Factors affecting DNA uptake by mammalian cells.

By

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Abstract.

The ability to introduce DNA and express custom DNA sequences in bacteria opened the door for improvements in a large number of fields including agriculture, pharmacology, medicine, nutrition, etc. The ability to introduce foreign DNA sequences into mammalian cells in an efficient manner would have a large impact on therapeutic applications especially gene therapy. The methods in use today suffer from low efficiencies and sometimes toxicity. In this work a number of factors were evaluated for their effect on DNA uptake efficiency.

The factors studied included exposure to sublethal concentration of hydrogen peroxide which have been show to lead to destabilisation of the lysosomes. These exposures have proven to be very toxic to cells when combined with either the calcium phosphate or the lipofectAMINE® transfection methods. Another factor evaluated was exposure to Electro-Magnetic Fields (EMF). This was fuelled by the fact that EMF have been shown to mediate a number of effects on cell structure and/or physiology. EMF exposure by itself was not sufficient to induce the cells to pick up the DNA, therefore its effect on calcium phosphate and lipofectAMINE® was tested. Although some positive results were obtained, the variability of these results exceeded by far any observed enhancements which discouraged any further work on EMF. Also tested was the possible effect the presence of the cytomegalovirus (CMV) sequence might have on DNA uptake (based on previous results in this lab). It was found that the presence of CMV in the DNA

sequence does not enhance uptake or slow down degradation of the internalised DNA. The final factor tested was the effect of basic amino acids on transfection efficiency. It was found that arginine can enhance DNA uptake by about 170% with calcium phosphate and about 200% with LipofectAMINE®. A model was proposed to explain the effect of arginine as well as the lack of effect from other amino acids.

Dedication:

To Nedal, Mariette, Sarkis and Toufic.

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List of abbreviations.

3-MA: 3-Methyl Adenine.
5-HT: 5-hydorxytryptamine.
ATP: Adenosine triphosphate.
cAMP: cyclic Adenosine Mono-Phosphate.
CAT: Cationic Amino acid Transporter.
CMV: CytoMegalo Virus.
CNS: Central Nervous System.
DMRIE: dimyristooxylpropytl dimethyl hydroxyethyl ammonium bromide.
DMTU: dimethylthiourea.
DODAB/DODAC: dioctadecyl dimethyl ammonium bromide/chloride.
DOGS: dioctadeyl amido glyceyl spermine.
DOPE: Dioleoyl phosphatidylethanolamine.
DOTMA: [2,3-bis(oleoyl)propyl] trimethyl ammonium chloride.
EMF: Electro-Magnetic Field.
EMF: Electro-Magnetic Field.
FCCP: Carbonyl cyanide *p*-trifluoromethoxy phenyl hydrazone.
GABA: Gamma - Aminobutyric Acid
GAT: Gaba transporter.
GFP: Green fluorescent protein.
IGF-II: Insulin like Growth Factor II.
LDH: Lactase dehydrogenase.
MPO: Myeloperoxide.
OMP-F/C: Outer Membrane Protein F/C.
PCR: Polymerase chain reaction.
PMN: Polymorphic Neutrophils.
SV-40: Simian Virus 40

Chapter 1: Transfection protocols.

With the advent of molecular biological techniques in the early 70's, the foundation was laid for a new science to be born. This science is the multidisciplinary biotechnology. One of the most exciting aspects of biotechnology is the ability to manipulate DNA molecules to create "custom" DNA sequences and introduce these especially designed molecules into living organisms. While the techniques for the introduction of DNA into bacteria and other unicellular organisms have been perfected (Cohen *et al.*, 1972) techniques for the introduction of DNA into mammalian cells have proven to be very elusive.

Most, if not all, methods used in the transfection of mammalian cells require the use of a vector system that facilitates the entry and/or expression of the foreign DNA. Based on the nature of the vector used, these methods can be separated into two major categories:

Viral mediated DNA Transfer:

In these methods the DNA of interest is integrated into the viral genome, and the engineered virus is used to infect the target cells. The most commonly used viruses are the retroviruses and adenoviruses. The use of viruses to deliver the gene of interest into the cell takes advantage of the viral machinery to overcome the different barriers to DNA transfer. However these systems have disadvantages that become most apparent when they are used in gene therapy applications. The most notable of these drawbacks are the following:

Safety:

Retroviruses integrate their DNA into the host cell's genome. While this ensures that the DNA is continuously transmitted to the daughter cells, it also carries with it the risk of insertional mutations which can lead to cancer.

Recombination:

Adenoviruses used in gene delivery are first rendered replication deficient by deletion of the early region (E1) which is required for replication. The packaging process allows for homologous recombination which could result in the production of viruses capable of replication.

Immune response:

The immune responses launched by the body in response to the viral introduction, reduced the efficiency of the repeated doses, as well as the elimination of the cells expressing the inserted gene.

For a more detailed review of these techniques and their respective advantages and disadvantages please refer to Cheikha-Douaihy (1996).

Non-viral methods.

These methods rely on chemical and/or physical means to deliver the DNA into the cells. The number of techniques in this category is growing daily, this review however will concentrate on two in particular.

Calcium Phosphate:

This method was first developed by Graham and Van der Eb (1973). It consists of mixing DNA with $CaCl_2$ solution which is then added to a phosphate buffer (usually HEPES). This results in the formation of a $CaPO_4$ precipitate that is associated with the DNA. The mixture is then placed on the cell culture and the cells are incubated for 2-6 hours. The calcium phosphate is then removed (by changing the media). This method is one of the most widely used methods in laboratories around the world due to its ease of use, simplicity and low cost.

The main problem with this technique is the low transfection efficiency and the variability it gives. A number of enhancements/modifications have been introduced in an attempt to increase the efficiency of transfection. Among the most notable of these are the following:

a) Glycerol shock: The incubation of cells in 10-25% glycerol for a short period of time (less than one minute) was shown to increase transfection efficiencies in certain cell lines. It was believed that the shock itself is necessary and it causes the cells to pick up DNA more efficiently although the mechanism is still not well understood (Frost and Williams, 1978). More recent work has shown that the presence of glycerol (as opposed to the chemical shock itself) is responsible for the increased transfection. In fact Wilson and Smith (1997) have shown that the addition of 50% of the glycerol concentration used in the shock to the DNA-$CaPO_4$ complex prior to addition to the cells, resulted in the same transfection levels as when the shock method was used.

b) Temperature: Jordan *et a.*(1996) found that maximum transfection can be obtained at 37°C. They attributed this difference in transfection efficiency to the difference in

the ability of the $CaPO_4$ to bind to the DNA. They found that at 0°C no DNA was bound by the precipitate while at 20°C the binding was reduced and took longer as opposed to a fast and complete binding at 37°C (Jordan *et al.*, 1996).

c) Concentrations: Chen and Okayama (1987) have shown that varying the plasmid concentration varies the transfection efficiency. These findings were supported by the findings of Jordan *et al.*(1996) who found the optimal DNA concentration to be 25 μg/ml. At the same time it was found that varying the concentration of $CaCl_2$ also affects the efficiency of transfection with the optimal concentration being 250 mM (Jordan *et al.*, 1996).

Liposomes and cationic lipids.

The discovery that liposomes can be used to deliver their contents into cells raised the possibility of their use in DNA delivery. Liposomes were expected to fuse with the cell's membrane liberating their contents into the cytoplasm. A large number of formulations have been tried to enhance efficiency while reducing toxicity.

Liposomes are colloidal particles composed of amphiphilic molecules that self assemble into vesicular structures. The shape and size of these vesicles is determined by, among other things, the size of the nonpolar part. A small nonpolar part will result in the formation of micelles. A large nonpolar group will result in the formation of a lipid bilayer that will close on itself forming a hollow bilayered vesicle. The shape of these vesicles is influenced by a number of different factors resulting in shapes ranging from

small multilamellar vesicles to cubic and inverse cubic and hexagonal shapes (Israelchvili,1985).

During the years a large number of lipids have been used, alone or in combination in order to try and maximise the transfection efficiency. The first systems tried were "conventional" liposomes made of neutral or anionic lipids. The most common lipids used were lecithins (phosphotidylcholines, Sphingomyelins and phosphotydylethanolamines (PE) (Kepholins). These lipids were zwiterionic at physiological pH values while phosphotidylserines, phosphatidylglycerols (PG) and phosphotidylinositols (PI) are negatively charged. All of the above mentioned lipids are natural lipids; synthetic lipids were also used and they include dimyristol, dipalmitoyl, distearoyl, dioleoyl and palmitoyl-oleoyl. In general cholesterol is added to provide mechanical stability. All of these different formulations reduce toxicity, with most attempts being driven by empirical procedures.

Another effort for enhancing DNA delivery was the development of pH sensitive lysosomes. At pH > 8, liposomes made of diloleoylphosphatidylethanolamine (DOPE) form lamellar structures that are unstable at pH <8. In order to lower this critical pH value to that of physiological levels, negatively charged cholesterol hemisuccinate is added bringing the critical pH to 5.5. These lysosomes have actually proven to be less effective in transfection although the reason for this are not well understood.

With the failure of conventional liposomes to live up to the expectations, attention was diverted toward liposomes made of cationic lipids. The main kinds of

lipids used to make these liposomes are chloride and/or bromide salts of alkyl of either dodecyl (C_{12}) or hexadecyl (cetyl, C_{16}) chains, (e.g. Dodecyl dimethyl ammonium bromide/chloride (DDAC/B) and cetyl trimethyl ammonium bromide/chloride (CTAB/C))

It was Berhn(1986) who first showed these lipids can complex DNA. The first transfection using these lipids was accomplished not long after by Flegner et al (1987) using liposomes containing dioleoxy propyltrimethylammonium chloride (DOTMA). A number of different lipids have been used over the years in an attempt to enhance efficiency including the use of dimyristooxy propyl dimethyl hydroxyethyl ammonium bromide (DMRIE) (Flegner et al.,1994) and the addition of natural cations such as spermine $^{4+}$ and spermidine $^{3+}$ giving formulations such as DOPSA and DOGS (Behr *et al.*, 1989). DC-Chol is an example of a positive charge being associated to the sterol backbone (Leventis and Silvius, 1990; Gao and Hung, 1991). The majority of other lipid formulation have been variations on the above mentioned ones (with respect to stereochemistry number of positive charges etc. The most common degradation reactions are oxidation and hydrolysis.

Interactions of liposomes with cell membranes can be categorised as lipid exchange, adsorption, fusion or endocytosis. The administration of liposomes in gene therapy application has proven to be one of the major obstacles toward their use. Oral administration results in the passage of the liposomes in the low pH of the digestive system which result in their destabilisation. Application of liposomes in a skin ointment results in them drying out and losing their structure before absorption can take place,

while subdermal injection in the mixture would result to destabilisation by ionic exchange. Direct injection in the blood stream results in the fast uptake of the liposomes by the reticuloendothelial system. The only administration route that has shown some success is the nasal spray which was used in the treatment of cystic fibrosis.

Another point that prevents the used of cationic lipids and liposomes in wide spread gene therapy is their toxicity. Germicidal action of cationic liposomes against bacteria, fungi, viruses, spores, protozoa and invertebrates is well known (Jungerman, 1970). On a colloidal level positively charged particles may cause the aggregation, flocculation, thrombosis or platelet aggregation. On the molecular level the lipids can act as surfactants causing membrane solubilisation, poration, hemolysis as well as changing the properties of the membrane and membrane proteins (such as kinase C) in the membranes in which these lipids insert (Bottega and Epand, 1992). Most molecular level toxicity is reduced by biodegradation while colloidal toxicity is unavoidable and may differ by lipid composition and by individual. This colloidal toxicity could probably explain the fact that the toxicity is relative to the positive charge on the lipid.

With all the formulations tried so far, endocytosis seems to be the main method of entry into the cell. This was established by electron microscopy and gold labelling studies by Zabner *et al* (1995).

Barriers to DNA transfer.

This review will focus on barriers to DNA transfer by non-viral methods, especially calcium phosphate-DNA co-precipitation and cationic lipids. Mammalian

cells do not have an innate capability to pick up DNA molecules that they encounter in solution. Most transfection methods however can overcome this initial barrier and the majority of cells pick up the DNA of interest even when using low transfection efficiency methods such as $CaPO_4$ (Orrantia and Chang, 1990). Once the DNA is picked up by the cells, the transfection efficiency becomes limited by the efficiency with which the DNA can move into the nucleus in an intact form.

With both $CaPO_4$ and cationic lipids, the main form of DNA entry into the cells is endocytosis. As a result the DNA is trapped in endosomes that eventually fuse to lysosomes resulting in the degradation of the DNA before it has a chance to escape into the nucleus. The low efficiency of transfection found with the different methods have been attributed to DNA escaping the lysosomes before being degraded although how this escape is brought about is not clear (Orrantia and Chang, 1990; Zabner *et al.*, 1995).

In the case of $CaPO_4$, the internalised DNA is found mainly in the cytoplasm. Four hours after transfection, the nucleus contained less than 6% of the total DNA found in the cell . The majority of this DNA was in a degraded form. These results were similar in both human fibroblasts (a low transfection cell line) and mouse LtK^- mouse cells (high transfection efficiency line). In the case of DMRIE/DOPE mediated transfection, as much as 80% of cells picked up the DNA but only less than 50% expressed the introduced gene (Luciferase). β-galactosidase assay has shown that nearly 100% of cells pick up the DNA but only about less than 10% expressed the gene as opposed to almost 100% with a viral vector (recombinant *vaccinia* virus). Furthermore,

when a DNA lipid complex was injected into the cytoplasm, no DNA expression of the gene was detected while injecting the same complex into the nucleus results in expression levels similar to injecting the protein into the nucleus directly. In addition injecting the protein into the cytoplasm gives similar results to injecting it to the nucleus. These results indicate that the inefficient expression is the result of the lipid/DNA complex being trapped in the cytoplasm and not being able to deliver the DNA into the nucleus. These results are confirmed by electron microscopy photographs. (Zabner *et al.*, 1995).

In an effort to overcome these barriers to DNA transfer it was decided to try a number of compounds/treatments that will help DNA escape the lysosomes or even completely avoiding the endocytotic pathway. These treatments include, exposure to hydrogen peroxide, EMF treatment, basic amino acids and effect of DNA sequence. The next section will review some of the literature pertaining to the above mentioned treatments.

Efficiency in different cell lines.

In the course of many studies done on different cell lines it became evident that the efficiency of transfection varies from cell line to cell line even with the same method and also the same cell line has different efficiencies with different methods.

During their study on DNA internalisation and subsequent barriers to DNA transfer, Zabner *et al.* (1995) transfected three difference cell lines (COS, HeLa, and C127). The transfection efficiency as well as the DNA uptake by COS and HeLa cells was similar (about 80% and 50% of all cells respectively). With C127 cells DNA uptake was about 50% while transfected cells were below 5% . Similarly studies by Jordan *et al.* (1996) on calcium phosphate optimisation have shown that Chinese Hamster Ovary cells (CHO) and 293 cells have markedly different transfection efficiencies even when the same method and setting are used on both of them. At certain settings the transfection efficiency of 293 cells was as much as 4 times higher, while at other settings CHO transfection efficiency was about 150% that of 293 cells.

During a study of the distribution of DNA in cells following transfection with calcium phosphate, Orrantia and Chang (1990), found that a human primary fibroblast cell line took up DNA almost as efficiently as the much easily transfected NtK^- mouse cell line, but there was a difference in the percentage of DNA that got into the nucleus (2.2% to 6.4%). These results indicate that the difference in transfection efficiency might be mediated in the difference in the amount of DNA that escapes to the nucleus. The results of Jordan *et al* (1996) however indicate that the difference is not just a function of the cell line but also of the conditions used at the time of transfection, as they showed that at certain conditions a cell line that is traditionally harder to transfect can give higher transient expression levels.

Nuclear transport.

The cell's nucleus contains not only the genetic information necessary for the cell's survival but also the machinery needed for the replication, transcription and repair of the DNA. This entails that the nucleus must contain a number of proteins that were originally assembled in the cytoplasm. This brings the question of how do things traverse the nuclear membrane in either direction. All nuclear membranes from fungi to human, have structural pores in them known as the nuclear pores. These pores are suspected to play a central role in nuclear transport. Any model for such transport has to account for the selectivity of this process. In fact the nuclear membrane acts as a barrier to the majority of proteins assembled in the cytoplasm, while nuclear proteins are accumulated in the nucleus against a concentration gradient. One of the most accepted models for nuclear transport is represented in figure 1.

Nuclear Localisation Signals.

One of the key elements of this system are the Nuclear Localisation Signals (NLS) which were first described by De Robertis *et al* in 1978. All NLS share at least two characteristics:

1) Deletions or mutations in the sequence leads to cytoplasmic accumulation of a usually nuclear protein.

2) Addition of the NLS to any cytoplasmic protein results in its accumulation in the nucleus.

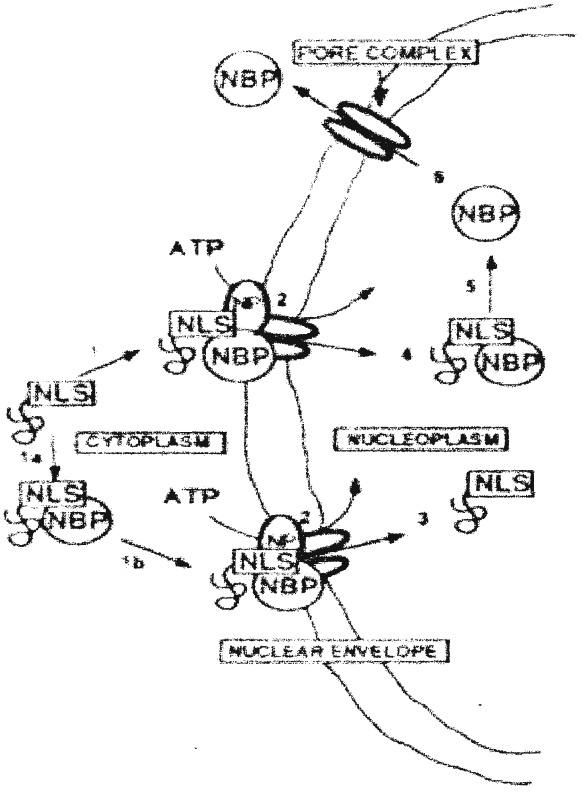

Figure 1: A model for nuclear transport. NBP= Nuclear binding Protein. NLS= Nuclear localisation signal. (Silver, 1991).

The first protein to provide clues as to the nature of NLSs was the nucleoplasmin protein of *Xenopus* oocytes. When the protein was injected in its intact state into the cytoplasm of intact oocytes, it migrated to the nucleus. If the C terminal tail of the protein was cleaved. The injected protein accumulated in the cytoplasm. Furthermore if

the C terminal was injected by itself into the nucleus, it migrated into the nucleus in the same way the intact protein usually does (Dingwall *et al.*, 1982). In an attempt at identifying the NLS of the SV40 T antigen, the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val was attached to the cytoplasmic protein pyruvate kinase which resulted in its localization into the nucleus (Kalderon *et al.*, 1984). Further studies have shown that even a single amino acid change in this sequence makes the T antigen non nuclear (Lanford and Butel, 1984; Kalderon *et al.*, 1984). As with the SV40 T antigen sequence, most other NLSs have a majority of basic amino acids in them, but that is not the only thing that defines the NLS as a substitution of a single amino acid with another basic amino acid (Lys 128 to Thr or Asn in the T antigen) results in reduction or abolition of the nuclear localisation effect (Lanford and Butel, 1984). While there seems to be no solid consensus on the kind or order of amino acids in the NLSs, the localisation of the sequence in the protein seems to play a major role. The addition of the SV40 T antigen NLS in the hydrophobic folds of the pyruvate kinase as opposed to its exposed surfaces results in no nuclear localisation of the protein (Roberts *et al.*, 1987; Nelson and Silver, 1989).

Many of the nuclear proteins have been shown to contain different NLSs (Hall *et al.*, 1984; Silver *et al.*, 1988). The question remains as to how exactly do the NLSs contribute to the transport of proteins to the nucleus and the exact mechanism that the proteins actually use to cross the nuclear membrane. Many models have been proposed for the actual transport of the nuclear proteins. Although some of the steps involved in this process remain undetermined or unresolved, others have been more or less established. Among the well established points are the following:

1) The existence of a receptor system on the surface of the nuclear membrane that binds the proteins before they are transported inside the nuclear membrane (Goldfarb *et al.*, 1986; Newmyer and Forbes, 1988; Richardson *et al.*, 1988).
2) Transport is ATP and temperature dependent. In the absence of ATP the binding occurs but the protein(s) are not transported across the membrane (Richardson *et al.*, 1988).
3) There are a number of proteins in the cytoplasm that bind NLSs (Richardson *et al.*, 1988; Breeuwer and Goldfarb, 1990).

One of the main arguments against the involvement of NLSs in nuclear transport is the fact that some nuclear proteins do not possess any NLS like signals. This however can be explained by the presence of proteins that act as shuttles by co-transporting these proteins into the nucleus (Dingwall *et al.*, 1982; Zhao and Padmanabhan, 1988). Some of the proteins that can interact with and bind to NLSs have been identified. In rat liver cells two such proteins have been identified, a 60 kD and 70 kD (Adam *et al.*, 1989; Benditt *et al.*, 1989), while a 66kd protein was identified in HeLa cells (Li and Thomas, 1989). This raises the question of how do NLS binding proteins recognise their signals. Like the majority of signals derived by consensus, the signals share some common characteristics. The majority of amino acids are basic ones, however replacing some the amino acids of the sequence with other basic amino acids reduces and/or eliminates the activity of NLS meaning that the acidity of the amino acids is not enough (Lanford *et al.*,

1988). Even reversing the order of the SV40 T-antigen results in a sequence that fails to bind to the rat liver cells' receptor (Adam *et al.*, 1989).

Role of nuclear pores.

Once bound to the nuclear envelope, proteins still have to cross the membrane. The most likely and most accepted possible site for their entry are the nuclear pores (Silver, 1991). In fact experiments done by Feldherr *et al.* (1984) have shown that gold particles coated with SV40 T antigen NLSs or the nuclear protein nucleoplasmin enter the nucleus only at the pores and that the pores can accommodate structures of as much as 250 Å. Studies done by Akey (1990) suggest that the pore might expand to accommodate such large structure using a model with an iris like mechanism on opposite sides of the nuclear envelope.

Nucleoporins are one of the subsets of proteins found in the nuclear pore on both sides of the membrane (Silver, 1991). Due to the N-acetylglucosamine found in these proteins, they can bind to Wheat Germ Agglutinin (WGA). The fact that antibodies directed against these nucleoporins block the transport of both RNA and nucleoplasmin (Featherstone *et al.*, 1988) supports the idea that these proteins play a role in nuclear transport. This idea is further supported by the findings of Akey and Goldfarb (1989) that proteins are bound by nucleoporins before there transport to the nucleus. Both these findings support previous findings that WGS blocks nuclear transport (Yoneda *et al.*, 1987; Debauvalle *et al.*, 1988), but does no do so by blocking the nucleopore's channels as evident by the fact that dextran diffusion into the nucleus is not stopped by WGA

(Finlay *et al.*, 1987). The fact that some proteins accumulate in the nucleus at certain stages of the cell cycle suggests the presence of a regulation process.

Regulation.

One of the most accepted models for regulating the transport of proteins into the nucleus is the masking/unmasking of NLSs model. Some examples where this model is more or less established are the glucocorticoid receptor and the NF-κB.

The glucocorticoid receptor is usually a cytoplasmic protein associated with the heat shock protein hsp90 until the receptor binds to the glucocorticoid hormone, at which time the hsp90 is dissociated from the receptor (Picard and Yamamoto, 1987). The NLSs of the receptor are located within the region that binds to the hsp90 protein, and the dissociation of the hsp90 leaves the NLSs exposed leading to the transport of the receptor to the nucleus (Sanchez *et al.*, 1985). Another example with similar regulatory mechanism is the NF-κB. The activity of NF-κB is dependent on its intracellular localisation. In its inactive form NF-κB is associated to IκB and it is located in the cytoplasm. Phosphorylation of NF-κB results in its dissociation from IκB and subsequent localisation in the nucleus where it is active (Ghosh and Baltimore, 1990). A similar system might regulate the ventralising activity of the *Drosophila*'s Dorsal protein (Rushlow *et al.*, 1989).

All three proteins mentioned above (glucocorticoid receptor, NF-κB and dorsal) have a high homology in a N-terminal region extending over 300 amino acids that

contains at the end of it a SV40 T-antigen type of NLS (Gilmore, 1990). This finding supports the idea that all three proteins are regulated by the same mechanism.

Chapter 2: Effect of electro-magnetic field on living organisms.

The driving force behind the first studies into the effect of EMF on humans and animals was the suspicion that they might be associated with increased incidence of certain cancers. These studies were epidemiological in nature and their results were controversial and inconclusive (Lacy-Hulbert *et al.*, 1998). More recent studies have focused on the experimental approach in order to try and elucidate the way in which EMF and Extremely Low EMF (EL-EMF) effect living beings. These studies can be divided into two major categories:

1. Whole animal experiments in which the animal is exposed to the experimentally generated field and then studied for any abnormal effects.
2. Cell culture experiments where the exposure is carried out on different cell lines and more basic effects (on the biological and molecular level) can be detected.

In this review only cell culture experiments are included as they help shed some light on cellular events and factors that might play a role relevant to this study. The studies will be organised based on which part of the cell morphology/physiology they affect.

Cellular and molecular effects of EMFs.

At our current level of understanding we don't know exactly at which point(s) do EMF act on the cells, however it is well established by now that the plasma membrane as well as other membranous organelles are one of the main points where the effects of EMFs can be seen (Goodman *et al.*, 1995).

Effect of EMF on Membranes.

Studies on the morphology of plasma membrane structure have shown that K562 cells (erythroleukemic cell line) exposed to a 50 Hz, 2.5 mT field resulted in bleb formation and fewer microvilli. In addition, exposed cells showed more orderly membrane structures (Paradisi *et al.*, 1993). Another notable change at the membrane level is the increased negative charge in response to electrical field exposure and a decrease in the membrane hydrophobicity. In addition these studies showed that the effect observed is the same with both pulsed and sinusoidal fields (Marron *et al.*, 1983, 1988; Smith *et al.*, 1991). These results can be explained by the observations made by Fisher *et al.* (1986). These studies showed a 100% increase in labelled glucosamine incorporation into existing plasma membranes as well as an increase in anionic residues (N-acetylglucosamine and N-acetylgalactosamine). This change in charge was proposed as the underlying mechanism for morphological changes in chick embryos exposed to electric fields (Hamada *et al.*, 1989).

The effect of electromagnetic fields extends beyond the structure of the membrane itself to affect membrane bound enzymes such as acetylcholinesterase; This effect on acetylcholinesterase is both time and temperature dependant. Stegemann *et al.* (1993) found that a stationary 1.4 T magnetic field reduced acetylcholinesterase activity by 80 % in 2 hr at 37°C, while a similar reduction at 27°C required a 3.5 hr exposure. This time difference was also evident in the time the exposed cells required to return to 93% of control (4 hr at 37°C as opposed to 15 Hr at 27°C). In the same fashion, ConA receptors distribution was altered by exposing *Xenopus* myoblasts to fields of 10 and 100 Hz and intensities of 0.3 and 3.0 V/cm (Lin-Liu *et al.*, 1984).

Several studies have shown alterations in mobility, orientation and/or shape of cells in response to EMF exposures (Hamada *et al.*, 1989; Dover and McCaig, 1989; Erickson and Nuccitelli, 1984). Further studies however with electric fields have suggested that all the above mentioned structural changes were due to Ca^{++} permeability changes which can be explained by the action of the electrical field component (Bedlack *et al.*, 1992). A topic related to the effect of EMFs on membrane is the effect of EMF on ion and charge movements across the membrane. The next section will review some of those effects.

Effect of EMF on Ions.

The three most important ions in maintaining the electrical potential of a cell are K^+, Na^+ and Ca^{++}, so it is no surprise that the effect of EMF on ion flow concentrates on these ions more than any others (Goodman *et al.*, 1995).

1. Calcium

One of the theories proposed to explain the results obtained with some EMF exposures was the fact that these settings coincide with Calcium ion cyclotron resonance frequencies (Liboff, 1985; Liboff *et al.*, 1987; Smith *et al.*, 1987). These explanations have been challenged though both on physics theoretical grounds (Tenforde, 1992; Halle, 1988) and due to negative experimental results (Parkinson and hanks, 1989; Parkinson and Sulik, 1992). The results regarding Ca^{++} intake in response to EMF are contradictory. Studies done by Lyle *et al.* (1992) and Walleczek *et al.* (1992) have shown no changes in intracellular Ca^{++} in response to 50 Hz, mV/cm and 50 Hz, 50 mV/cm fields respectively. On the other hand Lindström *et al.* (1993) have shown that a 50 Hz field resulted in a 400% increase in intracellular concentrations. Further studies by Liburdy (1992) showed that the observed differences in Ca^{++} are due to the electric component of the EMF field which acts by opening the calcium channel in the membrane rather than drive Ca^{++} into the cell by increasing its release from endoplasmic storage compartments.

2. Potassium, Sodium and analogs.

Much fewer studies have been done on K and Na ion movement in response to EMF. The studies done by Tsong's group (Teissie and Tsong, 1981; Serpersu and Tsong, 1983, 1984) have shown that high intensity fields (15-32 V/cm) resulted in enhanced Na/K transport against a concentration gradient. This effect was determined to be independent of any thermal effect the field might have had on the culture.

Effect of EMF on Nucleic acids and their expression.

As mentioned earlier, the first investigations into the biological effects of EMF were focused on their association with cancers so it is only normal for a large number of current studies to focus their attention on the effect of EMF on nucleic acids as they can be the earliest steps in cancer formation.

1. DNA.

Most studies dealing with effects of EMF on DNA deal with changes in rate of DNA synthesis. Studies by Liboff *et al.* (1984) showed a 60% increase in [^{3}H]thymidine incorporation in human fibroblasts exposed to a field of 76 Hz and 0.16 μT. Similar experiments resulted in a 13% increase in [^{3}H]thymidine incorporation by Chinese hamster ovary cells exposed to 10 Hz, 20 μT pulse and 25 μsec wide; an increase of the field strength to 200 μT resulted in up to 80% inhibition of [^{3}H]thymidine incorporation (Takahashi *et al.*, 1986). Similar experiments using human umbilical vein endothelial

cells (Goodman *et al.*, 1993) and other endothelial cells (Yen-Patton *et al.*, 1988) have shown no significant increase in [^{3}H]thymidine incorporation. Early [^{3}H]thymidine incorporation experiments have shown that the increase in response to EMF exposure is tissue specific and in some cases it can be blocked by Na^+ or Ca^{++} antagonists, which would mean that the increase in those cases are due to the electric component of these fields (Rodan *et al.*, 1978).

In a different kind of experiment, enhanced growth was observed when the cells were cultured in a medium obtained from cultures that were exposed to an electric field for 30 min. These results were interpreted to indicate the presence of a mitogen, which is released in response to the electric field. This mitogen has been determined to be an insulin like growth factor (IGF-II). In fact, a 14 Hz, 0.1 μV/m electric field resulted in growth enhancement that correlated with the increased release of IGF-II (25%) and IGF-II mRNA (Fitzsimmons *et al.*, 1992). In an attempt to isolate the effect of the electrical and magnetic exposures on DNA and cell proliferation, Schimmelpfeng and Dertinger (1993), exposed mouse fibroblasts to a 50 Hz magnetic field (2 mT) for 1 hr and observed the effect 6 hours later. The net effect was a significant decrease in DNA content, in contrast when a 0.25 mA/cm^2 was applied the opposite was observed. When the cell cycle population's distribution was studied it was found that the electric field exposure resulted in more cells being in the S, G_2 and M phases, while a magnetic field exposure resulted in more cells being in the G_1 phase. A concurrent exposure to both fields resulted in no net change in DNA content or cell cycle distribution which could be explained by the two fields neutralising each others effects (Schimmelpfeng and Dertinger, 1993).

A study on embryonic cells exposed to a bi-directional and unidirectional time-dependant waveforms, showed that exposed cells had a doubling period of about 16 hr compared to 21 hr for cells that were 2 to 3 days old. This decrease in the doubling period was reversed for cells that are between 3 and 4 days. This was interpreted as being due to the cells becoming more confluent (Guzelsu *et al.*, 1994).

2. RNA.

Early studies on effects of EMF exposure on RNA have showed an increase in [^{3}H]uridine incorporation that was generally thought to be due to an increase in mRNA levels (Goodman *et al.*, 1983; Goodman and Henderson, 1986, 1987). More detailed studies by Goodman's group (Goodman and Henderson ,1991; Goodman *et al,* 1992a,b) have shown an increase in the transcription levels of 13 different regions of *Drosophila melanogaster*'s 3R chromosome. Some of these regions correspond to heat shock proteins which were being produced in the absence of any increase in temperature. These findings prompted Blank *et al* (1993) to suggest that EMF fields may constitute an environmental stress factor to the cells. More complete studies on the rest of the *Drosophila* chromosomes revealed a total of 17 regions that showed increased transcription in response to EMF exposure. Most of these are involved in cell structure and development.

Expression levels of three different genes in *Saccharomyces cervisiae* exposed to a 60 Hz field at different intensities (0.8, 8.0 and 80 μT) and for various length of time (10 to 60 Minutes) were studied. The results showed that the weakest field resulted in the most significant and fastest response. The three genes examined were known to have

increased expression following a heat shock. Following EMF exposure the gene involved in uridine metabolism as well as the heat shock protein gene showed increased expression levels while the gene involved in meiotic regulation did not show any difference in expression levels (Weisbrot *et al.*,1993). Similar studies done on human cell lines (HL-60) showed that transcripts for histone H2B, actin and c-Myc were elevated about 20 min after exposure (Goodman *et al.*,1989, 1992 c, d; Wei *et al.*, 1990, Blank *et al.*, 1992). Based on their studies Goodman *et al.* were able to draw the following conclusions regarding effect of EMF on RNA:

1. Only a short exposure time is necessary to obtain the effects as 4 minutes are enough to cause the changes.
2. The enhanced transcripts are usually those that are already being expressed in the cell.
3. EMFs are perceived by the cell as a stress factor (Goodman *et al.*, 1993).

The above mentioned results are however in contradiction with results obtained from similar experiments conducted by Krause *et al.* (1991) and Parker and Winters (1992) that showed no increase in [^{3}H]uridine or *v-myc, v-fos or v-raf* respectively. Experiments on lymphobalstoid cells have shown an increase in [^{3}H]uridine incorporation into RNA was elevated as early as 30 min after exposure and stayed elevated 22 hr later. However incorporation into mRNA reached a maximum 2 hr after exposure and went back down to control levels over 14 hr (Philips and McChesney, 1991). This discrepancy in [^{3}H]uridine incorporation remains unexplained. Experiments taking into account the confluency state of the exposed cells have shown some enhancement in the levels of *c-fos, c-jun,* and *c-myc* (Philips *et al.*, 1992).

In an attempt at isolating the effect of the induced electric field from the magnetic field on RNA levels in exposed cells, Greene *et al.*(1991) exposed HL-60 cells cultured in dishes with concentric rings. In these dishes the cells in the outer ring are exposed to a higher induced electric field while being exposed to the same magnetic field as the ones in the inner ring. They found that RNA levels in the outer rings were 50% higher than those for the cells in the inner ring. In addition, when they varied the magnetic field while holding the electric current constant, they found no change in the levels of [^{3}H]uridine incorporation indicating that the electric filed is responsible for the changes in those incorporation levels. Studies on *E. coli* showed that a 72 Hz sinusoidal field generated an increase in the levels of RNA transcripts corresponding to the α subunit of DNA-dependant-RNA polymerase (Sustachek, 1992).

In an attempt to identify the regulatory sequence that mediates the response to EMF exposure of the *c-myc* gene, Lin *et al.*(1994) transfected HeLa and PX3 (mouse myeloma) cells with a plasmid containing the gene, its promoter and a 2329 bp sequence upstream of the promoter. The levels of transcripts were measured in response to a 20 min exposure to a 60 Hz, 8 and 80 μT fields. The upstream sequence was systematically shortened until the minimal region required was identified (-353 to -1257) from the promoter. Exactly how this region responds to EMF is not yet known.

Effect of EMF on Protein synthesis and enzymatic activity.

1. Protein synthesis.

With the number of changes in RNA levels observed in response to EMF exposure it was only natural for these studies to include protein synthesis. The first

studies done on this topic used *E. coli* as a model. Exposure of *E. coli* to a number of sinusoidal and pulsed magnetic fields resulted in an increase in the production of low molecular weight proteins and a decrease in the high molecular weight ones (Blank and Goodman, 1989). As with the enhanced RNA levels, some of the proteins with higher expression levels are related to the heat shock proteins. It is speculated that these changes are due to premature ending of transcription, and that they are influenced by the charge on the protein (Blank *et al.*, 1993). In total 36 different proteins showed field-induced changes (Goodman and Henderson, 1988). These include the outer membrane protein F and C (OMP-F and OMP-C), α subunit of RNA polymerase, NusA (a termination/ antitermination protein), topoisemerase II (E.M. Goodman *et al.*, 1994). Studies of *E. coli* linkage maps revealed that there is no specific regions or operons that are specifically susceptible to the effects of EMF exposure (Goodman *et al.*, 1995).

The alterations in the levels of subunit α expression have been confirmed with the use of a cell free transcription/translation system. [^{32}S]methionine labelling revealed an increase in protein synthesis although it could not determine if the enhancement is due to changes in translation, transcription or both (Goodman *et al.*, 1993a).

2. Enzymatic activity.

With fluctuation in DNA, RNA and protein synthesis it is only natural to expect fluctuations in enzymatic activity in response to EMF exposures. This section will include very short reviews of the effect of EMF on some enzymes.

- Ornithine Decarboxyalse (ODC): one of the most studied enzymes in respect to EMF exposure effects. ODC activity levels have been increased by 2 to 3

folds in human lymphoma (EM) cells and up to 5 folds in mouse myeloma cell line (P3) (Byus *et al.*, 1987).

- Protein Kinase C (PKC): the most significant increase in PKC activity was observed by Monti *et al.*(1991) and it could be eliminated by EGTA indicating that it is mediated by Ca^{++}. This leads to the possibility that any effect on PKC is due more to the electrical field than the electromagnetic one.
- Adenylate cyclase and cAMP: EMF exposure did not seem to affect the activity of adenylate cyclase although some changes in cAMP have been observed which were attributed to other effects of EMF such as receptor ligand interactions (Luben *et al.*, 1982, Luben, 1991).

Chapter 3: Effect of hydrogen peroxide on cells.

Hydrogen peroxide is one of the compounds released by activated Polymorphic neutrophils (PMN) and macrophages as a defence mechanism against bacteria and other infectious organisms. The H_2O_2 can be used to form hyperchlorous acid (HOCl) by interaction with the MPO- H_2O_2 halide system. Another mode of action of H_2O_2 is through its interaction with Fe^{++} to form the highly toxic hydroxyl radical (.OH). The PMN have a number of systems to protect them against the toxic effect of the H_2O_2 they use and generate including catalase and glutathione peroxidase systems (Clifford and Repine, 1987).

The main point of action of H_2O_2 on cells is the plasma membrane (Block, 1991). Using Lactase dehydrogenase (LDH) release as well as 5-Hydroxy tryptamine (5-HT) and conjugated dienes uptake assays in porcine pulmonary artery endothelial cells were used to study the effect of H_2O_2 on the plasma membrane's fluidity, permeability and function. These studies showed that an exposure to 50 μM H_2O_2 for 30 minutes increased 5-HT and conjugated dienes uptake as well as an increase in LDH release. These changes were observed as early as half an hour after exposure and lasted for at least 6 hours. These levels went back to normal 24 hours after exposure. All these changes were prevented when the cells were incubated with 1 mM or 10 mM dimethylthiourea (DMTU) or 50 μM alpha-tocopherol (Vitamin E) for as short as 1 hour but only if the incubation preceded the H_2O_2 exposure, leading to the conclusion that the damages are due to lipid peroxidation (Block ,1991).

A number of studies on the effect of H_2O_2 exposure on human erythrocytes has given us a better understanding of how the damage to plasma membrane by H_2O_2 exposure is mediated. When erythrocytes were exposed to H_2O_2, they change from dicocytes to echinocytic shape (Burnauer *et al.*, 1994), they become more susceptible to phagocytosis by macrophages, and the membrane becomes less deformable (Snyder *et al.*, 1988). It has been shown that the sulfhydryl groups in membrane proteins play a major role in mediating these damages as an incubation with 0.1-0.2 mM N-ethylmaleimide inhibited H_2O_2 toxicity toward red blood cells (Snyder *et al.*, 1988). Studies by Brunaeur *et al* (1994) showed that the phosphatidylserine content of the inner layer of the plasma membrane was reduced. This decrease was not associated with an increase in the amount of phosphatidylserine on the outside of the cell surface. However intracellular concentrations of phosphatidylserine were increased by H_2O_2 exposure suggesting that oxidative damage induces the extraction of inner monolayer compartments into the cytosol.

Similar studies done on neonatal rat cardiomyocytes showed that even a brief pulse of H_2O_2 can induce oxidative stress damages including disruption of the plasma membrane. These damages were accompanied by the phospholipid peroxidation, thiol oxidation and ATP loss. Another consequence of the H_2O_2 exposure is an increased turnover of the membrane proteins and phospholipids without a change in their overall concentrations which could be due to the replacement of oxidised membrane components. This observation was supported by the fact that antioxidant agents (and hydroxyl radical scavengers) protect the cell against H_2O_2 induced damage. These studies also showed that H_2O_2 exposure induced ATP loss in what seems to be non

peroxidative fashion (Janero *et al.*, 1991). This reduction in ATP could be related to the increase in cytosolic calcium as a result of increased membrane permeability. In fact it has been indicated that although increasing H_2O_2 concentration does not affect the myocyte's mitochondria to the point where ATP concentrations are reduced, a combination of H_2O_2 exposure and increased Ca^{++} concentration results in reduced ATP levels (Konno and Kako, 1991).

It has also been show that the combined exposure of cells to H_2O_2 as well as other membrane damaging agents results in a synergetic response (the combined effect is greater then the sum of the individual damages) (Ginsburg *et al.*, 1989). In addition to its effect on cell membranes, H_2O_2 can also induce single strand breaks in DNA as well as induce cellular proliferation. The latter effect is probably mediated either by interaction with receptors directly or by oxidation of molecules involved in growth signal transduction (Burdon, 1995).

Chapter 4: Amino acid transport systems.

The transport of amino acids through the plasma membrane is an active process. Different transport systems exist for the different amino acids present. The tissue distribution of these transport systems is not uniform throughout the tissues. Table 1 contains a summary of the systems identified so far and the corresponding amino acids specificity as well as tissue distribution. Also shown in Table 1 are the cloned transporters. Based on their physical characteristics, amino acid transporters can be divided into a number of different categories.

Table 1: A summary of amino acid transporters identified so far with their specificities. Also shown are the corresonding cloned transportes (from Castagna *et al.*, 1997).

Name of the cloned transporter	Tissue distribution	Corresponding transport system	Specificity
CAT-1	Widespread (except liver)	y^+	Lysine, histidine, arginine
CAT-2A	Widespread (including liver)		
CAT-2B	Widespread (activated in T-cells)		
EAAC1	Intestine, kidney, brain	X^-_{AG}	Glutamate, aspartate
GLAST	Brain, heart, lung, skeletal muscle, placenta	Glutamate transporter	
GLT-1	Brain, liver	Glutamate transporter	
EAAT4	Brain (cerebellum), placenta	Glutamate transporter	
ASCT1, ASCT2	Widespread	ASC	Small aliphatic amino acids
GAT1,2,3,4	Brain	GABA transporter	
GLYT-1a, 1b, GLYT-2	Brain	'System GLY-like'	Glycine
PROT	Brain		Proline
TAUT-R, M, D	Brain	β-like	Taurine
Transporter related proteins:			
D2/rBAT/NBAT	Intestine, kidney	$b^{0,+}$	Neutral and dibasic amino acids
4F2 hc	Widespread (activated in T-cells)	y^+L	Neutral amino acids: Na^+-dependent. Dibasic amino acids: Na^+-independent
Not cloned			
?	Widespread	$b^{0,+}$	Neutral and dibasic amino acids
?	Hepatocytes, fibroblast	x^-_c	Glutamate/cystine
?	Widespread	A	Small aliphatic amino acids
?	Intestinal epithelial cells	B	Most neutral amino acids
?	Widespread	$B^{0,+}$	Neutral and dibasic amino acids
?	Intestine	imino	Proline
?	Widespread	L	Branched chains/aromatic amino acids
?	Liver, muscle	N	Glutamine, asparagine, histidine

Cationic Amino Acid Transporter Family (CAT)

The first members of this family to be identified were the mCAT-1 and mCAT-2 (m standing for mouse) (Wang *et al*,1991; Closs *et al*, 1993a). Physical characteristics (specificity and Na^+ dependence) of the CAT family proteins are very similar to those described for the y^+ system. The y^+ system has a specificity for arginine, lysine and histidine in a Na^+ independent fashion. The system can also mediate the uptake of some neutral amino acids in the presence of Na^+ but to a lesser degree than the cationic ones (White, 1985). Since the original description of the mCAT-1 and mCAT-2 a number of isoforms have been identified (mCAT-2A; Closs *et al*, 1993a and mCAT-2B; Closs *et al*, 1993b). These isoforms appear to be the result of differential splicing (Closs *et al*, 1993b). These transporters appear to have up to 14 putative membranes spanning domains (Wong *et al*, 1991; Kim *et al* 1991; Closs *et al*, 1991). Expressions of this transport system is very widespread.

The transcriptional control of this transporter family is rather complex and not yet well understood. Finley *et al* (1995) identified at least 3 different promoters that contribute to the control of an upstream, untranslated region of this gene. One of the consequences of the activation of this gene is the expression of another widespread amino acid receptor/transporter the 4F2 heavy chain (4F2hc) antigen.

Another group of amino acid transporters that mediate the transfer of cationic and neutral amino acids includes P2/NABAT, 4F2hc, b^{o+}, L. The most notable of these

systems is the 4F2hc antigen. This membrane bound 125 kDa heterodimer is made of the 4F2hc itself which is a glycosylated heavy chain (85kDa) and 41 kDa light chain (non-glycosylated) that are linked by disulfide bonds. This antigen has a simple membrane spanning domain and a short cytoplasmic tail (Hemler and Strominger, 1982; Eisenbarth *et al.*, 1980). The expression of this protein in *Xenopus* oocytes resulted in the Na^+-dependent uptake of neutral amino acids and Na^+-independent uptake of cationic amino acids in a fashion similar to the y^+ system. It has been proposed that the light chain of 4F2hc is an endogenous amino acid transporter found in the oocytes membrane and it is this binding that activates it (Bröer *et al*, 1995). Ogimoto *et al*, (1995) determined that 4F2hc is involved in both monocytes and virus mediated fusion which are processes involving the activation of the integrin system.

Gamma - Aminobutyric Acid (GABA) Transporter Family.

GABA is an inhibitory neurotransmitter in the brain and the loss of glutamate induced release of GAB has been implicated in temporal lobe epilepsy (During *et al*, 1995). So far 4 different GABA transporters have been identified (mainly in the brains of mice, rats and humans), they are GAT-1 to GAT4. While GAT -1 and GAT-4 are high affinity GABA transporters, GAT-2 and GAT-3 have a lower affinity (Liu *et al*, 1993). Other differences have been observed in the specificity (GAT-3 and GAT-4 can transport β-alanine and taurine) and pharmacology (GAT-1 and GAT-4 are blocked by nipecotic acid and guvacine) (Liu *et al*, 1993).

Proline and glycine transporters (PROT and GLYT types) are also members of the GABA transporters family (Shofogot *et al*, 1995; Fremeau *et al* , 1992). Although the role of proline in the central nervous system (CNS) is not well understood, it has been shown to have neurotoxic effects (Nadler *et al*, 1998) and the PROT transporters are thought to provide the proline for the mitochondria of neural cells for the krebs cycle (precursor to the α-ketoglutarate).

All members of the GABA transporter family are dependent on Na^+ and Cl^- cotransport. They also share homologies in their cytoplasmic domain (kinase C sites) and some of the extracellular domains (N-glycolysation sites). In general they have 12 membrane spanning domains.

Glutamate transporter family.

This is one of the best studied amino acid transporter families. It consists of 4 high affinity transporters: EAACI (Heiger and Rhoods,1994), GLT-1 (Donbolt *et al*, 1992), GLAST (Stor *et al*, 1992), FAAT4 (Fairman *et al*, 1995). These transporters play a major role in the normal function of the brain. In fact glutamate is considered the major excitatory neurotransmitter in mammalian brain (Ottersen, Storm and Mathisen, 1984). The concentration of glutamate is in effect highest in nerve terminals (10mM) (Storm- Mathisen *et al*, 1992)

The importance of glutamate transporters in normal brain function is two-fold.

1) Removal of excess glutamate from the synaptic cleft improving the noise/signal ratio
2) Preventing continuous activation of glutamate receptors which could lead to cell death

Detailed studies (using voltage-clamp, pH sensitive electrode and ion substitution experiments), revealed that the transport of one glutamate by EAACI is coupled to the transport of 2 Na^+ ions (Kanoi *et al*, 1995), one H^+ (Bouvier *et al*, 1992) and one K^+ (Nursberger *et al*, 1995). Studies investigating the regulation of glutamate transporters revealed that at least 3 different regulation mechanisms exist.

1) protein-kinase C phosphorylation (Cosodo *et al*, 1993)
2) oxygen free radicals (Volterra *et al*, 1994)
3) 3 arachidonic acid (Trotti *et al*, 1995; Zerongere *et al*, 1995).

As expected these transporters are expressed mainly in the brain and in astrocytes throughout the CNS, but they have also been found in the kidneys, liver and heart (Lehre *et al*, 1995; Rothstein *et al*, 1994).

Chapter 5: Materials and methods.

Cell culture.

Cells were maintained in Eagle's minimum essential media (Gibco©) supplemented with 10% Bovine Donor serum (Gibco©) , and 2% of an antibiotic-antimycotic mixture (Streptomycin 10,000 μg/ml, Penicillin 10,000 units/ml and amphotericin B25 μg/ml in 0.85% saline. Purchased from GibcoBRL). Cells were passaged at least twice before being used after being taken out of liquid nitrogen. Cells were grown overnight in a CO_2 incubator (5% and 37°C). Cells were allowed to form a uniform monolayer in the plate before being subcultured. To subculture cells, the monolayer was rinsed twice with Versene (1.4 M NaCl, 8.8 mM EDTA, 26 mM KCl, 15 mM KH_2PO_4, 15 mM Na_2HPO_4, 0.2% glucose [w/v]) and then incubated in 3 ml of Versene and 0.5 ml of trypsin (GibcoBRL) for 3 min at 37°C. The versene and trypsin were removed and fresh media was used to lift the cells off the plate and the volume was split into new culture plates (ratio for propagation was between 1:2 to 1:4 depending on confluency) (Burleson *et al.*, 1992).

Calcium Phosphate precipitation.

The protocol for calcium phosphate precipitation used was the one developed by Graham and Van Der Eb (1973) and modified by Jordan *et al.* (1996). In brief, DNA was added to a sterile Eppendorf tube (at the concentration of 5 μg/35 mm dish). Calcium

chloride was then added to a concentration of 500 mM. The mixture was brought to its final volume with autoclaved water (the final volume was 100 µl/35 mM dish). This mixture was gently mixed and centrifuged briefly to bring everything to the bottom of the tube. This mixture was added drop wise to an equal volume of 2X HEPES buffered saline (NaCl 274 mM, KCl 10 mM, $NaH_2PO_4.2H_2O$ 1.7 mM, dextrose 11 mM, HEPES 42 mM, pH 7.05). Air bubbles were introduced into the mixture for 2 minutes. The mixture was allowed to sit at room temperature for 20 minutes. The cells were transfected by addition of 200 µl/35 mm dish. The cells were incubated in the media-$CaPO_4$-DNA mixture for 2-4 hr as indicated for each experiment, at which time the media was removed and replaced with fresh one.

Lipofectamine® transfection.

Lipofectamine® reagent was purchased from Gibco© and used according to manufacturer's instructions. Briefly, DNA (at the concentration of 1 µg/35 mm dish) was mixed in serum free media (100 µl/35 mm dish) to make solution A. Solution B was prepared by adding 5 µl of Lipofectamine® reagent to 95 µl of serum free media (for each 35 mm dish). Solutions A was added to solution B and mixed gently. The mix was allowed to sit at room temperature for 30 minutes. The mixture was topped of with serum free media to give a final volume corresponding to 1ml/35 mm dish. Cells were washed with 2 ml of serum free media and then incubated in 1 ml of the final solution (Neumann *et al.*, 1987). Media was removed off the cells 4 hours later and the cells were exposed to H_2O_2 as indicated. For experiments not involving hydrogen peroxide

exposure, 1 ml of media containing twice the concentrations of serum and antibiotics was added to the plate.

H_2O_2 exposure.

Media was removed from the cultures and the cells were incubated with 2 ml of H_2O_2 at the desired concentration for 1-30 minutes as indicated for each experiment. At the end of the incubation period, H_2O_2 was aspirated off and the cells washed with 2 X 1 ml of PBS for each 35 mm dish. The cells were then put back into regular media.

EMF exposure.

Cells were exposed using an EMF generator manufactured by the electronics shop at Brock University. The exposure chamber consisted of a hollow cylinder with plastic shelves that rest on levels on the inside part of the exposure chamber. The EMF field was generated by passage of electrical current through a set of wires running in the walls of the chamber. The electrical current is controlled by an electronic circuit connected to an LCD display allowing for the control of the fields frequency (0-99.5 Hz), current intensity (0-5.0 A), time of exposure (0-999 sec) and the direction of the field (+ or -). The apparatus did not provide any control for temperature.

Cell culture plates (35 mm in diameter) were placed in the same spot (within a circle) on the plastic shelf. Cells were taken out of the incubator and placed in the exposure chamber. Each exposure consisted of 2 different cycles, one in each field

direction (e.g. a 2 minutes exposure means that the cells were exposed for 2 min with the field in the positive configuration and 2 min with the field in the negative configuration). In all cases the positive exposure was carried out first.

Preparation of Competent cells.

All DNA transformations were carried out in *Escherichia coli* cells of the strain DH5α which has the following genotype: F^- ϕ80dlacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rk-, mk- phoA supE44-thi-1λ^- gyrA96 relA1) (Gibco BRL). The bacteria were grown in Luria-Bertani (LB) broth (10% Bacto-tryptone (w/v), 5% bacto-yeast extract (w/v), 10% NaCl (w/v), pH 7.0) and all stocks were kept at -80°C in 15% Glycerol (w/v). DH5α cells were grown on agar plates and single colonies were isolated and grown in 3 ml of LB at 37°C overnight. From the overnight culture, 1ml was taken and added to 500 ml of LB and incubated at 37°C until the culture reached an Optical Density (OD) of 0.45-0.55 at wavelength 550 nm. The cells were than incubated on ice for 2 hours before being centrifuged at 4°C for 15 min at 2500xg. The cells were then resuspended in 20 ml of cold transformation buffer (5 mM TrisHCl, 75 mM$CaCl_2$, pH 7.6). Once the cell pellet was dissolved, the volume was brought up to 500 ml with the same transformation buffer and the cells incubated for 45 minutes on ice. The cells were collected by centrifugation at 1800xg for 15 min and resuspended in 50 ml of ice cold transformation buffer (Cohen *et al.*, 1972). If needed the cells were used at this time for transformation or glycerol was added to 15% of final volume (w/v) and the cells were divided into 1.5 ml centrifuge tubes (200µl/tube) and stored at -80°C until needed.

Transformation.

Transformation of competent bacterial cells was carried out as described by Cohen *et al.* (1972). In short, 1 μg of the DNA was incubated with 200 μl of competent DH5α cells on ice for 30 minutes and the mixture was gently mixed every 10 minutes. The cells were then heat-shocked at 42°C for 45 minutes. The cells were then supplemented with 800 μl of LB media and allowed to grow at 37°C for 45-60 minutes. The bacterial cells were then spread on agar plates containing 2x Kanamycin for selection of transformed bacteria (150-200 μl/plate) and the plates were incubated for 16 hours at 37°C. Single colonies were picked, grown overnight in LB and screened for the plasmid with small scale DNA isolation followed by restriction enzyme analysis and gel electrophoresis. The colonies of interest were further purified before stocks of them were stored in 15% glycerol in LB broth.

Large scale DNA preparation.

This procedure was adapted from the one described by Brinboin and Doly (1979). The plasmid used was pEGFP-N1 (Invitrogen©). This plasmid was transfected into DH5α *E. coli* cells. The cells were plated on Kanamycin agar plates. Single colonies were picked and grown overnight. The cultures were screened using a "miniprep" procedure for the presence of pEGFP-N1. Cells containing the plasmid were grown for 6 hr in flasks of 500 ml of LB nutrient broth. The cells were then pelleted by centrifugation at 8,000 rpm for 8 minutes. The pellet (from each 500 ml nutrient) was resuspended in 10 ml of lysozyme solution 2μg/μl (25mM Tris base, 50 mM Glucose, 10

mM EDTA, pH 8.0) The solution was left on ice for 20 minutes. To each 10 ml of lysozyme, 20 ml of alkaline SDS (5% NaOH 4M, 1 % SDS), mixed gently and incubated at room temperature for 15 minutes. Once the solution was clear, 15 ml of 3M NaAc was added and the mixture incubated on ice for 45 minutes. The mixture was then centrifuged for 15 minutes at 10,000 rpm. The supernatant was filtered through Watman© # 1 filter paper. The filtrate was supplemented with 2 volumes of cold 95% ethanol. The mixture was centrifuged at 10,000 rpm for 15 minutes. The DNA pellet was resuspended in 2-6 ml of water and then purified using the cesium chloride banding method.

Small scale DNA isolation "miniprep".

Cells grown overnight were placed in a sterile Eppendorf tube (1.5 ml). The cells were pelleted by centrifugation at high speed for 30 seconds. The pellet was resuspended in 100 µl of lysozyme solution (2µg/µl) for 5-10 minutes. 200 µl of alkaline SDS was added to each tube and the solution incubated on ice until clear. Once clear, 150 µl of 3 M NaAc was added per tube and the solution incubated on ice for 15 minutes. The mixture was then centrifuged at high speed for 5 minutes. The supernatant was transferred to a sterile tube and 2 volumes of cold 95 % ethanol was added to it. The mixture was centrifuged at high speed for 5 minutes. The pellet was kept and dissolved in 300 µl of autoclaved water. Two volumes of cold 95% ethanol were added and the centrifugation step repeated (Birnboim and Doly, 1979). The pellet was kept and all the ethanol removed from the tube before suspending the DNA in a total of 100µl of water or

TE buffer (10 mM Tris, 1 mM EDTA). The DNA was then subjected to restriction enzyme analysis with the appropriate enzymes.

Isolation of genomic DNA from mammalian cells.

Cells were disrupted by addition of 500 µl (per 35 mm dish) of lysing buffer (0.01 M Tris, 0.01 M EDTA, 0.5 % SDS (w/v)). Cells were left in the lysing buffer for 5 minutes or until no cells could be distinguished under the microscope. The lysate was collected into a clean Eppendorf tube and supplemented with 25 µl of pronase A. The mixture was incubated overnight at 37°C. The following day, 400 µl of phenol was added to the tube and the mixture rocked gently for 3-5 minutes. The tube was then centrifuged at high speed for 15 minutes. The clear supernatant was collected and transferred into a clean tube. An equal volume of a mixture of chloroform, phenol and isoamyl alcohol was added to the tube and after 3-5 minutes of gentle rocking it was centrifuged at high speed for 15 minutes. The supernatant was once again transferred to a new tube, supplemented with an equal volume of chloroform and centrifuged at high speed. The collected supernatant was supplemented with at least 2 volumes of ice cold ethanol (95%) and centrifuged at high speed. The pellet was resuspended in sterile water and 2 volumes of cold ethanol (95%) were added before the DNA was pelleted at high speed. The pellet was then resuspended in water.

Cesium chloride banding.

For each 2 ml of DNA solution to be purified, 150 µl of ethidium bromide was added to the solution in addition to 2.75 g of cesium chloride. The density of the solution was adjusted (using water and cesium chloride) to between 1.56-1.59. The solution was then transferred to centrifuge tube and centrifuged at 65,000 rpm for 20 hr. The tubes were then removed and the DNA band aspirated out using a 8 ½ gauge syringe. The ethidium bromide was removed by adding and equal volume of cesium chloride saturated isoamyl alcohol. Once the alcohol layer turned pink it was aspirated out and new layer put in until no further colour distinction is found between the two layers. The solution was diluted with 3 volumes of water and the centrifuge tube was filled (at least two volumes) with cold 95% ethanol (Sambrook *et al.*, 1989). The DNA was precipitated by centrifugation at 15,000 rpm for 15 minutes. Once the DNA is dissolved in TE buffer or water it was analysed by restriction enzyme analysis and gel electrophoresis.

Restriction enzyme analysis.

Typically DNA was added to the rate of 1µg per digestion in total volume of 20 µl. The enzyme were used according to the supplier's (NEB) specifications. In general the DNA was added to 1-100 units of enzyme (defined as being the amount of enzyme needed to digest 1 µg of λ DNA at 37°C in one hour if the reaction mixture is 50 µl) diluted with the 2 µl of the appropriate 10X restriction buffer.

Gel electrophoresis.

The DNA to be analysed was supplemented by a loading buffer (20% glycerol (w/v), 2% SDS (w/v), 0.05% w/v bromophenol blue) that helped stop any restriction reaction and/or allow the DNA to settle in the bottom of the agarose wells. The wells were made in gel containing 0.6-1.1% agarose (w/v) in TAE buffer (1mM EDTA, 20 mM acetic acid, 40 mM Tris pH 8.0). The gel also contained 0.1-0.5 μg of ethidium bromide/ml. The gel was placed in an electrophoresis chamber and were submerged in TAE buffer to cover the wells. The DNA was then separated by running 1-10V/cm current in the TAE buffer. Molecular weight markers (λ HindIII digest and/or 1 Kb ladder (Both from NEB)) were added to adjacent wells to facilitate the estimation of DNA fragments' sizes.

Radioactive labelling of DNA probes.

The Promega © Nick translation Kit ® was used to radioactively label DNA probes. It was used according to manufacturer specifications. In brief 1 μg of the DNA to be labelled was added to 10 μl of a nucleotide mix (300 μM of dCTP, dGTP and dTTP), 5 μl of 10X nick translation buffer (500 mM TrisHCl (pH 7.2), 1mM DTT, 100 mM $MgSO_4$), 7 μl of [α-^{32}P] dATP and 5 μl DNA Polymerase I (1u/μl)/DNAse I (0.2 ng/μl) mix. The total volume was brought to 50 μl using sterile dH_2O. The mixture was

incubated at 17°C for 1 hour. The reaction was stopped with the addition of 10 µl of stop solution (0.25 M EDTA).

Southern Blotting.

The procedure followed for transfer of DNA from gels to nylon membranes and subsequent hybridisations were done according to Southern (1975). Following restriction enzyme analysis and electrophoresis the gel was incubated in a shaking bath of 0.25 N HCl for 10 minutes resulting in partial acid depurination. Excess HCl was removed by a brief wash in H_2O and the gel was transferred to a solution of 0.4 N NaOH and 0.6 M NaCl for 30 minutes in order to denature the DNA. A final soaking in 1.5 M NaCl and 0.6 M Tris-HCl (pH 7.5) for 30 minutes neutralised the gel.

A capillary blot device was made by saturating a sponge with 10X SSC and two layers of blotting paper (length and width were 2 cm greater than those of the gel) were placed on top of the sponge. All air bubbles were removed with a glass rod. The gel was placed on top of the blotting paper and the air bubbles were removed again. A nylon membrane (pre-soaked in 10X SCC for 15 minutes) was place on top of the gel.. The membrane was covered with 3 layers of blotting paper and the air bubbles were removed again. The blotting paper was topped with 10-20 cm of paper towels and about 1 K of weight was placed on top of the paper towels. The gel was allowed to sit overnight. Once the membrane was removed it was rinsed in 6X SSC, air dried and crosslinked in a UV oven.

Once the membrane was crosslinked, it was incubated in a pre-hybridisation buffer (10% dextran sulphate (w/v), 1 M NaCl, 1% SDS) for 15 minutes at 65°C. The

radioactive probe was then added to 0.9 ml of dH_2O and 0.1 ml of carrier DNA (Sheared herring sperm at 5 mg/ml) and denatured by incubation for 10 minutes in boiling water (100°C). The denatured probe solution was added to 10 ml of the pre-hybridisation buffer and the so formed hybridisation buffer was used to replace the pre-hybridisation buffer in the cylinder containing the membrane. The cylinder was then incubated in a hybridisation oven overnight at 65°C (with continuous rotation). The following day the membrane was then washed with 2X SSC (while still inside the cylinder), followed by a wash with 2X SSC with 1 % SDS (w/v) for 60 minutes at 65°C. The membrane was then washed twice with 0.1X SSC. The membrane was allowed to dry at room temperature and exposed to an X-ray film (Biomax Film, Kodak®) inside a light proof container overnight at -80°C. The film was later developed according to manufacturer specifications and solutions.

Polymerase chain reaction.

The polymerase chain reaction (PCR) (Mullis *et al.*, 1988) was performed using Taq DNA polymerase (MBI Fermentas) according to manufacturer specifications. The primers used were designed using the software Vector NTI Deluxe © version 4.0 by Informax Inc ®. The primers used to amplify the CMV promoter sequence were: sense primer 5'TGATTCTGTGGATAACCGTA 3', the antisense primer was 5'GTGGCCGTTTACGTCGCCGT 3'. The sense primer for the amplification of the GFP gene was 5'TCAAGCTTCGAATTCTGCAG 3' while the antisense primer for the same gene was 5' TCGCGGCCGCTTTACTTGTA 3'. These primers were obtained from Procyon Biopharma Inc.

The PCR consisted of 50 ng of template DNA (pEGFPN1), 1 unit of Taq DNA polymerase, 5 µl of 10X buffer (500 mM KCl, 0.8% Nonidet P40 (v/v), 100 mM Tris-HCl (pH 8.8)), 1-3 mM $MgCl_2$ (this amount was optimised for each fragment), 0.2 mM of each of the four dNTP and 1 µM of each primer. The volume for the reaction was brought to 50 µl using sterile dH_2O. For the amplification of the GFP gene, the $MgCl_2$ was added 2 minutes after starting the first step of PCR (DNA denaturation was started) a procedure known as Hot start PCR.

The Single Block Easy Cycler (Ericomp Inc.) was used to provide the temperature cycling. The initial denaturation step was 10 minutes long at 95°C. It was followed by 35 repeats of a cycle consisting of three steps:

1) a denaturation step: 45 sec at 92°C.
2) Annealing step: 90 sec at 55°C (for CMV amplification) or 42°C (for GFP amplification).
3) Extension step: 135 sec at 72°C.

This was followed by one final elongation step at 72°C for 10 minutes. Once the PCR reaction was over, the DNA was subjected to electrophoresis analysis to verify the presence and size of the DNA fragment amplified.

Cell count (GFP) and statistical calculations.

The cells were grown in 35 mm dishes with cover slips (22x22 mm) in the bottom of the dish. At the time of the count the cover slip was placed on a microscope slide.

Using the co-ordinates of the microscope's plate as point of guidance, co-ordinates for 33 fields were generated for each slide to be counted. These numbers were generated using a random number generating program and they were recalculated for each slide. The count was done using a fluorescent microscope at a magnification of x400. The diameter of the field of view was determined to be 2 mm. Taking into account that the diameter of one culture dish was 35 mm, it was determined that each plate contains the equivalent of 306.25 fields of view. So in order to obtain the total number of cells transfected the average number of cells per field should be multiplied by 306.25.

Once the data was collected it was plotted using Microsoft's© Excel®. The statistical analysis was done using Jandel's© SigmaStat®. The test used was ANOVA on ranks in order to determine the statistical significance of any observed differences.

Chapter 6: Electro-Magnetic Field exposure.

EMF as sole transfection mechanism.

Most of the effects of EMF have been reported in association with the plasma membrane. This raises the question of the ability of EM force to drive DNA across the membrane or lead to transfection through membrane modification. To study this possibility 3μg of DNA (pEGFP-N1) were added to each of the 10 mm dishes. One of the plates was not exposed to EMF and served as positive control while a similar plate was left without DNA and served as a negative control. The nine remaining plates were exposed to the frequencies and settings in table 2. No green cells were found in any of the control or experimental plates indicating that EMF cannot be used as a transfection method by itself.

Frequency(Hz)	99.5			50			10		
Intensity (A)	5.0	3.0	1.0	5.0	3.0	1.0	5.0	3.0	1.0

Table 2: Standard "wide-scan" settings

The effect of EMF on established Transfection methods.

The next logical step was to study the effect of EMF on previously established transfection methods. This was limited to 2 techniques.

Calcium Phosphate.

The effect of EMF on Ca PO_4 transfection efficiency was studied with respect to variations in time of exposure, length of exposure and intensity variations.

Time of Exposure.

This was done using the standard wide scan at 4 different time points:

T=2hrs.

EMF exposure was carried out 2 hours after Ca PO_4 addition and the Ca PO_4 was removed 4 hours after its addition. The efficiency observed varied from inhibition (20%) to significant enhancement (180%). The highest transfection efficiencies were observed with all three 99.5 Hz settings, but no discernible pattern was observed.

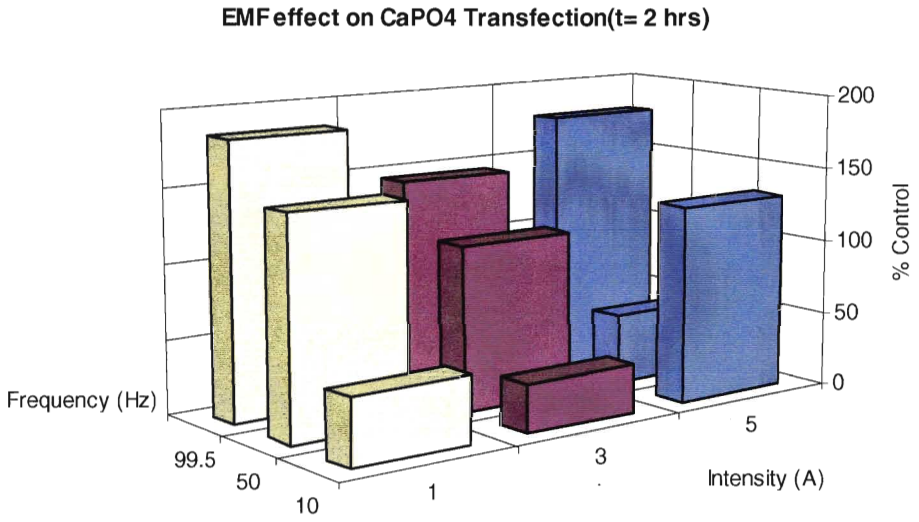

Figure 1: Effect of EMF exposure on efficiency of CaPO4 when exposure was done 2 hours after CaPO4 addition. All efficiencies are expressed as percentage of the positive control done that day (not shown on graph).

Table 1: The effect of EMF on the transfection efficiency of $CaPO_4$. Exposure is done 2 hours after DNA addition. The counts are the averages of 33 different fields. The column headings indicate the frequency (Hz) while the rows correspond to the intensity (A). N= negative control. P= Positive control.

	N	P	99.5	50	10
5	0	1.51	2.69	0.75	1.96
3			2.18	1.69	0.48
1			2.78	2.21	0.66

T=3.5hrs

In this case the EMF exposure was carried out 3.5 hours after Ca PO_4 addition (0.5 hours before removal). More discernible patterns were observed with this time of exposure, the efficiency enhancement was lower than for T=2hrs. A pattern similar to bell shaped curve was observed for the 99.5 Hz and 50 Hz settings in response to intensity variation. While 10 Hz showed a steady decrease, the bell shaped curves showed peaks at 120% and approximately 80% (99.5 Hz) as for the 10 Hz the peak was 155%.

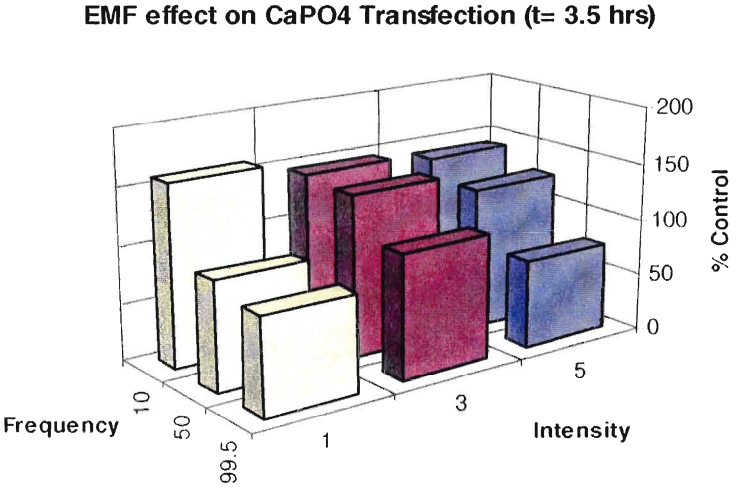

Figure 3: Effect of EMF exposure on CaPO4 transfection efficiency when exposure was done 3.5 hours after CaPO4 addition. All efficiencies are expressed as percentage of the positive control done that day (not shown on graph).

Table 4: Effect of EMF exposure 4 hours after addition of DNA. The column headings are the frequencies (Hz) while the rows are for the intensities (A). N= negative. P= Positive control.

	N	P	99.5	50	10
5	0	1.60	1.21	2.03	2.27
3			1.72	2.30	2.36
1			1.33	1.51	2.57

T=6hrs

For this experiment the exposure was done 2 hours following Ca PO_4 removal. The highest transfection efficiencies were observed with this setting. In fact none of the tested points showed inhibition. Enhancements as high as 300% were observed (99.5 Hz and 3A).

Table 5: Effect of EMF exposure on transfection efficiency of cells transfected with $CaPO_4$ when exposure was done 6 hours after DNA addition. Frequencies are indicated in column headings (Hz) while intensities are in row headings (A). N= negative control. P= Positive control.

	N	P	99.5	50	10
5	0	1.81	3.18	3.51	2.72
3			5.87	3.63	2.45
1			4.63	3.0	2.09

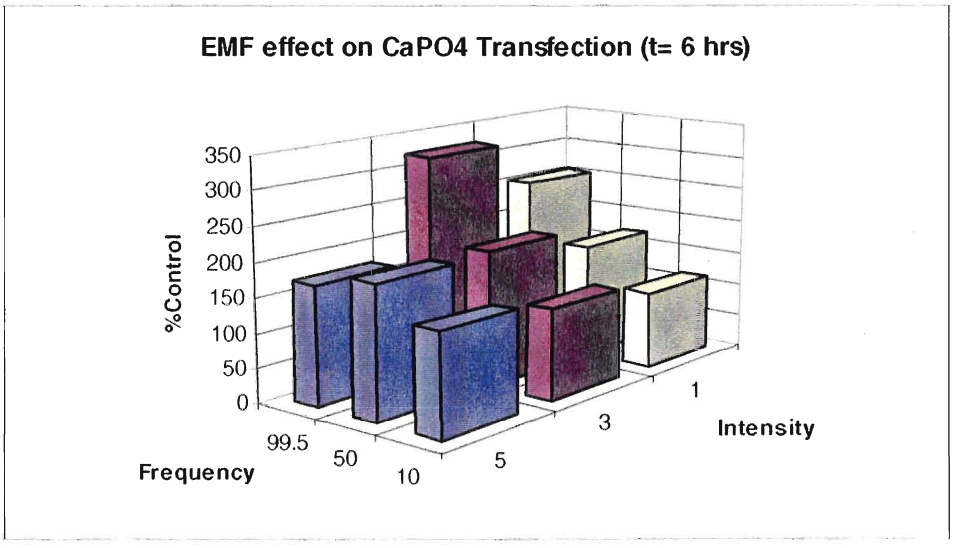

Figure 4: Effect of EMF exposure on CaPO4 transfection efficiency when exposure was done 6 hours after CaPO4 addition. All efficiencies are expressed as percentage of the positive control done that day (not shown on graph).

T=8hrs

For this experiment, EMF exposure was done 4 hours after Ca PO_4 removal and resulted mainly in inhibition of transfection with the exception of 2 (10Hz, 1A and 10Hz, 3A) where enhancement was over 15%.

Table 6: Effect of EMF exposure on $CaPO_4$ transfection efficiency. Exposure was done 8 hours after DNA addition. Column headings contain the frequency settings in Hz while intensities are in the row headings (A). N= Negative. P= Positive.

	N	P	99.5	50	10
5	0	1.75	1.87	0.96	1.3
3			1.96	0.57	3.75
1			1.33	1.12	3.42

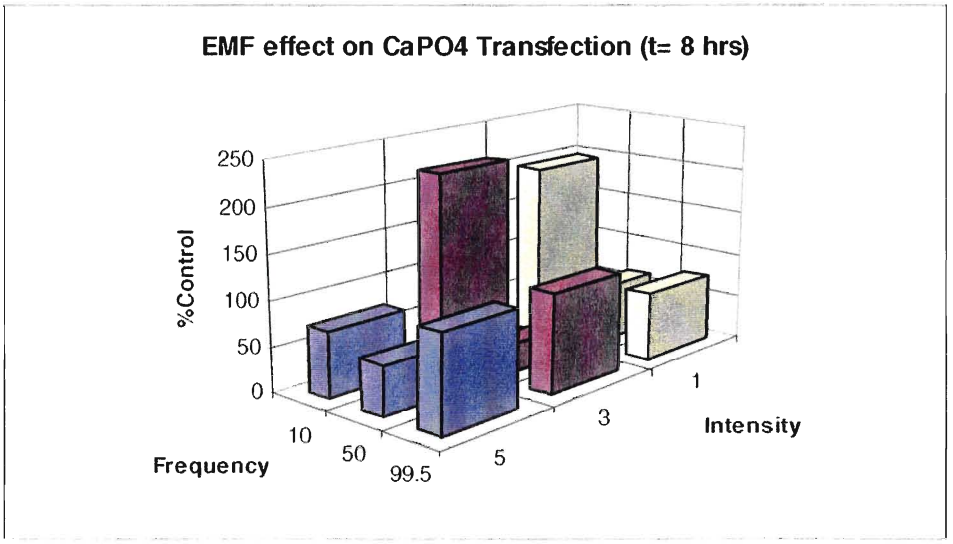

Figure 5: Effect of EMF on CaPO4 transfection when exposure was done 8 hours after CaPO4 addition. All efficiencies are expressed as percentage of the positive control done that day (not shown on graph).

Length of exposure.

The best five points from the exposure at T=2 hours were selected for further study in respect to the length of time the cells are exposed to EMF. The selected frequencies were all three 99.5 Hz settings, 50 Hz, IA and 10Hz, 5A and the cells were exposed for 1, 2 and 4 minutes in each direction. The patterns discerned varied from decreasing to increasing and bell shaped response curves, with the highest enhancement being approximately 200%. These results are represented in figures 6.

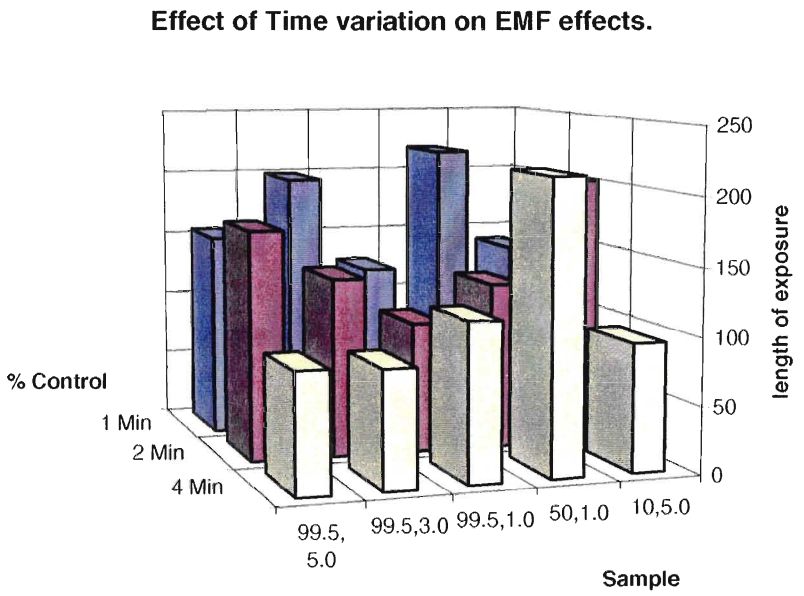

Figure 6: Effect of different lengths of exposure on EMF effect on CaPO4 efficiency. The best settings from the original scan at 2 hours post CaPO4 addition were used and the time factor was reduced by a factor of 2 (1 minute), left intact (2 minutes) or increased by a factor of 2 (4 minutes). All times represent exposure length in each direction of the field (+ and -).

Table 7: Variation in time of exposure on the settings with best results from exposure at 2 hours after transfection. The settings in column headings are displayed as frequency(Hz)/intensity(A). The rows indicate the time of exposure in each direction of the EMF field. N= Negative control. P= Positive control.

	N	P	99.5/5.0	99.5/3.0	99.5/1.0	50/1.0	10/5.0
1	0	1.78	2.75	3.54	2.24	3.9	2.48
2			3.06	2.39	1.75	2.24	3.6
4			1.57	1.54	2.09	3.84	1.69

Intensity Variation.

The same 5 settings were chosen for a narrow intensity variation scan at an exposure time of 2 hours and for 2 minutes in each direction. The results did not show any consistent pattern, with all points showing an inhibition, with the exception of 99.5 Hz, 2A which showed approximately 100%.

Table 8: A scan of narrow intensities (0.5 A increments) of the 5 best settings from exposure 2 hours after transfection. Cells with X in them were not tested. The column headings indicate the intensities (in A) while rows indicate the frequency tested in Hz. P= positive control. N= negative control.

	P	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5
99.5	1.69	0.81	0.84	1.03	1.51	1.24	0.78	1.78	1.21	1.33	X
50	X	X	X	X	X	X	X	X	1.42	1.18	1
10	1.66	0.81	1.39	X	X	X	X	X	X	X	X

Reproducibility.

In the course of the above mentioned experiments a number of measurements were repeated. A comparison among them showed a large variability from one trial to another (Table 9) as a result a reproducibility test was conducted.

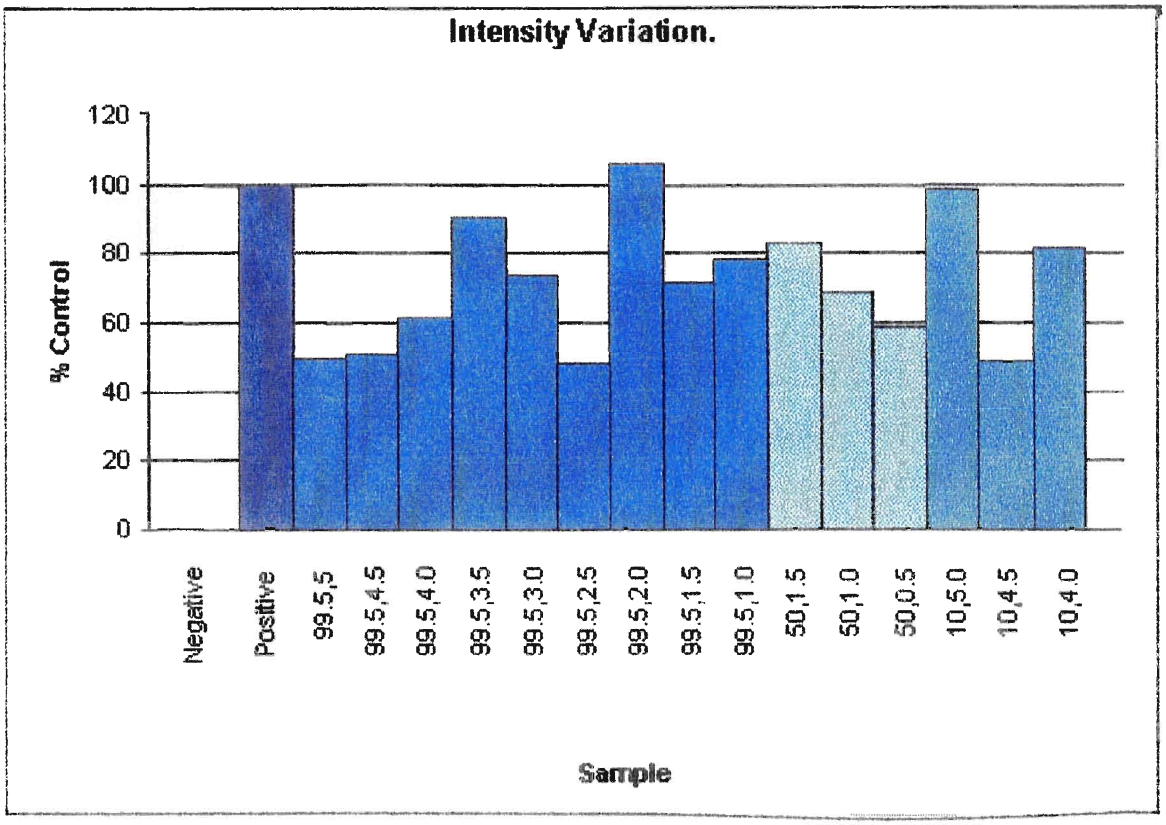

Figure 7: Effect of intensity variation on EMF's effect on CaPO4 transfection efficiency. The best frequency settings were selected as described earlier and the intensity was varied by 0.5 A in each direction.

For this test the setting chosen was 50 HZ, 5A. The test was conducted over a period of 3 days. Each day 3 plates were exposed. All 3 plates were from the same original plate, and those of the following day were from a plate seeded with the other half of the original culture. The same media, trypsin and versene bottles were used throughout the test and care was taken to handle the cells in an identical fashion. All cell splitting , transfection, exposure and counting were done at the same exact times during the day.

Table 9: Repeated measures of the best 5 settings from exposure time = 2 hours.

	99.5Hz/5A	99.5Hz/3A	99.5Hz/1A	50Hz/1A	10Hz/5A
First	180	145	185	145	130
Second	190	150	115	145	220
Third	50	75	105	65	95
Max. Var.	380%	200%	176%	223%	232%

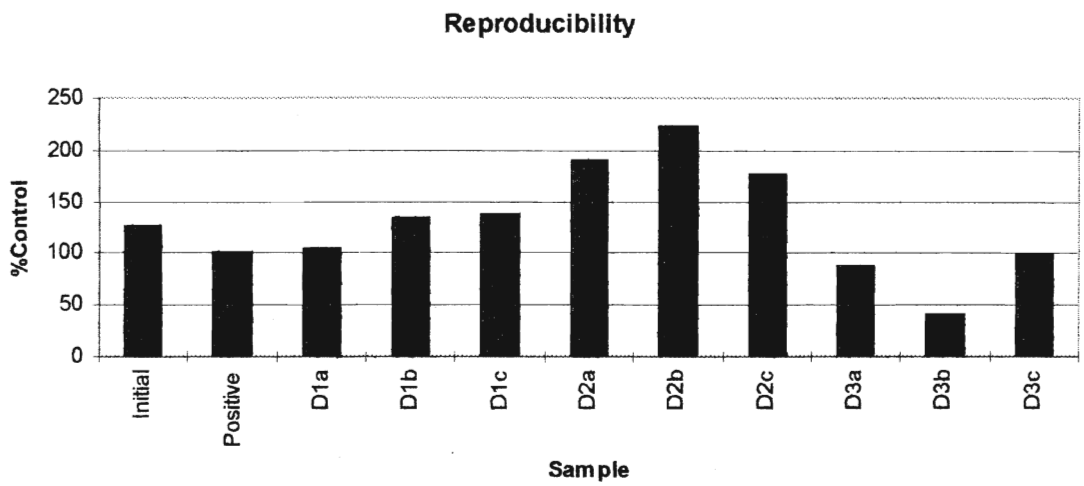

Figure 8: Reproducibility of EMF exposure effect on CaPO4 transfection efficiency. The test was conducted on three different days with three samples a day. Each day's samples were compared to a positive control done on the same day. The values in this figure represents these comparisons. Initial indicates the original reading at this setting.

The results show that daily variation from one plate to another can be as high as 200%, while day to day variation can reach over 500%. These variations raised the question of how much did the calcium phosphate method itself contribute to these variations. The three positive controls from each of the three days were compared and

showed that the day to day variation was very limited and could not account for the variations observed in fig 8. To further investigate the variability of the calcium phosphate method, a reproducibility test similar to the one done for the EMF effect was conducted. The results are graphed in fig 9. When it was determined that the variability in EMF results is not due to variability in calcium phosphate precipitation method itself, further investigations of the EMF effect on Calcium Phosphate were stopped.

Table 10: Table showing the average counts from the reproducibility test. Each count is the average of 33 different fields. N= negative control. P= positive control.

	N	P	1	2	3
Day 1	0	1.27	1.3	1.69	1.75
Day 2	0	1.18	2.24	2.63	2.09
Day 3	0	1.48	1.30	0.6	1.45

LipofectAMINE®.

The effect of EMF on the efficiency of Lipofectamine transfection was measured in the same manner as with $CaPO_4$. The settings investigated were less than in the case of $CaPO_4$. The results obtained with the original scan did not indicate any improvements in efficiency that justified any further studies based on the reproducibility results obtained later with $CaPO_4$. The tested settings are graphed in fig 10.

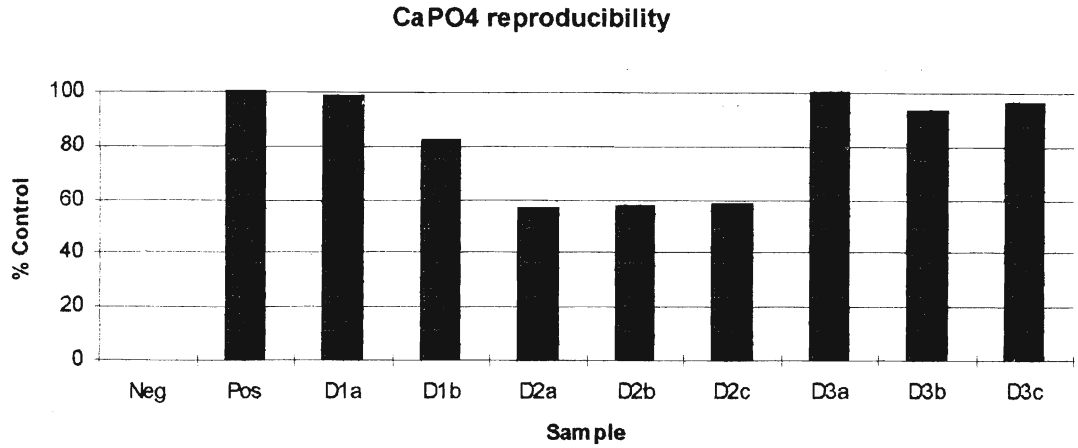

Figure 9: Reproducibility of the Calcium phosphate precipitation method. The test was conducted over 3 days with 3 samples used each day. One of the samples on the first day was used as the positive control.

Table 11: Average counts from $CaPO_4$ reproducibility test. One of the samples from the first day was used as positive control (P). N= negative control.

	N	1	2	3
Day 1	0	2.57 (P)	2.54	2.12
Day 2	0	1.45	1.48	1.51
Day 3	0	2.57	2.39	2.48

Table 12: Effect of EMF on lipofectAMINE® transfection efficiency. Settings are displayed in column headings in the format Frequency(Hz)/Intensity(A). N= negative control. P= positive control.

	N	P	99.5/5	99.5/3	50/5	50/3	10/5
Av.	0	12.6	4.6	9.45	5.36	4.33	4.24

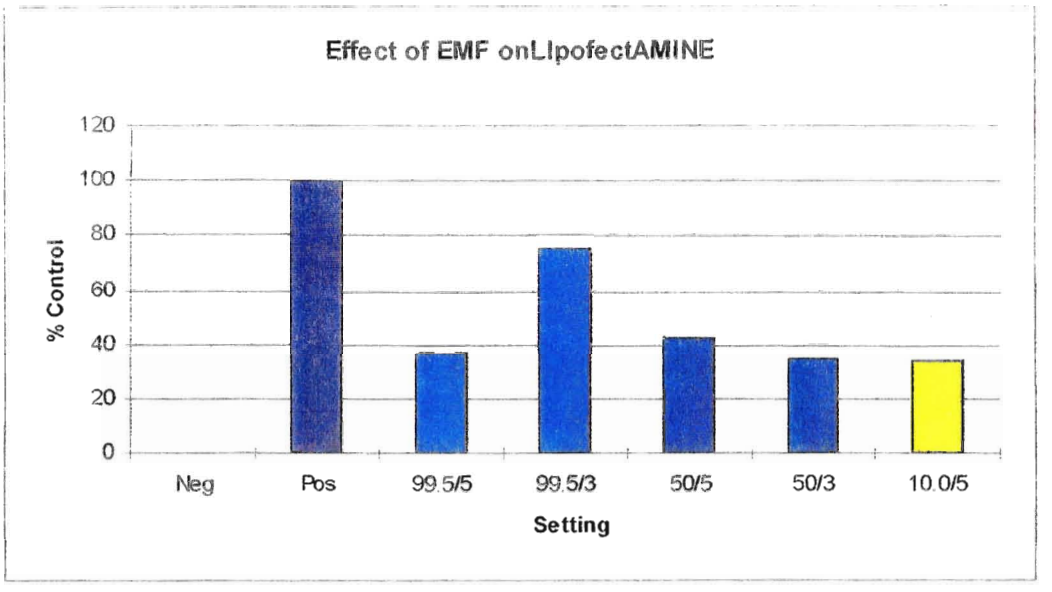

Figure 10: effect of EMF exposure on LipofectAMINE® transfection efficiency. The settings are shown in the format Frequency/Intensity. All samples are graphed as percentage of the positive control.

Chapter7: Effect of the DNA sequence on its uptake.

Previous results in this lab have shown that the transfection of HeLa cells with a plasmid containing the β-galactosidase gene under the control of the CytoMegalo Virus (CMV) promoter resulted in transfection rates that are much higher than those observed when the CMV promoter was replaced with the SV-40 promoter. In order to investigate the possibility that this difference is due to an increase in DNA uptake and/or enhanced release from the lysosomes, HeLa cells were transfected with PCR amplification products 790 bp in length that contained either the CMV promoter sequence or the GFP protein.

DNA was collected from the cells at 5 different time points. The first point was just prior to DNA addition. These two plates served as negative control. Five minutes after DNA addition two plates (one transfected with the CMV and one with the GFP sequence) were lysed and the DNA was collected. These served as the control for DNA present in the media (extracellular compartment). Two more plates (one for each DNA sequence) was lysed 4 hours after DNA addition (same time as media change). DNA from the next two plates was collected 8 hours after DNA addition. The last two plates were lysed 12 hours after DNA addition. The DNA was transferred to nylon membranes that were later probed with a radioactive probe (same DNA sequence as was used in transfection). The membranes were then exposed to an X-ray film. The results are represented in figures 12 and 13. The total DNA collected from each 35 mm dish was

around 90 μg. These 90 μg were suspended in 100 μl of water. Thirty μl of the total volume were loaded from each plate. Each plate was transfected with a total of 1 μg of DNA. As 1/3 of the total isolated DNA was loaded into the gel, one third of the transfected DNA would be expected to be present corresponding to 333 ng. According to the intensity of the bands compared to the standard lanes, it was determined that the lane corresponding to the DNA isolated 4 hours after transfection contained about 8 ng (as determined by band intensity comparison) of transfected DNA (for both GFP and CMV). This corresponds to a recovery rate of 2.4%. These results indicate that there is no difference between the uptake of DNA containing the CMV or GFP sequence.

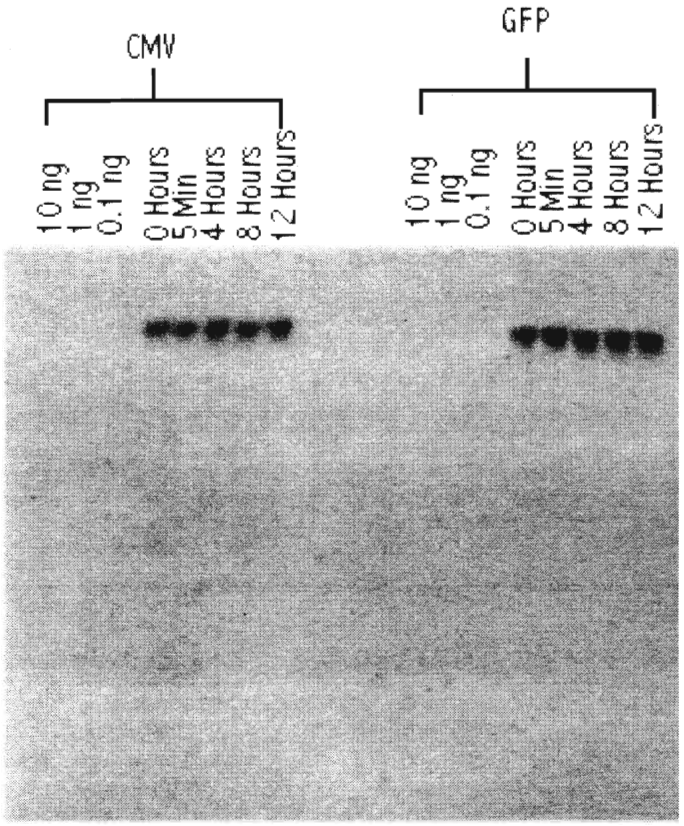

Figure 11: The gel showing the genomic DNA bands and the lanes for the standard lanes (not visible due to small quantities). The gel was stained with ethidium bromide and visualised with an ultraviolet light source. The picture was captured using a digital system.

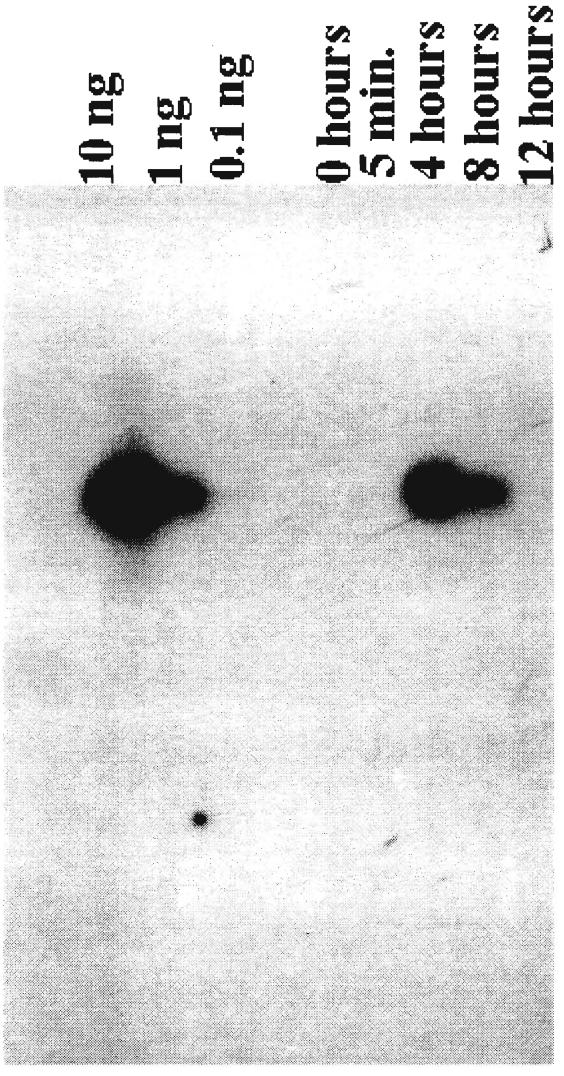

Figure 12: Radiograph of gel containing DNA isolated from cells transfected with the GFP sequence. The legend on top of each standard lane identifies the concentration loaded into that lane. The time indications on top of the experimental lanes indicates the time at which the DNA contained in that lane was collected.

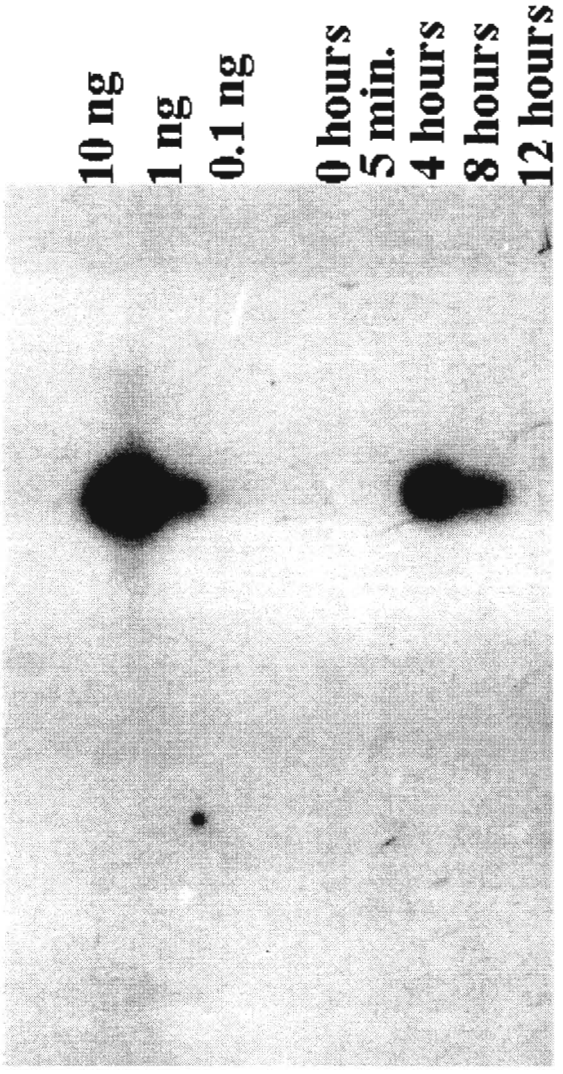

Figure 13: Radiograph of gel containing DNA isolated from cells transfected with the CMV sequence. The legend on top of each standard lane identifies the concentration loaded into that lane. The time indications on top of the experimental lanes indicates the time at which the DNA in that lane was collected.

Chapter 8: Hydrogen peroxide exposure.

HeLa cells viability in H_2O_2.

HeLa cells were exposed to concentrations of H_2O_2 between 10 mM and 500 mM for 10, 15 and 30 minutes for each concentration. Using Trypan blue exclusion tests to detect cell death, it was found that HeLa cells can survive in H_2O_2 concentrations as high as 250 mM if exposure time is kept to 10 minutes (Table 13).

Table 13: HeLa cells viability in H_2O_2 as a function of time. A + indicates normal cell growth. - indicates extensive cell death.

	10 mM	**50 mM**	**100 mM**	**150 mM**	**200 mM**	**250 mM**	**500 mM**
10 minutes	+	+	+	+	+	+	-
15 minutes	-	-	-	-	-	-	-
30 minutes	-	-	-	-	-	-	-

Effect of H_2O_2 on Calcium phosphate transfection efficiency.

Cells were transfected with 5 μg DNA/35 mm dish 24 hours after seeding. The original concentrations used were the same as those used in the viability test (10-250 mM) of H_2O_2 for 10 minutes. The combination of $CaPO_4$-DNA transfection and the H_2O_2 treatment (both known to cause damage to cytoplasmic membranes) proved to be

extremely toxic to the cells and even cells exposed to 10 mM were completely killed. Following these results lower concentrations were tried that ranged from 0.01 μM to 10 mM. Cells exposed to concentrations over 10 μM showed extensive or complete cell death. The results are shown in figure 14. In another attempt to decrease the combined toxic effect of H_2O_2 exposure and calcium phosphate transfection, the exposure was done after 2 hours of incubation with calcium phosphate-DNA mixture. This resulted in enhanced viability of the cells but no transfection was observed.

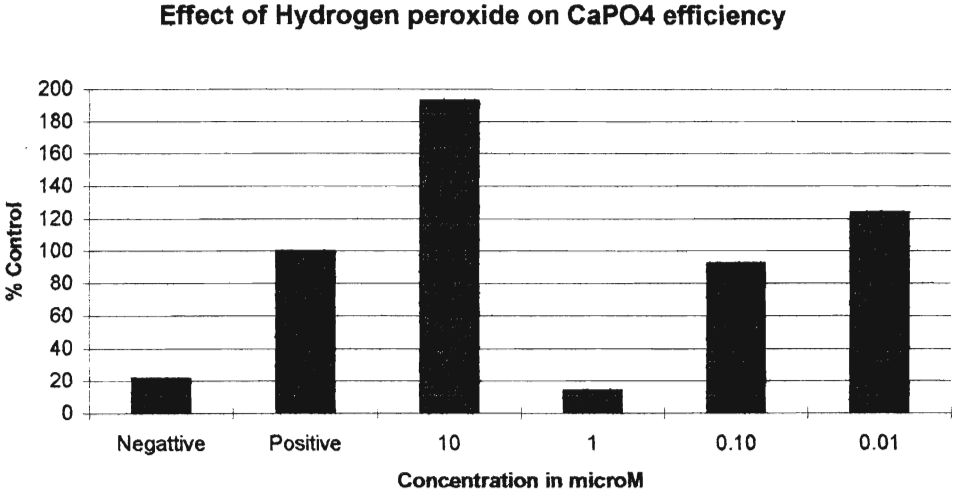

Figure 14: Effect of Hydrogen peroxide on $CaPO_4$ transfection efficiency. The samples are presented as percentage of the positive control. All 5 concentrations tested showed no significant difference from the control.

Statistical analysis using ANOVA on ranks (Student-Newman-Keuls method) of the data sets revealed no significant difference ($P>0.05$) between the positive control and

0.01 μM. The reduced transfection efficiency found at 0.1 μM was consistently encountered over 3 repeats of this experiment.

Table 14: Average counts from cells exposed to H_2O_2 following $CaPO_4$ transfection. The concentrations are shown in microM. The exposure was done 4 hours after DNA addition. N= negative control. P= Positive control.

	N	P	10	1	0.1	0.01
Av.	0.60	2.84	5.48	0.39	2.63	3.51

Effect of H_2O_2 on Lipofectamine® transfection efficiency.

The effect of H_2O_2 on Lipofectamine® transfection was observed for concentrations varying from 0.01 μM to 250 mM. As with the Calcium phosphate precipitation method, exposure of the cells to H_2O_2 concentration higher then 10 μM in combination with transfection proved to be too toxic. The results for concentrations between 0.01 μM and 10 μM are presented in figure 15.

These results clearly show that H_2O_2 has no positive effect on Lipofectamine® transfection efficiency but in facts inhibits it. Therefore no further investigations into the effect of H_2O_2 on LipofectAMINE were carried out and all further studies were limited to the effect on $CaPO_4$ transfection efficiency.

Table 15: Average counts of cells exposed to H_2O_2 following lipofectAMINE® transfection. The concentrations are in microM and the exposure was done 4 hours after transfection. N= negative control. P= positive control.

	N	P	10	1	0.1	0.01
Av.	0	10.18	3.63	8	10.18	7.18

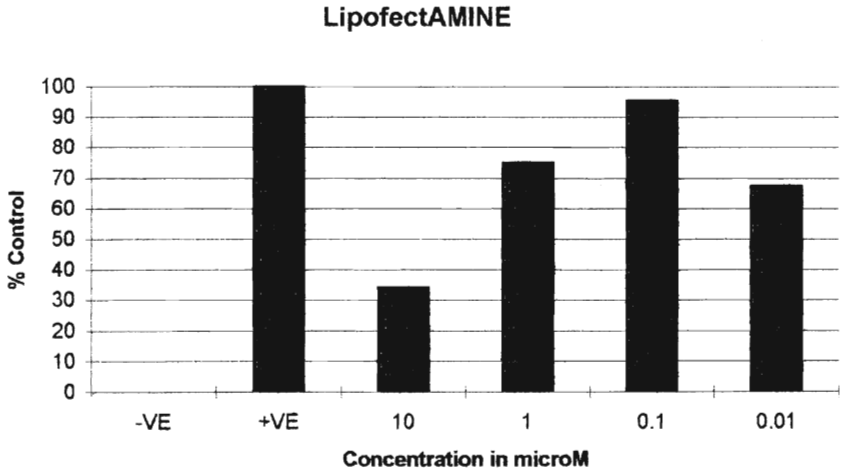

Figure 15: Effect of hydrogen peroxide on Lipofectamine's transfection efficiency. Each sample is represented as percentage of the positive control (Unmodified).

Variations of time and length of exposure.

In order to test for possible effect of H_2O_2 after the cells were given time to recover from the $CaPO_4$ treatment and to also investigate the possibility that a shorter exposure period might result in better viability and therefore better effect on transfection, the cells were exposed to H_2O_2 for 1, 2 and 4 minutes after 0, 2, 4 and 8 hours after

$CaPO_4$ removal. Concentrations tested at these times ranged from 10μM to 100 mM. Only cells exposed to 10 μM of H_2O_2 survived and therefore they are the only ones presented in the graph. The results are graphed in figure 16.

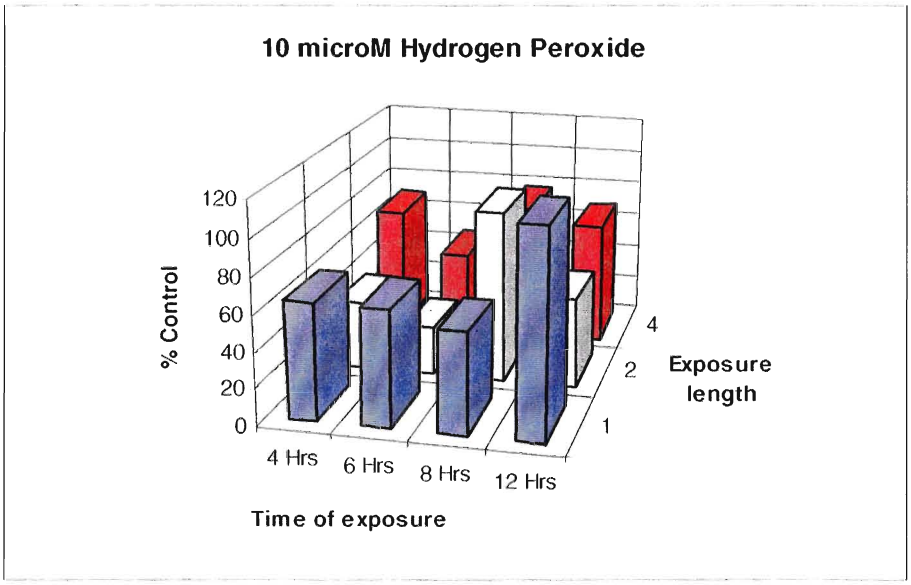

Figure 16: Effect of different exposure times and lengths on the efficiency of $CaPO_4$ transfection. All samples are shown as percentage of the positive control (not shown on graph). Cells exposed to concentrations higher than 10 microM are not shown.

Table 16: Summary table of the average counts of cells exposed to 10 microM H_2O_2 for 1, 2, or 4 minutes. The time of exposure is indicated in the row headings. The counts are average of 33 different fields. N= negative control. P= positive control.

	N	P	1	2	4
4 Hours	0	1.48	0.96	0.57	1.03
6 Hours	0	1.48	0.96	0.42	0.69
8 Hours	0	1.3	0.72	1.24	1
12 Hours	0	1.42	1.6	0.72	0.96

These results indicate that the application of hydrogen peroxide in combination with $CaPO_4$ transfection proved very toxic to cells in culture and all further investigation into this were stopped.

Chapter 9: Effect of basic amino acids.

In an effort to determine the effect of basic amino acids on the lysosomes' stability in which the DNA is getting trapped in, it was decided to add a number of different basic amino acids to the transformation mixture. The point behind adding these amino acids was to raise the pH in the lysosomes therefore reducing the activity of the lysosomal enzymes and giving the DNA a better chance of escaping. To this end three basic amino acids were tried: arginine, lysine and histidine. Each amino acid was tried with both $CaPO_4$ and LipofectAMINE®. Previous studies in this lab have indicated that addition of the amino acids to the DNA before addition of the transformation solution resulted in inhibition of transfection (Sywanicz, 1998), as a result all amino acid addition were done after the formation of DNA-$CaPO_4$ or DNA-LipofectAMINE® complexes.

1. Arginine.

1.1. Calcium phosphate.

Arginine was added to an Eppendorf tube and the volume was brought up to 200 μM before being supplemented with 200 μM of the already formed DNA-CaPO4 complex. All concentrations mentioned in this report are the concentrations in the 400 μM volume so formed. The concentrations tested ranged from 50 to 5000 μM. The results showed a bell shaped curve with the maximum enhancement occurring at 500μM (171% of unmodified control). These results are shown in figure 17 The effect of

arginine changed from positive to negative when the concentration used was above 650 μM (<84% of unmodified control) and are inhibitory at the higher concentration range (<17% of unmodified control). With the exception of the combination of 2000/950 μM and 500/350 μM, all differences are statistically significant ($p < 0.05$ as determined by an ANOVA on ranks, Student-Newman-Keuls Method).

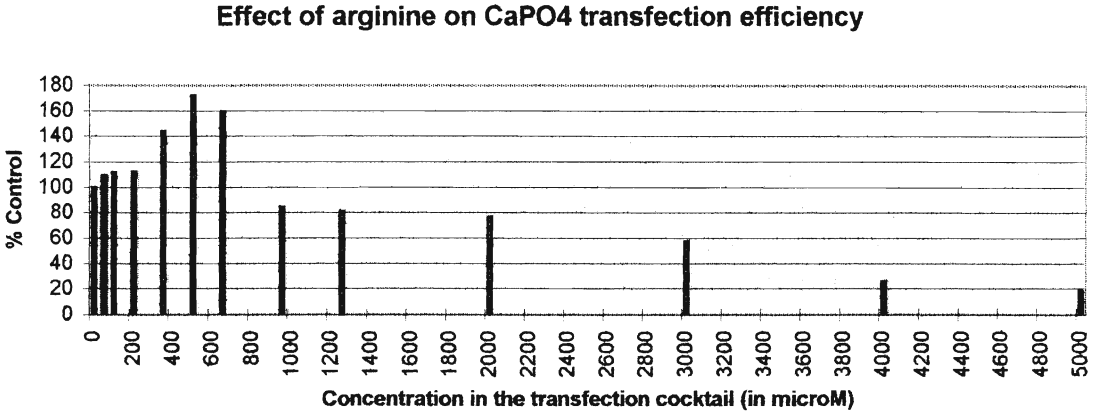

Figure 17: Calcium phosphate efficiency in response to addition of arginine. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

Table 17: Average count of the plates from arginine exposure. Each count is the average of 33 different fields each representing 1/306th of a 35 mm dish. The concentrations in the first row are the ones found in the transfection mixture (expressed in microM). P indicates the positive control (unmodified). N indicates negative control (not transfected).

	N	P	50	100	200	350	500	650	950	1250	2000	3000	4000	5000
Av.	0	2.69	1.72	1.75	3.03	3.87	4.63	4.30	2.27	2.18	2.06	1.54	0.69	0.48

1.2. LipofectAMINE®.

In the case of LipofectAMINE®, the amino acid was added to an Eppendorf tube and the volume brought up to 800 μM with serum free media. The tube was then supplemented with 200 μM of the already formed DNA-lipofectAMINE® complex. The range tested was between 50 and 1500 μM. The results fit a response curve similar in shape to that obtained with calcium phosphate; that is, there was an initial increase in efficiency followed by a decrease and eventually reaching an inhibitory level. With LipofectAMINE® the best efficiency was obtained with an arginine concentration of 50 μM (204% of unmodified control), while concentrations over 1000 μM resulted in inhibition as low as 19% of control. These results are shown in Fig 18. The results were tested by ANOVA on ranks and all differences were found to be statistically significant with the exception of the pair of 900 and 1500 μM (Student-Newman-Keuls method).

Table 18: Average counts from plates transfected with LipofectAMINE® and supplemented with arginine. Each count is the average of 33 different counts covering a total of over 10% of the plate. All concentrations are in microM and corresponds to the concentrations found in the transfection mixture. P2 and the concentrations after it represent additional concentrations tested in light of the first results obtained. N indicates negative control (Not transfected) while P is the positive control (unmodified).

	N	P	50	150	300	600	900	1500	P2	5	15	30
Av.	0	23.5	48.2	33.6	10.18	3.6	5.21	4.6	24.8	27.3	37.0	45.0

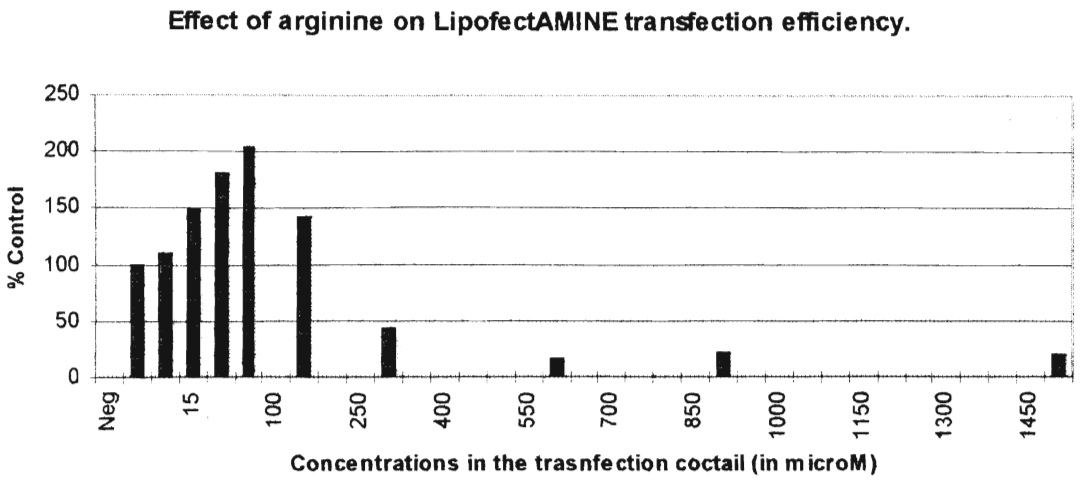

Figure 18: LipofectAMINE® transfection efficiency with the addition of arginine. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

2. Histidine.

2.1. Calcium phosphate.

The concentrations tested for histidine fall in the same range as arginine (50 to 5000 μM). The response curve is very similar to those obtained with arginine. The best efficiency was observed with 50 μM histidine at 118% of the unmodified control. The inhibition levels observed with histidine did not reach as low as those observed with arginine (40% opposed to 19%). These results are shown in figure 19. Statistical analysis using the Student-Newman-Keuls method (ANOVA on ranks) showed that the observed differences were significant ($p<0.05$) except for the results of 150/Pos and 450/5000 μM.

2.2. LipofectAMINE®.

The range of concentrations tested for histidine's effect on LipofectAMINE® efficiency was between 50 and 1500 μM as well. The best result was obtained with a concentration of 50 μM (87% of unmodified control). Surprisingly all concentrations tested showed a decrease in the transfection efficiency. These results are summarised in figure 20. All observed differences were found to be statistically significant with the exception of 600/900 and 150/300 μM (ANOVA on ranks using Student-Newman-Keuls method).

Table 19: Counts from cells transfected with calcium phosphate and exposed to histidine. All counts are the average of 33 different fields and the concentrations are in microM and indicate the concentrations in the transfection mixture. The unmodified control (positive) is represented by P while the negative control (not transfected) was indicated with N

	N	P	50	150	300	450	600	900	1100	1550	2000	3000	4000	5000
Av.	0	1.81	2.15	1.84	1.66	1.21	1.18	1.09	1	1	0.97	0.93	0.75	0.72

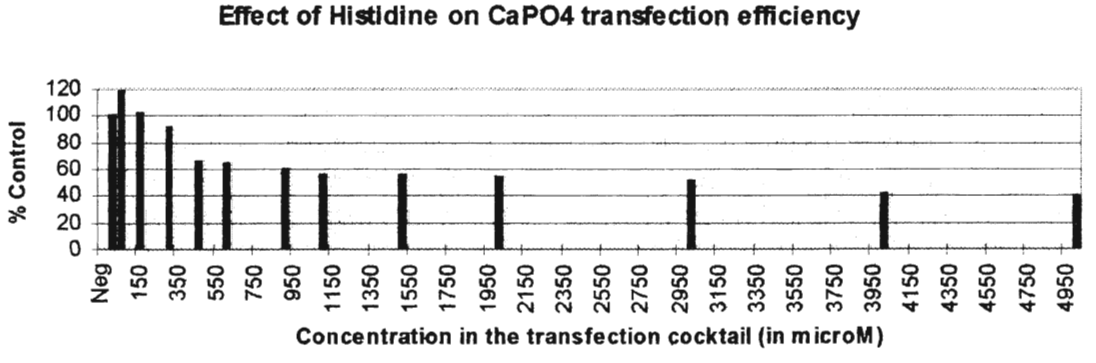

Figure 19: Effect of histidine on calcium phosphate efficiency. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

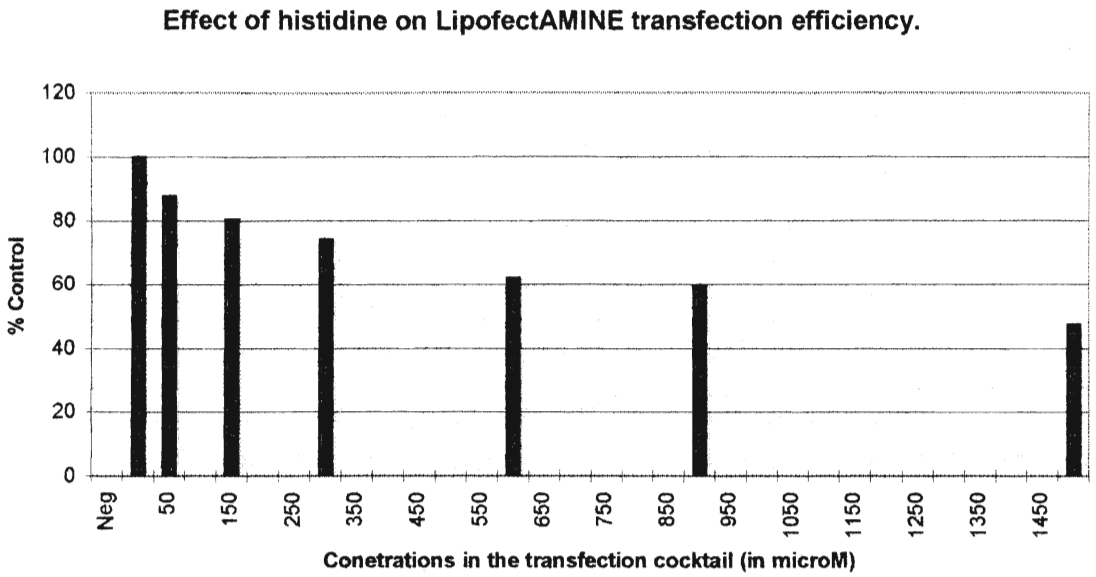

Figure 20: Effect of histidine on LipofectAMINE® transfection efficiency. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

Table 20: Summary of the counts from cells transfected with lipofectAMINE® and exposed to histidine. Each count is the average of 33 fields and the concentrations of histidine in the mixture cocktail are represented in microM. Unmodified control is represented by P while the negative control is represented by N.

	N	P	50	150	300	600	900	1500
Av.	0	38.6	33.8	31.1	28.6	23.9	23.0	18.3

3. Lysine.

3.1. Calcium phosphate.

As with the other two amino acids tested, the range of concentrations evaluated for the effects lysine on calcium phosphate's efficiency varied from 50 to 5000 μM. The best efficiency obtained was with a concentration of 150μM. (109% of unmodified control). The curve is very similar to that obtained with arginine with an inhibition level of 15% of the control. The results are graphed in figure 21. With the exception of the difference between 50 and 1300 μM all other differences were found to be statistically significant ($p<0.05$ using the Student-Newman-Keuls method of the ANOVA on ranks test).

3.2. LipofectAMINE®.

With lysine the effect of concentrations ranging from 50 to 1500 μM were tested for there effect on LipofectAMINE® transfection efficiency. The best result was obtained with a concentration of 900 μM (130% of unmodified control). The results fit a curve that is opposite to the ones seen with both arginine and histidine. The lowest level was observer with a concentration of 150 μM (<2% of control). These results are represented in figure 22. By applying the ANOVA test (Student-Newman-Keuls method) it was determined that all differences (with the exception of the difference between 900 and 1500 μM) are statistically significant.

Table 21: Counts from cells exposed to lysine following calcium phosphate transfection. Each count is the average of 33 counts. Concentrations are of lysine in transfection mixture. P= positive control (unmodified), N= negative control (Not transfected).

	N	P	50	150	300	450	600	900	1100	1550	2000	3000	4000	5000
Av.	0	1.81	2.15	1.84	1.66	1.21	1.18	1.09	1	1	0.97	0.93	0.75	0.72

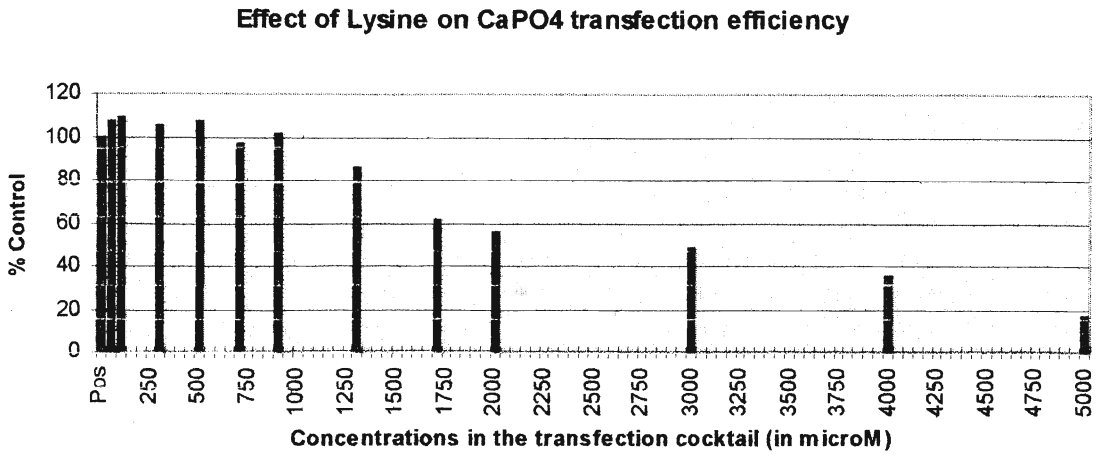

Figure 21: Effect of lysine on the efficiency of calcium phosphate transfection efficiency. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

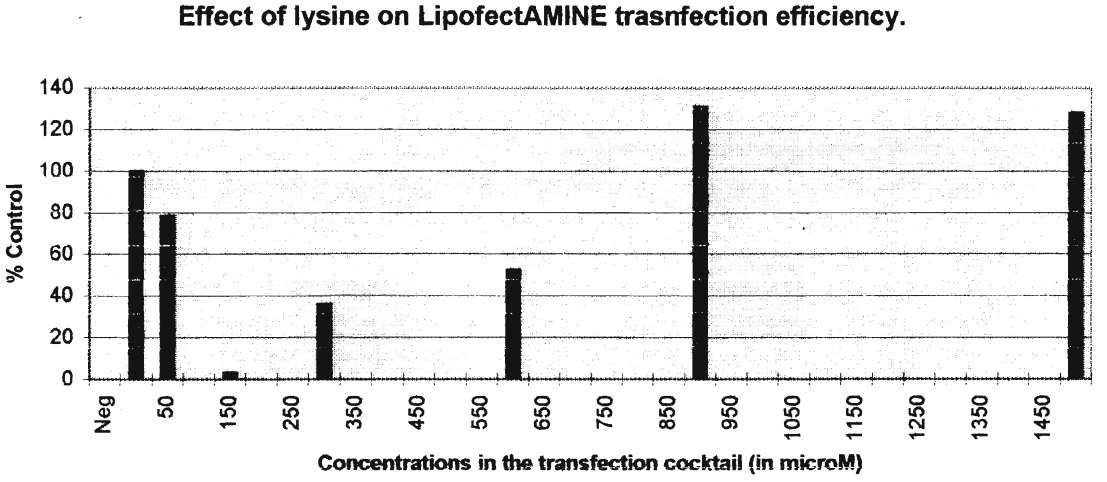

Figure 22: Effect of lysine on LipofectAMINE® transfection efficiency. The samples are represented as percentage of positive control. Each sample's count was the average of the count of 33 different fields.

Table 22: Average counts (from 33 different fields) of cells transfected with lipofectAMINE ® and exposed to Lysine. Concentrations are for the cocktail mixture. P= unmodified control. N= negative control.

	N	P	50	150	300	600	900	1500
Av.	0	38.6	33.8	31.1	28.6	23.9	23.0	18.3

4. Reproducibility.

In an attempt to determine the reproducibility of the results obtained so far with amino acids, a reproducibility test was performed in which the Arginine 500 μM was tested repeatedly over three days (three samples each day). The results obtained from this test (fig 23) indicate that the results are very reproducible. The maximum variation between 2 samples done on the same day was 106% (178.57% (D1c) Vs 167.85% (D1a)) while the highest variation level between samples transformed on 2 different days was 105% (178.57 % (D1c) Vs 168.75 % (D2b)).

5. Possible mechanisms.

The fact that some amino acids influenced transfection efficiency raises the question of how exactly they bring about this difference. This section will present the results of some experiments done in an effort to determine the mode of action of these amino acids.

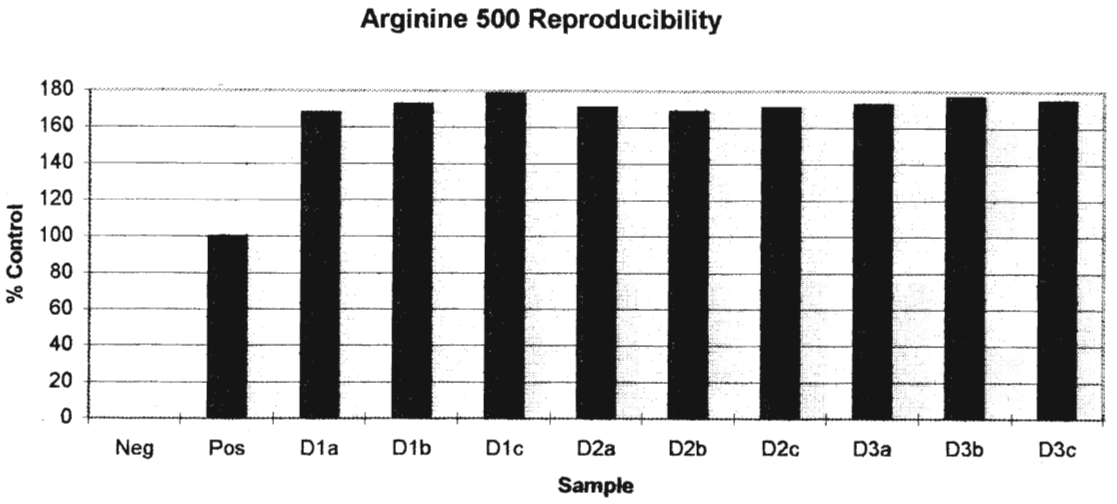

Figure 23: Reproducibility of Arg 500 effect on $CaPO_4$ transfection efficiency. Each sample is represented as percentage of the positive control (unmodified) counted on the same day. Each day three different plates were counted.

Table 23: Reproducibility of arginine results. The three samples are indicated in columns and the days are arranged in rows. P= positive control. N= negative control.

	Negative	Positive	a	b	c
Day 1	0	254	4.27	4.39	4.54
Day 2	0	1.45	2.48	245	2.48
Day 3	0	1.45	2.51	2.57	2.54

5.1. Modification of the calcium phosphate precipitate.

In an effort to determine if the amino acids act on the calcium phosphate-DNA complex that has already formed or not, a comparison was done between the efficiency of Arg 500 μM when the acid was added to the complex first or directly to the cells just before the addition of the transfection mixture. These results are graphed in figure 24.

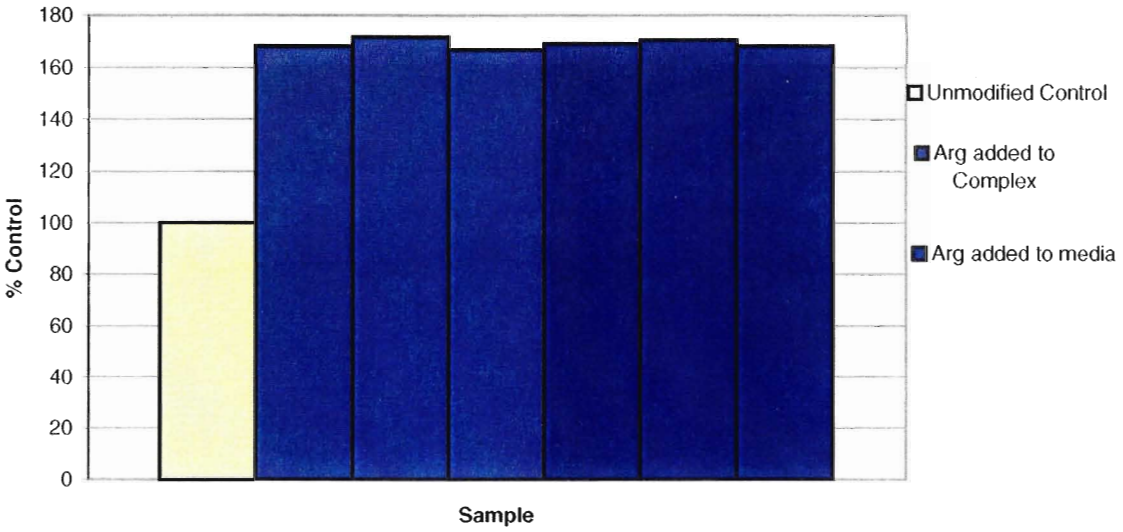

Figure 24: Effect of addition of arginine to the complex or directly to media, on $CaPO_4$ transfection efficiency. The samples are graphed as percentage of the positive control. All samples were treted the same except for the order of addition of the amino acids. The arginine was added to the DNA complex first which was then added to the cells or the arginine was added to the cells at the same time but seprately from the DNA complex.

Table 24: Effect of order of addition of the amino acid and the DNA-$CaPO_4$ complex. Complex = arginine was added to the transformation cocktail before being added to the media. Media = arginine added to the media at the same time as the DNA. All counts are the average of 33 fields.

	Negative	Positive	1	2	3
Complex	0	2.57	4.33	4.42	4.30
Media	0		4.36	4.39	4.33

Statistical analysis showed that the all differences are not statistically significant (except when compared to the two controls (using the Student-Newman-Keuls method o the ANOVA test). When the data for all 6 plates were pooled and analysed by a paired t-

test it showed that the results are not significantly different either (p=0.887). The results show no significant difference between addition of the amino acid to media or the transformation complex, indicating that the action of the amino acids is not due to a modification of the $CaPO_4$ mixture but to their action on cells.

5.2. Change in pH.

The addition of acid even in the small amounts required to achieve the concentrations under evaluation in this study should result in a change in the pH of the media. In order to determine the effect of the addition of arginine (500μM) on the pH, nine 35mm plates were filled with 3 ml of media (the same volume used in culturing the cells for the experiments) and left in the incubator overnight. Three of those plates had no cells in them. The media of the three plates with no cells had a pH of 6.72 while the media of plates with cells growing in them was 6.76. The media from the last three plates (with cells growing in them) was 6.77 and the addition of 20μL of arginine (10 mM which is the same amount added to the $CaPO_4$ mixture to bring it to 500 μM) brought the pH to 6.78. This change is too small to account for any changes that were observed in transfection efficiency.

5.3. Time of amino acids addition.

The goal of this experiment was to determine if the addition of amino acids had to be done at the same time as the transformation mixture to enhance transfection or if the exposure of the cells to the amino acids prior to the transfection would give the same results. For this goal 6 plates were transformed. For 2 of them the amino acids were added 1 hr before transfection. Out of these two, one plate had the media changed on it

before addition of the transfection mixture while it was kept the same for the other one. The last plate had the amino acids added just prior to the addition of the transfection mixture. A positive control plate (just $CaPO_4$) and a control for the addition of the new media (Media replaced just before the addition of the $CaPO_4$). The results of this test are represented in figure 25. The addition of amino acids needed to be done at the same time as the $CaPO_4$ for best results. All other methods resulted in no significant changes in transfection efficiency.

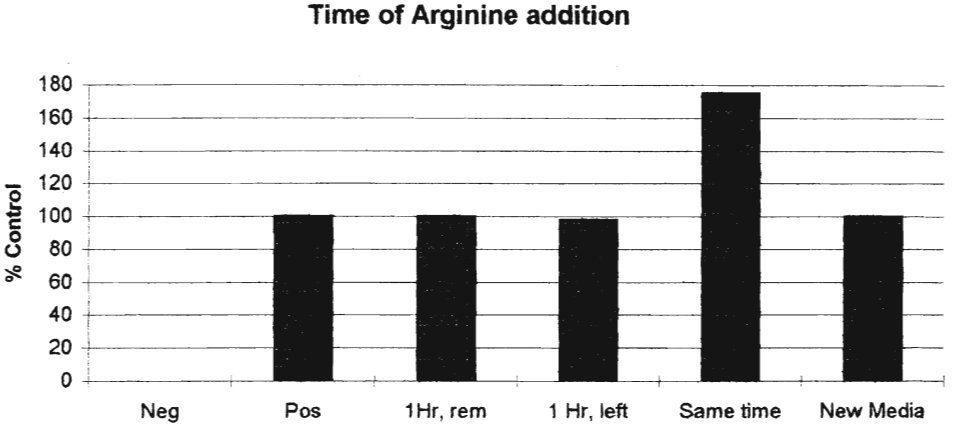

Figure 25: Effect of time of addition of arginine on transfection efficiency. The arginine was added to two plates one hour before the transfection mixture was added. In one plate the media containing the arginine was removed, in the other it was left. The third plate had the arginine added at the same time as the DNA complex.

Table 25: Effect of time of addition of the amino acid. 1 hr, rem = the media was supplemented with arginine 1 hour before transfection and the media changed just prior to transfection. 1 hr, left = same as 1hr, rem but the media was left on the cells and the DNA mixture added to it. Same time = both DNA and arginine were added at the same time. P = positive control. N = negative control. New media = control for replacing media prior to transfection.

	N	P	1 hr, rem.	1 hr, left	Same time	New media
Av.	0	1.45	1.45	1.42	2.54	1.45

5.4. Effect of the length of amino acid side chain.

The fact that arginine showed an increase in $CaPO_4$ transfection efficiency while the other basic amino acids used had no significant effect raised the question on the mechanism of action of arginine and if it is indeed the basic properties of arginine that leads to this improvement or if it is something else. In order to investigate this a number of other amino acids were used to try and investigate the other possibilities. The first point tested was the effect of the length of the amino acid's side chain on the effect on transfection efficiency. Glutamic acid and Aspartic acid differ only by the length of their side chain (same charge but Glutamic acid has one more CH_2). If side chain length was the determining factor we would expect the effect of these amino acids to be different. The results of the comparison of these two amino acids are graphed in figures 26 and 27. The results are very similar indicating that the side chain length does not appear to be the determining factor. These results are supported by the fact that lysine did not have the same results as arginine although the side chains are of similar length.

Statistical analysis of the data for both aspartic and glutamic acid show that all differences were statistically significant when evaluated by the Student-Newman-Keuls method of the ANOVA test ($p<0.05$).

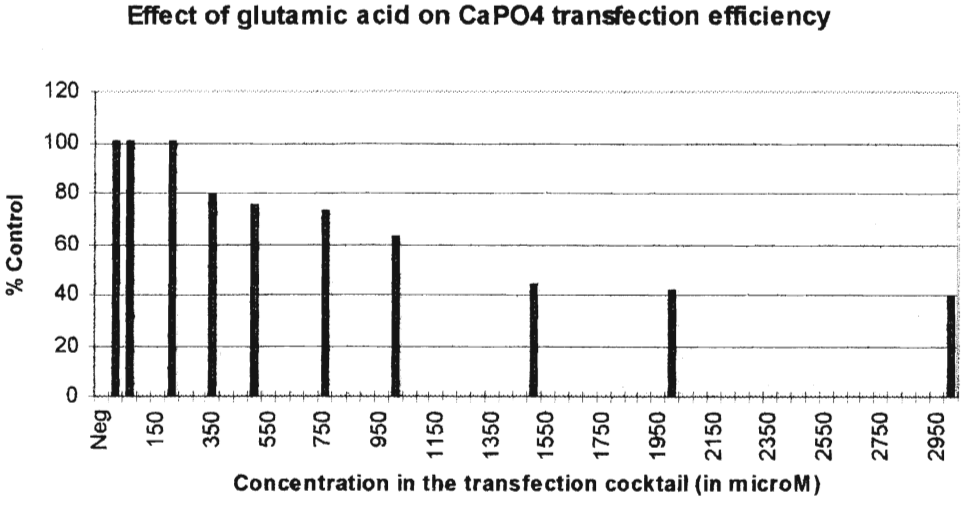

Figure 26: Effect of glutamic acid on transfection efficiency. The samples are graphed as percentage of the positive control. Each count is the average of 33 fields.

Table 26: Average counts of cells exposed to glutamic acid following transfection with calcium phosphate. N= not transfected. P = positive (unmodified). All concentrations are for the transfection cocktail (in microM) and the counts are the average of 33 fields each.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.45	1.45	1.45	1.15	1.09	1.06	0.90	0.63	0.60	0.57

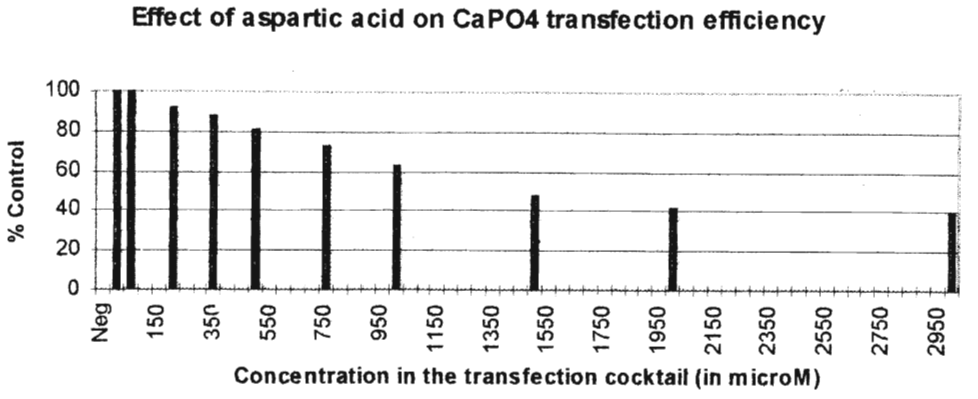

Figure 27: Effect of aspartic acid on Transfection efficiency. The samples are the average of 33 fields and are presented as percentage of the positive control.

Table 27: Effect of aspartic acid on $CaPO_4$ transfection efficiency. Counts are averages of 33 different fields. Concentrations are the ones in the transfection cocktail (in microM). P= positive control. N= negative control.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.54	1.54	1.42	1.36	1.24	1.12	0.96	0.72	0.63	0.6

5.5. The presence of an amino group.

One of the differences between arginine and Glutamic acid is also the presence of the NH_2 group at the end of the arginine side chain. To investigate the effect this might have on $CaPO_4$ transfection, the effect of glutamic acid and glutamine were compared. These 2 amino acids differ by the replacement of one of the oxygen groups at the end of glutamic acid's side chain with a NH_2 group. The results (fig 26and 28) show no

significant difference. This would indicate that the NH_2 group itself has little effect on the transfection efficiency. Furthermore all differences with the control were shown to be statistically significant.

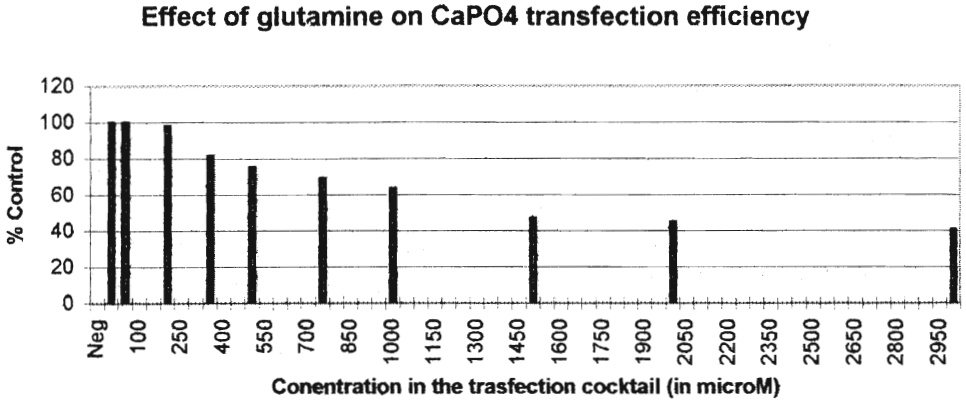

Figure 28: Effect of Glutamine on transfection efficiency. The samples are the average of 33 counts of random fields. The samples are presented as percentage of the positive control.

Table 28: Average count of cells exposed to glutamine following $CaPO_4$ transfection. The concentrations are the ones found in the transfection cocktail and they are expressed in microM. N= Negative control. P= positive control.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.48	1.45	1.45	1.21	1.12	1.03	0.93	0.39	0.66	0.60

5.6. Effect of amino acid mass.

One of the physico-chemical properties that distinguish arginine from the other amino acids used so far is the fact that it has a higher mass than any of them. To test the effect of amino acid mass, phenylalanine and tryptophan were studied in respect to their effect on transfection efficiency. These amino acids have masses similar to arginine.

The results (fig 29 and 30) show no improvement in transfection efficiency indicating that mass plays a little role if any in transfection improvement. All the observed differences in transfection efficiency were found to be statistically significant.

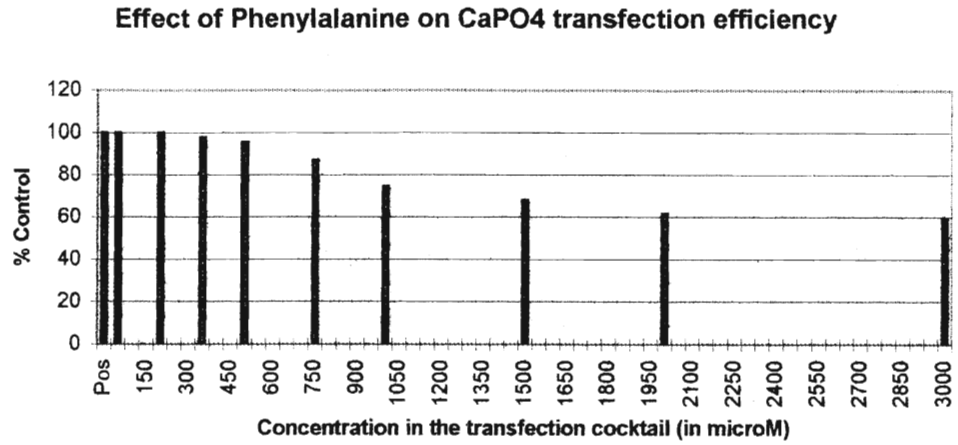

Figure 29: Effect of phenylalanine on transfection efficiency. The samples are presented as percentages of positive control and are the average of 33 different random fields.

Table 29: Average counts (from 33 fields) of cells transfected with calcium phosphate and exposed to phenylalanine. Concentrations are expressed in microM and they represent concentration of phenylalanine in the transfection mixture. N= negative control. P= positive control.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.4	1.4	1.4	1.39	1.36	1.24	1.06	0.97	0.88	0.85

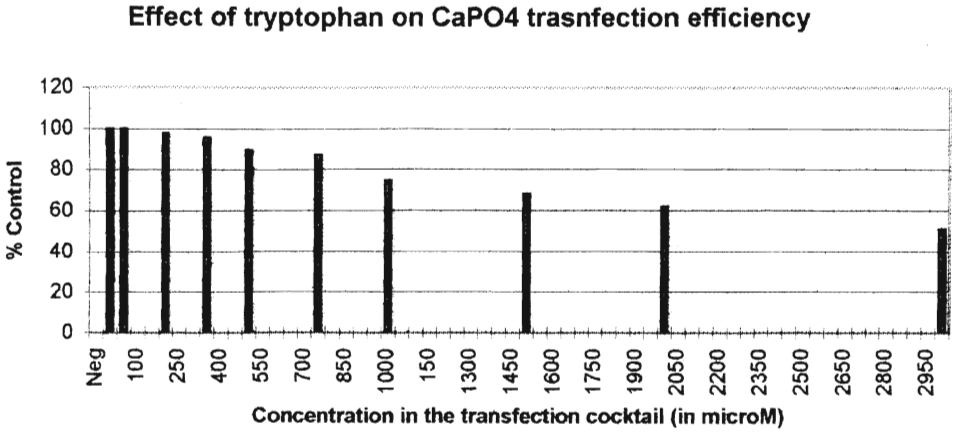

Figure 30: Effect of tryptophan on transfection efficiency. Thirty three different fields were counted and the averages represented in the form of percentages of the positive control.

Table 30: Counts from cells transfected with calcium phosphate and exposed to tryptophan. The counts are averages of 33 different fields. Concentrations are the ones in transfection cocktail expressed in microM. N= negative control. P= positive control.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.42	142	1.39	1.36	1.27	1.24	1.06	0.96	0.87	0.72

5.7. Effect of a negative charge.

The effect of the presence of a negative charge in the side chain was investigated by comparing the effect on transfection of phenylalanine and tyrosine that differ only by the presence of an OH group on the phenyl group in tyrosine. Once again no detectable difference was observed between these amino acids (fig 29 and 31) indicating that the presence of a negative charge does not result in any difference in transfection efficiency.

Using Student-Newman-Keuls method of the ANOVA on ranks test it was determined that the differences observed with respect to control were all significant ($p<0.05$).

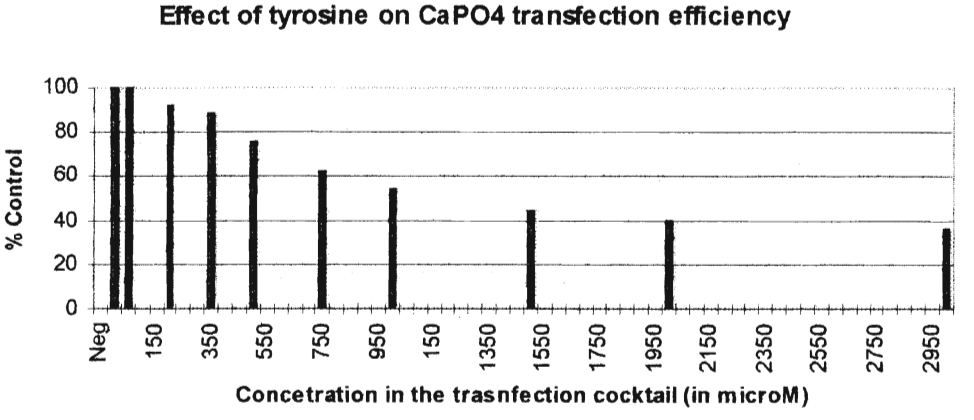

Figure 31: Effect of Tyrosine on transfection efficiency. The results of 33 different fields were averaged and presented as percentage of positive control.

Table 31: Average counts of cells transfected with $CaPO_4$ and exposed to tryptophan. The averages are from 33 different fields. The concentrations are in microM and are the ones found in the transfection cocktails. N= negative control. P= positive control.

	N	P	50	200	350	500	750	1000	1500	2000	3000
Av.	0	1.51	1.51	1.39	1.33	1.15	0.93	0.81	0.66	0.60	0.54

Combination of amino acids.

In order to gain a better insight into the possible mechanisms of action of arginine on transfection efficiency, a number of combinations of arginine and histidine were tested for their effect on calcium phosphate transfection efficiency. The results are

presented in table 32. All of the results obtained showed statistically significant differences when analysed by the Student-Newman-Keuls Method (ANOVA on ranks).

Another combination of amino acids tried is 3 different acidic amino acids. The concentrations chosen were those that had no effect on transfection efficiency by themselves. They were aspartic acid (50 μM), tyrosine (50 μM) and glutamic acid (200 μM) in combination with 500 μM arginine. The result was an 80% transfection efficiency compared to the unmodified positive control (1.09 compared to 1.36).

Table 32: Effect of a combination of amino acids on the transfection efficiency of calcium phosphate. The numbers in parentheses are the average of 33 different fields. The positive control had an average of 1.36 cells/field. Negative control showed no cells.

		ARGININE		
		350 μM	500 μM	650 μM
Histidine	300 μM	130 % (1.78)	141 % (1.93)	110 % (1.51)
	450 μM	102 % (1.42)	125 % (1.75)	98 % (1.36)
	600 μM	93 % (1.24)	112 % (1.51)	93 % (1.24)

Effect of arginine 500 μM on transfection efficiencies in other cell lines.

In order to test if the effect of arginine on transfection efficiency was specific to HeLa cells, it was tested with 4 different cell lines. The cell lines chosen were: 293 cells (transformed human embryo kidney cells) which are know to have a high transfection efficiency, MDBK, CCL-44 and CRL-6072 (bovine origin). While the transfection efficiency of the CCL-44 and CRL-6072 was not known before the experiment was conducted, MDBK are known to have a very low transfection efficiency. The test included 4 plates of each cell line. Two plates of each cell line were transfected with

calcium phosphate or lipofectAMINE® and left unmodified (positive controls) while the other two were exposed to arginine 500 μM in a fashion identical to HeLa cells.

The results of the transfection with 293 cells were very hard to quantify as the majority of the cells were green (e.g. for lipofectamine over 600 cells were found in one field compared to a maximum of about 70 with HeLa cells). Another cell line that proved impossible to quantify transfection efficiency for was the MDBK cell line as no transfected cells were observed in any of the 4 plates. The other 2 cell lines (CCL-44 and CRL-6072) showed enhancement levels between 150-170%. The results are graphed in fig 32 and the average counts from the different fields are tabulated in table 33.

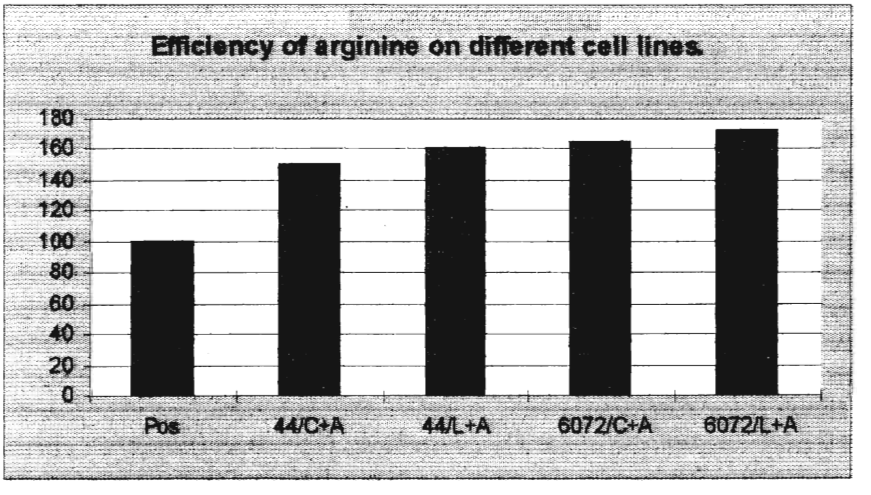

Figure 32: effect of arginine 500 microM on transfection efficiency of different cell lines. The cell lines are CCL-44 (shown as 44) and CRL-6072 (shown as 6072). The effect was measured in association with calcium phosphate precipitation (C+A) and lipofectAMINE® (L+A). Each cell line is expressed in percentage of the positive control for the transfection method. The positive control is shown for reference.

Table 33: Effect of arginine 500 microM on CCL-44 and CRL6072. The counts are averages of 33 different fields.

	$CaPO_4$	$CaPO_4$ + Arg	LipofectAMINE®	LipofectAMINE® + arginine
CCL-44	2	3	16.60	25.12
CRL-6072	2.45	4.03	30.12	51.84

Chapter 10: Discussion.

The increase of DNA uptake efficiency by mammalian cells would have far reaching consequences in a number of applications ranging from protein expression systems to gene therapy of hereditary disorders. The techniques in use today suffer from low efficiency and sometimes moderate to high toxicity. Among the methods that have been used most often are calcium phosphate precipitation and cationic liposomes. The first method uses relatively inexpensive products while the second shows better efficiency of transfection in most cell lines. However even with the use of expensive cationic liposomes transfection kits, the majority of cell lines show very low transfection efficiency. The main obstacle faced by these methods is the entrapment of DNA in lysosomes and its subsequent destruction without it reaching the nucleus for it to be expressed. Attempts at destabilising the lysosomes and/or reducing the lysosomal enzymes' activity (by raising the pH) are expected to result in a higher transfection level.

One of the methods aimed at destabilising the lysosomes was the use of sublethal concentrations of hydrogen peroxide. This kind of exposure had been shown to trigger a cell repair mechanism. The first step in this mechanism has been shown to be the destabilisation of lysosomes (Brunk *et al.*, 1995). Due to its known toxic effects, the first step was to determine the maximum exposure time that the cells can tolerate. It was empirically determined that HeLa cells can survive in H_2O_2 of concentrations up to 250 μM and exposure time of 10 minutes. Combinations of a 250 μM/10 minutes exposure following transfection with $CaPO_4$ or Lipofectamine proved to be too toxic for the cells.

Reducing the incubation time with $CaPO_4$ to 2 hours instead of 4 resulted in a better survival rate but the transfection efficiency was below detectable levels. Exposure to concentrations between 0.01 μM and 200 mM were examined and the highest concentration that can be used following $CaPO_4$ or Lipofectamine transfection was found to be 10 μM. Based on this, all further tests were done with concentrations of 10 μM or less. The initial screening of the H_2O_2 effect on $CaPO_4$ efficiency revealed an apparent increase in transfection efficiency after a 10 μM exposure. This increase however was not statistically significant. An interesting observation about the effect of H_2O_2 on $CaPO_4$ transfected cells is the low efficiency observed with the 1 μM, which was consistent in over 3 experiments. Hydrogen peroxide exposure following Lipofectamine exposure resulted in a marked decrease in transfection efficiency with all tested concentrations which could be explained in part by the damage that on elevated free radical content would cause to the lipid structures.

In an attempt at improving survival rate following H_2O_2 exposure, shorter exposure times (1-4 min) were tested with H_2O_2 concentration between 10 and 100 μM. Once again all cells exposed to H_2O_2 over 10 μM did not survive. This was also observed when cells were allowed to recover for 2 ,4 or 8 hours after $CaPO_4$ removal. The cells exposed to 10μM H_2O_2 did not show any significant increase in their transfection efficiency.

These results are the opposite of what was expected. The main reason behind the lack of enhanced transfection efficiency is most likely a combination of limited

destabilisation of the lysosomes due to the relatively low H_2O_2 concentrations used (10 μM compared to the reported 350 μM) and to the enhanced toxicity levels of H_2O_2 exposure following transfection. It has been shown that there is synergy between the toxicity of hydrogen peroxide and other membrane active compounds. Calcium phosphate precipitates have been show to induce cell death and to cause extensive damage to the cell membrane if left in the media for over 6 hours. Another reason for the increase in toxicity could be the fact that calcium phosphate induced damage to the cell membrane had increased its permeability to hydrogen peroxide leaving the intracellular domain exposed to the peroxide which can induce single strand breaks in the DNA contributing to cell death.

With the failure of the hydrogen peroxide to elicit the desired enhancement in transfection efficiency the attention was shifted towards another method to destabilise the lysosomes namely the use of basic amino acids to attempt and raise the lysosomal pH. The amino acids chosen for this attempt were the ones with the most basic side chains; namely: arginine, lysine and histidine. The initial screening for effect on both $CaPO_4$ and Lipofectamine showed a slight increase in efficiency in response to arginine exposure at an initial concentration of 500 μM. Both histidine and lysine gave less significant enhancements. The effect of arginine and lysine on LipofectAMINE® transfection was similar while histidine showed a U shaped response curve. The most likely explanation for this is a possible effect of the amino acid on Lipofectamine - DNA complex structure. With the results from the original screening with $CaPO_4$ and lipofectamine being similar, all subsequent work was done on $CaPO_4$ and Arginine 500 μM was investigated further, being the exposure with the highest efficiency. The first point tested was the

reproducibility of the results obtained with Arg 500 μM. For this purpose a reproducibility test was done over 3 days that showed very limited variation. The next question to answer was how does arginine mediate this effect.

The first question asked was whether or not the effect of arginine is due to its action on the formed $CaPO_4$- DNA complex or the cells, and it was determined that arginine does not affect the formation of the complex. It was also determined that the change in the pH was too low to allow for such change in transfection efficiency. The time of addition of Arginine proved to be essential for maximum efficiency (only works when amino acid is added at the same time) as $CaPO_4$.

In order to investigate which of arginine's physico-chemical properties plays the most important role in the observed enhancement, the effect of different amino acids was examined. Phenylalanine and tryptophan were used to study the effect of mass while phenylalanine and tyrosine were compared for any effect of the extra positive change. Aspartic acid, glutamic acid and glutamine were used to determine the effect of chain length, net charge in a side chain as well as the presence of the NH_2 group at the end of the side chain. All the previous studies showed that none of the other amino acids had any positive influence on transfection efficiency. The only characteristic left that distinguished Arginine from other amino acids is the presence of a highly basic side chain.

Higher concentrations of amino acids resulted in a reduced transfection efficiency. The levels of inhibitions are not proportional to the levels of enhancements (e.g. Arg 4000 resulted in a 20% efficiency while Histidine 4000 resulted in 40% efficiency even though Arg 500 gave a much better efficiency than Histidine 500).

The above mentioned results prompted the development of a model for the way arginine and the other amino acids mediate their effect on transfection efficiency. In this model, amino acids bind to receptors on the cell surface (high affinity receptors from the amino acids transport systems and lower affinity ones as the concentrations are increased). As the amino acids are added at the same time as the calcium phosphate-DNA complex, the internalisation of the latter by endocytosis would be accompanied by a number of amino acid molecules. Upon fusion of the endocytosis vesicle with a lysosome, the basic amino acid results in pH increase that would reduce and/or stop the activity of lysosomal enzymes giving the DNA a better chance of escaping the lysosome intact. As the number of amino acids attached to the cell surface increases with the increase in the added concentration, the interaction between calcium phosphate-DNA complex and the cell membrane becomes less probable to occur as more and more of the cell membrane becomes covered with amino acids.

Based on this model a number of predictions can be made. The first prediction is that the amino acids have to be added at the same time as the DNA for the amino acids to have any effect. This has already been shown to be the case. Another prediction relates to the fact that the response curve should be bell shaped as the number of amino acids on the cell surface reaches an optimal concentration giving the DNA maximum chance to

escape the lysosomes. The efficiency would start to decrease after this as the number of amino acids on the cell surface starts to hinder DNA-membrane interactions. This was also determined to be the case with the 3 basic amino acids tested. The only amino acids that showed an increase in transfection efficiency carry a positive charge on them, which would correlate with another prediction of this model. While this model was able to explain all the data that was collected, it was decided to conduct two experiments to test some of the predictions of this model.

The first experiment involved the study of the effect on calcium phosphate efficiency of a combination of two amino acids. The amino acids chosen were arginine and histidine. The prediction based on the different possible mechanisms of action are tabulated in table 34. The first row indicates the expected values if the amino acid contributed equally at all concentrations and was calculated by obtaining the average of the efficiencies of the amino acids at each concentration (e.g. for the combination arginine 350 μM and histidine 300 μM, the average efficiency was (143.82 + 91.66)/2=117.33). As the two amino acids tested had different efficiencies at different concentrations, a more accurate prediction would need to take into effect the proportionality of the amino acids mixtures. The calculations for the expected results based on this method were done by multiplying the proportion of the amino acid in the mixture by its efficiency at that concentration and adding the two numbers up (e.g. for arginine 350 μM and histidine 300μM, the efficiency was calculated as: 143.82 X (350/600) = 83.95 + (91.66 X (300/600)) = 83.9 + 45.83 = 129.73). The theoretically expected results are presented at the same time as the expected results based on the model outlined before. The observed results are consistent with the model's prediction.

model outlined before. The observed results are consistent with the model's prediction. According to the model it would be expected that each amino acid would have an effect proportional to its fraction in the final mixture for low concentrations and for these numbers to start falling bellow the proportions as the inhibitory effect of elevated amino acid concentrations starts to have a greater effect then the enhancement.

Table 34: Effect of a combination of arginine and histidine on $CaPO_4$ transfection efficiency. The top row for each concentration indicates the result expected by calculating the average of the two efficiencies obtained when used alone. The second row indicates the efficiencies expected if the amino acids acted in an way proportional to their relative concentrations. The last row indicates the expected efficiencies based on the proposed model and the experimental efficiencies.

			Arginine		
			350	500	650
Histidine	300	Average	117.74	131.78	128.23
		Proportional	129.78	140.4	148.73
		Model/Observed	129/131.11	140/142.22	< 130/111.11
	450	Average	105.24	119.28	113.11
		Proportional	101.28	124.71	119.3
		Model/Observed	100/104.44	125/128.88	< 110/100
	600	Average	104.41	118.45	112.27
		Proportional	93	110.03	112.27
		Model/Observed	93/91.11	110/111.11	< 105/91.11

According to the model depicted addition of acidic amino acids in combination with arginine should result in higher inhibition levels then the addition of a basic amino acid with the same concentration. This prediction was tested by the addition of three different amino acids in concentrations that did not have any effect on calcium phosphate efficiency by themselves (tyrosine 50 µM, glutamic acid 200 µM and aspartic

μM resulted in a transfection efficiency 80% of control. This would be the same as adding 750 μM of arginine, while the addition of 300 μM of histidine resulted in a transfection similar to 950 μM arginine or the addition of 450 μM of arginine. This in accord with the model's prediction. The model thus presented not only supports all the results obtained with the preliminary experiments but also is validated by a number of experiments designed to test its validity.

Finally to test whether this enhancement is specific to the HeLa cell line or is a general phenomenon, 293 and MDBK cells were transfected with the same plasmid and exposed to arginine 500 μM. The cell lines were chosen because 293 cells are known to show higher transfection efficiencies then HeLa cells while MDBK are known to have very low transfection efficiency. The result indicate that the number of transfected 293 cells was increased by the exposure to arginine 500 μM, with the enhancement being less than that found with HeLa although this could not be quantified easily due to the large number of 293 cells that were transfected as a result of 293 cells' higher transfection levels. The transfection efficiency of MDBK cells could not be assessed as no transfected cells were observed. This is in accord with previous experiments done in this lab that showed MDBK cells to have a very low transfection efficiency. As the generality of the effect of arginine on transfection efficiency could not be determined with 293 and MDBK cells, two other cell lines were tested, namely CCL-44 and CRL-6072 (both of bovine origin). The enhanced transfection efficiency observed with both cell lines indicates that the effect is not limited to just HeLa cells. The above mentioned model is depicted in figures 33 to 37.

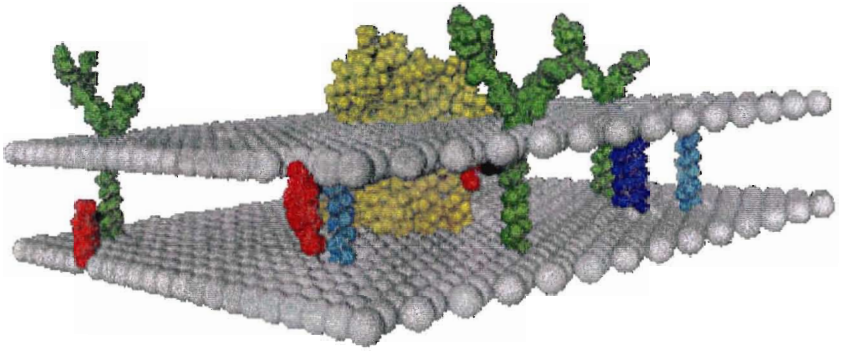

Figure 33: A three dimensional model of a plasma membrane. The phospholipids tails have been removed for clarity.

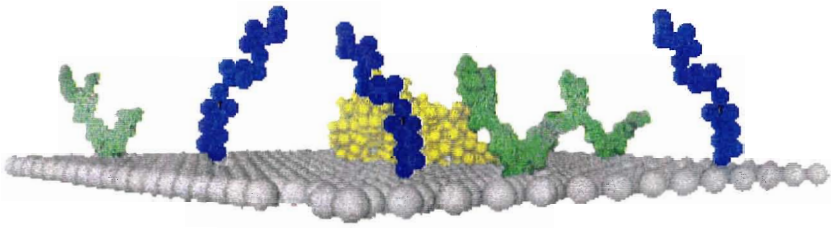

Figure 34: A simplified version of figure 30 showing the top half of the bilayer. The blue receptors added represent amino acid receptors.

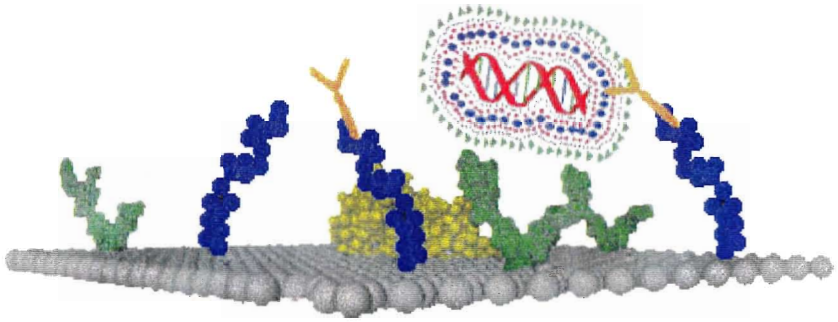

Figure 35: DNA-$CaPO_4$ complex interacting with the DNA in the presence of low concentrations of amino acids added.

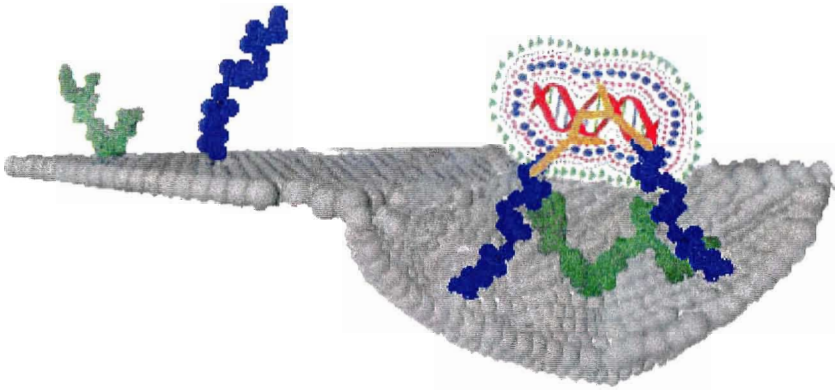

Figure 36: Internalisation of the DNA by the cell. Co-internalisation of amino acids is shown as well.

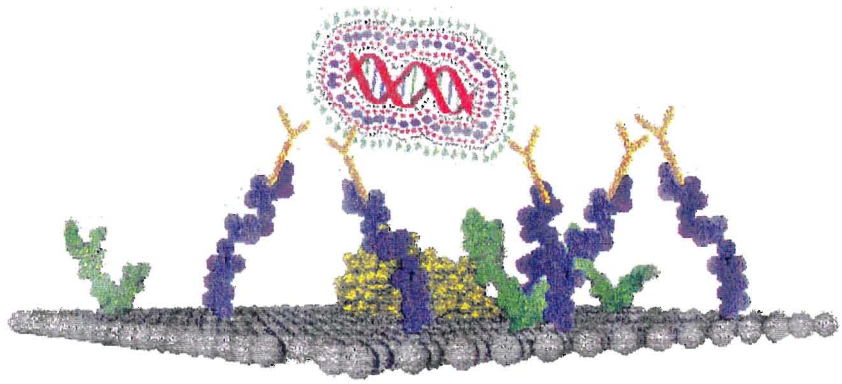

Figure 37: A schematic representation of an excess of amino acids interfering with the interaction between the plasma membrane and the subsequent internalisation of the $CaPO_4$-DNA complex. Not all amino acid receptors were shown in the previous figures for clarity.

Although the increase in lysosomal pH was never directly measured, previous reports in the literature have shown that basic compounds such as NH_4Cl, carbonyl cyanide *p*-trifluoromethoxy phenyl hydrazone (FCCP), 3-methyl adenine as well as monensin (6 microM), FCCP (10 microM), chloroquine (140 microM), ammonia (5 mM), methylamine (10 mM) are capable of inducing an increase in the lysosomes pH. These studies have also shown that the low pH in the lysosomes (4.5 - 5) is maintained by a number of proton pumps as opposed to a buffering system (Maxfield, 1982; Ohkuma and Poole, 1978; 1981a; 1981b). The addition of NH_4CL, FCCP and 3-MA resulted in a 10 fold improvement of transfection efficiency using calcium phosphate precipitation in rat 2 tk^- cells (Ege *et al.*, 1984). These previous findings support the idea that small amounts of a weak base can alter the pH considerably (over pH 6.7 from pH 5).

The model illustrated so far explains the enhancements that we have seen with transfection efficiency as well as to why arginine would have the best results. For this model to be acceptable it would have to provide an explanation for the different inhibition levels that were seen with the different amino acids. According to the model the inhibitory effect of an amino acid is the result of its binding to receptors on the cell surface that will prevent the DNA complex from interacting with the cell membrane and therefore preventing its internalisation. Based on this amino acids inhibitory effect would be related to the affinity at which they bind the cell membrane. As outlined previously (table 1), there are a number of amino acid receptors that are more or less specific to certain amino acids. Also, for each amino acid there are a number of different receptors with different affinities. As the concentration of the added amino acid increases, receptors of lower affinity will bind the amino acid while receptors with higher affinity would be at or near saturation. As the number of amino acid receptors that are occupied increases DNA interaction with the cell membrane becomes increasingly difficult. The fact that the highest inhibition levels were obtained with the three basic amino tested (arginine, histidine and lysine) would support this explanation as receptors for these amino acids are numerous and more widespread then the receptors or any of the other amino acids tested (including the y^+, L, and b^{0+} systems). With the development of a model that explains the results obtained (both enhancement and inhibition) and the maximum enhancements with arginine already identified, no further work into this line of investigation was conducted.

Another of the factors tested for their effect on transfection efficiency was electro-magnetic field exposure. Electro-magnetic fields have been studied extensively in the past few years due to the suspicion that they are involved in cancer formation. On a cellular basis EMFs have been shown to have many effects on cell physiology and structure. As the major effect of EMFs has been shown to be associated with the cell membrane it was decided to see if EMF exposure can drive the DNA across the plasma membrane which would result in high efficiency transfection due to bypass of endocytosis and low toxicity. However this was shown not to be the case as no transfection was observed. The effect of EMFs on calcium phosphate and lipofectamine were examined.

EMFs effects are known not to have linear response curves so the screening for any effects had to look for windows of action. To do this it was decided to use a "wide scan" that included a high frequency setting (99.5 Hz), a medium frequency (50 Hz) as well as a low frequency (10 Hz). For each frequency 3 intensities were tested that covered a high, medium and low setting as well (5.0 A, 3.0 A and 1.0 A respectively). This scan was used to try and spot any possible points that would show any positive results and further studies into those points were carried out. As it was not known at which point of the transfection procedure EMFs might act, it was necessary to examine their effect at different time periods to test their effect at different points. The initial wide scan was carried out with exposure times at 2, 4, 6 and 8 hours after the addition of calcium phosphate precipitate. The results indicated that the best results were obtained with exposure at 6 hours. It was decided to start a systematic investigation of 5 settings that showed good results when exposure was carried out 2 hours after transfection

started. These settings were all three points of high frequency, as well as two of the lower ones. The first parameter tested was the effect of the length of exposure period on EMF effect. The time period was increased or decreased by a factor of 2. To better test the effect of intensity on EMFs effect, the best 5 settings were used again and the intensity variation was varied by 0.5 A in each direction. It was hoped that analysing the results of the detailed tests would help in elucidating the exact way EMFs are having their effect on transfection efficiency. A close examination of the data however revealed a large variation in the results for some of the settings that were repeated during the course of the different tests. These variations prompted an investigation into the reproducibility of the EMF effect.

The reproducibility test showed that the results obtained on the same day can vary by as much as 200% and those obtained on different days can vary by over 500%. The three positive controls used on the three days of the test showed no significant variability among them, leading to the conclusion that the EMF results obtained showed a variability that is higher than any enhancement obtained with any setting. The reasons behind this variability are not easily determined. Similar problems with reproducibility of EMF results have already been pointed out in the course of this work. While it appears that no one reason can be put forward to explain this variability a number of different reasons can be acting in concert. Some of these reasons that have been suggested before include variations in the earth's magnetic field (this is especially relevant with experiments with extremely low-EMFs experiments), variations in the humidity and temperature levels as well as ionic content of the air in the room. Other reasons that have been put forward include the distribution of cells in the different cell cycle phases as well as the number of

divisions of the cells in question before the exposure was carried out (at least in the case of primary cells). In addition to these reasons that have been mentioned so far, there could be some other factors that have not been identified yet that play a major role in mediating the effect of EMFs but that have not been controlled for. The results obtained with the reproducibility test discouraged any further studies into the effect of EMFs on calcium phosphate efficiency. When the effect of EMFs on lipofectamine mediated transfection was examined it did not show any enhancement over the variability results obtained with calcium phosphate. As a result no further studies into this were carried out.

The final parameter tested for its effect on DNA uptake was the influence of the CMV-promoter on DNA uptake. Previous experiments in this lab have indicated that transfection with a plasmid containing the β-galactosidase gene under the control of the CMV promoter resulted in better transfection efficiency compared to a similar plasmid with β-galactosidase gene under the control of the SV-40 promoter. These results can be explained by either a higher expression of the galactosidase gene under the influence of the CMV promoter resulting in a higher number of the transfected cells accumulating protein levels above the threshold of detection by the X-gal staining test, or the results can be due to an enhanced uptake of DNA by the cells when the plasmid contained the CMV-promoter's sequence. To test which of the two explanations is the most likely answer, HeLa cells were transfected with a linear piece of DNA. Half of the cells were transfected with a DNA region containing the CMV-promoter while the other half was transfected with a piece of DNA of the same length containing the gene for the GFP protein derived from the same plasmid as the CMV-promoter (pEGFP-N1). The

transfection mixture was removed off the cells after 5 minutes, 4, 8, or 12 hours after addition. A southern blot analysis of the total DNA isolated from the transfected cells indicates that the amount of DNA taken up by cells was similar in both cases indicating that the presence of the CMV promoter sequence does not effect the amount of DNA taken up by cells. This observation could be explained by the fact that the DNA itself is usually coated with calcium phosphate precipitates and the interaction of the complex with the cells is most likely to be mediated by the calcium phosphate and not the DNA itself.

In this work, a working model for transfection enhancement using arginine as a modifier of the calcium phosphate and lipofectAMINE® methods was developed. Although the maximum enhancements with calcium phosphate appear to have been determined (171% for calcium phosphate and 201% for lipofectAMINE®) this leaves the door open for a number of questions. It would be of interest to try and determine if other molecules with higher basidity can give better results then those seen with arginine. For any molecule to act in the same way arginine did, it would have to have a structure that allows arginine receptor on the cell surface to recognise it. The molecule should also have a low cytotoxic effect. The most obvious attempt would be to try and replace the COOH group on the C_α with another more basic group. Care has to be taken however to make sure that the addition does not abolish the ability of the molecule to bind to the arginine receptor.

Summary.

The investigation of the effect of EMF on transfection efficiency revealed that even though a number of changes are brought about in the plasma membrane following such exposure, EMF can not be used as a transfection method by itself. The effect of EMF exposure on the transfection efficiencies of both calcium phosphate and lipofectAMINE® showed some positive results especially in the case of calcium phosphate where enhancements of over 300% were observed. These positive results however were still lower then the variability levels observed in samples treated the same way over a period of 3 days. This large variability prevented the drawing of any conclusions and all further studies in this line of investigation were abandoned.

Another line of investigation dealt with the effect of the presence of the CMV promoter sequence in the transfected DNA and the possibility that it might enhance DNA uptake and/or delay its degradation. Time lapse detection of the fate of DNA following transfection showed that the level of DNA uptake and its degradation levels were very similar for a sequence of DNA containing the CMV promoter and another of the same size containing the GFP protein. This leads to the conclusion that the CMV sequence does not have to have an effect on DNA uptake.

The next factor to be investigated was the effect of exposure to sublethal concentrations of hydrogen peroxide. The combination of transfection followed by hydrogen peroxide exposure proved to be very toxic to cells. The concentrations that

allowed cell survival following transfection were not able to induce any significant increase in DNA uptake.

The final parameter that was evaluated was the effect of basic amino acids on transfection efficiency. The addition of arginine has proved to provide a significant increase in DNA uptake efficiency. This enhancement reached about 170% with calcium phosphate and about 200% with lipofectAMINE®. The other basic amino acids tested (lysine and histidine) showed lower enhancement levels if any. All other amino tested showed no detectable enhancements. A model based on the ability of arginine to bind to the surface of the cell membrane and subsequently increase the pH of the lysosomes in which the DNA was being trapped thus reducing the activity of lysosomal enzymes and allowing DNA to escape intact into the nucleus was developed to explain arginine's effect.

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