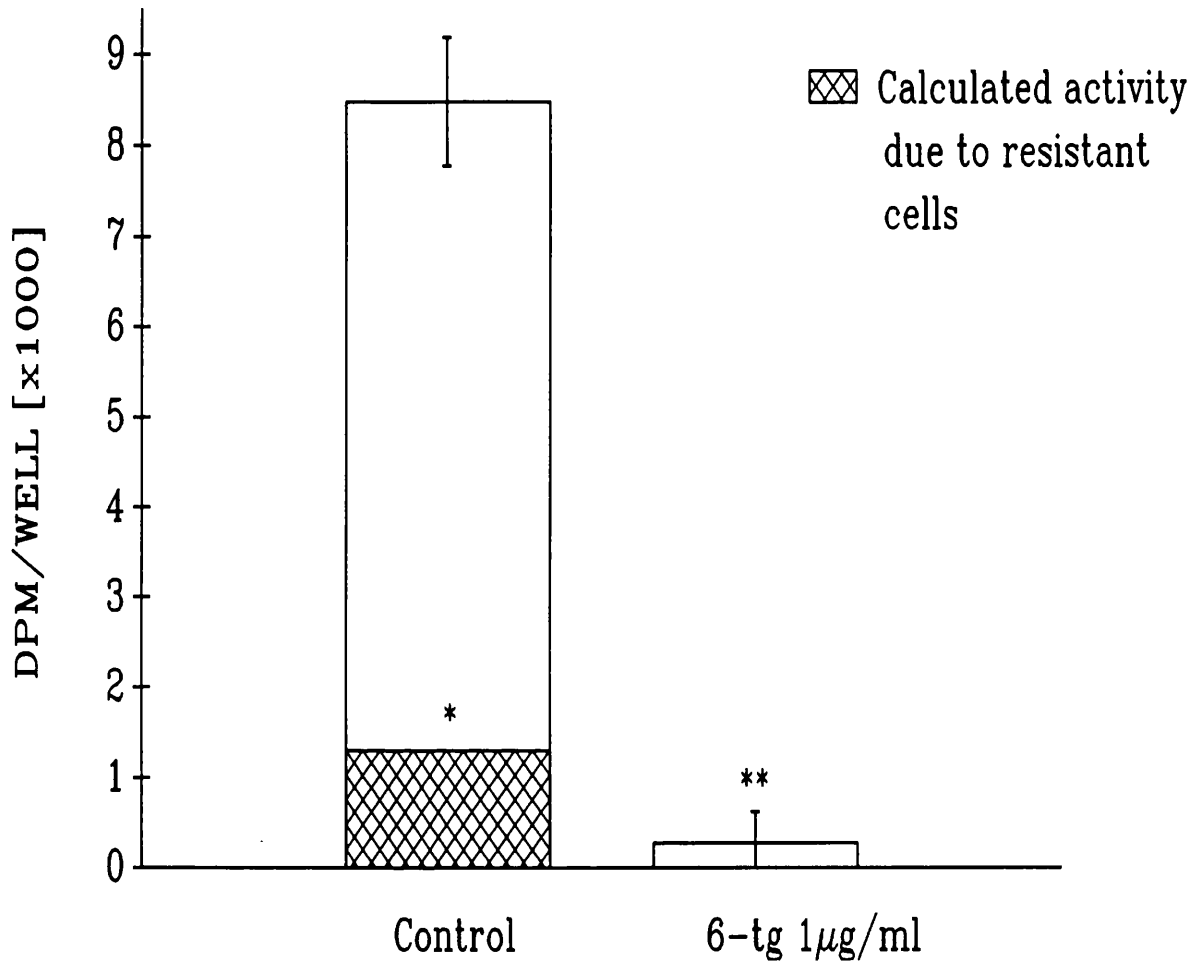
To demonstrate intercellular communication, resistant cell DNA synthesis has to be inhibited by the toxic metabolite transferred through gap junctions from sensitive cells in contact with the resistant cells. In order to demonstrate this one resistant cell to every five sensitive cells were plated to ensure adequate transfer of the metabolite. The co-culture was then incubated for 24 hours for the cells to stick down on the tissue culture surface, grow together and form gap junctions. 6-Tg was then added to the co-culture at 1 $\mu$ g/ml and the culture was incubated for a further 24 hours. In the absence of cell communication, the radioactive counts from the resistant cells would be expected to amount to about 17% of the control value because this is the percentage of resistant cells in the co-culture. However figure 3.7 shows that treatment of the co-culture with 1 $\mu$ g/ml 6-tg reduces the resistant cells capacity to synthesis DNA to about 3%. This shows that resistant cells are affected by 6-tg profoundly and intercellular communication is taking place.

The experiment gave a result demonstrating intercellular communication. However, since the number of resistant cells plated is small the potentially incorporated radioactivity is also small compared to control values. Plating errors and errors in recovery of incorporated radioactivity could produce large variability in the results. It would be better if a 1:1 ratio could be used to demonstrate junctional communication and then be able to inhibit it. The greater number of resistant cells plated would incorporate more radioactivity if communication is inhibited and there would be less ambiguity in the results.

A dose response of 12-O-tetradecanoyl phorbol ester (TPA) on wild type cells was carried out to find a concentration of TPA to use for inhibiting junctional communication. This showed (figure 3.8) that TPA upto 50 ng/ml does not adversely affect cell growth and DNA synthesis in these cells.

Figure 3.7

### Demonstration of intercellular communication

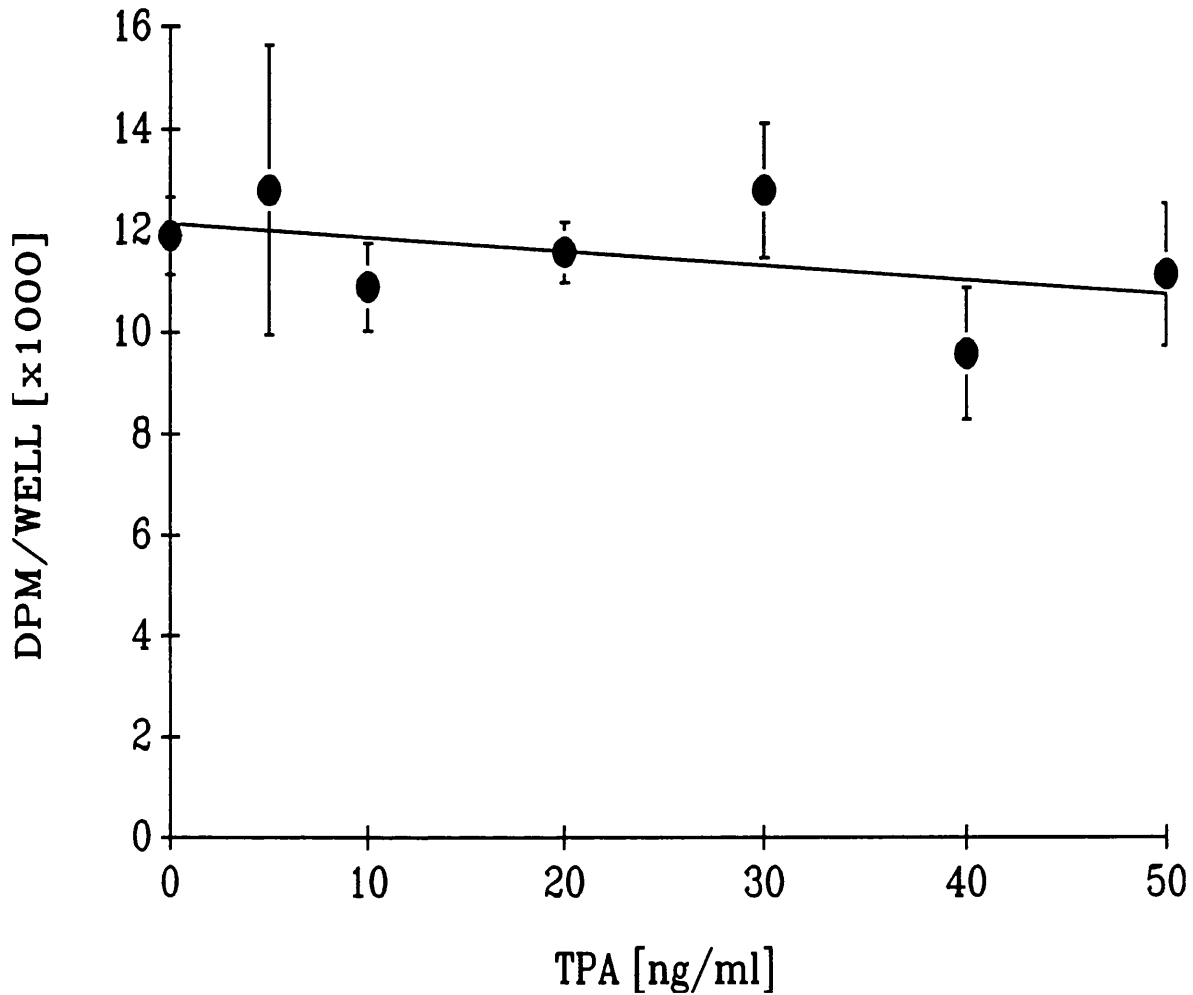


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 5:1 ratio at 30 K total density of cells. 24 hours later 1µg/ml of 6-tg was added to appropriate group of 4 wells. Controls were treated with solvent. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis. The calculated resistant cell value (\*) and the 6-tg treated group (\*\*) are significantly different.  $p=0.018$



Figure 3.8

Effect of TPA on growth & DNA synthesis of V79 cells



Wild type cells were plated at a density of 50 K per well and left overnight. Old medium was removed and medium containing different concentrations of TPA was added to appropriate groups of wells. Controls were treated with solvent. After 24 hours exposure, the TPA containing medium was removed and medium containing 0.1 $\mu$ Ci/ml tritiated thymidine was added. After 4 hours of incubation the amount of tritiated thymidine incorporated was then measured.

Figure 3.9a and 3.9b shows the results of an experiment using 1:5 and 1:1 ratio of resistant and sensitive cells at a total density of 30,000 cells. It can be seen that a 1:1 ratio gives results which demonstrate intercellular communication and show its inhibition using 50 ng/ml TPA. The next step was to find the optimum cell number to plate, the optimum 6-tg concentration to use and the optimum TPA concentration which will produce the maximal inhibition of gap junctional communication in this co-culture system.

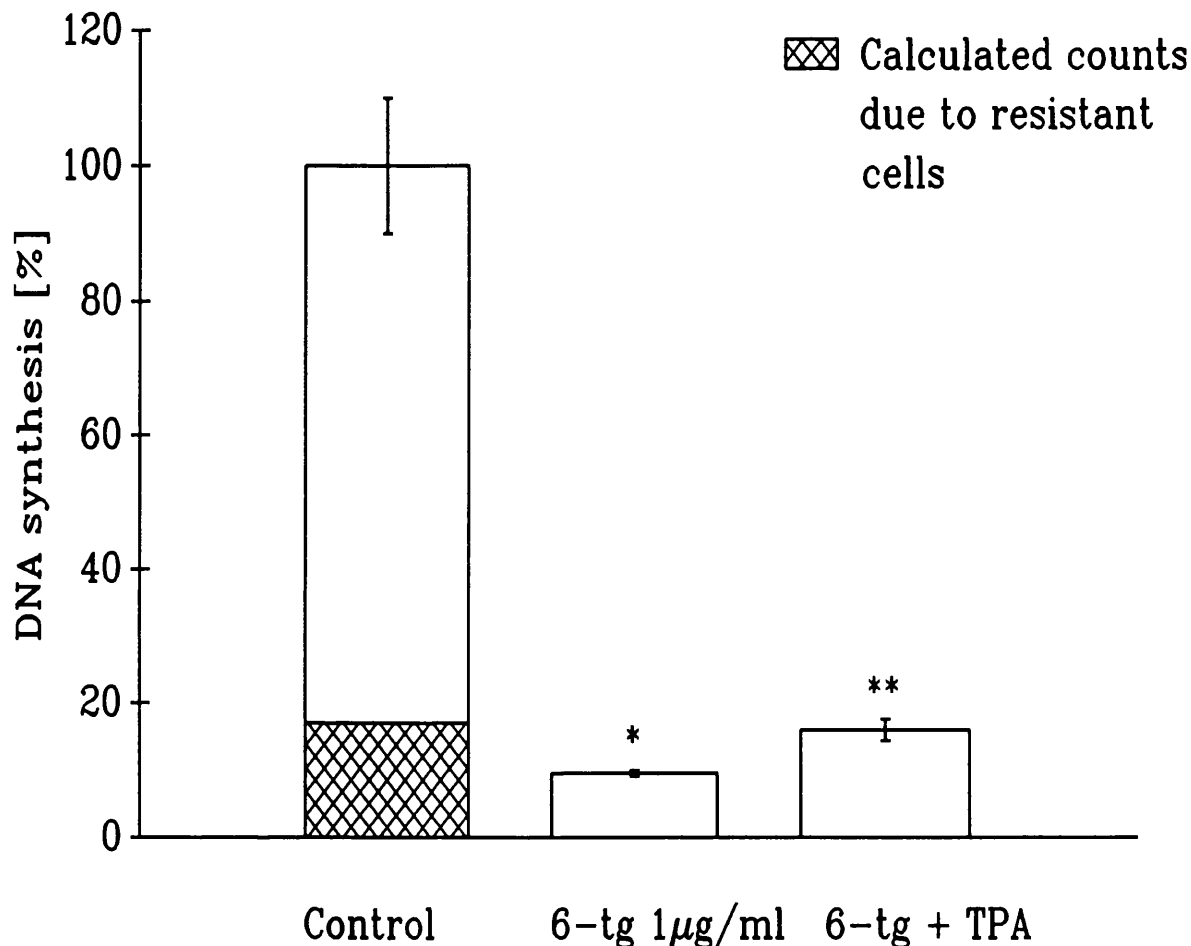
It was found by visual observation that plating  $6 \times 10^4$  cells on 24 well plates for 24 hours provided good cell to cell contact as seen in figure 3.10. The culture also has sufficient time to form gap junctions. Therefore resistant and sensitive cells were plated in a 1:1 ratio at a total density of  $6 \times 10^4$  cells. The cultures were grown for 24 hours before being treated with 6-tg or 6-tg and TPA.

Figure 3.11a shows that treatment of co-culture with  $1 \mu\text{g/ml}$  6-tg for 24 hours does not reduce resistant cell DNA synthesis to any appreciable degree below the 50% theoretical value that would be found if no resistant cell were affected. A separate culture of 30,000 resistant cells cannot be used as a baseline because the growth characteristics would be different from that of the resistant cells in a co-culture of 60,000 cells.

However,  $10 \mu\text{g/ml}$  6-tg reduces co-culture DNA synthesis to about 30% of control value as seen in figure 3.11b. Therefore,  $10 \mu\text{g/ml}$  6-tg is a suitable concentration to use. This experiment also shows the optimum concentration of TPA to use. Figure 3.11b shows that  $20 \text{ng/ml}$  TPA produces the maximal inhibition of gap junctional communication and this concentration of TPA was selected to be used in future experiments.

Figure 3.9a

Cell communication in 1:5 co-culture of V79 resistant and sensitive cells

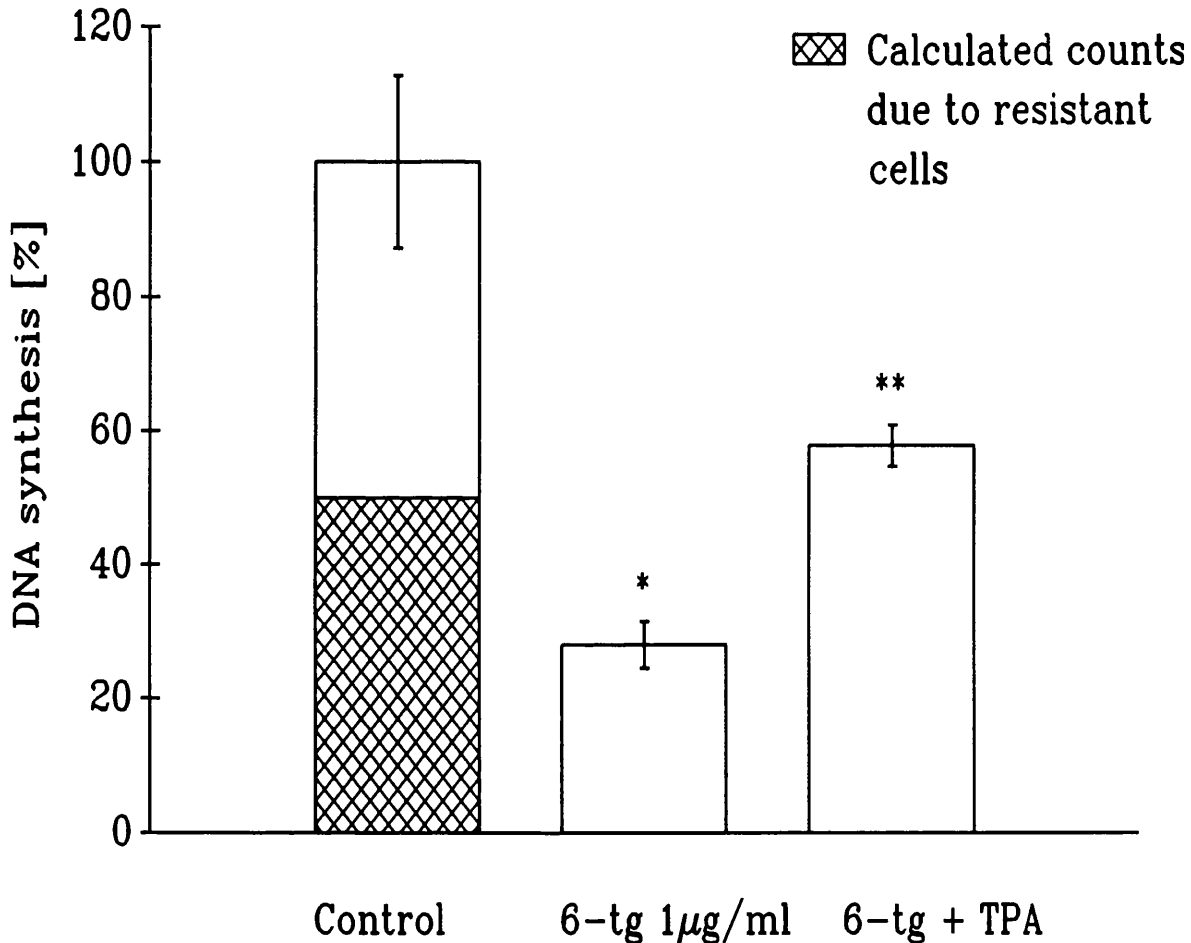


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:5 ratio at a total density of 30 K cells. 24 hours later 1µg/ml of 6-tg with 50ng/ml TPA was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and 1 ml of this medium was added to the cell culture. Solvent controls (0.02% v/v DMSO) and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA group (\*\*) are significantly different.  $p=3.3 \times 10^{-3}$

Figure 3.9b

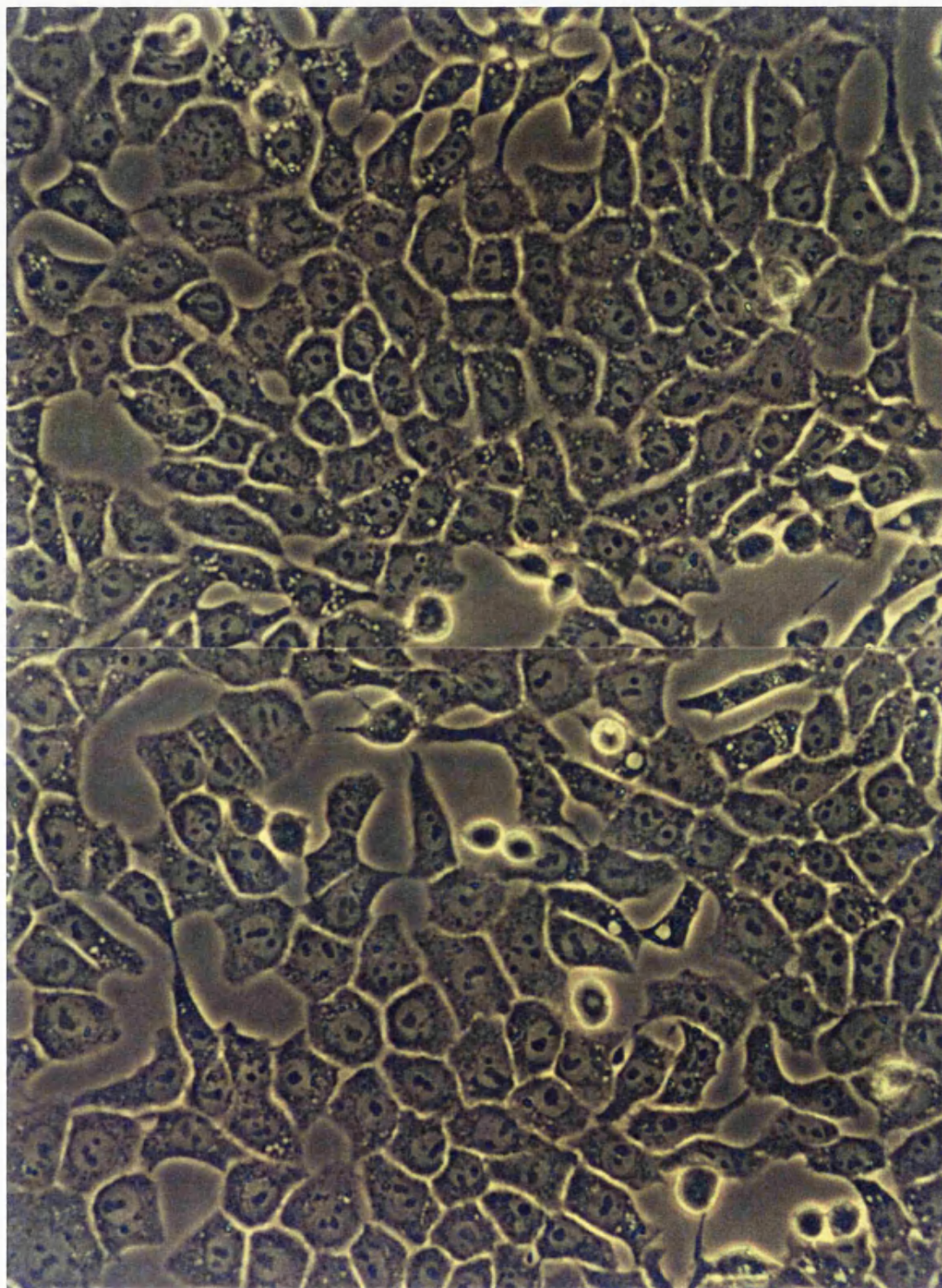
Cell communication in 1:1 co-culture of V79 sensitive and resistant cells



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 30 K cells. 24 hours later 1µg/ml of 6-tg plus 50ng/ml TPA was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA group (\*\*) are significantly different.  $p=5.1 \times 10^{-3}$

Figure 3.10

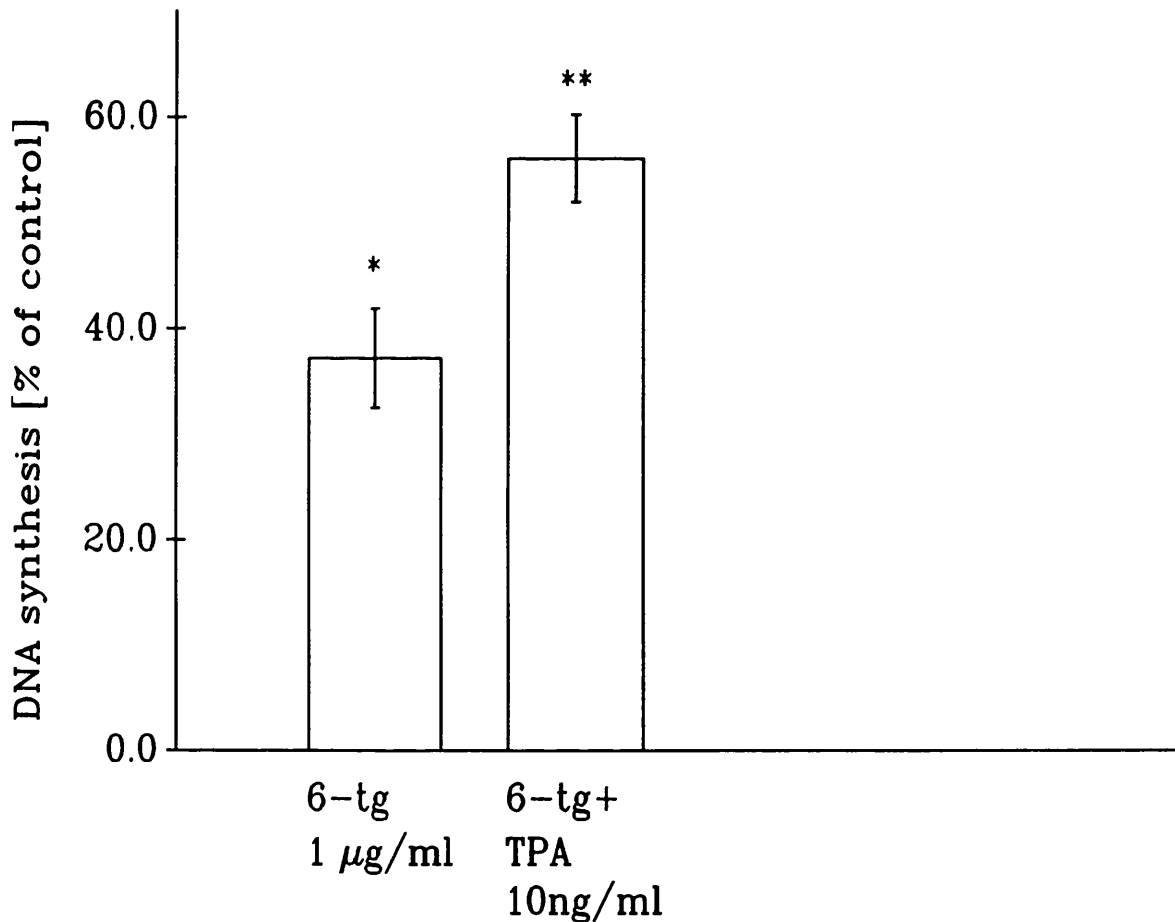


10  $\mu\text{M}$

V79 cells plated at a density of 60 K per well for 24 hours

Figure 3.11a

Effect of TPA on metabolic cooperation  
using  $1\mu\text{g}/\text{ml}$  6-tg

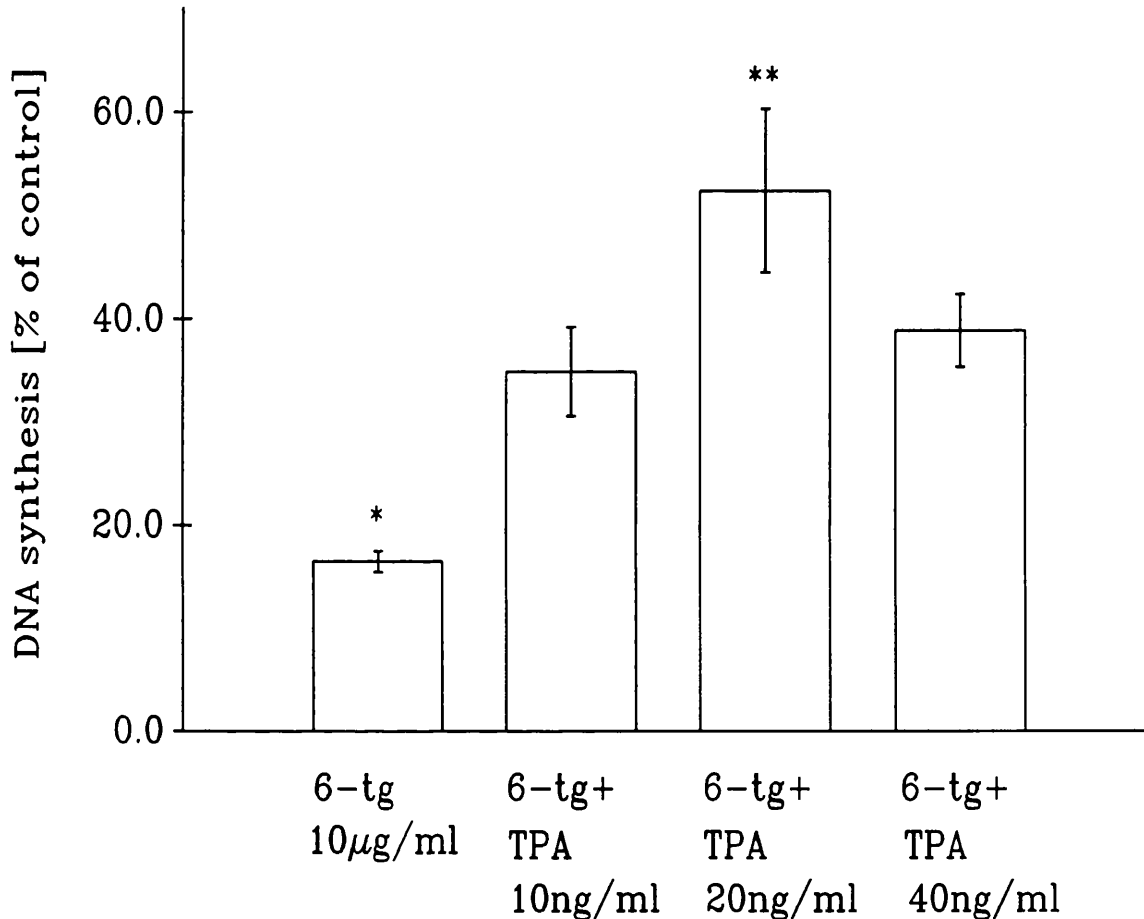


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later  $1\mu\text{g}/\text{ml}$  of 6-tg plus  $10\mu\text{g}/\text{ml}$  TPA was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing  $0.1\mu\text{Ci}/\text{ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA group (\*\*) are not significantly different.  $P=0.08$

Figure 3.11b

Effect of different concentrations of TPA on metabolic cooperation using 10  $\mu\text{g}/\text{ml}$  6-tg



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu\text{g}/\text{ml}$  of 6-tg plus a range of concentrations of TPA was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu\text{Ci}/\text{ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis.

The 6-tg control groups (\*) and the 6-tg+TPA 20ng/ml group (\*\*) are significantly different.  $P=0.01$

When passage number of cells increases their growth characteristics change. Hence, cells were discarded after passage number 40 had been reached. Replacement cells were thawed out from original frozen stocks. The sensitivity of the cells to TPA changed as the batches were changed. Generally, most batches responded to 20ng/ml TPA.

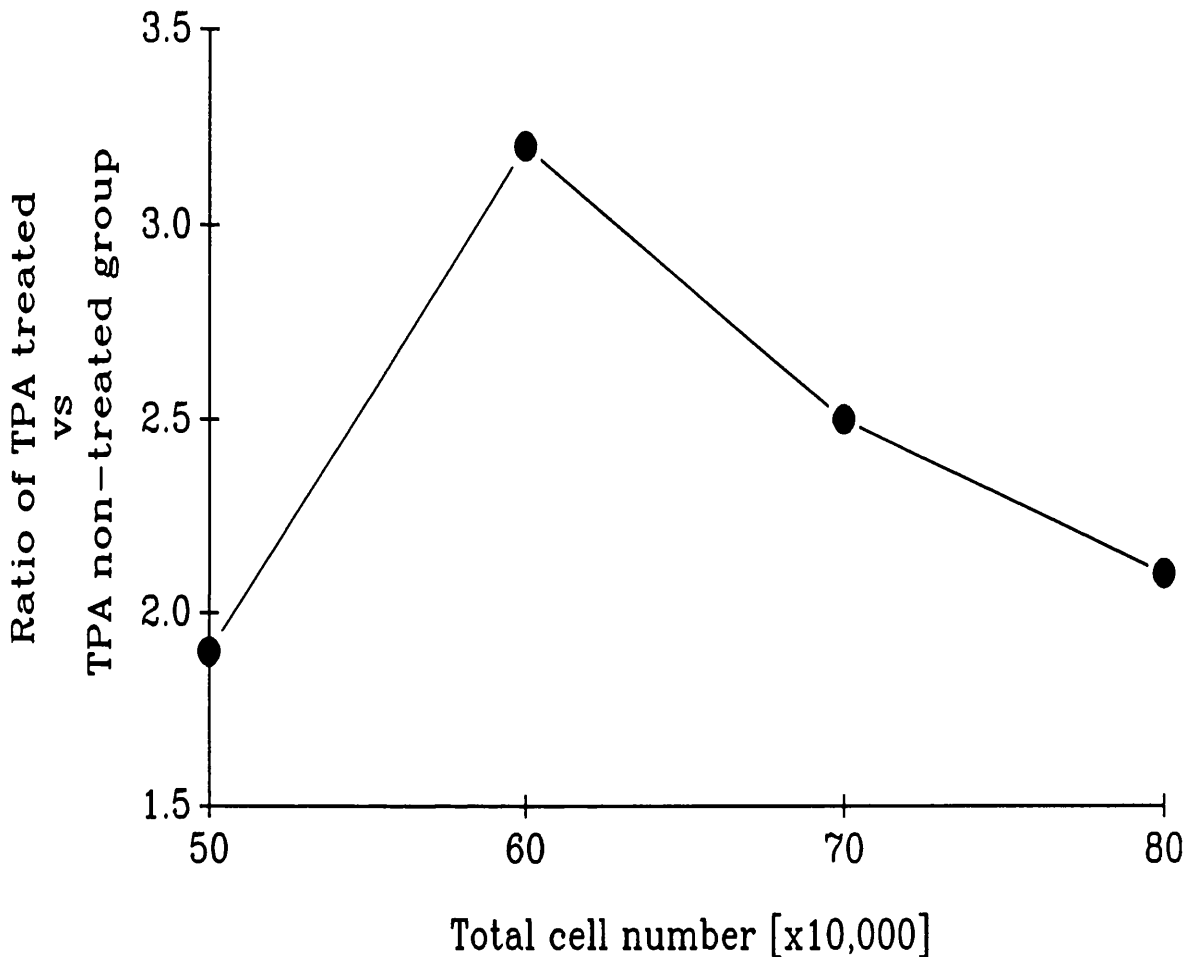
The biochemical confirmation of the visual observation that  $6 \times 10^4$  is a optimum cell number to plate can be seen in figure 3.12. In this experiment co-cultures were plated at increasing densities and communication was inhibited using TPA. It was seen that TPA produces the maximal inhibition of junctional communication when a total of  $6 \times 10^4$  cells is plated. At this density, the ratio of resistant cell DNA synthesis treated with 6-tg versus 6-tg plus TPA has revealed its maximum value.

Further to the characterization of the cells, the relative  $^3\text{H}$ -thymidine incorporation values of resistant and sensitive cells was investigated. Figure 3.13 shows that resistant and sensitive cells have similar incorporation values. A 1:1 co-culture of resistant and sensitive cells give a value similar to  $6 \times 10^4$  cells of either type. Therefore both cell lines have similar plating efficiencies. This finding is important because it shows that co-plating both cell types does not inhibit the growth of the co-culture. This suggests that when TPA does not inhibit junctional communication maximally, the reduced inhibition of communication is a real effect and not due to too few resistant cells present in the co-culture.



Figure 3.12

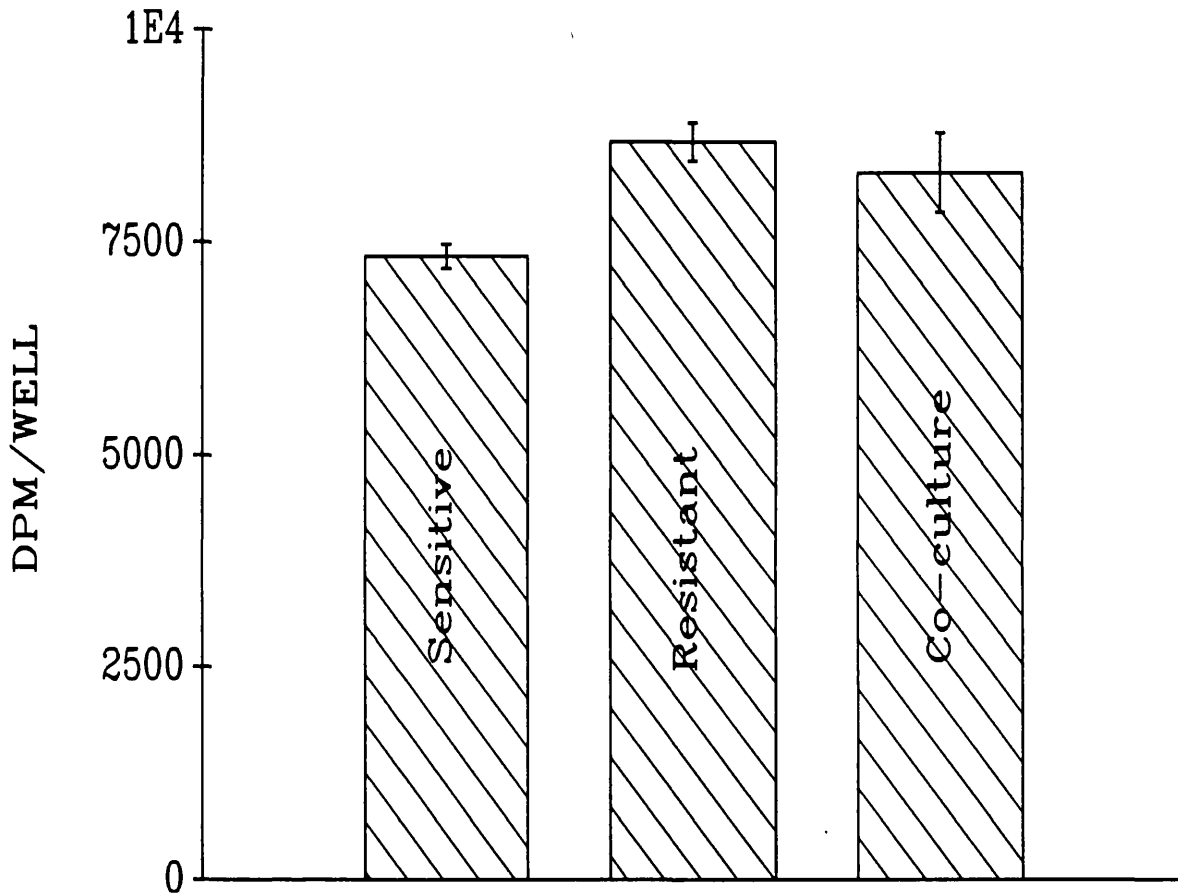
Effect of density on ability of TPA to inhibit cell communication



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at various densities. 24 hours later 10 $\mu$ g/ml of 6-tg plus 20ng/ml TPA was added to groups of 4 wells for each density. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu$ Ci/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative DNA synthesis.

**Figure 3.13**

Relative <sup>3</sup>H-thymidine incorporation by  
60 K sensitive, resistant and co-culture  
of V79 cells



Wild type, resistant and 1:1 co-culture was plated at a total density of 60 K cells per well for 24 hours. Tritiated thymidine was then incorporated for 4 hours and the incorporated activity was then measured.

### 3.10. Timecourse Of Toxic Metabolite Transfer

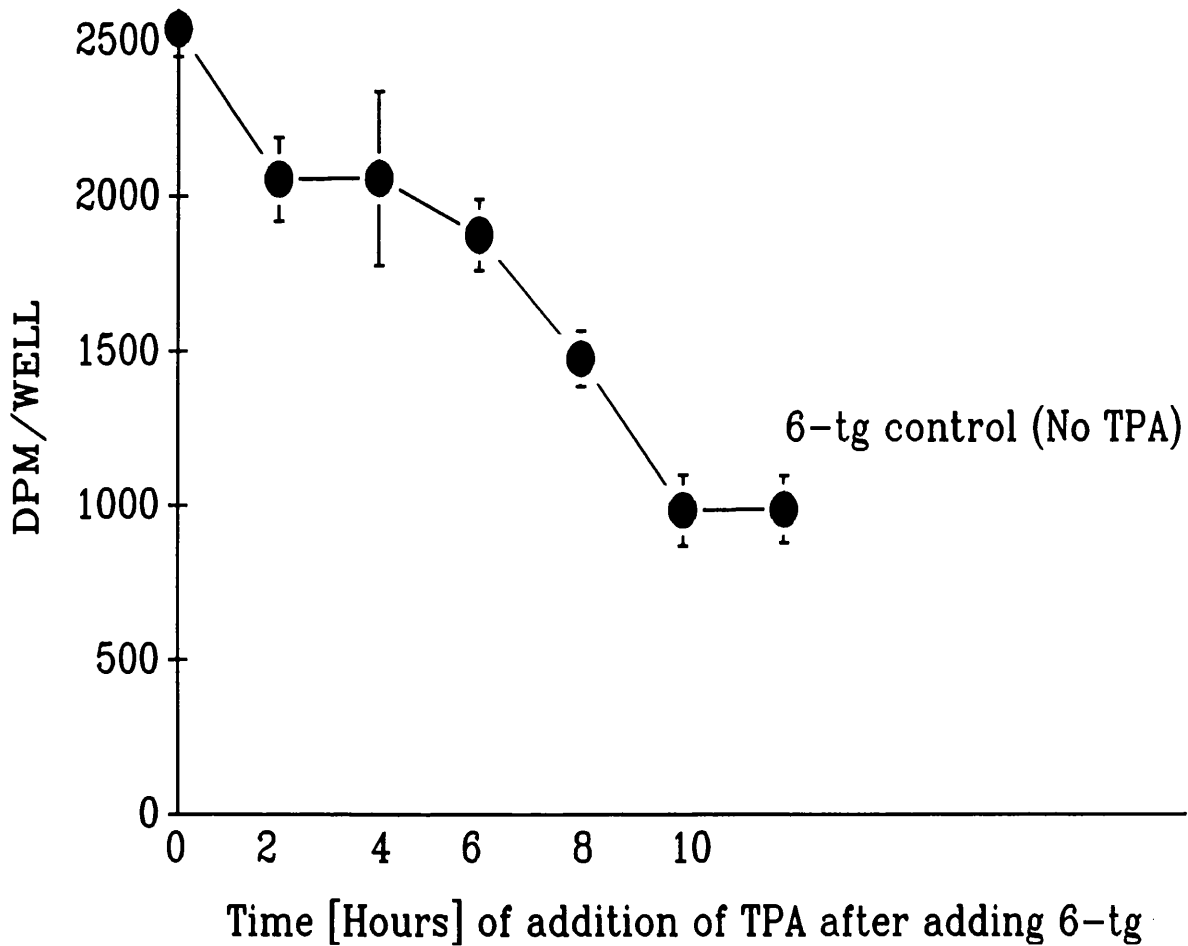
The drug exposure protocol in the metabolic cooperation assay developed by Yotti et al. (1979) requires that four hours after plating, the co-culture should be pre-exposed to the compound of interest for one hour. 6-Tg should then be added.

Finding out how long it takes for the toxic metabolite to be transferred from the wild type to the resistant cells in the present system could be useful. If interrupting communication after 6-tg exposure were to have any effect on resistant cell DNA synthesis it would allow application of a chemical which proves toxic during prolonged exposure to be delayed and still be able to show the inhibition of junctional communication. Therefore, co-cultures were treated with 6-tg and communication was inhibited by adding TPA at various times afterwards. Figure 3.14 shows that the metabolite starts to be transferred to the resistant cells within two hours. It requires about ten hours after which interrupting communication has no protective effect on resistant cell DNA synthesis. This could be because sufficient metabolite has been transferred to inhibit DNA synthesis in these cells. Interrupting junctional communication after 5 hours of 6-tg exposure can still afford some protection to the resistant cells.

In the present metabolic cooperation method TPA is added with the 6-tg. This means that TPA must inhibit junctional communication within 2 hours. After 2 hours 6-tg starts to take effect.

Figure 3.14

Inhibition of cell communication in co-culture by adding TPA at intervals after the addition of 6-tg



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu$ g/ml of 6-tg was added to the wells. TPA at 20ng/ml was then added to groups of 4 wells at staggered intervals. Solvent and 6-tg controls were also treated. After 24 hours from start of 6-tg treatment, fresh medium containing tritiated thymidine was substituted and cultures were incubated for a further 4 hours. The incorporated radioactivity was then measured.

### 3.11. Comparison Of Colorimetric And <sup>3</sup>H-thymidine Incorporation Methods As Ways Of Measuring Cell Viability

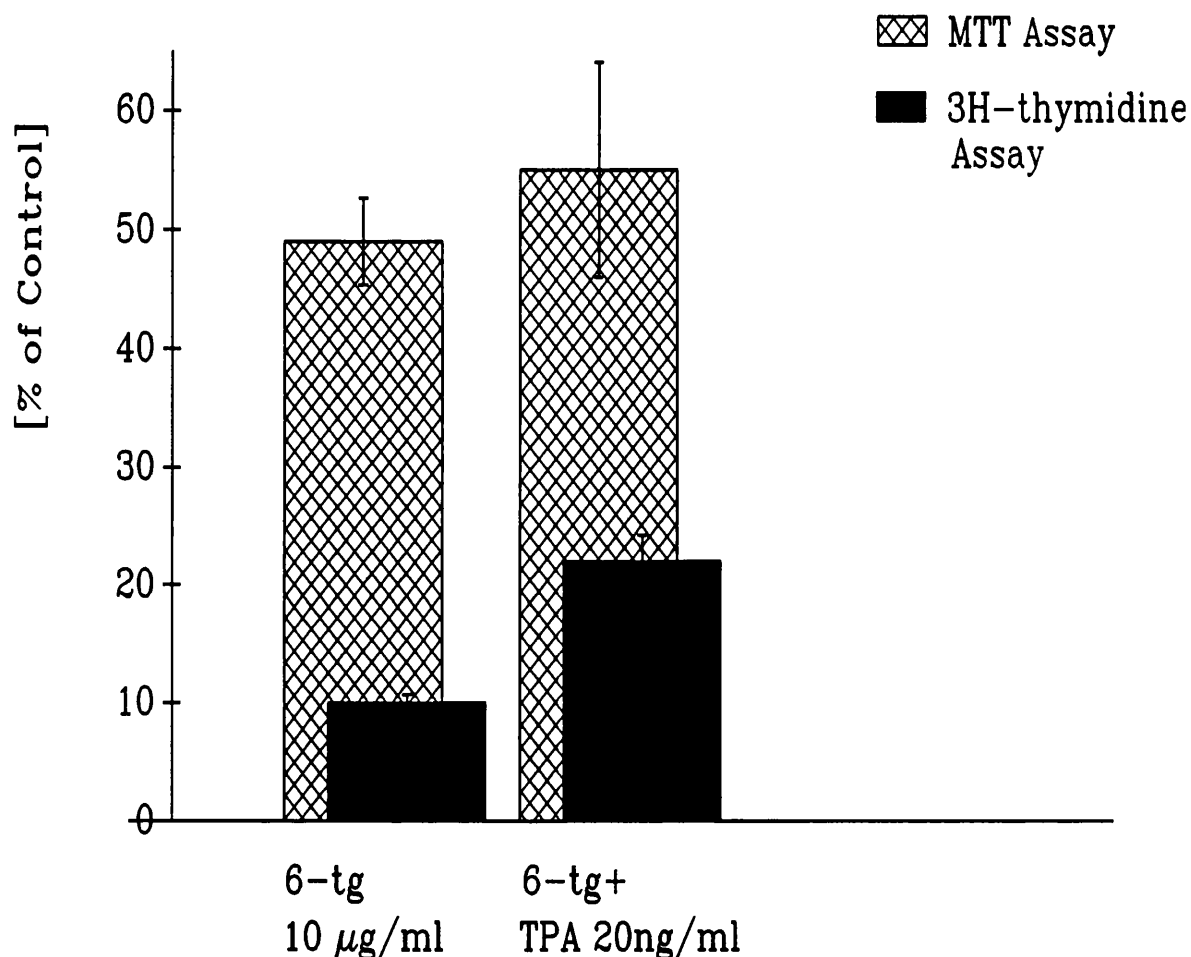
Alternative methods for measuring cell viability were investigated to see if they offer any advantages over DNA synthesis. There is a colorimetric assay available for measuring cell viability and is based on the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The tetrazolium ring of MTT is cleaved by dehydrogenase enzymes in active mitochondria to give a coloured compound and hence the reaction should only occur in living cells (Mossman 1983; Green et al. 1984). Measuring the amount of purple formazan produced using a manual spectrophotometer or an automated multiwell plate scanning spectrophotometer gives a measure of the cell cultures growth and viability. This colorimetric assay has the advantage of not using radioactive isotopes and large number of samples can be assayed.

**Figure 3.15 shows the tritiated thymidine and MTT assay data together.** The uptake of radioactive thymidine and hence DNA synthesis by cells in co-culture is reduced by treatment with 6-tg to about 10% of control. The MTT assay shows cell viability in the 6-tg treated group to be about 50% of control. This would suggest that only the wild type cells which constitute 50% of the culture are being injured.

When the base analogue 6-tg is incorporated into DNA it prevents further replication of the cellular DNA (Kornberg & Baker 1992). Therefore, DNA synthesis measurement by <sup>3</sup>H-thymidine incorporation is a better method to use because it measures the first step leading to cell death. Mitochondria may take a longer time to stop functioning though the cell may be unable to replicate DNA.

Figure 3.15

### Comparison of MTT and 3H-thymidine assays



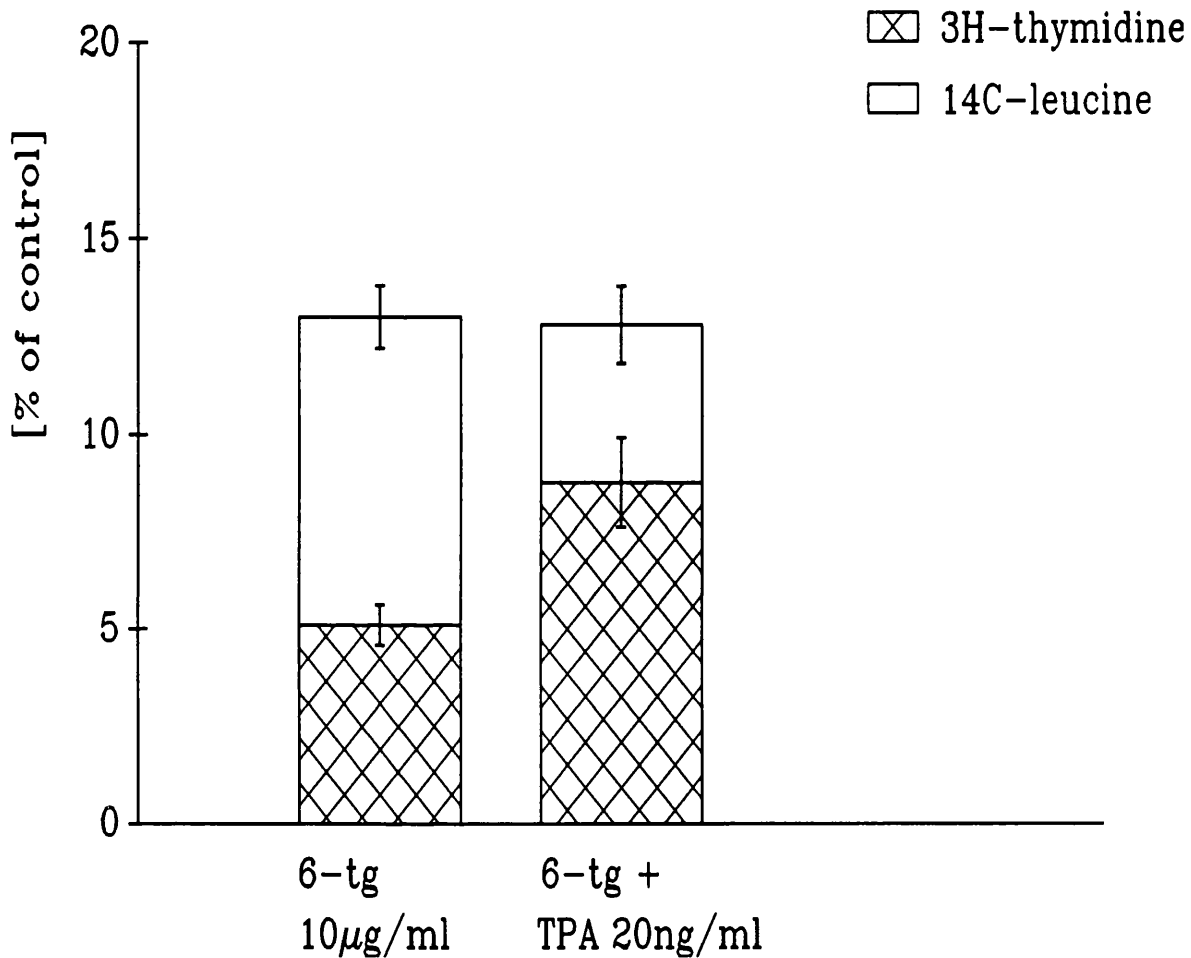
A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10μg/ml of 6tg was added to one group of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls were also treated. A positive control group was treated with 20ng/ml TPA plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1μCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured. The MTT reagent was added to wells in a parallel experiment for two hours. The amount of formazan dye produced was then measured.

### 3.12. Protein Synthesis As A Measure Of Cell Viability

Measuring protein synthesis by  $^{14}\text{C}$ -Leucine incorporation could be an alternative method for assaying cell viability. Protein synthesis may be a less sensitive indicator of transfer of toxic nucleotide analogue but may have some advantage. It was found that protein synthesis is not as sensitive as DNA synthesis when looking at transfer of 6-tg metabolite from wild type to resistant cells as seen in figure 3.16. Measuring protein synthesis shows greater degree of cell viability than measuring DNA synthesis. This was expected because any treatment which affects DNA synthesis is going to take time to affect protein synthesis. Protein synthesis does not always require new synthesis (transcription) of messenger RNA from DNA as there is a pool of transcribed Messenger RNA in the cells. Given sufficient time protein synthesis may show the same trend as DNA synthesis measurement because protein synthesis is a few steps downstream from DNA. Therefore, it is better to use a method which measures the early effects of toxic 6-tg metabolite transfer. This way the final outcome will be realized earlier.

Figure 3.16

Comparison of  $^3\text{H}$ -thymidine and  $^{14}\text{C}$ -leucine incorporation



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu\text{g}/\text{ml}$  of 6tg was added to one group of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls were also treated. A positive control group was treated with 20ng/ml TPA plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu\text{Ci}/\text{ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured. A parallel experiment was conducted in which 0.5 $\mu\text{Ci}/\text{ml}$   $^{14}\text{C}$ -leucine was incorporated for 30 minutes instead of thymidine.



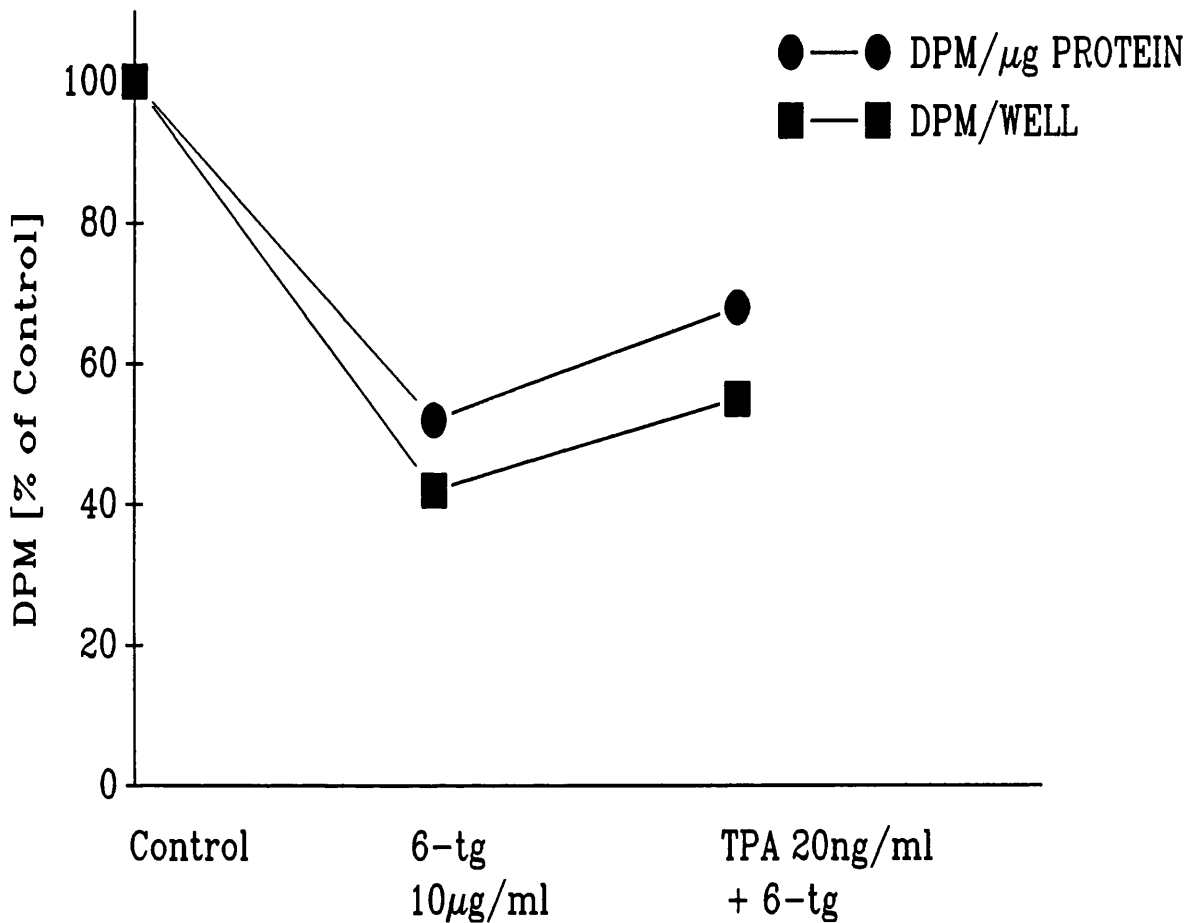
### 3.13. Effect Of Quantifying Results Per Total Protein

Expressing incorporated radioactivity in relation to total protein could help in the interpretation of results. If the cells are not injured by 6-tg but merely inhibited from incorporating tritiated thymidine or float off into the medium then this would show up in the data. Experiments were carried out to find out if measuring protein content of wells and expressing the results as per  $\mu\text{g}$  protein has an impact on the trend of the results.

Figure 3.17 shows that expressing the results as DPM/well or DPM/ $\mu\text{g}$  protein has no significant effect on the results. The overall information that can be derived from the data is not altered by expressing the results in either way. This means that measuring protein content of each sample for every experiment would be superfluous.

Figure 3.17

Choice of denominator does not affect trends in results



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10μg/ml of 6tg plus 20ng/ml TPA was added to group of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1μCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis. A fraction of the cell solution was taken and protein determination was carried out.

### 3.14. Discussion

TPA has wide ranging and diverse effects on cells. It can act as an agonist in the inositol triphosphate cellular signal transduction pathway to activate protein kinase C (Berridge & Irvine 1984) which is one of the intracellular receptors for this phorbol ester (Nishizuka 1984a; 1984b; 1988). Activated protein kinase C phosphorylates cellular proteins thereby regulating their activity (Cohen 1982). TPA increases phospholipid metabolism in cultured human epidermal keratinocytes in culture (Deleo et al. 1986). There are some genes which are directly induced by TPA without the need for protein synthesis. These genes are also induced by growth factors as well (Epner & Herschman 1991).

The effects of TPA on junctional communication are also diverse. Fitzgerald et al. (1983) have shown that TPA reversibly inhibits dye transfer in mouse epidermal HEL-37 cell line. TPA also rapidly activates the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  pump in these cells. The inhibition of gap junctions by TPA is followed by increase in tight junction permeability. Exposing V79 cells to TPA for 18 hours leads to a decrease in the number of gap junctions in the cell membranes as revealed by freeze-cleave techniques (Yancey et al. 1982). But in intact mouse skin, four hours of TPA treatment did not inhibit junctional communication. Moreover, gap junctional communication was increased by TPA application between the dermal and the epidermal layers of the skin as revealed by dye coupling (Kam & Pitts 1988). In another study TPA enhanced DNA synthesis and inhibited dye coupling in quiescent 3T3-L1 cells but cAMP enhanced dye coupling and inhibited serum induced DNA synthesis (Shiba et al. 1989). It seems that TPA and cAMP play an antagonistic role but Yoshimasa et al. (1987) have shown that TPA enhances adenylate cyclase activity in frog erythrocytes and increases cAMP levels. The effects of TPA are variable from one system to another. Cell

communication in V79 cells is not affected by TPA when these cells are grown in medium supplemented by horse serum rather than the usual bovine serum (Miller et al. 1987). Dorman & Boreiko (1983) found that the communication inhibitory effects of TPA were transient and after four hours of exposure metabolic cooperation had returned to greater than 50%.

It has become apparent that TPA inhibits junctional communication in some cell systems but not in others. Pitts & Burk (87) have shown that TPA inhibits junctional communication in V79 cells but not in BHK Syrian hamster fibroblasts in the same system. The rate of junction formation in BHK cells is much faster than in V79 cells. They postulate that TPA prevents gap junction formation but once those junctions have formed then TPA is less able to affect their function.

A study with mouse epidermal cells shows that treatment with TPA inhibits junctional communication and the level of expression of the main gap junction protein (connexin 43) was found to be reduced (Jongen et al. 1991). This supports Burk & Pitts (87) hypotheses that TPA would have the greatest inhibitory effect on gap junctional permeability in systems which have a lag time between treatment with TPA and the growth of the culture to confluency and hence greater cell contact. The time lag would allow reduction in connexin expression by TPA before the cells make contact.

There are numerous effects of TPA on cellular physiology besides the effects on gap junctions. In light of the present studies it would be prudent to exercise caution when using TPA as a standard inhibitor of cell communication. If TPA does not inhibit junctional communication maximally in a cell communication system then it would not necessarily mean that the system does not work. This present method of assessment depends on inhibition of DNA synthesis in resistant cells by transfer and incorporation of the 6-tg metabolite into

DNA to demonstrate cell communication. If any treatment prevented the metabolism of 6-tg in the wild type cells or prevented the incorporation of the metabolite into mutant cells the outcome would be to increase resistant cell DNA synthesis. This would give a false positive inhibition of metabolic cooperation. Therefore, it is important to ensure that any positive results obtained are not due to inhibition of 6-tg metabolism or incorporation by using appropriate controls.

After characterizing the different parameters of the present metabolic cooperation method it was concluded that this multiwell plate assay provides a faster way for investigating gap junctional intercellular communication. This present method takes less than four days whereas the colony counting method of Yotti et al. (1979) takes upto two weeks. In the colony counting method the size of the resistant cell colonies varies. The investigator has to identify colonies containing fifty or more cells to count. This inevitably leads to some subjectivity in the counting. The present method makes use of automated sample counting which reduces subjectivity when collecting data. The following work was done using the present multiwell plate method.

## Chapter 4. The Effect Of Different Chemicals On Junctional Communication

### 4.1. The Effect Of Calcium Ionophore A23187 On Junctional Permeability

#### 4.1.1. Introduction

It is known that rise in intracellular calcium affects closure of gap junctions. Rose & Loewenstein (75) have shown that increasing calcium near junctions reduces junctional coupling in chironomus salivary glands. The permeability of junctions can be regulated in a graded way such that selective blockage of molecules with respect to size can occur (Rose et al. 1977; Spray et al. 1982).

Unwin & Ennis (1983;1984) has shown that calcium ions can directly modulate the opening and closing of the gap junctions. Calcium dependent proteins such as E-Cadherin bring about cell to cell adhesion (Nagafuchi et al. 1987) and Jongen et al. (1991) has demonstrated that increase in E-cadherin in cells correlate with increased junctional communication and this E-Cadherin expression is calcium dependent.

Increasing intracellular calcium by using the calcium ionophore A23187 would be a good way to test this assay and the effect of this treatment on junctional communication. A23187 binds divalent cations and facilitates their transport across membranes (Pressman 1976). Other workers who used metabolic cooperation to measure junctional cooperation have been unable to find inhibition of communication by A23187 (Davidson et al. 1985). Metabolic cooperation methods which rely on cell viability as a measure of junctional communication by necessity require time for the cell viability to decrease or not. These methods may not readily detect compounds which produce effects lasting only short periods

of a few hours.

Intercellular calcium is maintained at about  $10^{-7}M$  and DMEM culture medium contains 1.8 mM calcium. Therefore adding an ionophore to the culture would lead to increase in intracellular calcium. This approach was used in the experiments which follow.

#### 4.1.2. Materials And Methods

Tissue culture materials used were as stated previously. Metabolic cooperation method employed was as outlined in chapter 3. Calcium ionophore A23187 (Sigma) was dissolved in DMSO to give a 10 mM stock solution which was used at different concentrations. The final concentration of DMSO in culture medium was 0.05%. A23187 is virtually insoluble in aqueous solution and if less DMSO is used then the ionophore comes out of solution as soon as it is added to the culture medium and forms globules. Three different protocols were employed to apply A23187 to the co-culture:

#### 4.1.3. Protocol 1.

Cells were plated in co-culture for 24 hours. The medium was exchanged for one containing 10  $\mu g/ml$  6-tg and a specified concentration of A23187. The culture was incubated for a further 24 hours after which the medium was removed and fresh medium containing 0.1  $\mu Ci/ml$   $^3H$ -thymidine was added. Four hours later, the cells were detached using trypsin and processed to assay the amount of tritiated thymidine incorporated.

#### 4.1.4. Protocol 2.

Cells were plated in co-culture for 24 hours. The 6-tg and 0.4  $\mu\text{M}$  A23187 were applied as previously. After 0.5, 1 or 2 hours of exposure the medium containing the A23187 was removed and fresh medium containing 6-tg was added. 24 hours after start of A23187 treatment, tritiated thymidine incorporation was carried out for 4 hours and the culture was processed as previously to assay incorporated radioactivity.

#### 4.1.5. Protocol 3.

The co-culture was plated for 24 hours as previously. Medium containing 6-tg was then added. 5 hours were allowed to elapse before medium containing 0.4  $\mu\text{M}$  A23187 and 6-tg was added. Different experimental groups were treated for 0.5, 1 or 2 hours and then the ionophore was removed. 6-tg containing medium was added and culture was incubated until 24 hours had elapsed. Tritiated thymidine was incorporated and measured as previously.

#### 4.1.6. Results

In order to ascertain which concentrations of A23187 would be tolerated by V79 cells, different concentrations were applied for a 24 hour period after which cell capacity to synthesize DNA was determined. A dose response curve was constructed. From the dose response curve presented in figure 4.1.1 it became apparent that concentrations of A23187 of 0.1  $\mu\text{M}$  or above were toxic to the cells when applied over a 24 hour period. Concentrations of A23187  $\geq 0.5 \mu\text{M}$  reduce thymidine incorporation to less than 8% of the



solvent control. Therefore, experiments probing the communication modulatory effects of A23187 would have to be carried out with concentrations of about 0.1  $\mu\text{M}$ . Even 0.1  $\mu\text{M}$  A23187 reduces cell DNA synthesis by about 26%.

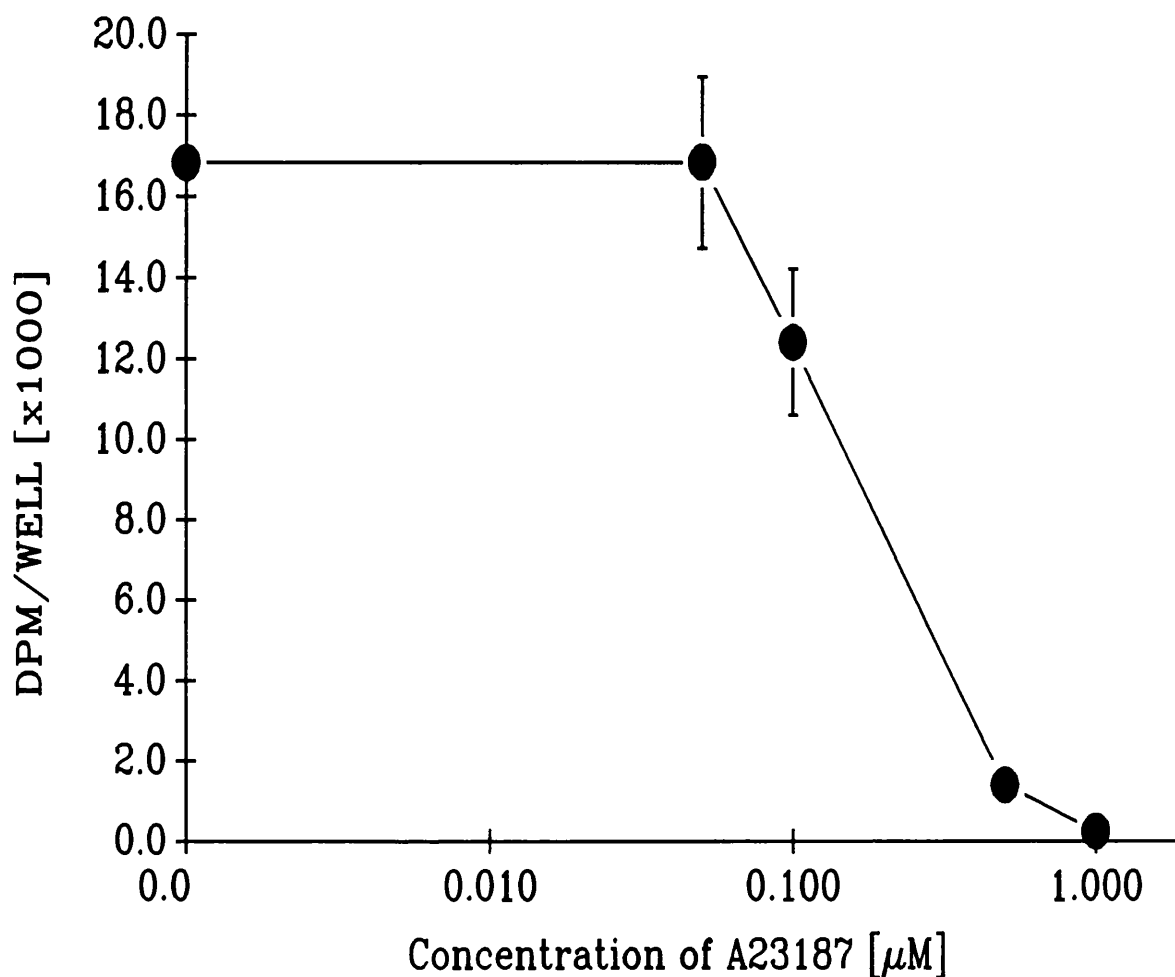
Using the metabolic cooperation method described previously, it was found that A23187 did not inhibit junctional communication or that the communication inhibitory effects were short lived and not detected by this method. Figure 4.1.2 demonstrates that A23187 at concentrations of 0.1  $\mu\text{M}$  and 0.5  $\mu\text{M}$  did not allow DNA synthesis to proceed in resistant cells by inhibiting junctional communication. In the same experiment TPA at the normal dose of 20 ng/ml did inhibit junctional communication. This shows that junctional communication could be inhibited in this particular batch of cells. The DNA synthesis of co-cultures exposed to 6-tg plus 0.1  $\mu\text{M}$  ionophore and 6-tg plus 0.5  $\mu\text{M}$  ionophore was the same as 6-tg controls. The higher toxicity of 0.5  $\mu\text{M}$  A23187 in the dose response curve is not apparent in this experiment nor are effects on junctional communication to be seen.

Since calcium plays an important role in cellular physiology its levels are highly regulated (Rose et al. 1977; Williamson et al. 1985). A concentration of A23187 which sustains an increase in intracellular calcium for a long time may kill the cell and any inhibitory effects on junctional communication may not be manifested. Therefore it was decided to use a concentrations of A23187 between 0.1  $\mu\text{M}$  and 0.5  $\mu\text{M}$  for a very short period and then remove the ionophore. This treatment protocol may inhibit communication and if the inhibition persists it will be detected by the metabolic cooperation method. Figure 4.1.3 shows that this strategy did not produce an inhibitory effect on junctional communication. A trend is seen in the results in that when the exposure to A23187 is increased from 0.5 hour to 1 hour and then to 2 hours, the cell survival is decreased in a stepwise manner. The 0.5 hour treated sample is significantly different from the 2 hour

treated sample when tested statistically. This indicates that longer exposures lead to more cell death but no effect on junctional communication is apparent. Control co-cultures were treated with A23187 for short periods as in the previous experiment to see what effect just A23187 exposure would have on cells in the absence of 6-tg. The results of this experiment presented in table 4.1.1 show that A23187 appears to be cytotoxic even with short exposures. However, the difference between the different groups is not significant when tested statistically.

Figure 4.1.1

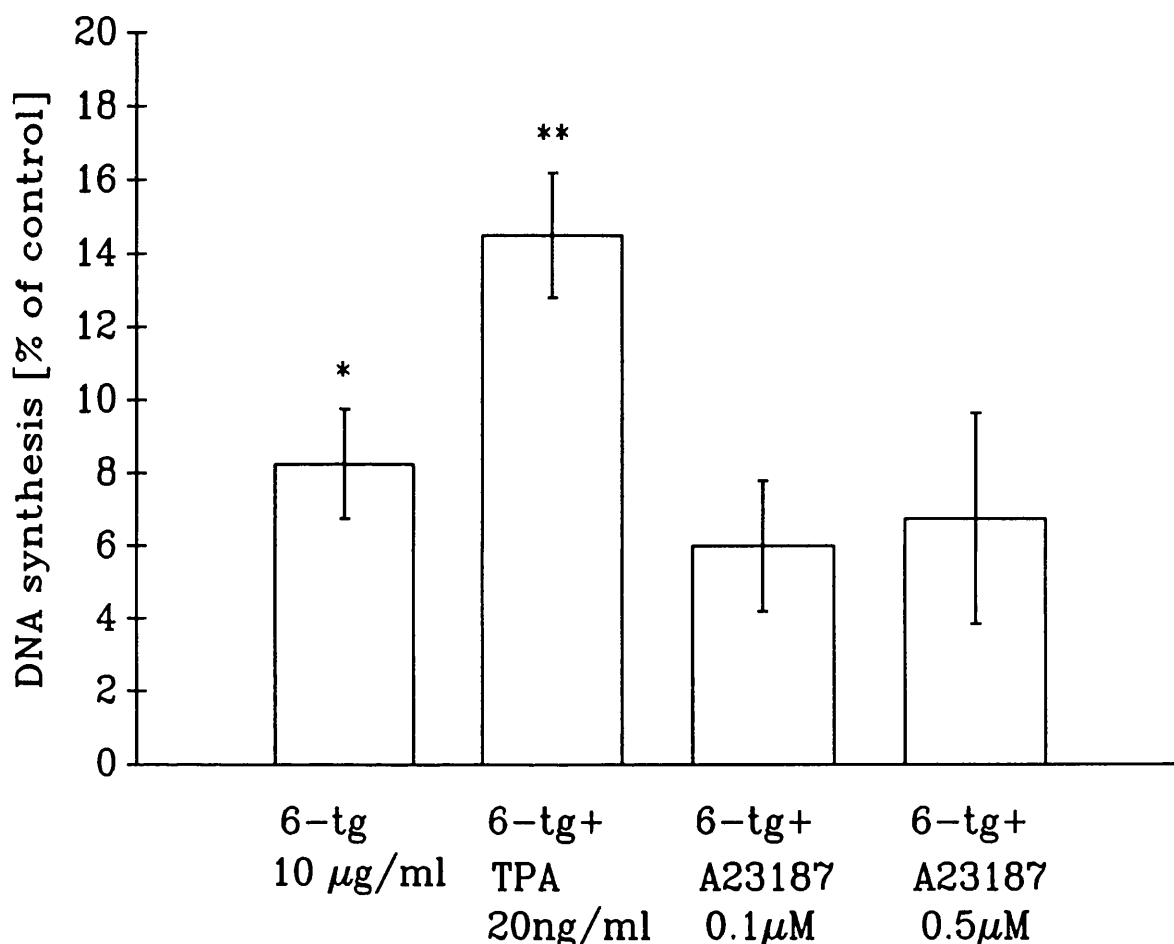
Effect of exposure to different concentrations of A23187 on cell growth and DNA synthesis



V79 wild type cells were plated at a density of 60 K per well and incubated for 24 hours. Different concentrations of A23187 were made up in culture medium which was then added to the cell cultures. The culture was incubated for a further 24 hours. Tritiated thymidine was incorporated for 4 hours and the incorporated radioactivity was assayed to give a measure of DNA synthesis. Solvent control cultures were exposed to 0.05% DMSO.

Figure 4.1.2

### Calcium ionophore A23187 does not inhibit metabolic cooperation

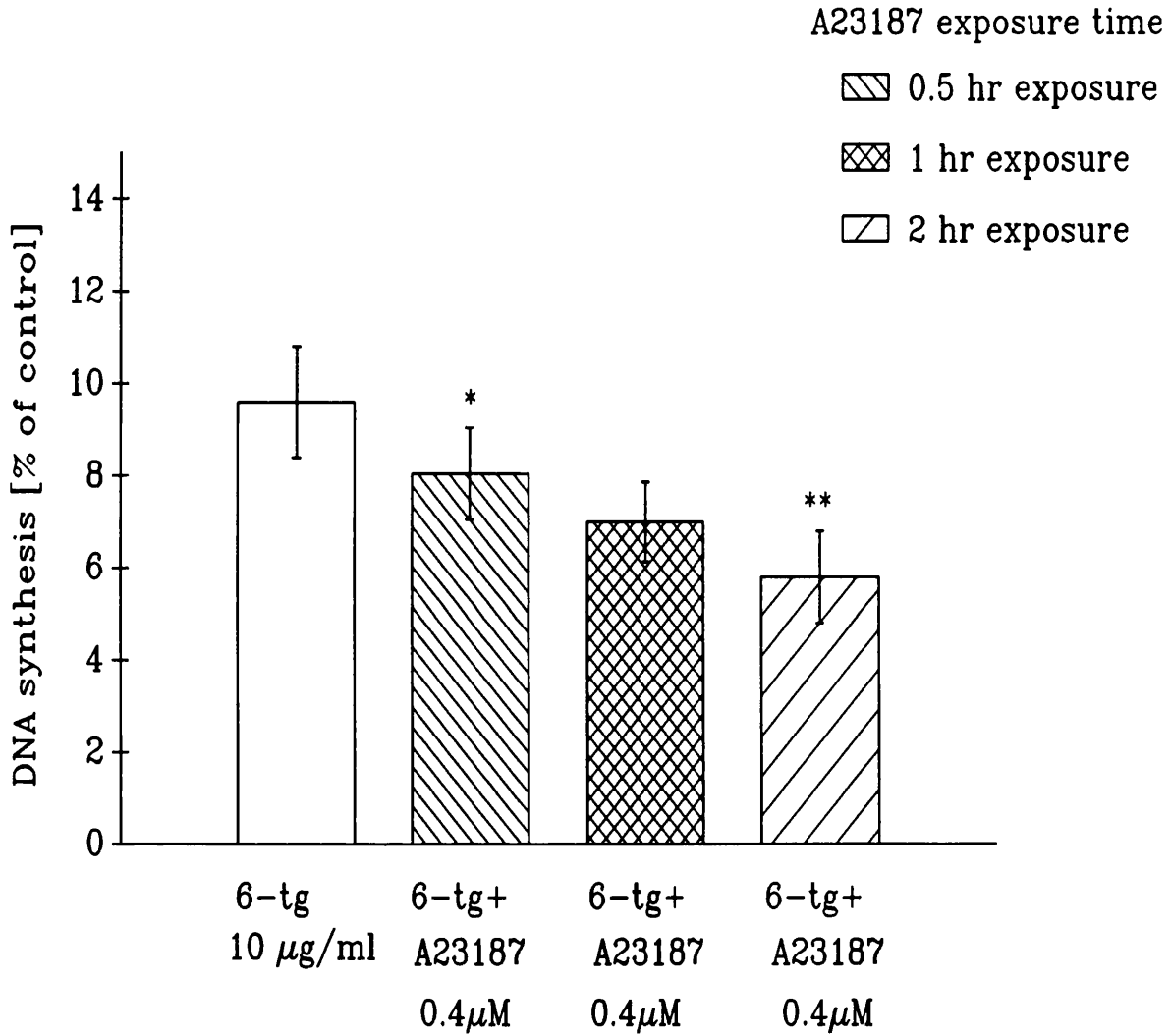


Wild type and 6-tg resistant cells were plated in a 1:1 ratio at a total density of 60 K per well and left to grow for 24 hours. Separate groups of wells were treated with either 10 µg/ml 6-tg or 6-tg plus A23187 at different concentrations. A solvent control group was also treated. After 24 hours had elapsed, tritiated thymidine was incorporated for 4 hours. The incorporated radioactivity was then determined.

The 6-tg control group (\*) and the 6-tg+TPA group (\*\*) are significantly different. P=0.027

Figure 4.1.3

Short exposures of A23187 do not inhibit junctional communication



Cells were plated in 1:1 co-culture at a total density of 60 K per well for 24 hours. 6-Tg at 10 µg/ml or 6-tg and 0.4 µM A23187 were applied to appropriate groups. Control group was exposed to solvents. After 0.5, 1 or 2 hours of exposure the medium containing the A23187 was removed and fresh medium containing 6-tg was added. 24 hours after start of A23187 treatment, tritiated thymidine incorporation was carried out for 4 hours and the culture was processed as previously to assay incorporated radioactivity.

The 0.5 hour group (\*) and the 2 hour group (\*\*) are significantly different. P=0.038

Table 4.1.1 showing the effect of exposure to 0.4 $\mu$ M A23187 without 6-tg for short periods.

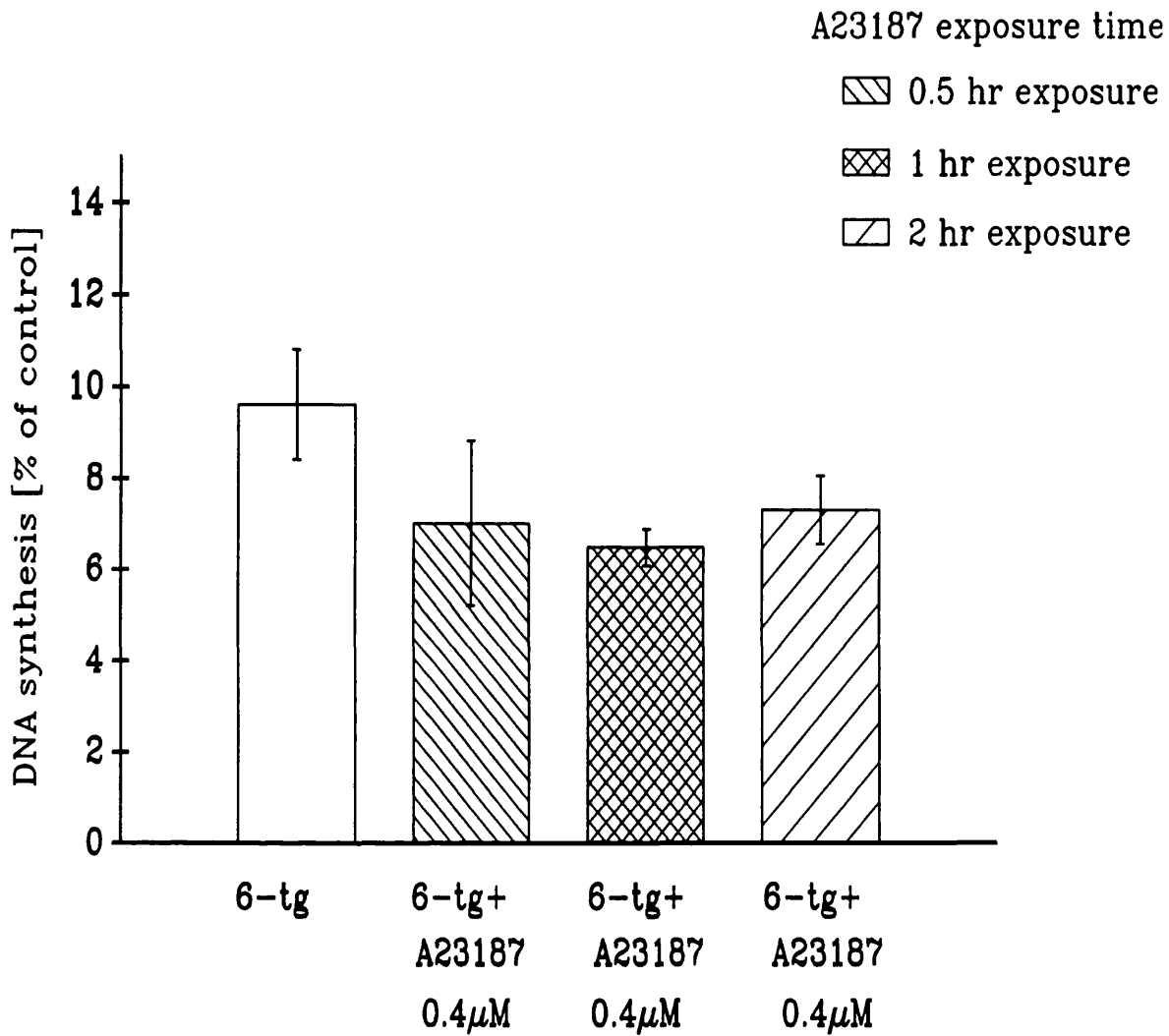
	Solvent control	0.5 hr exposure	1 hr exposure	2 hr exposure
DPM/Well	20657	18126	18363	17080
Standard Deviation	3244	1596	1439	2112

Cells were plated in 1:1 co-culture at 60,000 per well for 24 hours. Medium containing DMSO (0.05%) was added to solvent control wells and 0.4 $\mu$ M A23187 was added to other wells. A23187 was removed after 0.5, 1 and 2 hours and fresh medium was added. 24 hours later DNA synthesis was assayed by <sup>3</sup>H-thymidine incorporation. Four samples per treatment group.

It became apparent from earlier work (see section 3.10) that the transfer of the toxic metabolite takes more than five hours to take place. With the lack of success at inhibiting communication using previous regimes, it was decided to treat the co-culture with the ionophore five hours after addition of 6-tg for different short periods of time as outlined in protocol 3. If A23187 inhibits communication then it could still prevent transfer of the toxic metabolite five hours after the culture was treated with 6-tg. If the effects produced by A23187 wear off with time then the later the ionophore is applied during the 24 hour incubation period, the greater the possibility of seeing an effect in this metabolic cooperation method. This experiment was performed to find out if this could be so. As figure 4.1.4 shows the late application of A23187 did not inhibit junctional communication to a perceptible degree.

Figure 4.1.4

Delaying application of A23187 for 5 hrs followed by short exposures do not inhibit cell communication



This experiment was carried out in accordance with protocol 3. Cells were plated in 1:1 co-culture at a total density of 60 K per well for 24 hours. 6-Tg at 10 µg/ml was applied to all experimental groups for 5 hours. Control group was exposed to solvents. Then 0.4 µM A23187 were applied to appropriate groups. After 0.5, 1 or 2 hours of exposure to A23187 the medium containing the A23187 was removed and fresh medium containing 6-tg was added. 24 hours after start of 6-tg treatment, tritiated thymidine incorporation was carried out for 4 hours and the culture was processed as previous to assay incorporated radioactivity.

#### 4.1.7. Discussion

Increasing intracellular calcium with the aid of the ionophore A23187 did not inhibit junctional communication sufficiently to allow its detection by metabolic cooperation.

It is possible that the perturbation of calcium levels in the cell by A23187 are transient and therefore inhibitory effects on junctional communication are short lived. There is evidence in the literature to suggest this could be the case. Injection of calcium ions into intact cells produces only transient changes in junctional conductance. This shows that cells can rapidly restore their free calcium concentration (Spray et al. 1982).

Rose & Loewenstein (1975) found that 2  $\mu\text{M}$  A23187 increased intracellular calcium within one minute of application as detected by aequorin luminescence. They were using electrophysiological measurements to study junctional communication over minutes rather than hours. In the present study chemicals are applied to the culture for several hours.

A23187 proves toxic to the cells at prolonged exposures. In order to limit the toxic effects of A23187 concentrations below 0.5  $\mu\text{M}$  were used. It is possible that such concentrations of A23187 did not lead to an increase in intracellular calcium.



## 4.2. The Effect Of DDT On Junctional Communication.

### 4.2.1. Introduction

It has been shown that DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane) is a potent tumour promoter in the rat liver (Periano et al. 1975; Pereira et al. 1982; Oesterle & deml 1984; Flodstrom et al. 1990). DDT has also been shown to inhibit gap junctional intercellular communication in human fibroblasts (Davidson et al. 1985), rat hepatocytes (Sugie et al. 1987; Flodstrom et al. 1990), rat liver epithelial (WB-F344) cells (Hemming et al. 1991), V79 cells (Warngard et al. 1987;1989) and mouse hepatocytes (Klaunig et al. 1990). This has been demonstrated in both dye transfer and metabolic cooperation experiments.

The effects of DDT on the present metabolic cooperation system were investigated.

### 4.2.2. Materials And Methods.

DDT (Sigma) was dissolved in DMSO to give a 0.1M stock solution. Small volumes of stock solution were diluted in cell culture medium which was then added to the cell cultures. The final concentration of DMSO in the culture medium did not exceed 0.1%. Metabolic cooperation assay was carried out as outlined previously.

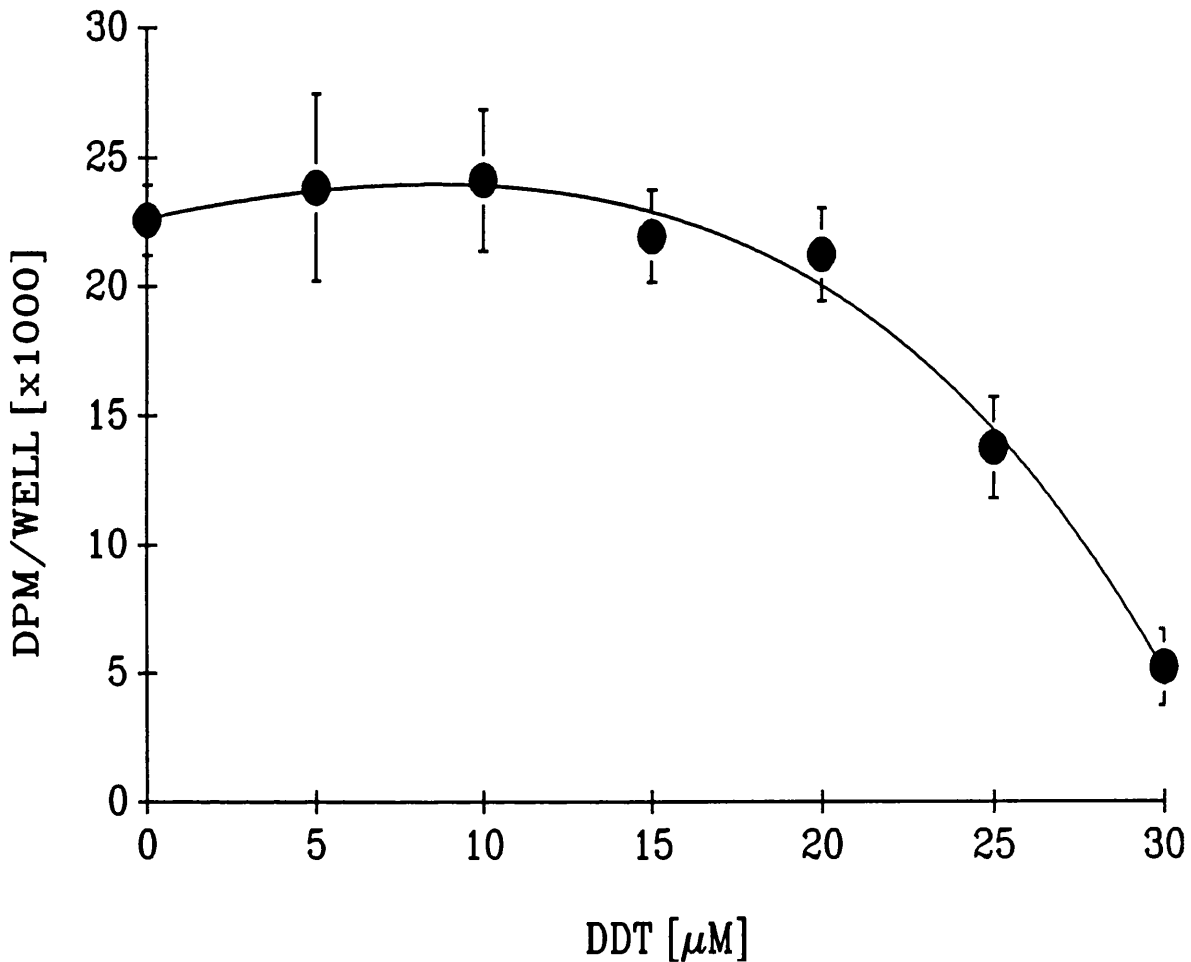
### 4.2.3. Results

A dose response study for DDT on V79 cells was carried out and provided the results shown in figure 4.2.1. These results show that DDT is not toxic to the cells at concentrations upto 20  $\mu\text{M}$  but becomes toxic to the cells when this concentration is exceeded. A range of different concentrations of DDT was tested to see if DDT inhibits junctional communication. Figure 4.2.2 shows that DDT does not have an inhibitory effect on junctional communication when metabolic cooperation is used as a basis for measurement. Inclusion of TPA as a positive control shows that the metabolic cooperation can be inhibited in this batch of cells. The possibility that DDT does not inhibit functional gap junctions but prevents the formation of gap junctions was tested by treating the co-culture with DDT before gap junctions are formed. This was done by applying 20  $\mu\text{M}$  DDT four hours after plating the cells. The culture was then incubated for a further 20 hours before 6-tg was added. This pre-treatment with DDT did not inhibit junctional communication as seen in figure 4.2.3. Pre-exposure to DDT does not increase DNA synthesis above the 6-tg control.

As a comparison with the metabolic cooperation method, wild type V79 cells treated with 25  $\mu\text{M}$  DDT were injected with fluorescent dye to assess whether dye transfer was effected by this compound. The photographs presented in figure 4.2.4 show that DDT does not inhibit dye coupling after 1 hour exposure to 25 $\mu\text{M}$  DDT whereas cells treated with 20ng/ml TPA did show blockage of dye coupling.

Figure 4.2.1

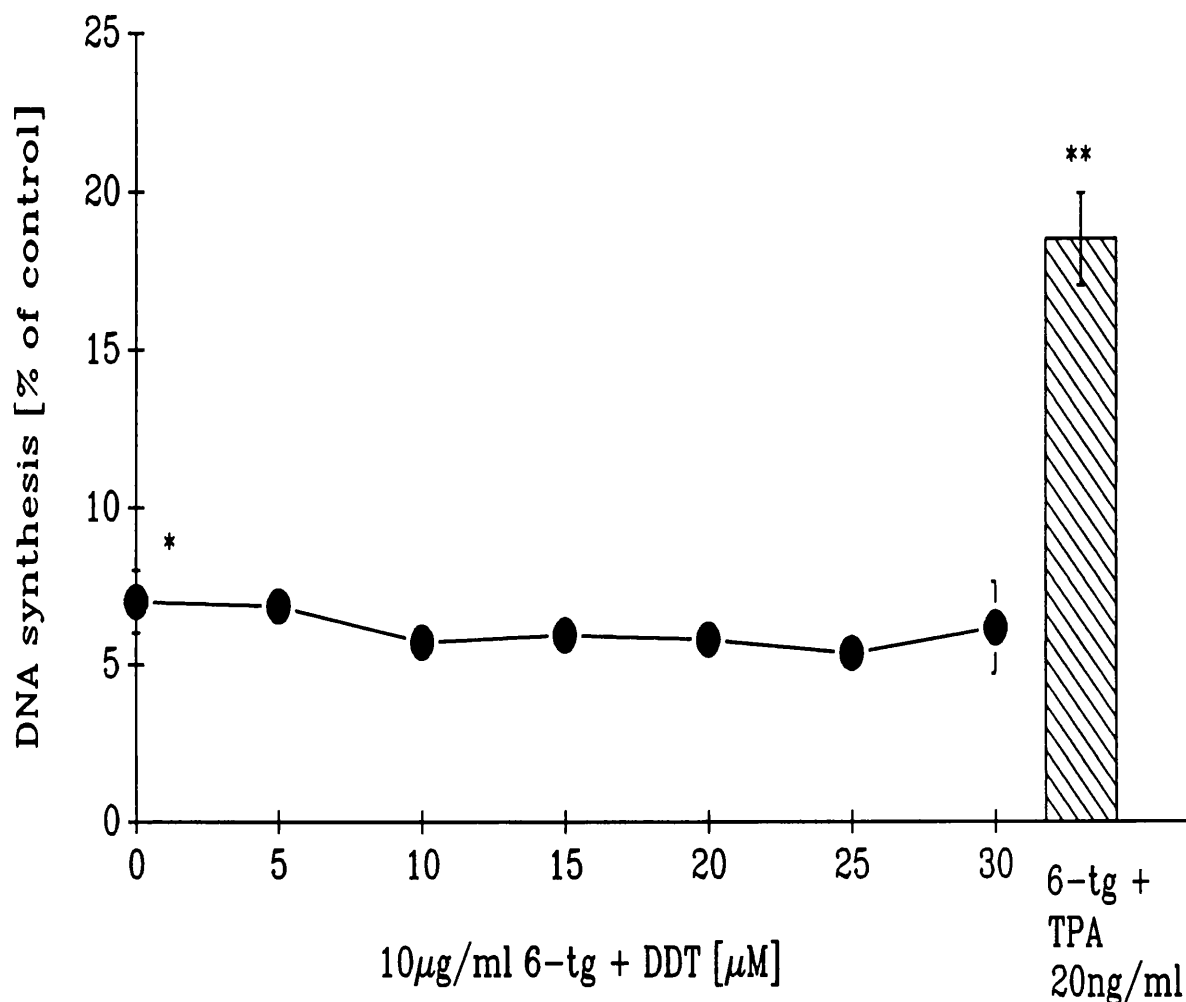
Effect of exposure to different concentrations of DDT on cell growth and DNA synthesis



V79 wild type and resistant cells were plated in 1:1 co-culture at 60 K total density in 24 well plates. After 24 hours incubation the culture medium was replaced with that containing a specified concentration of DDT. For every concentration used 4 wells were treated. After exposure to DDT for 24 hours the medium was removed and fresh medium containing  $0.1\mu\text{Ci/ml}$  of  $^3\text{H}$ -thymidine was added. After 4 hours incubation the incorporated radioactivity was measured as stated in materials and methods.

Figure 4.2.2

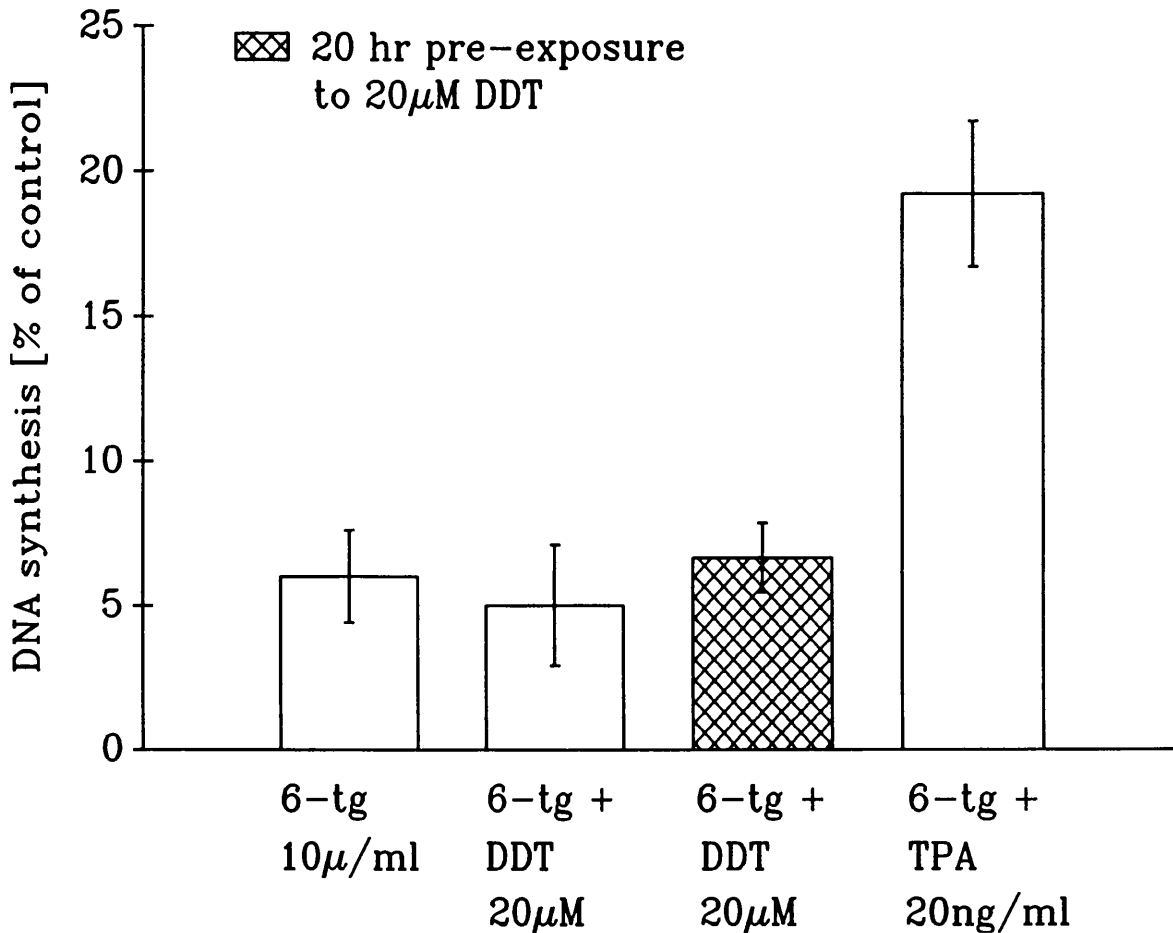
DDT at various concentrations does not inhibit junctional communication in V79 cells



V79 wild type and 6-tg resistant cells were plated in 1:1 ratio at a total density of 60 K per well in 24 well plates. After 24 hours incubation the culture medium was replaced with that containing 10μg/ml of 6-tg and a specified concentration of DDT. For each concentration of DDT 4 well were treated. Control group was exposed to solvents. The co-culture was incubated in this medium for 24 hours, after which fresh medium containing 0.1μCi/ml of <sup>3</sup>H-thymidine was added. After a further 4 hour incubation the incorporated radioactivity was measured to give a measure of relative DNA synthesis. The 6-tg control group (\*) and the 6-tg+TPA group (\*\*) are significantly different.  $P=9 \times 10^{-4}$

Figure 4.2.3

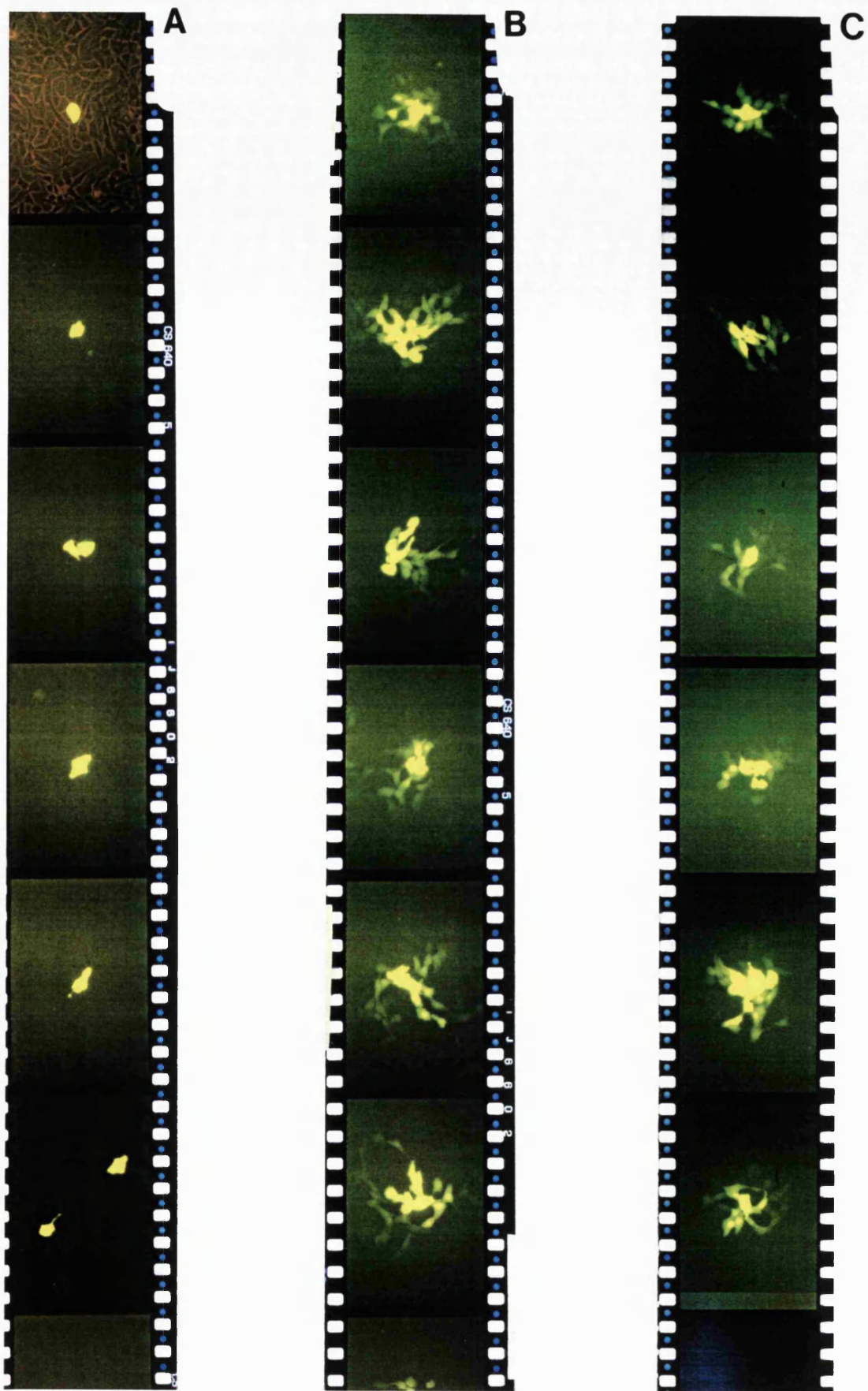
Pre-exposure to DDT does not inhibit junctional Communication



V79 wild type and 6-tg resistant cells were plated in 1:1 ratio at a total density of 60 K per well in 24 well plates. 4 hours after plating, 20 µM DDT was added to a group of wells. After a further 20 hours incubation the culture medium was replaced with that containing 10µg/ml of 6-tg plus 20 µM DDT and incubated for 24 hours. A group of wells was also treated with DDT for only 24 hours without pre-treatment. Control group was exposed to solvents. After this incubation, fresh medium containing 0.1µCi/ml of <sup>3</sup>H-thymidine was added. After a further 4 hour incubation the incorporated radioactivity was measured to give a measure of relative DNA synthesis.

Figure 4.2.4

Effect of DDT on dye transfer



#### Figure 4.2.4. Legend

V79 wild type cells were plated at a density of  $2 \times 10^6$  cells per 35mm plate and left overnight. Group A was exposed to 20ng/ml TPA. Group B was exposed to 0.3% solvent (DMSO) v/v. Group C was exposed to 25 $\mu$ M DDT dissolved in DMSO. After 1 hour exposure to the chemicals the cells were injected with lucifer yellow. The cell culture was then washed with fresh medium and the injected cells were photographed under fluorescent lighting conditions ten minutes after injection.

#### 4.2.4. Discussion

From the results presented it becomes apparent that DDT does not inhibit junctional communication in the present strain of V79 cells. If DDT inhibited junctional communication for a short period of time (few hours) then the metabolic cooperation assay would not have picked up the effect. Therefore, dye transfer was used as a comparison because this method is able to detect changes in junctional communication which only present themselves for a very short period of time such as an hour or less. TPA was used as a positive control in both the metabolic cooperation and the dye transfer assays for measuring junctional communication. In both assays TPA inhibited cell communication.

It can be concluded that in this strain of V79 cells DDT does not affect junctional communication.

#### 4.2.5. The Dye Injection Assay

Metabolic cooperation techniques can be used to monitor effects of compounds on gap junction function in a large population of cells. It is also necessary that the effects of substances on junctional communication are present for a sufficient duration to be detected. An alternative technique, transfer of fluorescent dye can be used to test compounds whose action only persist for a short period of time and therefore cannot be investigated using the metabolic cooperation assay. For this reason I have used dye injection to verify the results obtained using the metabolic cooperation assay. Using this system to assess the effect of paracetamol and other compounds on cell communication, a difficulty became obvious. Sometimes there was not a clear cut difference between controls and a treated group and



measuring the spread of dye in a culture was subject to ambiguity.

Dye transfer is useful if control cultures communicate and treated groups show a clear inhibition of dye transfer. Variation in the communication of dye injected cells in control cultures made quantitation of junctional communication difficult.

It was found that cells in control cultures sometimes either did not communicate with each other at all or the communication varied considerably from one area of the plate to another. The source of this problem became apparent when the cell culture was immunostained with connexin antibody (study kindly carried out by Colin Green, Dept. of Anatomy). He found that cells in some areas of the culture expressed connexins and hence gap junctions, while others did not (see appendix). This meant that when cells were injected with dye the communication was non-existent in some areas and present in others.

Zeilmaker & Yamasaki (1986) also found this problem when they used the V79 cell line. They assumed that it could be due to heterogenous culture of cells containing cells with different abilities to transfer dye. They isolated nine different subclones from the stock culture but found that the subclones also exhibited the same heterogeneity in dye transfer. It seems this is an inherent property of the V79 cell line because the Balb/C 3T3 cell line showed a homogenous distribution of dye spread from cell to cell. This aspect of the dye injection assay made quantitative assessment of communication difficult.

This problem of course does not occur in the metabolic cooperation assay as a large number of cells are sampled and the junctional communication is monitored over time and not at a specific instant. This means that gap junctions may open and close many times during the incubation period but only the cumulative effect of the communication is seen. This advantage of the metabolic cooperation assay allows better understanding of the role of junctional communication in organs and cell tissues.

### 4.3. Effect Of Paracetamol On Junctional Communication

#### 4.3.1. Introduction

Paracetamol is an analgesic which is readily available over the counter. When taken in overdose quantities, paracetamol causes liver damage. The damage or necrosis occurs mainly in the centrilobular area (Prescott et al. 1971). Electrophysiological measurements indicate that in the liver the cells seem to be linked in a three dimensional network (Graf & Peterson 1978). Another hepatotoxin which causes centrilobular necrosis in the liver is carbon tetrachloride and it has been demonstrated by Saez et al. (1987) using electrophysiological and dye transfer techniques that this compound blocks junctional communication at hepatotoxic doses. This blockage of communication is reversible.

There is evidence to suggest that chronic dosage of paracetamol may cause liver tumours in mice (Flaks & Flaks 1983). Given that blockage of junctional communication is implicated in tumourigenesis it would be interesting to find out if paracetamol inhibits cell communication. Also it is not known whether junctional communication is blocked by paracetamol during liver injury or indeed whether paracetamol has any effect on junctional communication at all. For this reason the effects of paracetamol on cell communication were investigated using the metabolic cooperation method.

#### 4.3.2. Materials And Methods

Tissue culture materials used were as previously. Paracetamol (Sigma) was dissolved in PBS to give a 20 mM stock solution. Dilutions of this stock were added to medium before

addition to cell cultures. Paracetamol used for dose response assessment was made upto 100 mM to avoid diluting growth medium too much with PBS. Fresh stock was made for each experiment to avoid the possibility of degradation of this compound in solution. The stock solution was filter sterilized prior to use. Metabolic cooperation methods used were as described previously.

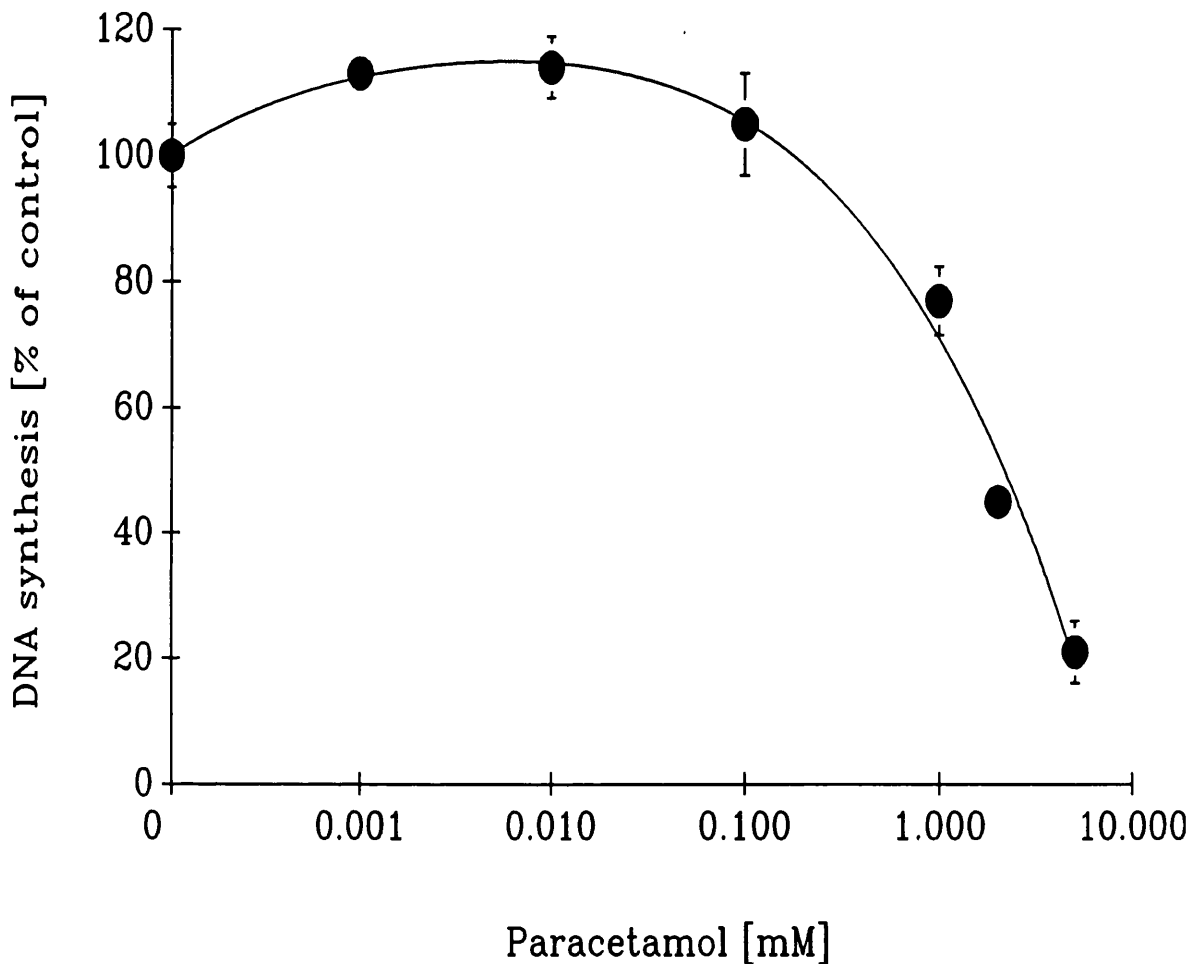
#### 4.3.3. Results

A dose response study was carried out to test the toxic effects of paracetamol on V79 cells. Figure 4.3.1 shows that 24 hour exposure to concentrations of paracetamol above 1 mM blocks DNA synthesis over the subsequent 4 hours. Even 1mM paracetamol reduces tritiated thymidine incorporation by 30% as compared to the control.

Having established a dose response curve and obtained an idea as to which concentrations of paracetamol are toxic to the cells, it was decided to investigate whether paracetamol inhibited junctional communication. A range of different concentrations of paracetamol were tested in the metabolic cooperation assay. It was found that paracetamol did appear to inhibit junctional communication in this assay. Moreover, the communication inhibition produced by paracetamol was comparable with that produced by the TPA positive control. Figure 4.3.2 shows that the maximal inhibition of 6-tg induced resistant cell injury by paracetamol occurs when 1 mM paracetamol is used.

Figure 4.3.1

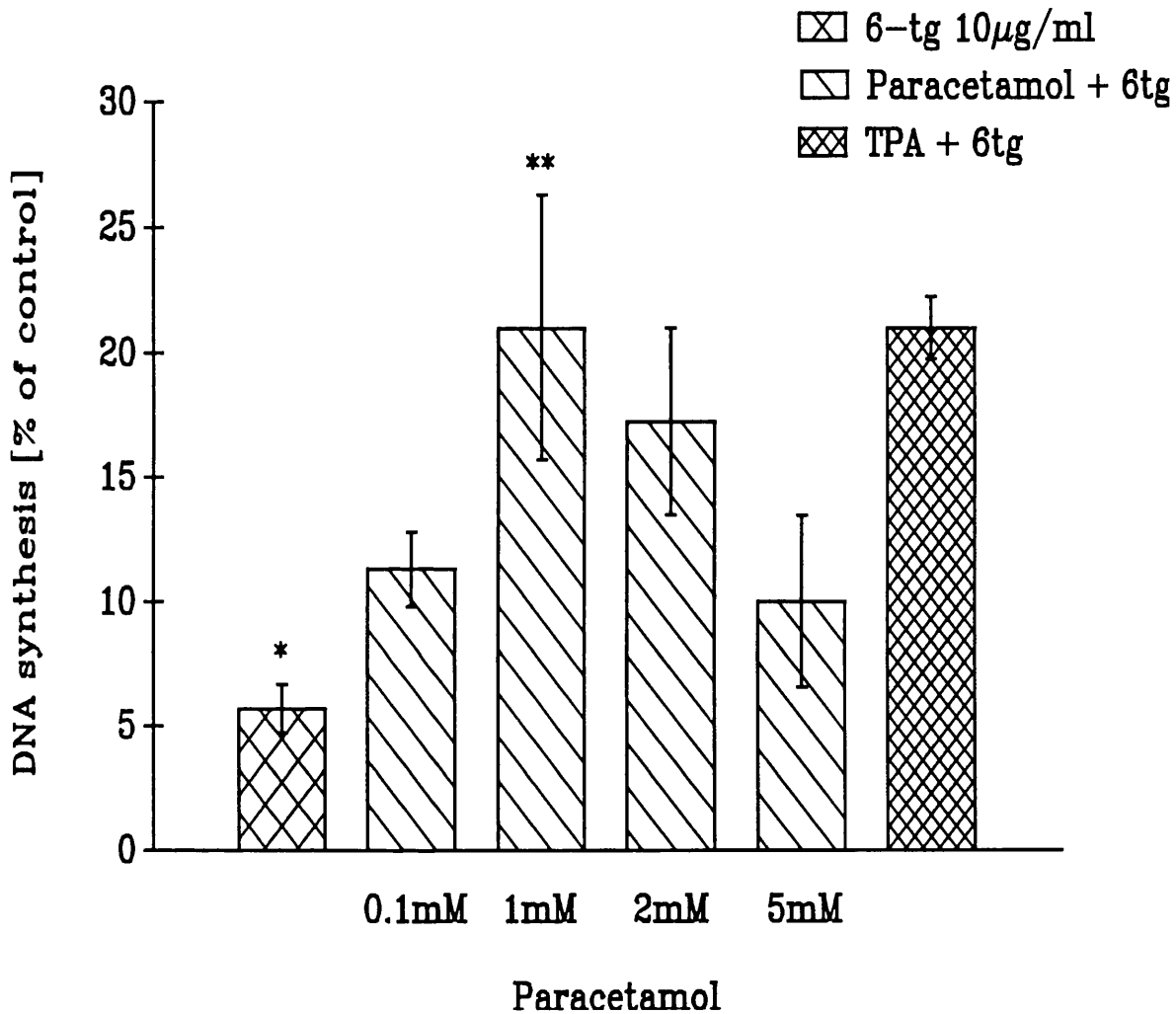
Effect of exposure to different concentrations of paracetamol on cell growth and DNA synthesis



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later paracetamol dissolved in PBS was added to culture medium at various concentrations and the medium was then added to the wells. 4 wells were treated for every concentration of paracetamol used. PBS was added to solvent control group at 5% final concentration. After 24 hours exposure the paracetamol containing medium was removed and medium containing 0.1 $\mu$ Ci/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

Figure 4.3.2

### Paracetamol inhibits junctional communication



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg plus a range of concentrations of paracetamol was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 20ng/ml TPA plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+1mM paracetamol group (\*\*) are significantly different.  $P=7 \times 10^{-3}$

#### 4.3.4. Discussion

The above results show that paracetamol appears to exert a powerful effect on gap junctional communication using this method. Looking at the dose response curve in figure 4.3.1, one can see that 1mM paracetamol reduces cell DNA synthesis by 30% compared to solvent controls. Figure 4.3.2 shows that the inhibitory effect of paracetamol on gap junction mediated injury has a far greater effect in rescuing resistant cells from 6-tg toxicity than the toxic effect of paracetamol has in reducing cell DNA synthesis. The protective effect of inhibition of metabolic cooperation on the resistant cells is reduced when concentrations of paracetamol exceed 1 mM. This is likely to be due to higher concentrations of paracetamol killing the cells.

In the present metabolic cooperation assay paracetamol has inhibited gap junctional intercellular communication. This has not been shown previously. The action of paracetamol on junctional communication may have an affect on liver injury. A greater understanding of the mechanisms behind this effect of paracetamol on gap junctions would be beneficial.

One question which immediately arises is whether paracetamol is actually affecting gap junctions or whether it is interfering with the metabolism of 6-tg` in some other way. If paracetamol prevented the conversion of 6-tg to its toxic metabolite then this effect would increase resistant cell DNA synthesis and would be presented as inhibition of junctional communication. The following work was carried out to investigate this.

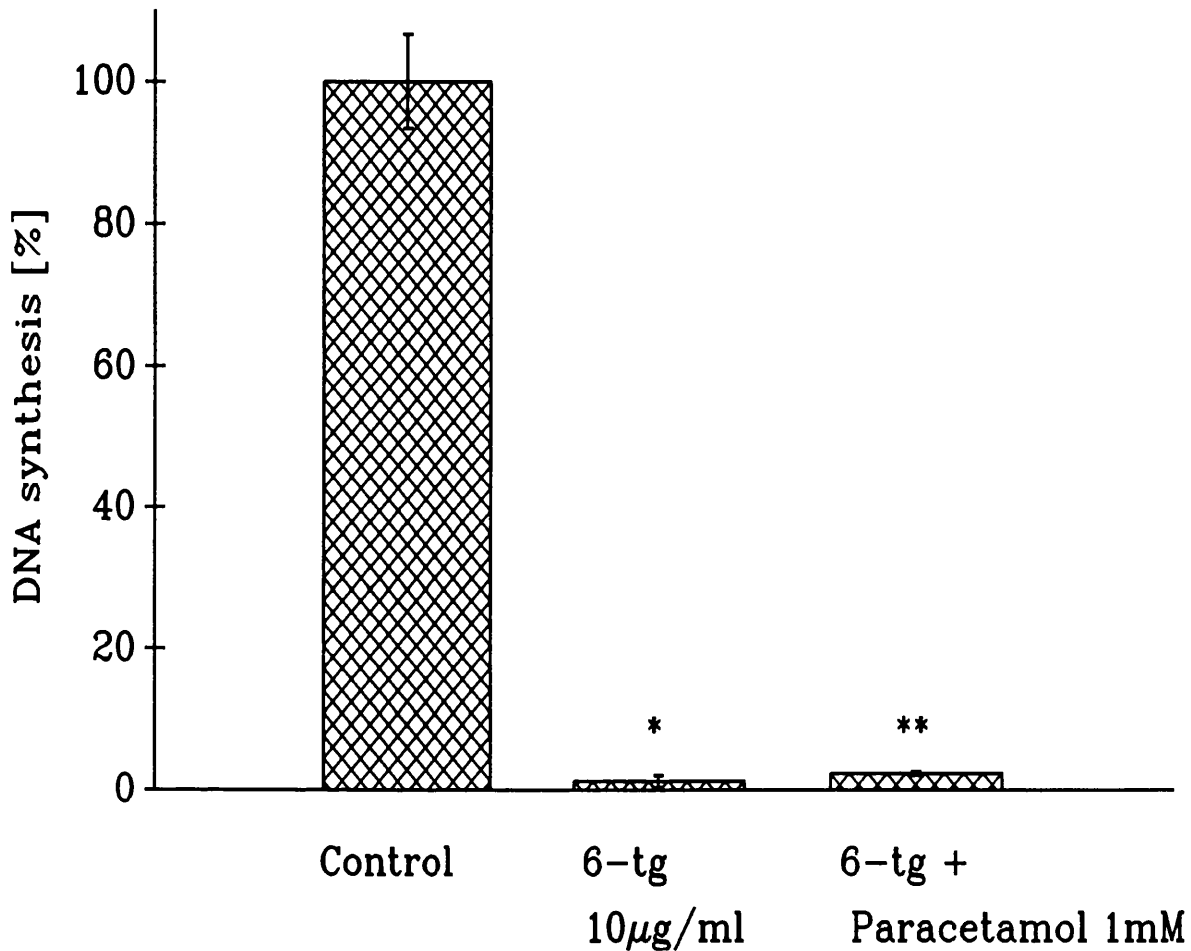
#### 4.4. Elucidation Of The Mechanism Of Paracetamol Action On Junctional Communication.

The effect of paracetamol on 6-tg toxicity on wild type V79 cells was investigated. This was done by treating one group of wild type cells with 6-tg only and another group with 6-tg plus 1mM paracetamol. Figure 4.4.1 Shows that paracetamol does not effect the action of 10  $\mu\text{g/ml}$  6-tg to any great degree. A more detailed investigation using various concentrations of 6-tg and a fixed concentration of paracetamol was also carried out. The results of this experiment presented in figure 4.4.2 show that at the lower concentrations of 6-tg, paracetamol inhibits the toxic effect when compared to the 6-tg only treated group.

In the present metabolic cooperation assay 10 $\mu\text{g/ml}$  6-tg is used and at this concentration of 6-tg paracetamol is unable to inhibit toxicity to the wild type cells to any great extent. One possible explanation could be that metabolism of 6-tg in a culture of wild type cells may be heterogenous. Some cells may convert more 6-tg to its toxic form than neighbouring cells. In a normal wild type cell culture without paracetamol, if gap junctions are functional then there would be an equilibration of the toxic metabolite in the culture. However, when paracetamol is also present in the culture and cell communication is inhibited, this equilibration may not take place and hence some wild type cells would synthesize more DNA. Therefore, the increased DNA synthesis of wild type cells treated with 6-tg and 1 mM paracetamol compared to the 6-tg only treated group could be explained by a cell communication inhibitory effect. Further investigation of the mechanism is required.

Figure 4.4.1

Paracetamol does not prevent in wild type cells the lethal effects of 6-tg



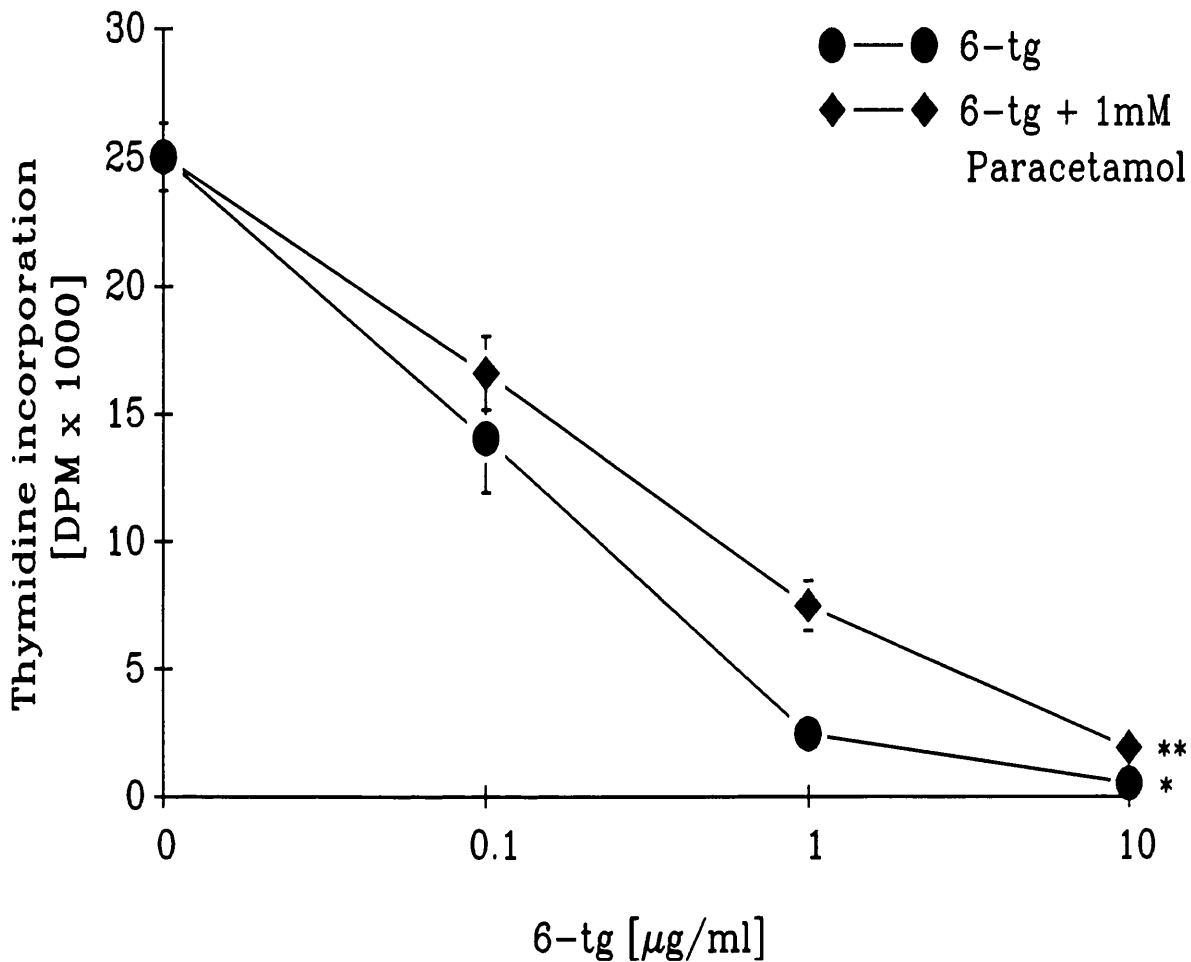
Wild type cells were plated at a density of 60 K per well for 24 hours. Old medium was removed and medium containing 6-tg at 10µg/ml was added to one group while medium containing 6-tg and 1mM paracetamol was added to another group. Solvent control group was also treated. After 24 hours exposure, the chemicals were removed and medium containing 0.1µCi/ml tritiated thymidine was added. After 4 hours of incorporation the amount of tritiated thymidine was measured.

The 6-tg control group (\*) and the 6-tg+1mM paracetamol group (\*\*) are not significantly different. P=0.2



Figure 4.4.2

Effect of paracetamol on 6-tg toxicity with different concentrations of 6-tg



Wild type cells were plated at a density of 60 K per well for 24 hours. Old medium was removed and medium containing 6-tg at different concentrations was added to appropriate groups of wells while medium containing various concentrations of 6-tg and 1mM paracetamol was added to other groups. Solvent control group was also treated. After 24 hours exposure, the chemicals were removed and medium containing 0.1µCi/ml tritiated thymidine was added. After 4 hours of incorporation the amount of tritiated thymidine was measured.

The 10 µg/ml 6-tg control group (\*) and the 6-tg+1mM paracetamol group (\*\*) are significantly different.  $P=1.7 \times 10^{-3}$

## 4.5. Effect Of Butylated Hydroxy Anisole On Junctional Communication

### 4.5.1 Introduction

Paracetamol is a phenolic compound which can exhibit a range of properties. One of these properties is that paracetamol can behave as an antioxidant and perhaps this is the property which causes gap junctional communication inhibition. Would other antioxidant compounds also inhibit junctional communication? In order to answer these questions the antioxidant compound butylated hydroxy anisole (BHA) was tested for its ability to inhibit junctional communication. This compound has been shown to inhibit dye coupling in V79 cells (Zeilmaker & Yamasaki 1986; Masui et al. 1988) and may well inhibit cell communication in the metabolic cooperation method used.

### 4.5.2. Materials And Methods

BHA (Sigma) was dissolved in DMSO to give a 0.5M stock. This was then diluted in further DMSO before being used. The final concentration of DMSO in cell culture medium never exceeded 0.1%. Metabolic cooperation methods used were as described previously.

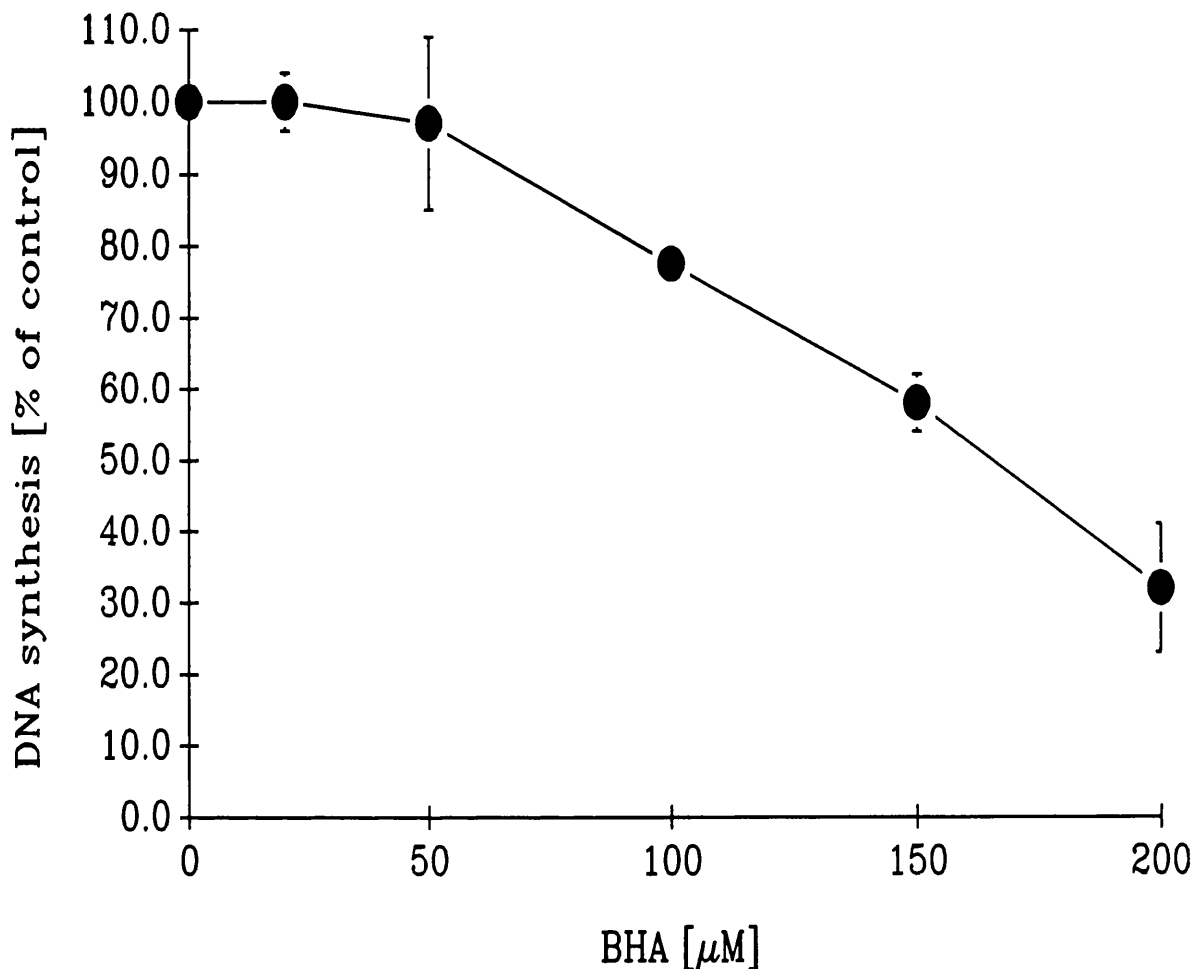
### 4.5.3. Results

Experiments were carried out to find a concentration of BHA which is not toxic to the V79 cells when exposed for 24 hours. BHA at around 50 $\mu$ M is not toxic to the cells as

shown in figure 4.5.1. Various concentrations of BHA were tested but did not inhibit junctional communication. Figure 4.5.2 shows that BHA at concentrations of 20, 40 and 80  $\mu\text{M}$  did not have any cell communication inhibitory effect. However, the capacity to synthesize DNA of the co-culture treated with BHA and 6-tg decreases as the concentration of BHA is increased as seen in figure 4.5.2. This trend highlights dose dependent toxicity of BHA. As a positive control paracetamol at 1mM in the same experiment was shown to inhibit junctional communication.

Figure 4.5.1

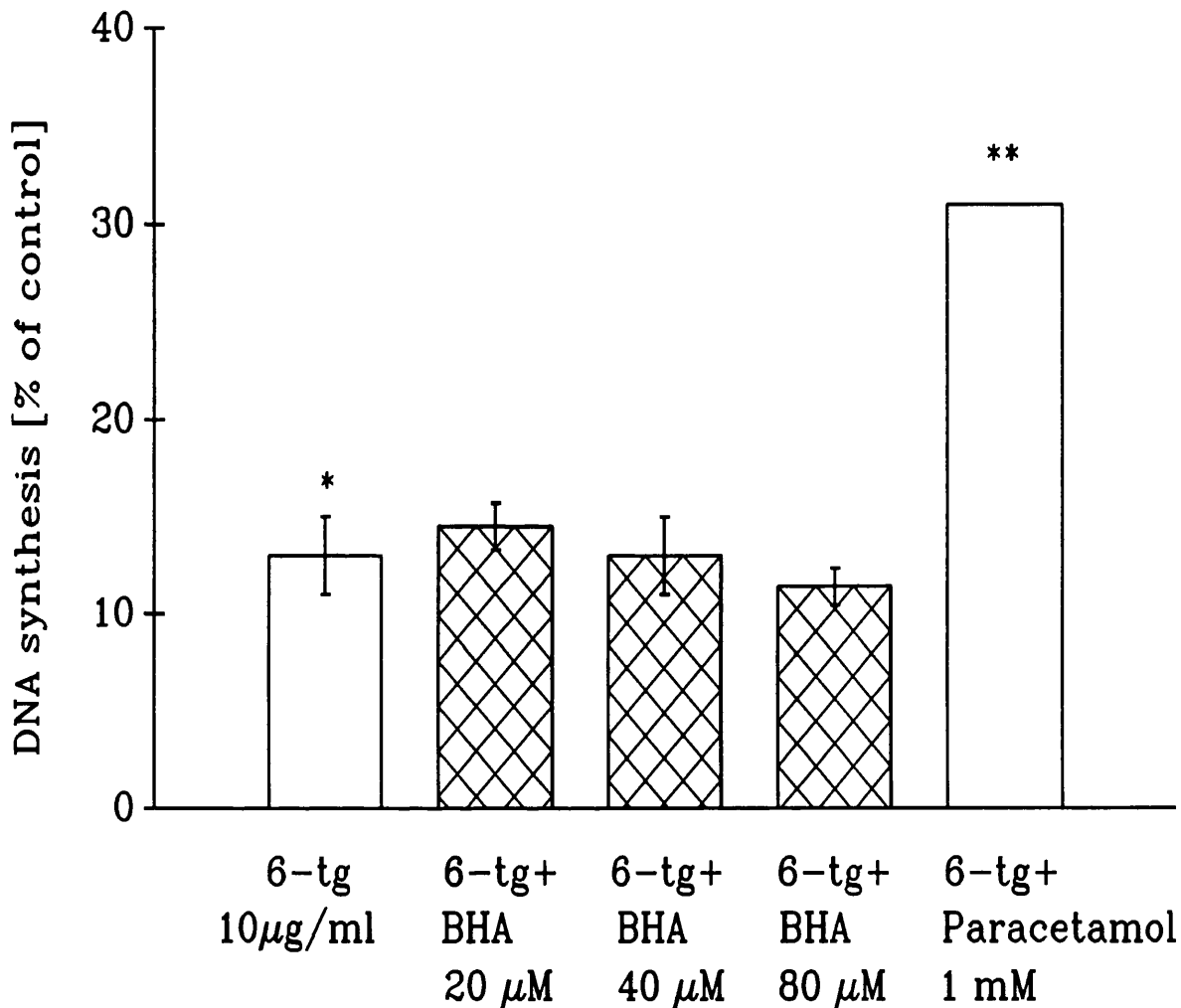
Effect of BHA on cell growth and DNA synthesis  
in V79 cells



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later BHA dissolved in DMSO was added to culture medium at various concentrations and the medium was then added to the wells. 4 wells were treated for every concentration of BHA used. DMSO was added to solvent control group at 0.05% final concentration. After 24 hours exposure the BHA containing medium was removed and medium containing 0.1 $\mu\text{Ci/ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

Figure 4.5.2

### BHA does not inhibit junctional communication



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg with a range of concentrations of BHA were added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 1mM paracetamol with 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+paracetamol group (\*\*) are significantly different.  $P = 3 \times 10^{-3}$

## 4.6. Do Aspirin And Indomethacin Inhibit Junctional Communication?

### 4.6.1. Introduction

Paracetamol is an inhibitor of lipoxygenase and prostaglandin synthetase enzymes (Rainsford 1988). It could be that the inhibitory effect of paracetamol on gap junctions is being exerted through inhibition of the prostaglandin synthetic pathway. There is evidence to suggest that accumulation of arachidonic acid in the cell can lead to gap junction closure (Giaume et al. 1989). Inhibiting the prostaglandin pathway could lead to accumulation of arachidonic acid and this could plausibly be the mechanism by which paracetamol acts. Would other compounds which affect prostaglandin synthesis also modulate junctional communication? Two compounds which also act as inhibitors of prostaglandin synthesis are aspirin and indomethacin. Therefore, the effect of aspirin and indomethacin was tested in the present metabolic cooperation assay for junctional communication.

### 4.6.2. Materials And Methods

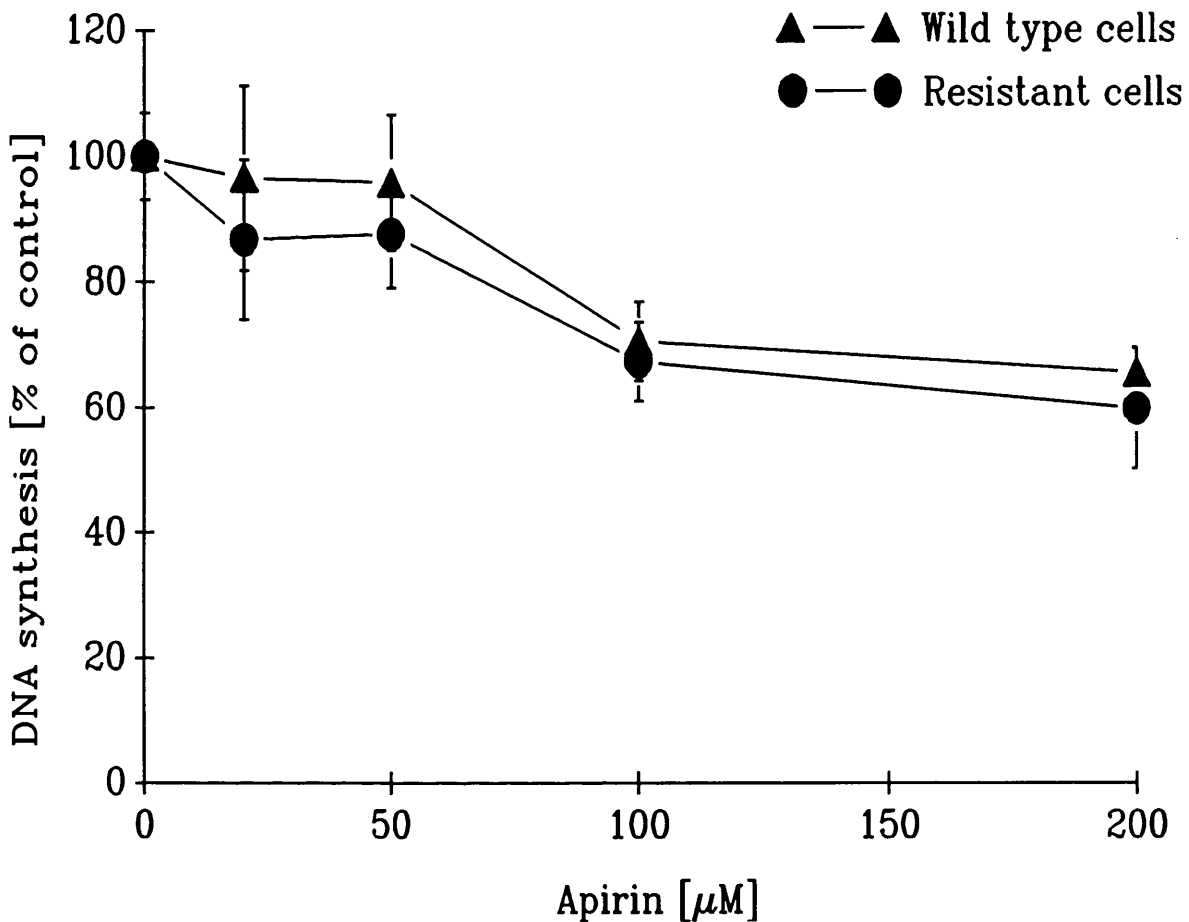
Stock solutions of aspirin (Sigma) 0.5 M and indomethacin (Sigma) 0.25 M were made up in DMSO. This stock was then diluted in cell culture medium to give the final concentration. Solvent concentration in culture medium did not exceed 0.1% . Metabolic cooperation methods used were as described previously.

### 4.6.3. Results

Figure 4.6.1 shows the effect of different doses of aspirin on DNA synthesis in both strains of V79 cells. It can be seen that there is a similar trend in the response of these cells to aspirin. The effect of aspirin was tested on both types of cells to ascertain if this compound has differential effects on either strain. If the two cells types had shown different susceptibilities to this drug then all drugs tested in this communication assay would have to be characterized in this way. However, there is no evidence to suggest that these cells behave differently. Figure 4.6.2 shows that aspirin at 50  $\mu\text{M}$  does not inhibit junctional communication. A more comprehensive representation of results in table 4.6.1 shows that aspirin between the concentrations of 20  $\mu\text{M}$  and 200  $\mu\text{M}$  does not affect junctional communication.

Figure 4.6.1

Effect of exposure to different concentrations of aspirin on wild type and resistant cell growth and DNA synthesis

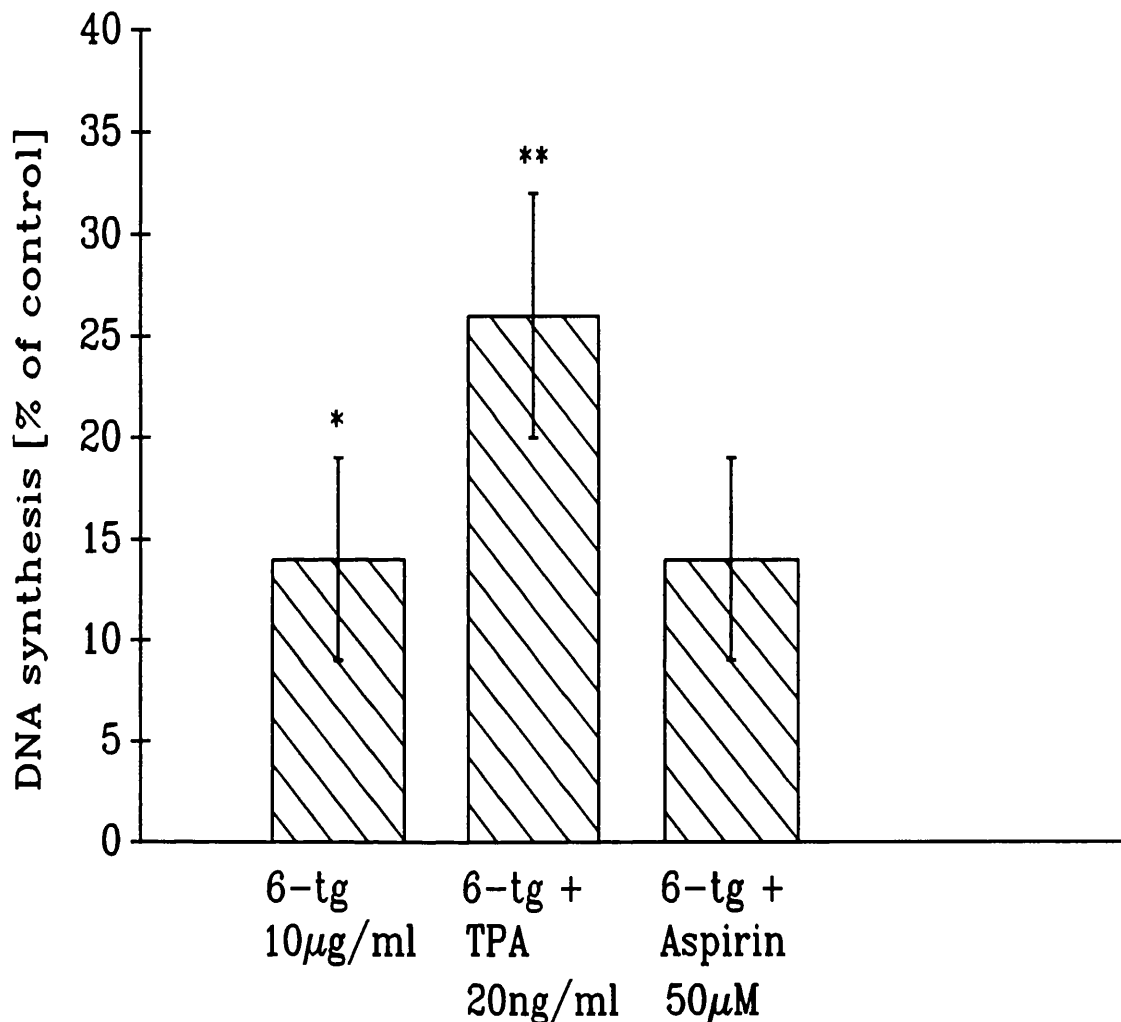


V79 wild type and 6-tg resistant cells were plated in separate 24 well plates at a total density of 60 K cells . 24 hours later aspirin dissolved in DMSO was added to culture medium at various concentrations and the medium was then added to the wells. 4 wells were treated for every concentration of aspirin used. DMSO was added to solvent control group at 0.1% final concentration. After 24 hours exposure the aspirin containing medium was removed and medium containing 0.1μCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.



Figure 4.6.2

### Effect of aspirin on metabolic cooperation in co-culture



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg with 50µM aspirin was added to a groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 20ng/ml TPA plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA treated group (\*\*) are significantly different.  $P=1 \times 10^{-3}$

Table 4.6.1 showing the effect of aspirin on junctional communication. Results expressed as DNA synthesis % of co-culture control without 6-tg.

6-tg 10 $\mu$ g/ml	Paracet- amol 1mM + 6-tg	20 $\mu$ M Aspirin + 6-tg	50 $\mu$ M Aspirin + 6-tg	100 $\mu$ M Aspirin + 6-tg	200 $\mu$ M Aspirin + 6-tg
5.7	18.5	6.27	5.7	5.4	5.47
$\pm$ 0.52	$\pm$ 2.37	$\pm$ 0.57	$\pm$ 0.2	$\pm$ 0.53	$\pm$ 0.76

A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu$ g/ml of 6-tg with a range of concentrations of aspirin were added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 1mM paracetamol plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu$ Ci/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

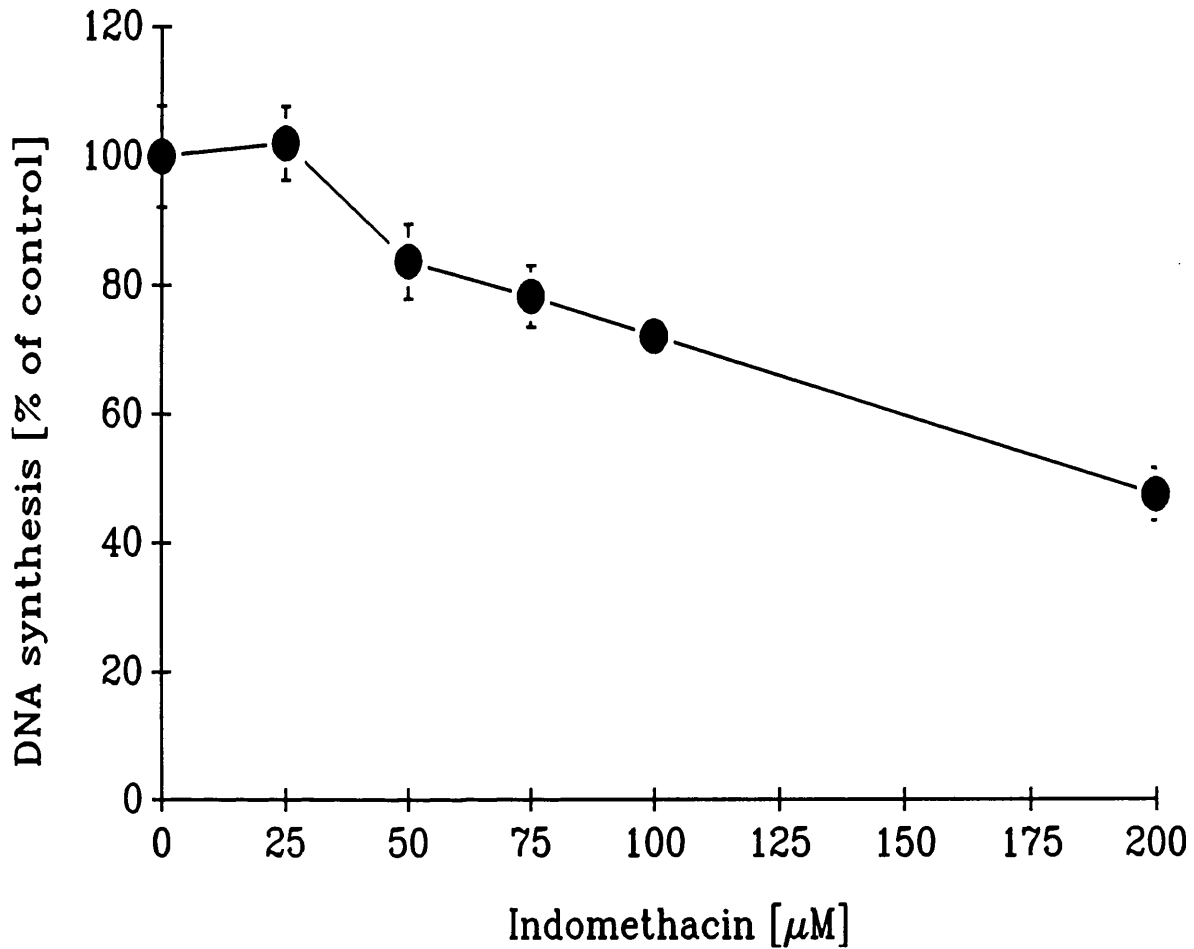
Similarly to aspirin, indomethacin does not inhibit junctional communication either. Figure 4.6.3 shows the effect of different doses of indomethacin on V79 cell DNA synthesis. Figure 4.6.4 demonstrates that while paracetamol has a significant effect on junctional communication, indomethacin does not. Table 4.6.2 further illustrates this point.

#### 4.6.4. Discussion

It would seem that neither aspirin nor indomethacin has an effect on junctional communication. Giaume et al. (1989) have demonstrated that indomethacin can reduce junctional conductance in some of their experiments but they found their results variable. Perhaps the effect of aspirin and indomethacin on gap junctions is not of a sufficient duration to allow detection by metabolic cooperation methods.

Figure 4.6.3

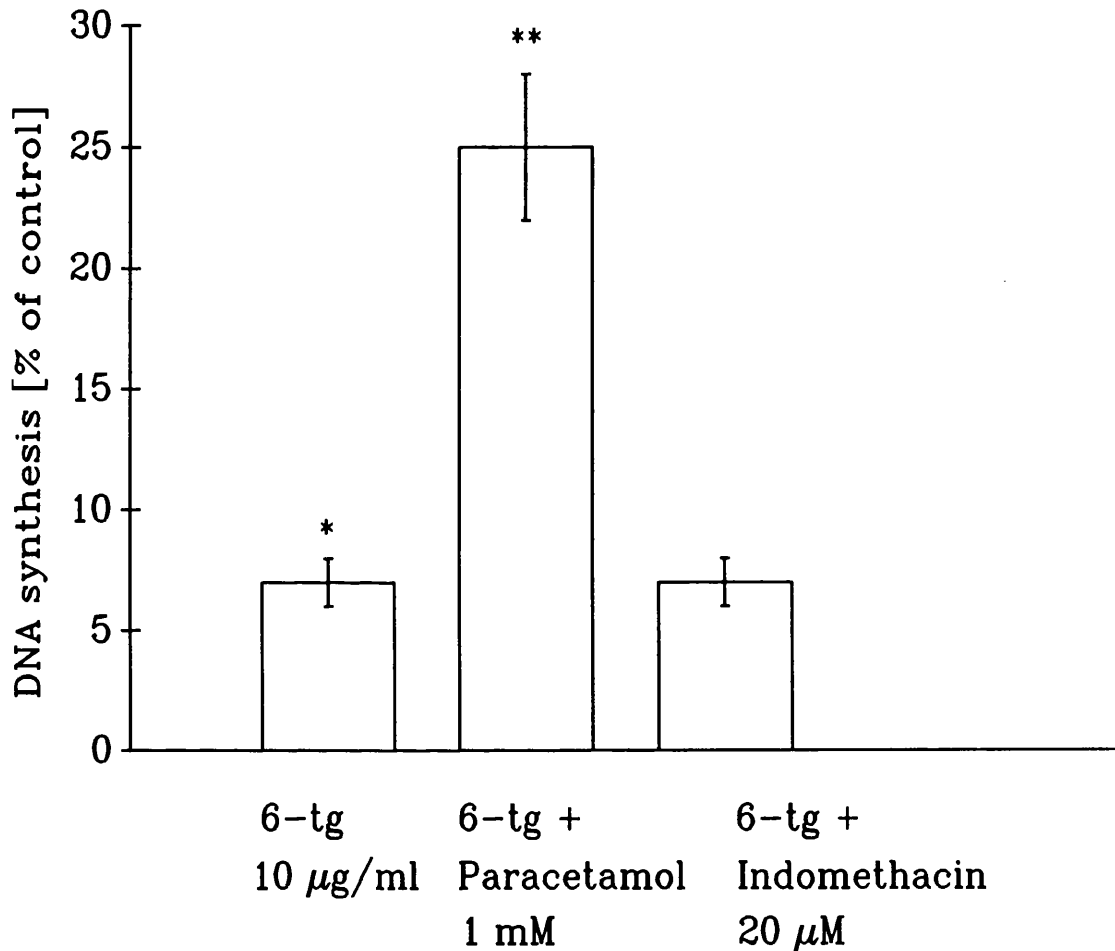
### Effect of exposure to different concentrations of Indomethacin on cell growth and DNA synthesis



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later indomethacin dissolved in DMSO was added to culture medium at various concentrations and the medium was then added to the wells. 4 wells were treated for every concentration of indomethacin used. DMSO was added to solvent control group at 0.1% final concentration. After 24 hours exposure the indomethacin containing medium was removed and medium containing 0.1 $\mu\text{Ci/ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

Figure 4.6.4

### Indomethacin does not inhibit junctional communication



A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg plus 20µM indomethacin was added to a groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 1mM paracetamol plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA treated group (\*\*) are significantly different.  $P=7 \times 10^{-3}$

Table 4.6.2 showing the effect of indomethacin on junctional communication. Results expressed as DNA synthesis % of co-culture control without 6-tg.

6-tg 10 $\mu\text{g/ml}$	25 $\mu\text{M}$ Indo+6-tg	50 $\mu\text{M}$ Indo+6-tg	100 $\mu\text{M}$ Indo+6-tg	200 $\mu\text{M}$ Indo+6-tg
26	21	20	18	13
$\pm 1.5$	$\pm 1.2$	$\pm 1.8$	$\pm 1.2$	$\pm 1.1$

A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu\text{g/ml}$  of 6-tg with a range of concentrations of indomethacin were added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu\text{Ci/ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

## 4.7. Does Nordihydroguaiaretic Acid Effect Gap Junctions?

### 4.7.1. Introduction

Aspirin and indomethacin are more selective at inhibiting the prostaglandin synthetase (cyclooxygenase) enzyme than the lipoxygenase enzyme (Rainsford 1988) and NDGA is a more selective inhibitor of the lipoxygenase enzyme. Giaume et al. (89) have found that this compound decreases junctional conductance. If NDGA produced an effect in the present metabolic cooperation system then it would provide some clues to the mechanism of action of paracetamol on junctional communication.

### 4.7.2. Materials And Methods

NDGA (Sigma) was dissolved in DMSO to give a 0.5M stock solution. This stock was then diluted in cell culture medium to the final concentration. Final solvent concentration was less than 0.1%. Metabolic cooperation methods used were as described previously.

### 4.7.3. Results

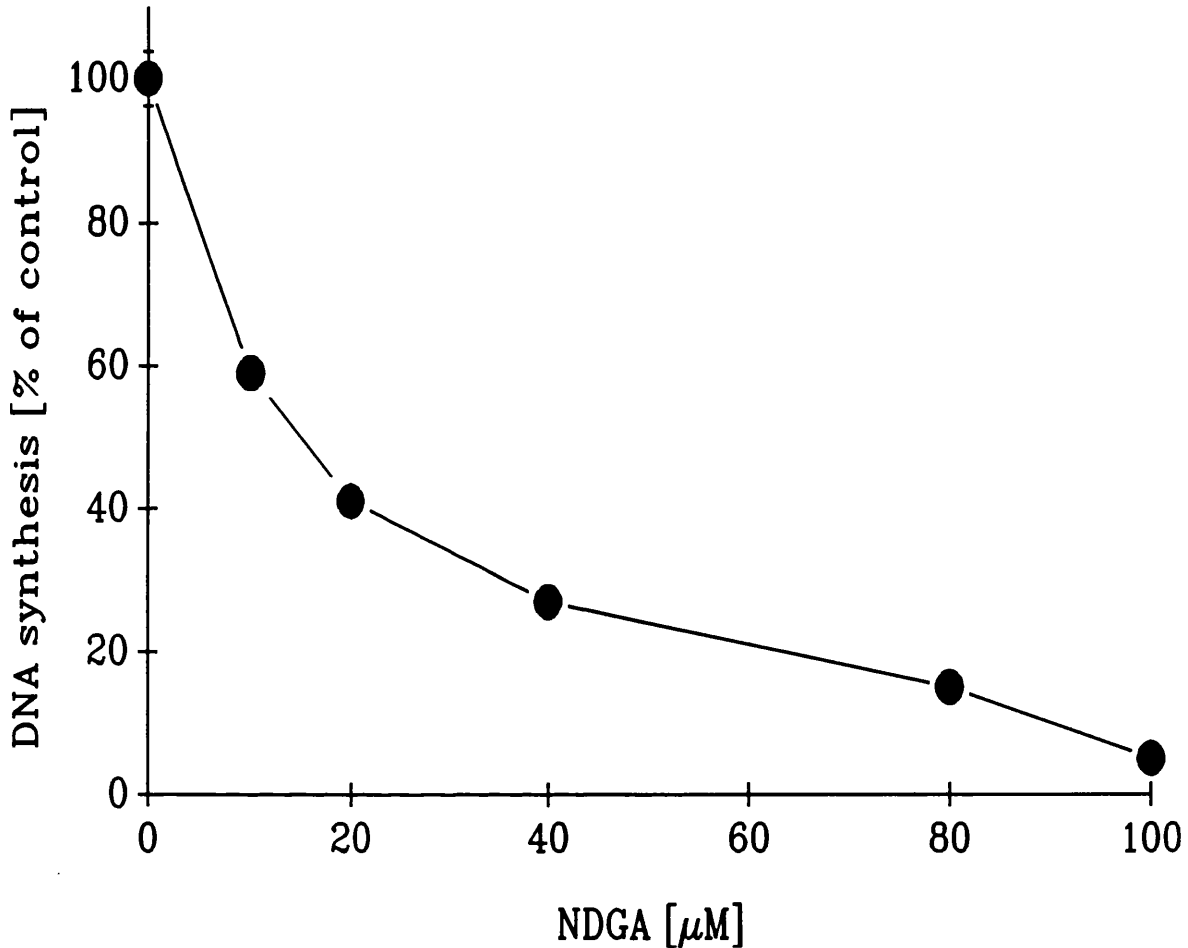
As figure 4.7.1 shows NDGA has a marked effect on cell capacity to synthesize DNA, even at concentrations as low as 20  $\mu$ M. The effect of NDGA was then tested on junctional communication and it was found that indeed this compound does inhibit junctional communication. Could it be that NDGA was preventing the metabolism of 6-tg to its toxic metabolite? In order to answer this question an experiment was performed whereby wild

type cell cultures were incubated with 6-tg only at various concentrations. In a parallel experiment cell cultures were treated with various concentrations of 6-tg and 20  $\mu$ M NDGA.

If NDGA prevented the conversion of 6-tg to the toxic metabolite then DNA synthesis in the wild type cells would not be inhibited. The results depicted in figure 4.7.2 show that NDGA increases cell capacity to synthesize DNA when 6-tg at concentrations of 1  $\mu$ g/ml is used but at the concentration of 10  $\mu$ g/ml 6-tg the effect is not great. These results are similar to that obtained for paracetamol and show that the two compounds could be acting through the same mechanism.

Figure 4.7.1

Effect of exposure to different concentrations of NDGA on cell growth and DNA synthesis

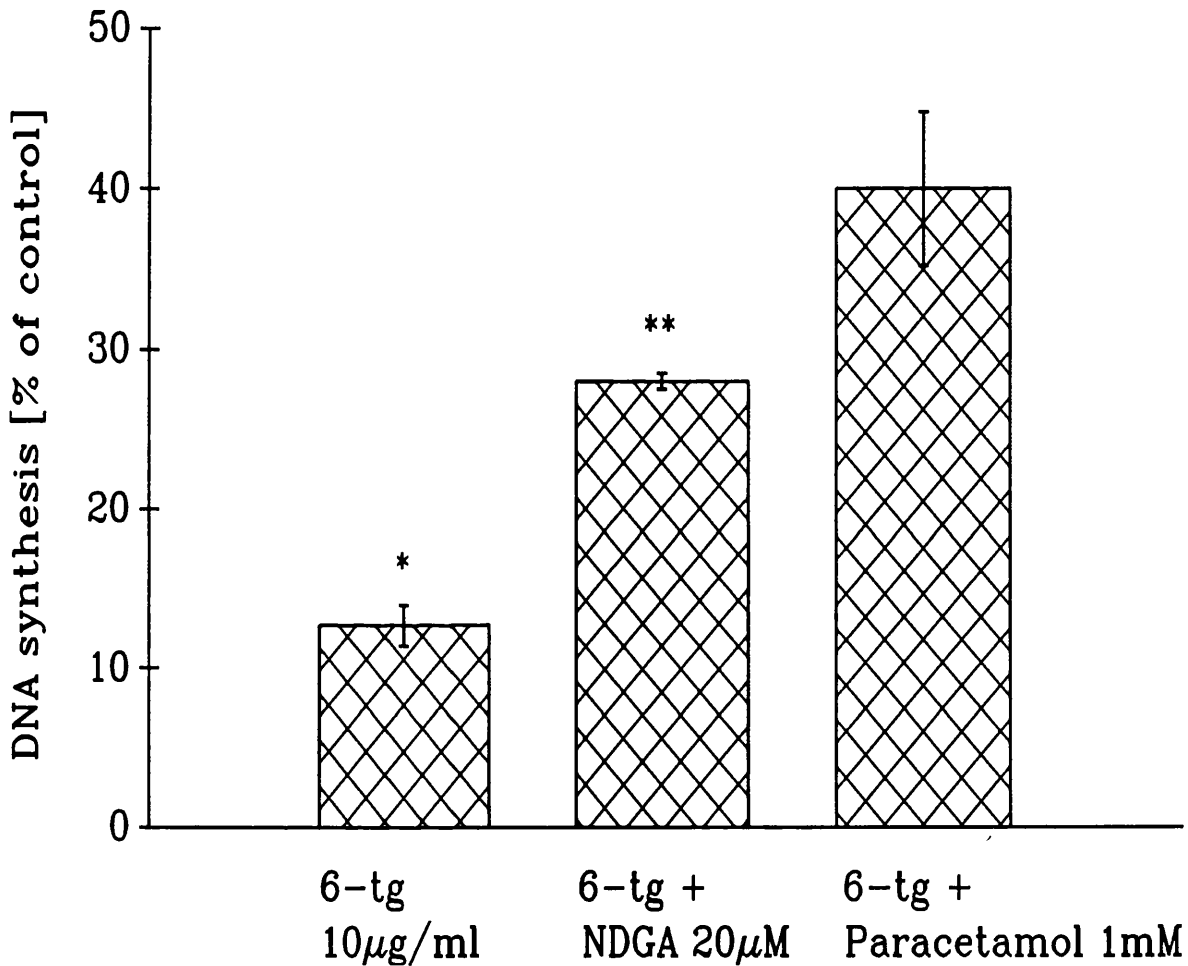


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later NDGA dissolved in DMSO was added to culture medium at various concentrations and the medium was then added to the wells. 4 wells were treated for every concentration of NDGA used. DMSO was added to solvent control group at 0.1% final concentration. After 24 hours exposure the NDGA containing medium was removed and medium containing 0.1 $\mu\text{Ci/ml}$  tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.



Figure 4.7.2

### NDGA inhibits junctional communication

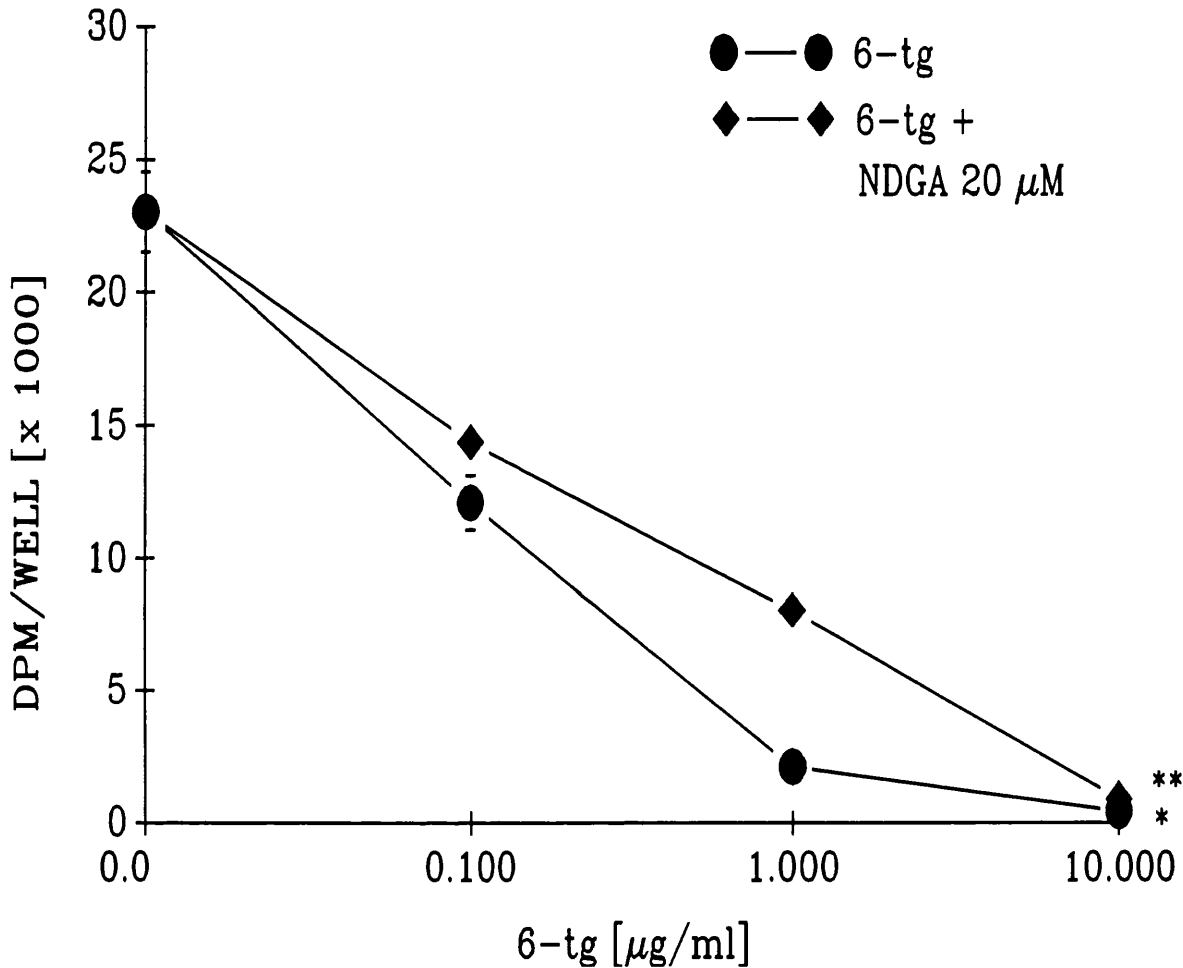


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg plus 20µM NDGA was added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 1mM paracetamol plus 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+TPA treated group (\*\*) are significantly different.  $P=7 \times 10^{-7}$

Figure 4.7.3

Does NDGA reduce the lethal effects of 6-tg on wild type V79 cells?



Wild type cells were plated at a density of 60 K per well for 24 hours. Old medium was removed and medium containing 6-tg at different concentrations was added to appropriate groups of wells while medium containing various concentrations of 6-tg plus 20µM NDGA was added to other groups. Solvent control group were also treated. After 24 hours exposure, the chemicals were removed and medium containing 0.1µCi/ml tritiated thymidine was added. After 4 hours of incorporation the amount of tritiated thymidine incorporated was measured.

The 6-tg control group (\*) and the 6-tg+NDGA 20µM group (\*\*) are significantly different.  $P=4.3 \times 10^{-6}$

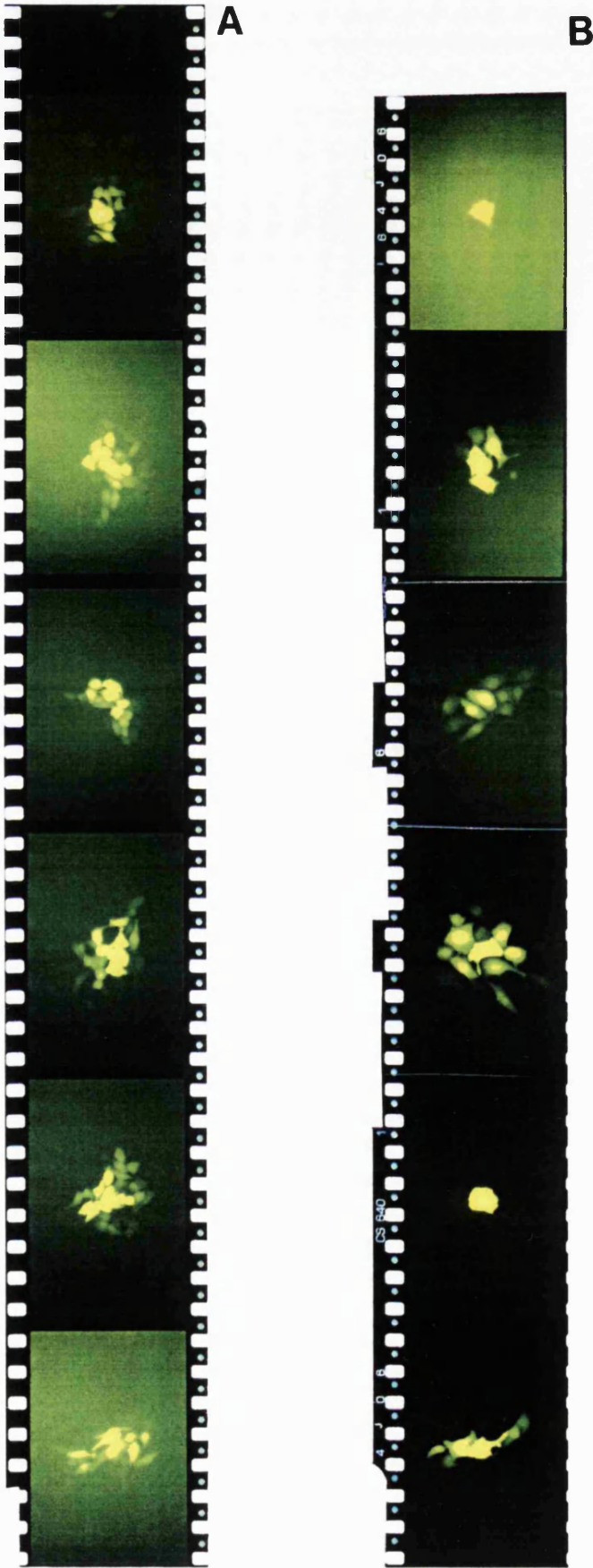
#### 4.7.4. Verification Of Metabolic Cooperation Results With Dye Transfer

Dye transfer is an alternative method that can be used to monitor junctional communication. Moreover, it is a direct way of visualizing the spread through gap junctions of a substance from one cell to its neighbours. Using two methods to measure junctional communication would give a greater insight into the phenomenon of junctional communication than just one method.

All techniques for measuring junctional communication have their own limitations. The results derived from metabolic cooperation experiments give information about communication in a relatively large population of cells. The main drawback with metabolic cooperation is that any change in gap junctions must remain for several hours to be detected. The dye injection assay however, can be used to measure the junctional communication status of a cell culture at specific instances. The main limitation of the dye injection assay is that a relatively small number of cells can be injected at any one time. Furthermore the problems explained in section 4.2.5, namely ambiguity in quantifying results from dye transfer experiments also apply.

The fact that V79 cells grow in monolayers means that dye injection can easily be carried out. Therefore the use of the dye transfer method could confirm the effects of paracetamol and nordihydroguaiaretic acid (NDGA) on junctional communication obtained using metabolic cooperation. When cultures treated with 1mM paracetamol for 8 hours were injected with lucifer yellow, it was found that there was no inhibition of communication. The results presented in figure 4.7.4 were rather surprising.

Figure 4.7.4 Effect of paracetamol on dye transfer



#### Figure 4.7.4 Legend

V79 wild type cells were plated at a density of  $2 \times 10^6$  cells per 35mm plate and left overnight. Group A was exposed to 5% solvent (PBS) v/v. Group B was exposed to 1mM paracetamol dissolved in PBS.

After 8 hours incubation the cells were injected with lucifer yellow. The cell culture was then washed with fresh medium and the injected cells were photographed under fluorescent lighting conditions ten minutes after injection.

With the powerful effect exerted by paracetamol on inhibiting metabolic cooperation it was expected also to see inhibition of dye transfer. Indeed paracetamol produced effects comparable to TPA in the metabolic cooperation assay. In the dye transfer assay TPA blocked dye transfer yet paracetamol did not. In the cultures treated with paracetamol there seemed to be fewer cells to which the dye had travelled. This could mean that perhaps paracetamol does not block gap junctions completely but reduces their permeability. The metabolic cooperation results cannot be explained this way since lucifer yellow has a molecular weight of 457.3 and the 6-tg metabolite has a molecular weight of 380. This means that the 6-tg metabolite should find it easier to traverse the junctional channels.

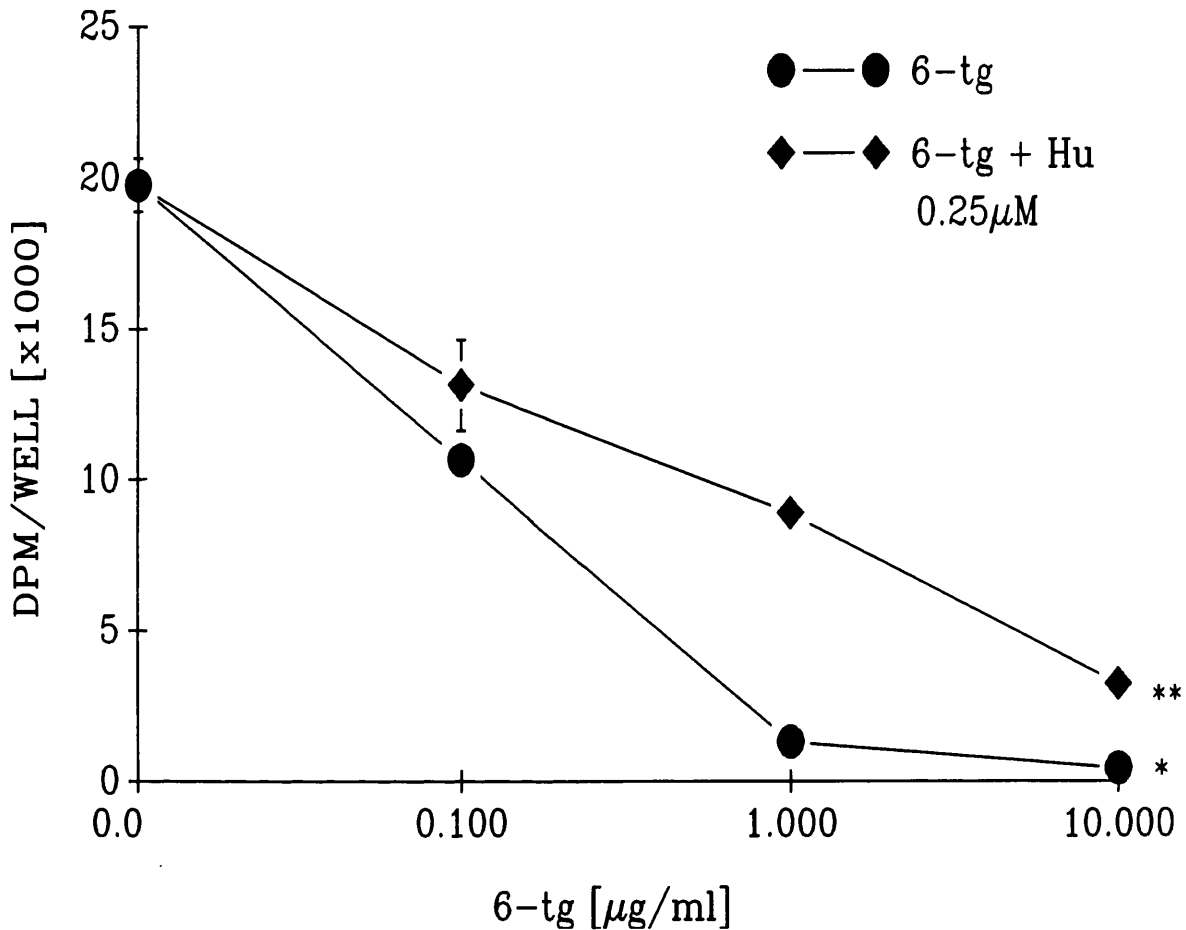
On closer examination of the dye transfer experiment, it was observed that the spread of dye in the paracetamol treated culture was over a similar area to that of the control culture. The paracetamol treated cells seemed to have spread out more than the control cells and looked larger. This could have happened if the cells were prevented from dividing and spread out to fill the culture surface available to them. This leads to the conclusion that perhaps paracetamol and NDGA inhibit DNA replication (This possibility was tested for, see section 4.4 and 4.7.). If this were so then 6-tg would not be incorporated into DNA and DNA synthesis in cells would not be inhibited. This would give a result that would appear to show that junctional communication was inhibited.

To test this theory the effect of a known inhibitor of DNA synthesis on metabolic cooperation was investigated. Hydroxyurea (Hu) is a compound which inhibits DNA synthesis by inhibiting the enzyme ribonucleotide reductase (Lagergren & Reichard 1987). Hydroxyurea was tested to find out if it would prevent the metabolism of 6-tg in V79 wild type cells and thereby prevent the action of 6-tg. In an experiment similar to that done for paracetamol and NDGA, it produced a significant reduction of 6-tg toxicity even when 10

$\mu\text{g/ml}$  6-tg was used as shown in figure 4.7.5. Next, the effect of hydroxyurea on metabolic cooperation was tested. The concentration of hydroxyurea which has a comparable effect with paracetamol in this metabolic cooperation assay was found by testing a range of concentrations as shown in figure 4.7.6. This finding points to the possible mechanism of action of paracetamol. Figure 4.7.7 shows that 0.25 mM hydroxyurea increases DNA synthesis in co-culture to a similar degree to that of 1mM paracetamol. This does not necessarily mean that hydroxyurea, NDGA and paracetamol do not inhibit metabolic cooperation. All of these compounds could exert an effect on junctional communication as well as being inhibitors of DNA synthesis. Therefore, further evidence is needed to reveal the mechanism involved. If hydroxyurea prevents DNA synthesis, how would it affect tritiated thymidine incorporation when added to the culture with the thymidine incorporation medium? In the following experiment all the compounds which inhibited junctional communication based cell damage in the metabolic cooperation assay were tested for their effect on tritiated thymidine incorporation. Figure 4.7.8 shows that paracetamol, NDGA and hydroxyurea all severely inhibited thymidine incorporation. However, TPA which has consistently inhibited metabolic cooperation and dye transfer did not. This shows that the effect of TPA on metabolic cooperation are genuine and that of paracetamol and NDGA are artifacts perhaps because of interference with 6-tg activation.

Figure 4.7.5

Hydroxyurea (Hu) reduces the lethal effects of 6-tg in wild type cells



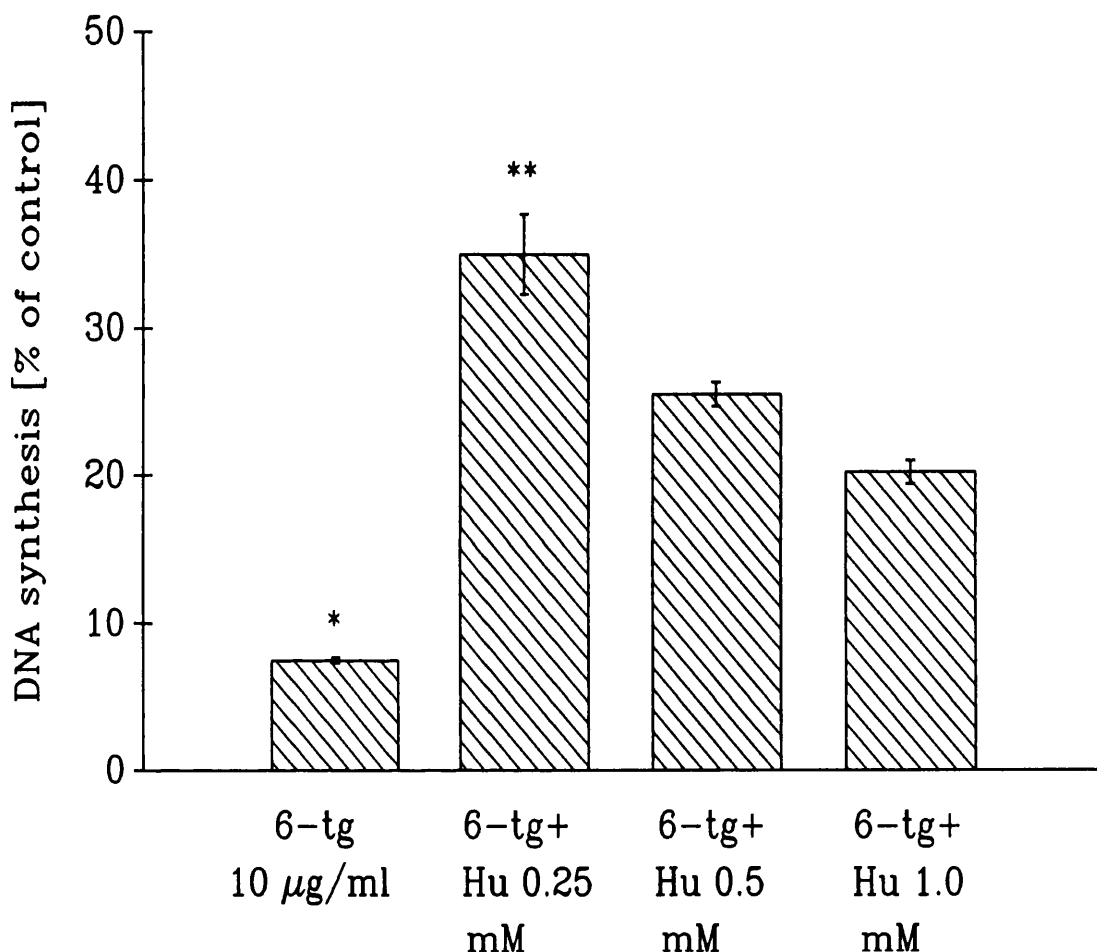
Wild type cells were plated at a density of 60 K per well for 24 hours. Old medium was removed and medium containing 6-tg at different concentrations was added to appropriate groups of wells while medium containing various concentrations of 6-tg plus 0.25mM hydroxyurea was added to other groups. Solvent control group was also treated. After 24 hours exposure, the chemicals were removed and medium containing 0.1µCi/ml tritiated thymidine was added. After 4 hours of incorporation the amount of tritiated thymidine incorporated was measured.

The 6-tg control group (\*) and the 6-tg+0.25mM hydroxyurea group (\*\*) are significantly different.  $P=2 \times 10^{-4}$



Figure 4.7.6

Effect of Hydroxyurea (Hu) on junctional communication based lethal effects of 6-tg.

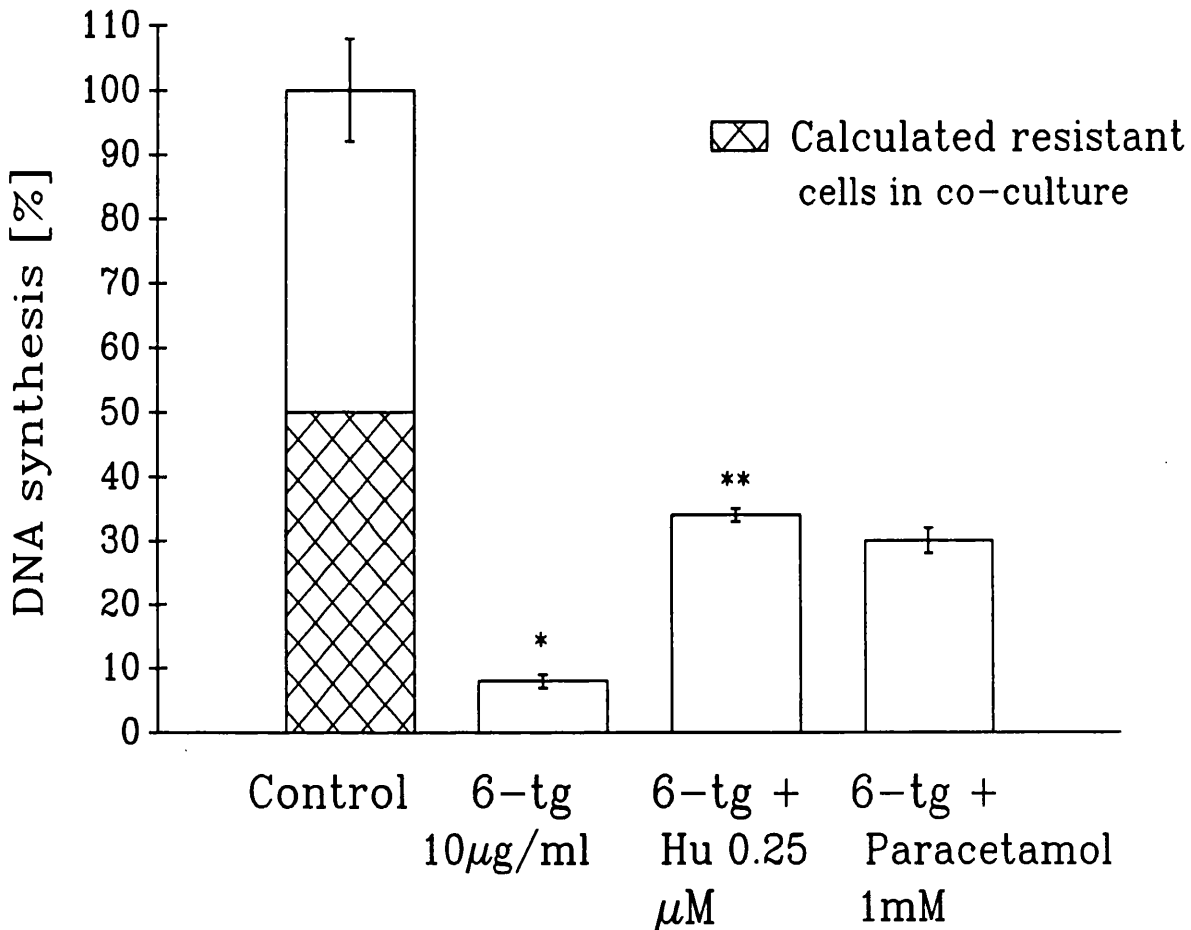


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10µg/ml of 6-tg plus various concentrations of hydroxyurea were added to groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1µCi/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+0.25mM hydroxyurea group (\*\*) are significantly different.  $P=8 \times 10^{-7}$

Figure 4.7.7

Hydroxyurea (Hu) increases resistant cell capacity to synthesize DNA in co-culture

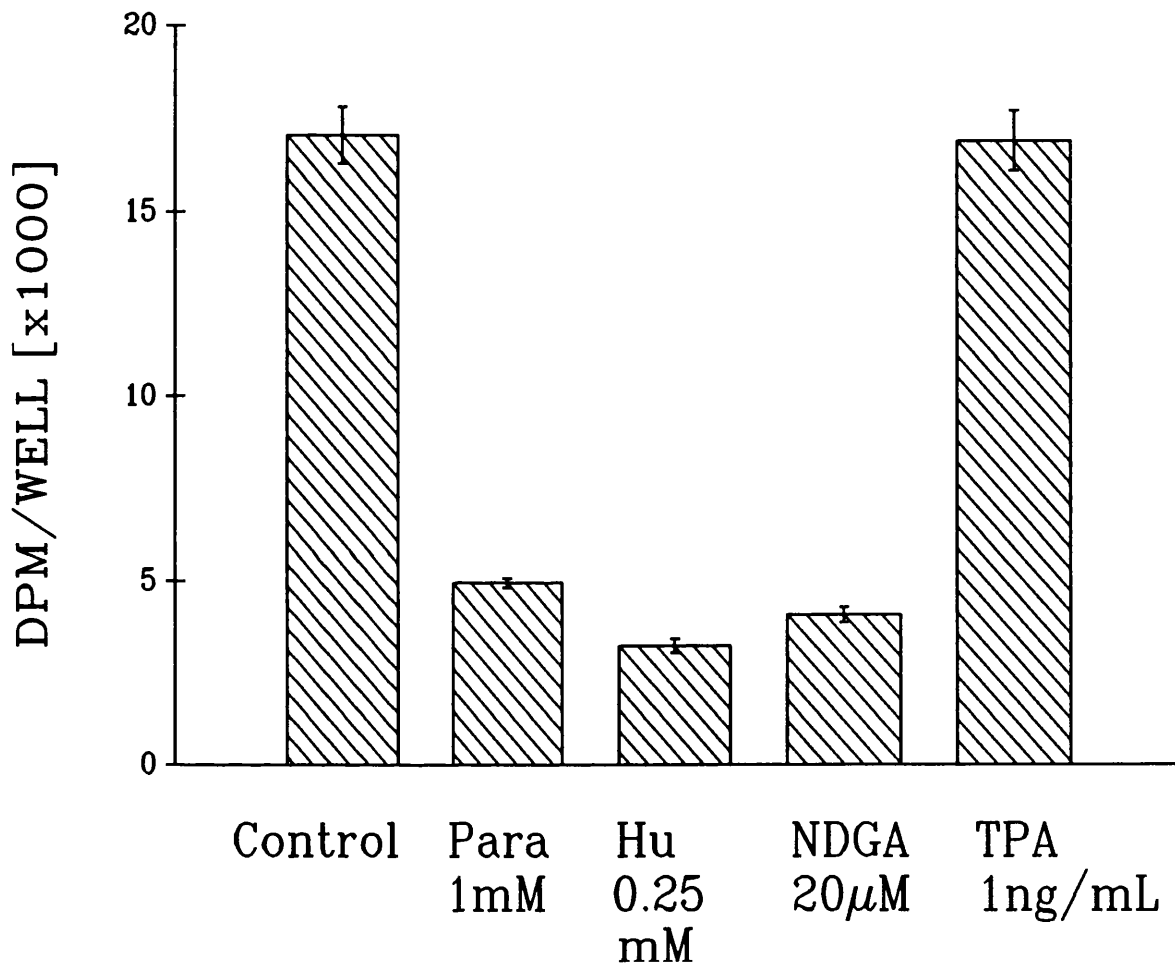


A co-culture of V79 wild type and 6-tg resistant cells was plated in a 24 well plate in a 1:1 ratio at a total density of 60 K cells. 24 hours later 10 $\mu$ g/ml of 6-tg plus 0.25mM hydroxyurea was added to a groups of 4 wells. All chemicals were made up to final concentration in growth medium and this medium was then added to the cell culture. Solvent controls and 6-tg controls were also treated. A positive control group was treated with 1mM paracetamol and 6-tg. After 24 hours exposure, the drug containing medium was removed and medium containing 0.1 $\mu$ Ci/ml tritiated thymidine was added. After a further 4 hour incubation the incorporated tritiated thymidine was measured to give a measure of relative cell DNA synthesis.

The 6-tg control group (\*) and the 6-tg+0.25mM hydroxyurea group (\*\*) are significantly different.  $P=8 \times 10^{-7}$

Figure 4.7.8

**<sup>3</sup>H-thymidine incorporation is directly inhibited by some potential cell communication inhibitors**



Wild type V79 cells were plated at a density of 60 k cells per well and the culture was incubated for 24 hours. Medium containing different drugs plus 0.1µCi/ml tritiated thymidine was then added to the culture and thymidine was incorporated for 4 hours. The amount of tritiated thymidine incorporated was then measured.

## Chapter 5. Discussion

### 5.1. Project Achievements

- 1) Development of a short metabolic cooperation assay to measure junctional communication based on toxic nucleotide analogue transfer through gap junctions.
  
- 2) Demonstration of positive effects by paracetamol and nordihydroguaiaretic acid in the short assay. Demonstration that these effects are due to inhibition of action of nucleotide analogue and not an effect on junctional communication.
  
- 3) Demonstration that using two methods for measuring junctional communication is more informative about the mechanisms involved rather than using one method just to catalogue effects.

### 5.2. Methodology Of Junctional Research

Gap junctional intercellular communication has taken prominence in recent years especially since it has been shown that junctional communication is modulated in tumour cells. Numerous methods have been utilized to measure junctional communication. When one looks through the published literature it becomes apparent that there are a multitude of compounds and treatments which inhibit junctional communication. Perhaps it is the fortuitous nature of gap junctional research that most compounds tested produce an effect, or perhaps we need to look closely at the methods we use to assay junctional communication.

All substances are toxic if the doses are high enough. Perhaps this also applies to effects on junctional communication.

One phenomenon which gives us information as to the way cells communicate via gap junctions is metabolic cooperation. This has in its many guises produced much data on gap junctional research. One particular method which utilizes metabolic cooperation as a basis for measuring gap junctional communication is the colony forming method developed by Yotti et al. (1979). Compared to other metabolic cooperation methods the method of Yotti et al. (1979) is easier to carry out, making it popular with research workers and a catalogue of compounds which affect gap junctions has been compiled.

One has to ask the question as to whether an effect exists or that the method used to measure the phenomenon is itself producing the effect? It is not possible to study junctional communication *in vivo* at the present time. It is however, possible to study junctional communication *in vitro* using tissue culture, with none of the problems associated with whole animal work. Hence most of the literature on junctional communication comes from *in vitro* experiments in cell or tissue culture. The most physiologically meaningful results have been obtained using whole organ and freshly isolated tissues, which were as near to the *in vivo* situation as possible. It becomes more difficult to extrapolate the results obtained *in vitro* in cell culture to the *in vivo* situation which prevails in the organism. For example, Kam & Pitts (1988) have shown that TPA which inhibits junctional communication in tissue culture failed to do so when applied to intact mouse skin. They treated the mouse skin with TPA for a period then removed the skin and assayed for junctional communication *in vitro* using dye transfer. This approach does not measure junctional communication *in vivo* but is closer to the *in vivo* situation than cell culture. Since TPA is a tumour promoter in mouse skin the fact that it did not inhibit junctional communication is important. Alternatively the work of

Krutovskikh et al. (1991) supports the *in vitro* findings that tumour cells have diminished junctional communication. They induced liver tumours in rats and measured junctional communication using dye transfer in slices taken from those livers. They found that junctional communication and connexin 32 expression was down graded.

The ideal situation would be to study the rate of junctional communication *in vivo* but this is not yet possible. Hence, a model system which approximates nearest to the *in vivo* situation needs to be used. Dye transfer is one assay which can be used to measure junctional communication but it has its limitations. Communication in a relatively few cells can be monitored at any one time. It is hard to quantitate but it does reveal the existence of communication at that moment. Alternatively, metabolic cooperation can provide information about communication in a population of cells. For this reason, the metabolic cooperation method of Yotti et al. (1979) was selected for this project which started out to explore the relation between toxicity and cell communication.

### 5.3. Limitations Of Colony Counting Method And Development Of Modified Method

As the method of Yotti et al. (1979) stood it took two weeks to carry out and also required large culture dishes and large amounts of expensive medium. Hence, it was decided to modify the method such that the assay could be carried out over a shorter time period using multiwell plates thereby providing an easier method for screening junctional communication inhibitors. In the colony forming method 100 resistant cells are plated with about  $8 \times 10^5$  sensitive (wild type) cells in 6 cm dishes. Four hours after plating down the co-culture a compound of interest is added to the dishes to pretreat the co-culture for one hour. 6-Tg at  $10 \mu\text{g/ml}$  plus the compound is then added to the cultures and 3 days later the medium

is changed with fresh medium containing just 6-tg. The surviving resistant cells are allowed to grow into macroscopic colonies for 7 to 10 days and the colonies are stained and visually scored.

One notable feature of the Yotti et al. (1979) assay is the treatment of the co-culture 4 hours after plating down with the presumptive communication inhibitor. After 4 hours the cells attach themselves to the culture dish surface and would most likely not have formed gap junctions. Indeed Miller et al. (1987) found that in this metabolic cooperation assay the cells only cover 10% of the tissue culture dish surface area and thus are spread out. This means that the communication inhibitor is added to the co-culture before junctional channels have formed. This model does not reflect the situation in adult whole animals in which cells are essentially contiguous, but measures a combination of gap junction formation and function. A further problem with the colony forming assay is that one cannot control for the non specific membrane effects of chemicals which may not necessarily inhibit junctions but which may prevent the cells coming into close apposition so that they become able to form functional junctions.

The colony forming assay was modified to take these limitations into consideration and a more compact metabolic cooperation method was developed which can be carried out much faster. In this new method the cells are plated at a sufficient density such that the cells are already in close apposition before the communication inhibitor is added to the co-culture. This allows the co-culture to form gap junctions before any treatment is initiated.

Using the Yotti et al. (1979) colony forming assay a number of workers have shown that TPA has a powerful effect on junctional communication. In most cases TPA produces maximum inhibition in communication (Binder & Volpenhein 1987; Trosko et al. 1980). This is in contrast to the effects of TPA seen in most cases in the modified method where TPA

does not rescue all the resistant cells. How could it be that TPA does not have the same powerful effect in this assay? The reason is not clear but it could be due to the increased cell density in the new method. Indeed when the cell number is increased in the Yotti et al. (1979) colony forming assay, the ability of TPA to inhibit communication and thus rescue resistant cells is diminished (Jones et al. 1985). When the number of sensitive cells plated with the 100 resistant cells is increased from  $5 \times 10^5$  to  $1 \times 10^6$  the TPA induced recovery of resistant cells is reduced from about 80% to about 30%. This leads to the assumption that TPA is more efficient at preventing gap junction formation but less efficient at closing functional gap junctions. This explanation agrees with Pitts & Burks' (1987) hypothesis concerning the mechanism of action of TPA, namely that TPA prevents gap junction formation but is less able to inhibit existing junctions.

There is some experiment to experiment variation in the level of communication inhibition by TPA in the present method. This could be due to the differences in mixing of the two cell strains prior to plating down. When there is greater and even mixing, there is greater communication and hence the inhibition produced by TPA is less (the converse could also be true).

#### 5.4. Use Of New Method To Investigate Junctional Communication

Using the new method, the effect of increasing intracellular calcium on gap junctional communication was investigated. There were no real effects seen on junctional communication by increasing cell calcium using ionophore A23187. Since calcium is an important secondary messenger and participates in a host of physiological processes, prolonged increase in cell calcium could be detrimental to the cell. Consequently any



metabolic cooperation method which relies on transfer and incorporation of a toxic metabolite is not suitable for investigating the effects of calcium on junctional communication. This is because communication inhibition may be short lived and transfer of toxic metabolites may resume during the relatively long incubation period.

The next compound which was tested in this system was DDT. This compound has been shown to be a tumour promoter in the liver and an inhibitor of junctional communication in the Yotti et al. (1979) assay and dye transfer. DDT has inhibited junctional communication in human fibroblasts (Davidson et al. 1985), rat hepatocytes and rat liver epithelial (WB-F344) cells (Flodstrom et al. 1990), V79 cells (Warngard et al. 1989) and mouse hepatocytes (Klaunig et al. 1990). Therefore it was surprising to find out that DDT did not inhibit junctional communication in this system even though in the colony forming assay other people have shown V79 cells to be susceptible. DDT did inhibit gap junctions in rat liver epithelial cells as measured by dye transfer (Hemming et al. 1991). It was decided to test the effect of DDT on this particular strain of V79 cells using dye transfer as a measure for communication. Again the results were negative. Hemming et al. (1991) found that 25 $\mu$ M DDT inhibited gap junctions after 30 minute incubation but it was found that even after 1 hour incubation with 25 $\mu$ M DDT there was no apparent effect on junctional communication. There is no obvious explanation for the lack of effect seen in the strain of V79 cells used. If DDT was only able to inhibit junction formation rather than close existing functional gap junctions then pre-exposing the co-culture to DDT before the cells had grown together would have shown an inhibitory effect on metabolic cooperation. As figure 4.2.3. shows, pre-exposure to DDT did not inhibit junctional communication.

The next compound tested was paracetamol. A number of compounds including paracetamol are hepatotoxins. Indeed carbon tetrachloride is a hepatotoxin as well as being

an inhibitor of cell communication (Saez et al. 1987). Could paracetamol which is a hepatotoxin also inhibit junctional communication? When the effects of paracetamol were investigated in the present plate assay it was found that it produced a powerful inhibitory effect on metabolic cooperation and presumably on junctional communication. This was an exciting result as paracetamol had not been shown previously to affect gap junctions. One had to ascertain if the communication effect observed was just an effect on the cell's ability to metabolize 6-tg. If paracetamol inhibited metabolism of 6-tg then it would appear as if junctional channels were being closed. It was found that paracetamol did reduce 6-tg toxicity on wild type cells when low concentrations of 6-tg were tested. However, when the normal concentration of 6-tg which is used in the metabolic cooperation assay was used there was no great effect. But how could it be explained that at the lower 6-tg concentrations there was an inhibitory effect on 6-tg induced toxicity? It could be possible that metabolism of 6-tg in a culture of cells may be heterogenous. Some cells may convert more 6-tg to its toxic form than neighbouring cells. In a normal culture without paracetamol, if gap junctions are functional then there would be an equilibration of the toxic metabolite in the culture. However, when paracetamol is also present in the culture and cell communication is inhibited, this equilibration may not take place and hence some wild type cells would survive the lethal effects of 6-tg longer. Therefore, the increased DNA synthesis of wild type cells treated with 6-tg only compared to the group treated with 6-tg and paracetamol could be explained by a cell communication inhibitory effect.

In an effort to elucidate the mechanism by which paracetamol inhibits junctional communication, other compounds which share properties with paracetamol were tested. One such compound is BHA. Paracetamol has antioxidant properties and is a phenolic compound. BHA is also a phenolic antioxidant. BHA had already been shown to inhibit junctional

communication as measured by dye transfer (Zeilmaker & Yamasaki 1986; Masui et al. 1988). BHA has also been classed as a tumour promoter (Zeilmaker & Yamasaki 1986) yet BHA is able to inhibit the tumour promoting activity of TPA in mouse epidermal cells by counteracting oxidant generation (Fischer et al. 1986). It seems BHA can have varied effects in different systems. When the effects of BHA were investigated in the present plate system, it was found that BHA did not inhibit metabolic cooperation. In this system the co-culture was treated with BHA for 24 hours continuously. However, Zeilmaker & Yamasaki (1986) found that BHA had the strongest effect on the cells after 48 hour exposure to the compound. The cell used by these investigators were Balb/C 3T3 and although different from the V79 cells they are also hamster fibroblasts. It seems that observation of junctional communication inhibition in one cell type cannot be extrapolated to another cell line.

With these results one can tentatively say that the mechanism of apparent action of paracetamol on junctional communication perhaps does not depend on the antioxidant effect. Perhaps other analgesics could have a similar effect on gap junctions. Indeed indomethacin had been reported to inhibit junctional communication in rat lacrimal gland cells as measured by electrical conductance measurements even though the effects were not long lasting (Giaume et al. 1989).

The effects of aspirin and indomethacin were tested on this metabolic cooperation system. It was found that neither of these compounds had any effect on junctional communication in the present metabolic cooperation assay. Paracetamol is also an inhibitor of arachidonic acid metabolism (Rainsford 1988). Perhaps this could be the mode of action of paracetamol. Giaume et al. (1989) had found that arachidonic acid inhibits junctional communication and the lipoxigenase inhibitor nordihydroguaiaretic acid (NDGA) also inhibits junctional conductance in rat lacrimal gland cells. Inhibiting the lipoxigenase enzyme could

lead to a build up of arachidonic acid in the membranes. The prospects of observing such an effect in V79 cells seemed promising. When the metabolic cooperation experiment was carried out it was found that NDGA did inhibit junctional communication. This result lends some credibility to the theory that paracetamol could be inhibiting junctional communication by inhibiting arachidonic acid metabolism. Giaume et al. (1989) have shown that arachidonic acid causes gap junctions to close directly. This means that if paracetamol and NDGA inhibit the enzymes which catabolize arachidonic acid and hence cause the accumulation of this compound then one could observe gap junctional closure.

As with paracetamol when the ability of NDGA to prevent the toxic effects of 6-tg on wild type cells was tested, it was found that there were no great effects at the concentration of 6-tg used in the metabolic cooperation assay.

At this stage the effects of paracetamol on dye transfer were investigated to confirm the work done using metabolic cooperation. The results obtained were surprising in that dye transfer was not inhibited by paracetamol. On closer observation of the dye transfer experiment it was found that the paracetamol treated cells were larger in size than in the control culture. The conclusion reached was that paracetamol may be preventing the cells from dividing hence they grew larger. If paracetamol prevented the cells from dividing the toxic metabolite would not be incorporated into the DNA and DNA synthesis would not be inhibited. The increased DNA synthesis of the resistant cells after removal of paracetamol would give the impression that junctional communication was being inhibited. But the experiments on wild type cells have shown that at the dose of  $10\mu\text{g/ml}$  6-tg,  $1\text{mM}$  paracetamol does not prevent the lethal effects of 6-tg and hence does not show increased DNA synthesis. Neither does NDGA at  $20\mu\text{M}$ . The next step was to look at the effect of a known inhibitor of DNA synthesis in this metabolic cooperation system. Hydroxyurea which inhibits the enzyme ribonucleotide

reductase and thereby inhibits DNA synthesis was selected (Reickard 1988). When this compound was tested for its effects on metabolic cooperation it gave a positive result comparable with the other inhibitors. This of course did not prove that paracetamol and NDGA were not inhibitors of junctional communication. Hydroxyurea could also have been an inhibitor. An experiment was carried out to further prove that the effects seen were due purely to inhibition of incorporation of the toxic metabolite into the DNA.

Tritiated thymidine was usually incorporated without the communication inhibitory compound being present. In this experiment tritiated thymidine was incorporated in the presence of paracetamol, NDGA, TPA and hydroxyurea in separate experimental groups. It was found that all of these compounds inhibited thymidine incorporation severely except TPA. This experiment suggested strongly that the effects of paracetamol, NDGA and hydroxyurea on the metabolic cooperation assay were indeed due to inhibition of 6-tg metabolism.

One again asks as to how these compounds did not prevent the lethal effects of 6-tg in wild type cells yet prevented those lethal effects in resistant cells present in co-culture?

In order for the resistant cell DNA synthesis to be inhibited the 6-tg has to be taken up by the wild type cells, metabolized, passively transferred through gap junctions to the resistant cells where the metabolite would be incorporated into the DNA and then the resistant cell DNA synthesis would be inhibited. There are clearly quite a few steps involved. Inhibiting not just junctional communication but any of these steps would increase resistant cell DNA synthesis and give the impression that gap junctions were being closed. It can be argued that the inhibitory effect of paracetamol on DNA synthesis may not be complete. Sufficient DNA synthesis may be taking place in the wild type cells in the presence of paracetamol which would allow the lethal effects of 6-tg to take effect.

A small decrease induced by paracetamol in incorporation of a large amount of accumulated

toxic 6-tg metabolite in wild type cells would not have a great effect on wild type cell DNA synthesis. But a decrease in the amount of toxic metabolite in wild type cells will reduce the amount transferred to resistant cells if the toxic metabolite is transferred in limiting amounts. This may well have a great effect on resistant cell DNA synthesis. This could then explain the results obtained.

Evidence in the literature that paracetamol does inhibit DNA synthesis became clear while this work was being done (Hongslo et al. 1988; 1990). There is no previous evidence that NDGA is an inhibitor of DNA synthesis yet this compound inhibited incorporation of 6-tg metabolite into DNA. NDGA has also been shown to inhibit junctional communication (Giaume et al. 1989). It was entirely possible that paracetamol could inhibit DNA synthesis and also be a communication inhibitor. However this was shown not to be the case in the dye transfer experiment. Since the present method and the colony counting method rely on metabolism, transfer and incorporation of 6-tg into resistant cells to demonstrate junctional communication, both methods would be subject to the same errors.

There has been extensive research carried out using metabolic cooperation systems requiring the metabolism of a toxic nucleotide analogue and the subsequent incorporation of the analogue into cellular DNA. The possibility that artefactual positive results could occur through inhibition of DNA synthesis have not been discussed previously.

Could it be that the method employed in the present investigation is the only one to produce these artifacts? One can answer this question by comparing this method with other metabolic cooperation methods such as that developed by Yotti et al. (1979). Both of these methods rely on the metabolism, transfer and subsequent incorporation of 6-tg into the DNA. This means that compounds which affect 6-tg metabolism or DNA synthesis in one, could in theory also affect the other system. In light of the evidence presented, one must exercise

caution when interpreting data derived from such metabolic cooperation assays. Perhaps existing results need to be re-evaluated. In contrast the dye transfer method is not subject to errors due to inhibition of metabolism of nucleotide analogues and offers an alternative method for checking metabolic cooperation results. The combined use of the present multiwell plate metabolic cooperation assay and the dye transfer assay provide a more powerful tool for investigating junctional communication.

#### 5.5. Awareness Of Factors Other Than Disruption Of Junctional Communication Which May Affect Carcinogenesis

It has been shown that disrupting cell to cell communication causes abnormal development in embryos (Warner et al. 1984). It has also been documented extensively that TPA inhibits junctional communication (Yamasaki 1991). Livingston & Wilt (1992) have shown that treatment of sea urchin embryos with TPA causes altered development. Although these authors did not look at junctional communication in these embryos, if junctional communication was inhibited it could be said that the abnormal development was due to inhibition of junctional communication if other parameters were not investigated. However, Livingston & Wilt found that TPA caused an increase in the number of cells which differentiate as endoderm and mesoderm relative to ectoderm. They found that TPA caused an increase in accumulation of RNAs specific to endoderm and mesoderm with a concomitant decrease in RNAs specific to ectoderm.

An investigator looking at the effects of cell communication on embryogenesis using TPA as an inhibitor without looking at other parameters would perhaps make a causal connection between cell communication disruption and abnormal development. However, communication

inhibitors like TPA may have other more subtle effects on cellular physiology which may alter embryonic development or promote tumourigenesis as shown by Livingston & Wilt (1992). Lack of junctional communication in abnormal tissue may not necessarily be the cause. Rather, it may be an effect of the underlying cause. Miller et al. (1987) express similar sentiment.

#### 5.6. Significance Of Interrupting Junctional Communication.

There are a multitude of compounds which inhibit junctional communication. Since inhibition of junctional communication has been implicated in tumourigenesis does it mean that all of these compounds are potential tumour promoters?

In one study cells incapable of junctional communication were transfected with a gene for an adhesion molecule. Transfected cells did express the adhesion molecule and also began to communicate (Yamasaki 1991). This means that it is not necessary to modulate gap junctions directly to affect cell communication. Any treatment which reduces cell adhesion or perturbs cell membranes could inhibit junctional communication. In the colony forming metabolic cooperation assay the co-culture is treated before the different strains of cells have time to grow together and form gap junctions.

Consequently, treatments which interfere with the process of the cells growing together and adhering but not necessarily having any specific effect on junctional channels could give a misleading impression. This kind of error would be limited greatly in the new method because cells are allowed to grow together before being treated with chemicals. The logical extension of this argument is that compounds which give a positive result as inhibitors of cell communication in the colony forming assay will not necessarily give the same result in the



new method. The new method is a closer model of the *in vivo* situation of adult organisms when substances are tested because the cells are in close contact whereas this is not the case in the colony forming assay. The colony forming assay may reflect the events during embryogenesis when spatially separated cells migrate and come together.

### 5.7. Future Of Gap Junctional Research

In 1964 Kanno & Loewenstein showed that cells were coupled with respect to dye transfer. This was a novel, visually appealing method at that time. Great progress in junctional research has been made since, yet investigators are still testing the effects of new and old compounds on junctional communication solely using dye transfer as an assay. This strategy is useful for classifying whether compounds affect junctional communication or not but does not tell us a great deal about the mechanism or the role of junctional communication. It is recognized that every assay for junctional communication has limitations. What we need is comparative studies using different methods to compare and contrast the effects of substances with a view to delineating the role of this form of communication.

Brummer et al. (1991) used dye transfer and ionic coupling to look at the effects of retinoic acid on junctional permeability. They found that although dye transfer was greatly affected, ionic coupling was not. This is an interesting result because it shows us that restricted transfer of dye does not mean that gap junctions are closed. The present project is another example where two different assays for junctional communication allowed the elucidation of a mechanism of action of paracetamol which could not have been discovered by only using one method.

The reductionist approach to biological research is useful inasmuch it allows problems to be divided into manageable projects. The ultimate goal is to understand how living organisms function. Gap junctional communication is a dynamic process present in all tissues except adult skeletal muscle. The ubiquitous nature of junctional communication means that research workers attempting to understand the functional processes in any tissue should also look at junctional communication. The tools available for investigating junctional communication should be utilized by research workers, whether they are investigating the underlying causes of cell injury or atherosclerosis for example. Integration of what we have learnt of biological systems is the real future of gap junctional communication and biological research.

#### 5.8. Conclusions

The development of a shorter, faster technique for measuring metabolic cooperation means that screening of substances for their effects on junctional communication can be carried out reliably and with ease. The present method models more accurately the interactions between cells in adult organisms than the metabolic cooperation methods widely used previously. The testing of paracetamol in this assay and subsequent elucidation of the mechanism of action of this compound on junctional communication has brought forward certain issues. The fact that paracetamol was not inhibiting junctional communication but preventing the incorporation of the nucleotide analogue into DNA has highlighted the vulnerability of metabolic cooperation assays to such artifacts. The use of more than one assay made this realization possible and shows that comparative studies are necessary.

For future use the new metabolic cooperation method requires that controls which test for

the ability of compounds to inhibit DNA synthesis should be performed. This can be accomplished by adding any compound of interest to the cell culture medium along with the tritiated thymidine. If any compound was to inhibit tritiated thymidine incorporation then the junctional communication effects of that compound would then be best tested with dye transfer for example. With adequate controls the present metabolic cooperation method offers a short semi-automated assay for measuring junctional communication which would facilitate the screening of compounds for their communication inhibitory effects.

The presence of junctional communication in organisms from simple sponges to man means that this form of communication has some value, to enable organisms to adapt to changing internal and external environments. Junctional communication could only play this role if it was labile and adjustable with change in situation. Changes in gap junctional communication have been associated with carcinogenesis and failure of embryonic development. Inhibition of junctional communication in organs and cell tissues may not always be detrimental to the organisms.

Could it be that change in junctional communication on exposure to chemicals is just an adaptation to the changed environment?

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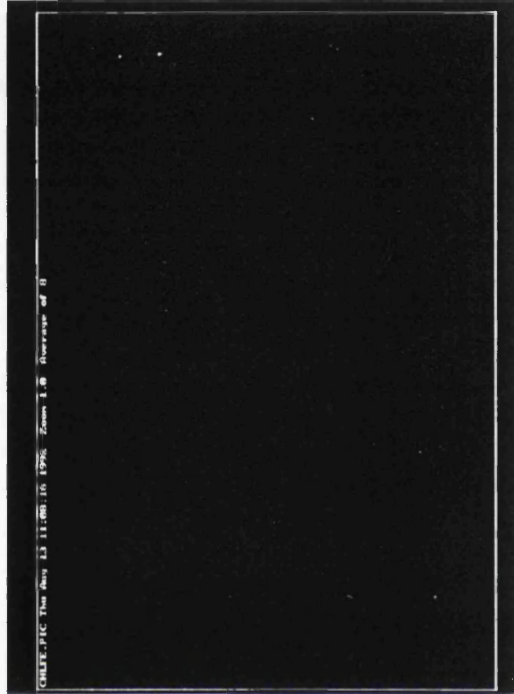
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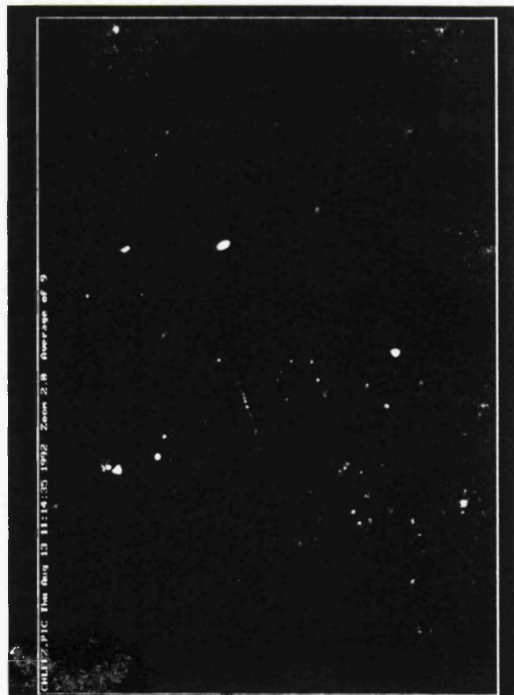
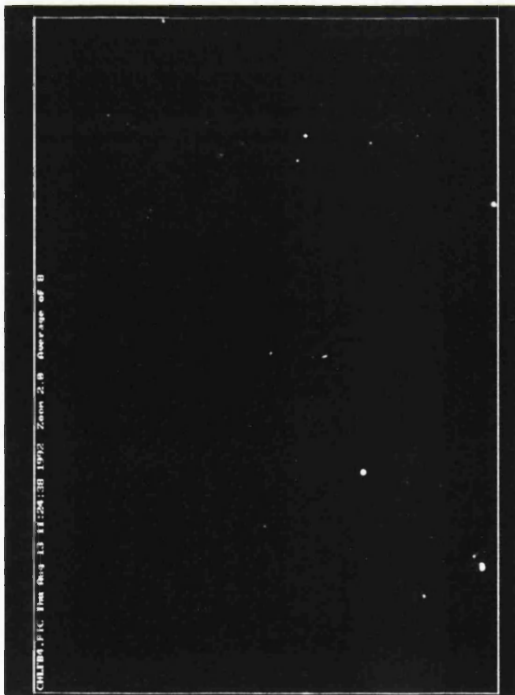
★

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Appendix



Control



Recall

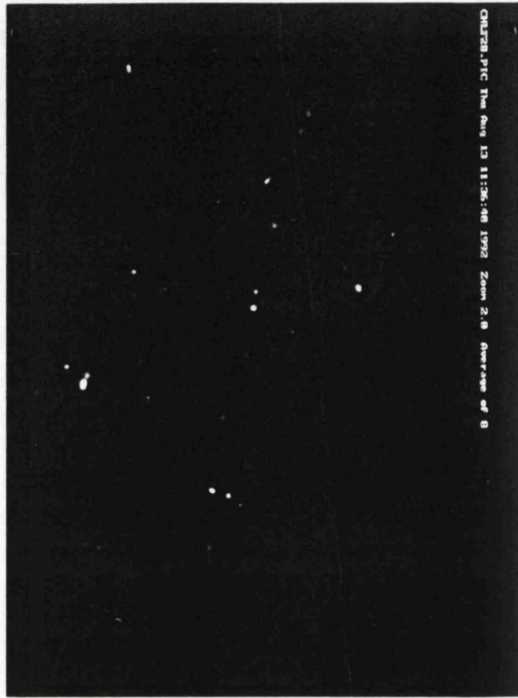


10 μM

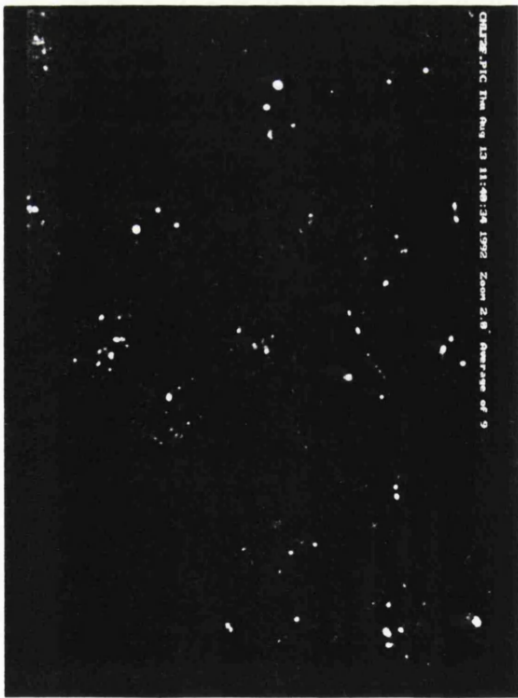




OM202.P1C Thu Aug 13 11:04:20 1992 Zoom 2.0 Average of 8



OM202.P1C Thu Aug 13 11:05:40 1992 Zoom 2.0 Average of 8



OM202.P1C Thu Aug 13 11:09:24 1992 Zoom 2.0 Average of 9

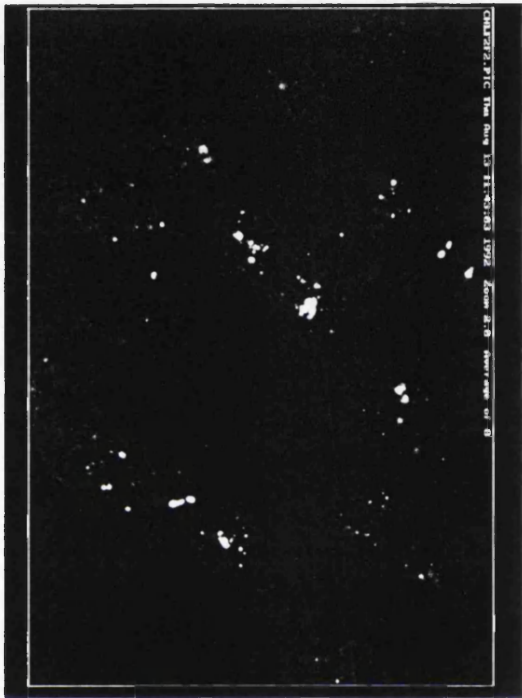


OM202.P1C Thu Aug 13 11:20:58 1992 Zoom 2.0 Average of 9

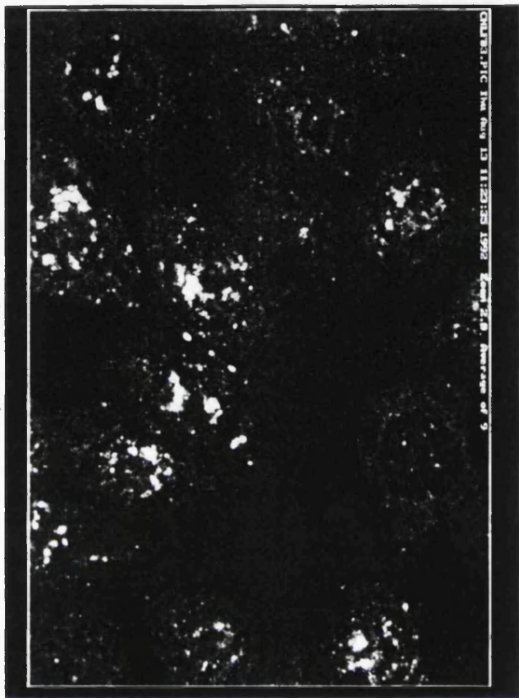
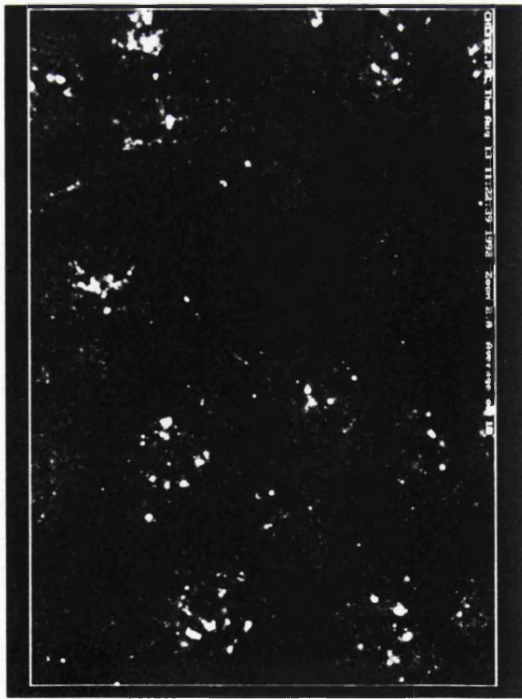
note above of  
smaller 9's

10 μm

V. lye particles



particles



note label  
no label  
towards cell



10 μM



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21 August 1992

Dear Muhammad,

With reference to your CHLF cells which I have labelled for gap junctions using site-specific anti-peptide antibodies. Antibodies recognising three gap junction connexin types (connexin43, connexin32 and connexin26) were used with only connexin43 antibodies giving positive results. This connexin type was labelled using our HJ antibody made to a peptide recognising a portion of the cytoplasmic loop (see Harfst et al reference enclosed).

The labelling observed was of two types:

1. Occasional large junctions between cells. Where these occurred two or three cells could be seen to be expressing large junctions, and often two or three junctions between cells. However, such labelling was not widespread and had a patchy distribution over the culture dish.
2. Long processes overlying cells had fine specking indicating the presence of small junctions between the processes and the cells below. In these cases a cell could be in contact with another which was not a direct neighbour, but possibly two or three cells distant.

In general there was not a large amount of labelling indicating that communication between cells was patchy to say the least. Even where a cell was forming large junctions with one neighbour it would have none with another. Lack of label can not exclude the possibility that there are very small junctions not detectable by immunohistochemistry, although there would still be a large variation in the size of junctions if this were the case.

I enclose a few video print images showing the types of labelling seen.

Hope this is helpful.

(Colin Green)