

ISOLATION OF AND INTERACTION OF NUTRIENTS WITH
THE LINOLEOYL-CoA DESATURASE COMPLEX

THESIS

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ABSTRACT

The terminal enzyme in the linoleoyl-CoA desaturase enzyme complex, delta-6-desaturase was implied in the control of cell proliferation in cancer cells. One of the aims of this study was to isolate the terminal enzyme. It was decided that in order to isolate this enzyme it was first necessary to isolate the entire complex and then to enzymatically solubilise the first two components of the complex i.e cytochrome b₅ reductase and cytochrome b₅ from the complex resulting in a pure delta-6-desaturase. The first two components were isolated and purified using simplified and easily reproducible methodologies which could be utilised in the final purification of delta-6-desaturase. The entire enzyme complex, linoleoyl-CoA desaturase was also isolated in a pure form and this pure complex was used to attempt to isolate delta-6-desaturase. The terminal enzyme was isolated with some cytochrome b₅ still bound to it. The methods used had proven to be successful and with some modifications should yield a pure enzyme.

Zinc and GLA were known to play a role in the inhibition of cancer cell proliferation and zinc was hypothesised to inhibit cell growth by stimulating the activity of the linoleoyl-CoA desaturase enzyme complex which is involved in the regulation of cell proliferation. GLA is the product of the reaction that this enzyme complex catalyses and GLA has been shown to inhibit cancer cell growth. The effect of

GLA on cell growth and linoleoyl-CoA desaturase activity was thus investigated. Results showed that both zinc and GLA inhibited cell growth and that the combined addition of zinc and GLA generally resulted in the inhibition of cell growth and the activation of linoleoyl-CoA desaturase activity in the BL-6 cells while having a less pronounced effect on the LLCMK cells. The results of this study support the hypothesis that zinc may be a cofactor of linoleoyl-CoA desaturase.

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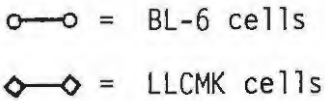
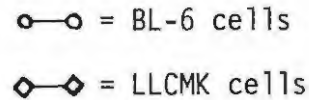
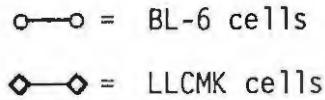
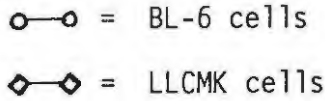
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CHAPTER 1

LITERATURE REVIEW

1.1 The Aetiology of Cancer

Cancer is defined by the Concise Medical Dictionary as "arising from the abnormal and uncontrolled division of cells that then invade and destroy the surrounding tissues". A tumour is "any abnormal swelling in or on a part of the body" (1). A tumour may be either malignant or benign. A malignant tumour is one "which invades and destroys the tissue in which it originates and can spread to other sites in the body i.e metastasises". Benign tumours are defined as "those that do not invade the tissues in which they originate or spread" (1). From these definitions it can be seen that cancer refers only to malignant tumours and not to benign.

Malignant tumours metastasise from the primary tumour via the bloodstream, the lymphatic canals or across body cavities such as the pleural and peritoneal spaces to initiate secondary tumours. There are three main types of malignant tumours namely; carcinomas, sarcomas and a heterogeneous group. The carcinomas originate in the epithelium of the skin, and internal organs of the body, for example, the respiratory tract, gut, breast, pancreas and thyroid. It is possible, but not normally concurrently, for an organ to manifest more than one type of carcinoma, for example, in the cervix an adenocarcinoma and a squamous carcinoma may occur. The sarcomas originate in connective tissue. They can arise in fibrous tissue,

muscle, fat, bone, cartilage, synovium, blood, lymphatic vessels and other tissues. These tumours arise in the tissues that constitute an organ rather than being restricted to a particular organ. The third type of malignant tumours consist of a heterogeneous group of tumours which originate in the cells that produce the leucocytes and the lymphocytes i e, the white blood cells and cells involved in the immune system. This third group includes the leukemias, the lymphomas and Hodgkin's disease. The leukemias are classified according to the type of white blood cell from which they originate i e, that cell type which is proliferating abnormally. The lymphomas are classified as "any malignant tumour of the lymph nodes excluding Hodgkin's disease". Hodgkin's disease is a disease of the lymphatic tissues which involves the lymph nodes (1,2).

Cancer is the result of a combination of factors exerted on the body by procarcinogens, carcinogens, initiators, deoxyribonucleic acid (DNA) - repair drugs, promoters, cocarcinogens and anticarcinogens (see Fig.1). A procarcinogen is a substance that requires a chemical modification in order to act as an inducer of cancer. A carcinogen is a chemical, physical or biological agent with an initiator effect that increases the incidence of cancer. An initiator is an external stimulus or agent producing a cell that becomes malignant under certain conditions while a promotor is an agent that causes an initiated cell to produce a tumour. A cocarcinogen is an agent that augments tumour induction and an anticarcinogen is a substance

that inhibits or eliminates the activity of a carcinogen (3-8).

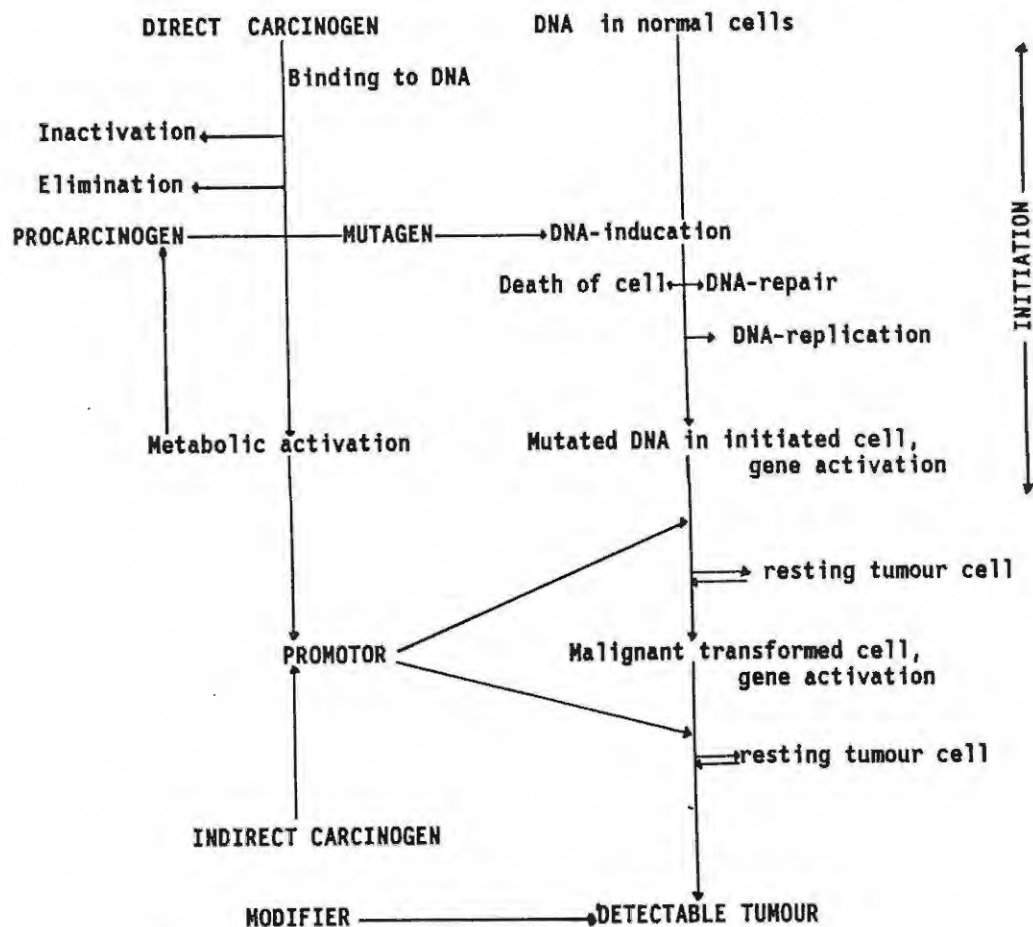


Figure 1. The chain of reactions including interaction between external influences and the response of the organism which can result in cancer (4).

The aetiology of cancers is known to be multifactorial with the result that it is a complex problem to decide the significance of certain agents and their roles in the initiation and promotion of cancers. The initiation of malignancy of a cell results from a genetic mutation. The process of initiation is considered to be irreversible. These genetic mutations can be in the form of point mutations, frame shift mutations or transposed DNA. An initiated cell is considered to be a latent tumour cell. This cell will only

become malignant if it is consequently acted upon by a promotor. Promotion of the cancer process can only occur on an initiated cell. The process of promotion is considered to be a reversible process and cannot by itself produce a tumour. The malignant transformation of a cell can be halted by DNA-repair mechanisms and anticarcinogens.

There are a variety of initiators and promoters and these include various forms of radiation, such as ultra-violet, X-rays and radioactivity; chemicals such as benzpyrene, aromatic dyes and aflatoxins; viral oncogenes such as those carried in the retroviruses and for example, the src oncogene; mammalian oncogenes; and viruses (1-9).

It is believed that factors from the environment, including diet, are responsible for 80-90% of all forms of cancer found in man. From this statement it can be seen that diet plays a very significant role in the study of cancer and will be discussed in more detail in subsequent sections in this chapter (3-11).

1.2 Differences between Tumours and Normal Tissues

In the treatment of cancer, it is preferable to find a characteristic unique to the cancer cells and to utilise this characteristic in the fight against the disease. This would allow drugs, etc to be targeted to the cancer cells only, and thus would cause no harm to the normal host cells. Some of the possible characteristics are cell

division (2,3,12-16), membranes (15,17-20), metabolic changes in, for example, carbohydrates, proteins and lipids (12,13), tumour vascularisation (14,21) and metastasis (12-14). Cell division and lipid metabolism, with respect to its role in membrane structure and function, are two characteristics of great interest in the treatment of cancer and of particular interest in this study.

1.2.1 **Membranes and Lipids**

The protein and lipid composition of membranes is greatly altered in cancer cells which drastically alters the membrane characteristics since phospholipids and proteins are important components of membranes. Some of the membrane alterations are unique to specific tumours. The membrane alterations generally allow greater permeability of nutrients into, and products out of, cancer cells (15,17-20).

In cancer cells the mitochondrial function is almost completely lost because of membrane changes. The shape of cancer cells is totally different to that of normal cells, as the rigidity of the cell membranes has been destroyed. By altering membrane structures, the activities of various enzymes are either modified or destroyed. This modification of enzyme activity is a specific result of the alteration of the viscosity of the membrane lipid component, the degree of lipid peroxidation, the composition of polyunsaturated fatty acids (PUFAs) within membranes, the relative amount of phospholipids within a membrane and the ratio of lipid weight to protein weight within a

membrane (15,17-20). The significance of an alteration of a specific enzyme activity, linoleoyl-Coenzyme A desaturase (linoleoyl-CoA desaturase) is of particular interest to this study and will be discussed in detail later.

1.2.2 Cell Division

While it is true that a large proportion of cancers have a greatly increased rate of cell division, it is not true for all cancers (3,15,16). In normal mature cells, cell division usually occurs to replace those cells that have died, but in tumours there is generally an increased cell growth (2,12,13). It is also true that the growth of cancer cells can be out of synchrony with normal cells, which have a synchronous growth (12).

If there is increased cell division, this is shown by increased DNA synthesis and an elevated activity of the enzymes involved in this synthesis. The enzyme DNA polymerase III has an activity which is five to six times greater in certain cancer cells than normal cells. It is not known whether the concentration of this enzyme is increased or if its activity is increased (2,3,12-16). Thymidine kinase is an enzyme involved in the salvage pathway of DNA synthesis. A pool of nucleosides resulting from DNA turnover is found in most cells. When this pool reaches a critical level, the excess nucleosides are normally catabolised and excreted, but when cells divide rapidly or greater DNA synthesis is required, these nucleosides can be phosphory-

lated to form nucleotides. The nucleotides can then be used to synthesise DNA. Thymidine kinase is one of the phosphorylating enzymes, and it appears to be a rate controlling enzyme in this pathway. The enzyme thymidine kinase has little activity in normal cells but a larger quantity of activity is present in most cancer cells. The increased activity of the salvage pathway results in an increased synthesis of DNA. This enzyme has, as a result, become a target for cancer therapy (12,13).

Another enzyme that is thought to play a role in the control of cell division is linoleoyl-CoA desaturase. This enzyme is the rate controlling enzyme in the essential fatty acid (EFA) synthetic pathway. Horrobin (5) has stated that this enzyme appears to be absent in cancer cells. It has since been shown that it is present in certain cancer cells at least, but even when it is present it has reduced activity compared to normal cells (22-24). Linoleoyl-CoA desaturase plays a role in the control of cell division since the eicosanoids (e g prostaglandins (PGs)) are synthesised from intermediates in this essential fatty acid pathway and certain prostaglandins regulate cyclic adenosine monophosphate (cAMP) which in turn has been shown to influence the rate of cell division in many cells (cited in 22,25-30). (See Fig. 2).

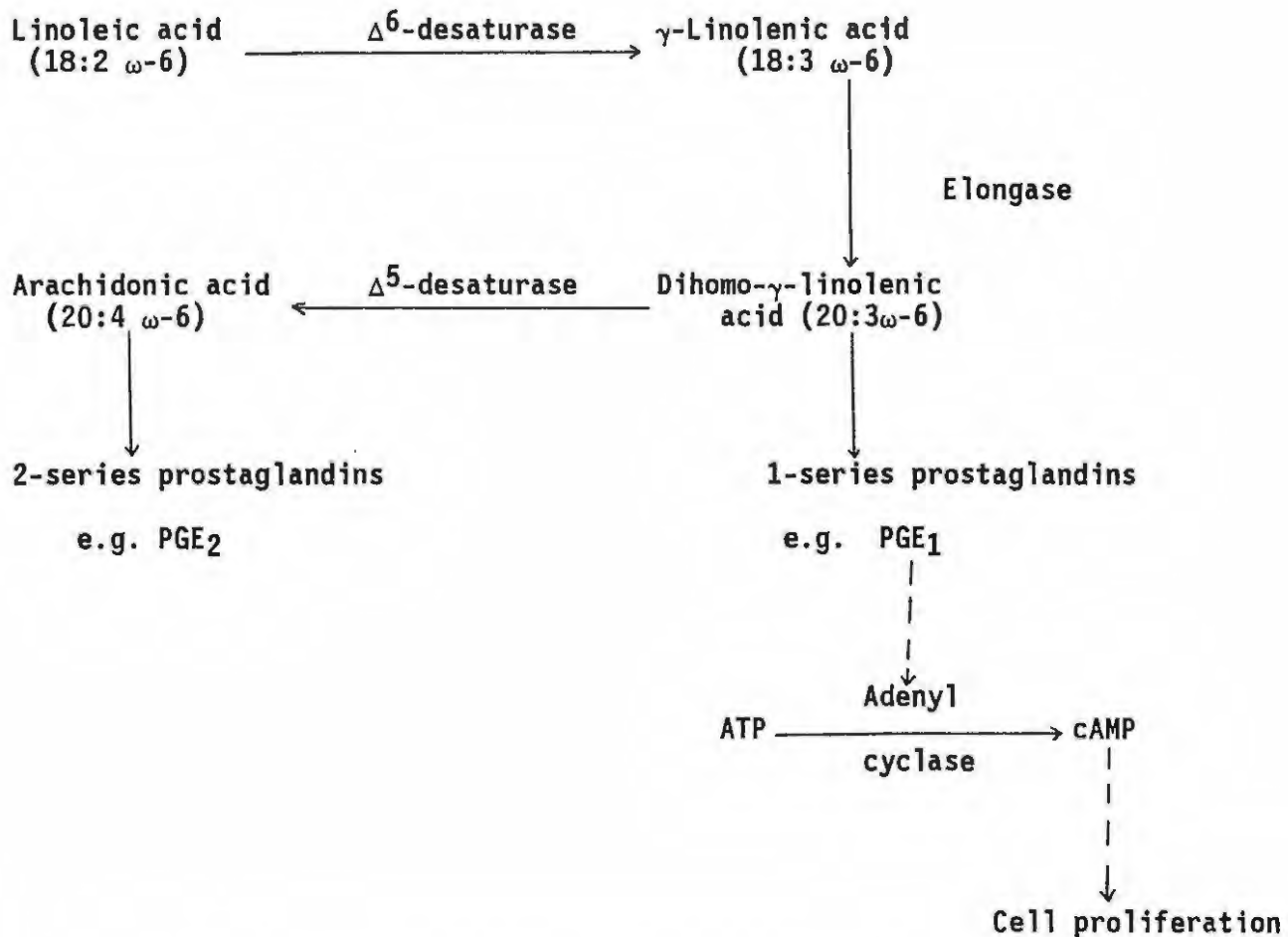


Figure 2. An outline of the conversion of ω -6-EFAS to prostaglandins indicating the link with cAMP synthesis and cell proliferation (25).

————→ = metabolic pathways
- - - -> = suggested effects

The role that linoleoyl-CoA desaturase plays in the control of cell proliferation and the methods by which this control is maintained will be discussed in much greater detail later in this review.

Since this study concentrates largely on the role of essential fatty acids in cancer therapy and the control of EFA synthesis, the general pathway of EFA and PG synthesis as well as the possible involvement of zinc in this synthesis, and the importance will now be considered.

1.3 The Metabolism of Dietary EFAs and Zinc

Two nutrients of interest in this study, with respect to their involvement with cancer, are EFAs and zinc. The general background and metabolism of these nutrients will be discussed and their relationship to tumour growth will then be considered.

1.3.1 The Omega-6-Essential Fatty Acids and the Eicosanoids

There are two groups of naturally occurring EFAs in the body, the ω -6- and the ω -3- EFAs. The ω -6- EFAs are derived from linoleic acid (18:2 ω -6) and the ω -3- EFAs are derived from α -linolenic acid (18:3 ω -3), and differ in the carbon number at which the first double bond occurs within the EFA numbering from the of the EFA chain. The eicosanoids encompass both the lipoyxygenase and cyclo-oxygenase products of the two EFA pathways. One of the lipoyxygenase products are called leukotrienes and the products from cyclo-oxygenase are called prostaglandins, thromboxanes and

prostacyclin (31-42). In this review only the ω -6- EFA pathway will be considered in detail and henceforth the term EFA will be used referring to the ω -6- EFAs. When ω -3- EFAs are discussed they will be specifically referred to.

1.3.1.1 The Biosynthesis of the Omega-6-Essential Fatty Acids and the Eicosanoids

Figure 3 shows the biosynthesis of the ω -6- EFAs, ω -3- EFAs and the eicosanoids (35). As can be seen from Fig. 3, the EFAs are derived from their parent fatty acids via desaturations and chain elongations. All the double bonds are in the cis form and are methylene interrupted (34,38).

As illustrated in Fig. 3, both of the EFA pathways utilise the same enzymes. The conversion of linoleic acid (LA) to gamma-linolenic acid (GLA) is catalysed by the enzyme complex linoleoyl-CoA desaturase [EC 1.14.99.25]. This enzyme complex was only classified in 1986 at the request of the author. It should be noted that there is an error in the classification, in that it is stated that the more common name for this complex is delta-6-desaturase (Δ^6 -desaturase). Δ^6 -desaturase is in fact only a component of the complex being the terminal enzyme in the linoleoyl-CoA desaturase complex, the complex consisting of cytochrome b₅, cytochrome b₅ reductase [EC 1.6.2.2] and Δ^6 -desaturase (43-45).

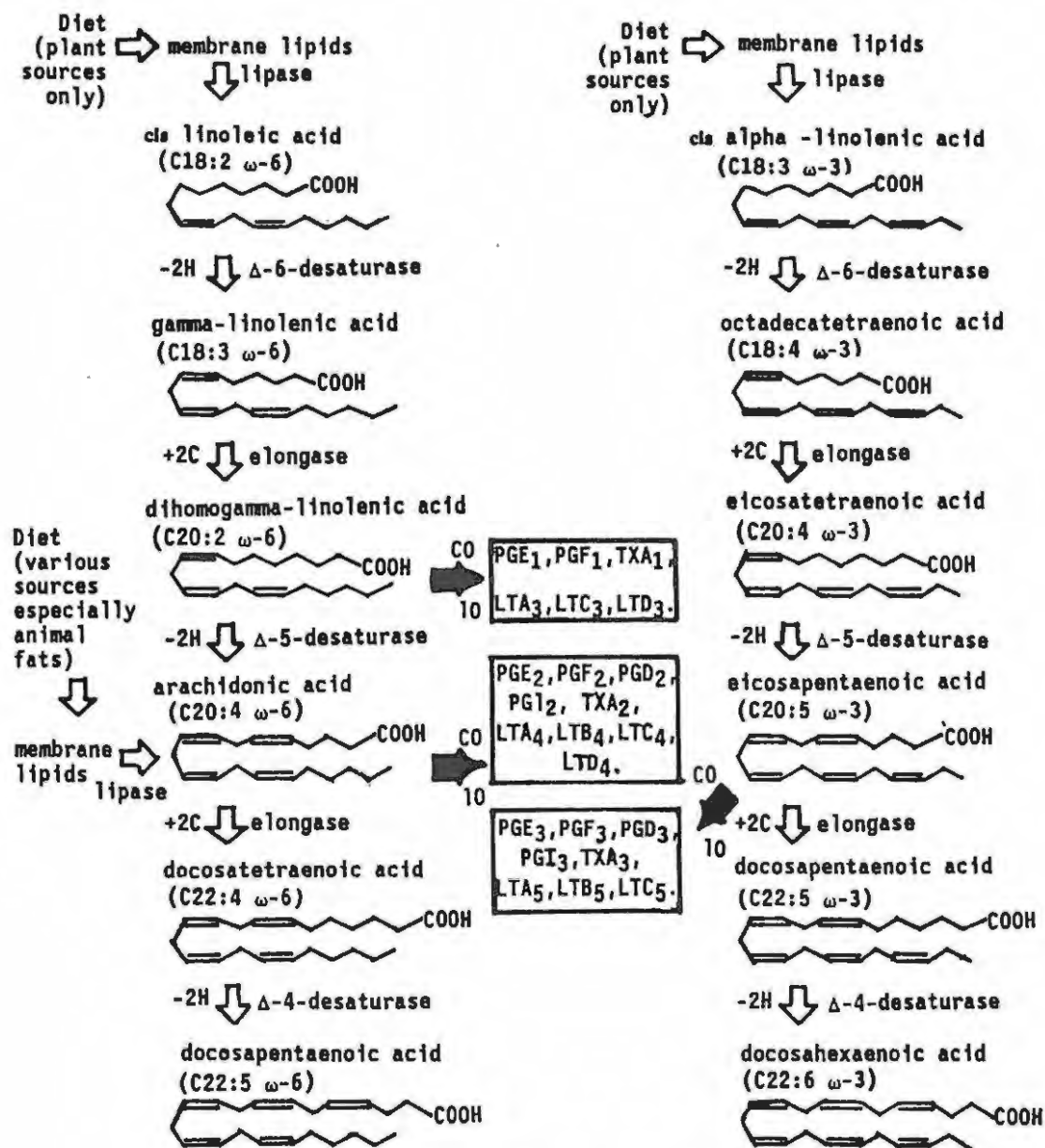


Figure 3. Essential Fatty Acid and Eicosanoid Metabolism (35).
The terminal enzymes in the desaturase complexes are cited in this figure and not the entire complexes.

PG = prostaglandin
TX = thromboxane
LT = leukotriene
CO = cyclo-oxygenase
IO = lipoxygenase

In the biosynthesis of the ω -6- EFAs and the eicosanoids the rate limiting step is the conversion of LA into GLA (43-45). The linoleoyl-CoA desaturase enzyme complex catalysing this step and the effects of zinc and GLA on the complex are the subjects of this thesis.

LA is unable to be synthesised in mammals and as such has to be present in the diet (34,38-40). For omnivores the main source of LA is plants; for carnivores the only source is the LA present in the meat eaten. LA is also present in dairy products, organ meats e g liver, human milk and many vegetable seed oils (33,39). The average adult daily intake is 23g in Europe and North America. GLA is present in human milk as 0.3-1.0% of its fat. It is also found in very small quantities in cows milk. This is generally more than an adequate source of the EFA for a baby. GLA is known to occur in oats and barley. GLA is found in large quantities in the seed oil of the evening primrose, but makes up only 0.2% of the total fat intake of an adult, which is an insufficient intake. Dihomo-gamma-linolenic acid (DGLA) is found in moderate amounts in human milk, liver, the adrenals, the testes and the kidneys. Arachidonic acid (AA) is present in moderate amounts in human milk, in small amounts in cows milk and substantial amounts in meat, egg yolks, some seaweeds and shrimps. The average daily intake of AA in several European cities is 100-190mg which is more than adequate to account for the total daily production of PGs, which is estimated to be approximately 1mg. However, this intake of AA is insufficient to meet all the requirements for AA (39).

Animal experiments show that males require 2-5 times as much EFAs as do females, but little research in this regard has been done on humans. Trans-fatty acids are known to interfere with EFA metabolism and result in a greatly increased requirement for EFAs. It is thought that the trans-fatty acids interfere directly with linoleoyl-CoA desaturase. In general, therefore, adult humans require a dietary supply of LA from which the other EFAs can be synthesised (33,34,36,38-40).

It has been shown that the rate controlling enzyme in this system, linoleoyl-CoA desaturase, has defective function in the following human diseases; diabetes, hypercholesterolemia, Crohn's disease, hepatic cirrhosis, cystic fibrosis, Sjogren-Larsson syndrome, atopic eczema, non-cancerous breast diseases, Sjogren's syndrome, scleroderma, and ulcerative colitis. It has also been shown to be defective in aging cells, in the premenstrual syndrome and in the irritable bowel syndrome (5,39,46-49).

1.3.1.2 The History of the Omega-6- EFAs and the Eicosanoids

There are various reasons why the importance of EFAs has only been elucidated in the last century. It has been known since 1877 that carbohydrates can be converted into fat in the body, so it was not obvious that fat deficiency could occur. In 1918 Aron deduced that fats must have a specific role in nutrition as he showed that rats would grow when fed a diet consisting of casein, wheat bran, wheat starch and butter, but if butter was removed from the diet the rats no longer grew. Fatty acids are required in very small quantities and

this made it difficult to determine which were necessary i e essential. EFAs are PUFAs which are essential for growth, but cannot be synthesised by the body. In the initial experiments carried out to determine whether fat was necessary in the diet, it was not realised that certain supposedly fat reduced diets contained EFAs, and this led to erroneous results (1,cited in 50).

Evans and Burr in 1928 further purified diets and utilised an experimental diet which contained pure casein, purified sucrose and salts, a small amount of yeast and three drops of cod liver oil daily. This diet produced subnormal growth and ovulation in young rats. This indicated that researchers were getting closer to a fat deficient or fat free diet. It was confirmed in 1929 that rats on a very low fat diet did not grow normally and half of the rats had kidney complications. In 1929 Burr and Burr further purified the diet they had used previously and showed that the rats did not grow normally on this diet and they also developed a scaly skin, caudal necrosis of the tail and lesions were found in the kidneys and urinary tract. Burr and Burr were therefore the first researchers to really achieve fat deficiency and to show that these fats were essential in the diet (cited in 50,51,52). In the following year they noticed that there was a large increase in water consumption by rats on their fat deficient diet, but there was not the expected increase in urine. The deficiency symptoms were found to be cured by LA. LA, thus became the first EFA to be discovered. They also tried to cure the

deficiency symptoms by using oils which did not contain LA, but these oils had no effect. They hypothesised that warm blooded animals could not synthesise any appreciable quantities of LA. The fats necessary for growth had by this time been shown to be PUFAs (cited in 31,51,52,cited in 53). In 1932 a breakthrough was made when Evans utilised sucrose in experimental diets instead of starch, which was shown to contain EFAs (1, cited in 50).

Burr and Burr initially suggested that alpha-linolenic acid (ALA), which they had also found to be an EFA, was as effective in curing the fat deficiency symptoms as LA. It was however found that ALA is only about one-sixth as effective as LA in curing the deficiency symptoms. Turpeinen stated that the most important fat deficiency symptoms were a retardation and a gradual complete cessation of growth, scaly skin, renal lesions (often resulting in blood in the urine), excessive water consumption and ovulation irregularities. Gestation, lactation and male reproductive functions were also shown to be greatly impaired (54).

In 1909 Hartley named the PUFA that he isolated as AA. This was a 20 carbon molecule with four double bonds (55). AA has been found to be widely distributed throughout the body. It was subsequently found that AA depressed the growth of rats on a fat deficient diet which was supplemented with LA and ALA (cited in 31, cited in 50). This work was repudiated in 1938 by Turpeinen who showed that AA was more effective than LA in curing deficiency symptoms (54). As has been

In the late 1940's it had been noted that liver enzyme activities could be dramatically altered by dietary conditions. It was then suggested that the deficiency symptoms resulting from a fat free diet noted by Burr and Burr could be a result of altered enzyme activities. If this was true, then the addition of EFAs could result in the resumption of normal enzyme activities. Further evidence for this hypothesis came from the results of an experiment which showed that rats suffering from fat deficiency had a much higher metabolic rate than rats on a fat sufficient diet. It was found that starvation resulted in a lowering of the activity of plasma phosphatase. This activity reverted to normal upon the addition of PUFAs, but saturated fatty acids, proteins and carbohydrates had no effect on the enzyme level (cited in 63). Kunkel and Williams in 1950 showed that the rate of transport on the electron transport chain and of oxidative phosphorylation were linked to PUFA deficiency. When a rat was suffering from PUFA deficiency there was greatly increased activity of cytochrome oxidase. This increase in activity could be reversed if the rat was supplemented with LA (64, cited in 65).

LA and ALA were suggested to possibly belong to different PUFA families and that LA and ALA were the parent PUFAs of these families. It was noted that LA and other PUFAs which cured skin lesions, as a result of fat deficiency, had their first double bond (counting from the methyl end) after the sixth carbon atom and that the other PUFAs had their first double bond after the third carbon atom (cited in 50, 62).

infants developed spasmodic bronchitis and eczema. Von Chwalibogowski in 1937 fed two new born babies on a diet consisting of skimmed milk, sugar and cereal. These babies were maintained on this diet for thirteen and fifteen months. Fruit and carrots were added later on to the diet, although this did not seem to make the diet any more palatable to the babies. They both became anaemic and had repeated infections (cited in 50). There are no follow up reports concerning the growth of any of these infants, but the ethics behind these studies still remain extremely dubious.

A more ethical experiment was conducted in 1938 when Brown fed himself a low fat diet for six months. Brown seemed to benefit from this diet, as he stated he was less tired and had fewer headaches. If young rats were fed the same diet that Brown had they developed deficiency symptoms. Six months, however, was not considered a sufficient period of time for the experiment to be carried out if any comparison is to be drawn between human and rat reactions to a low fat diet. Six months in a human life is the equivalent of one week in the life of a rat, and this could indicate that deficiency symptoms could take a much longer time to occur in humans. This experiment also showed that it is easier to induce deficiency symptoms in younger animals than in adults. The EFAs in Brown's serum at the end of the six months had dropped by 50% indicating that the low fat diet was starting to have an effect (cited in 50, cited in 53). This evidence strongly suggests that EFAs are also essential for man.

In 1963 Hansen et al (53,cited in 68) published a paper concerning the role of LA in infant nutrition. This study was conducted over a period of four years and involved more than four hundred infants. The infants were maintained on diets which were fat free, or had different fatty acid compositions. The diets were fed in the form of milk mixtures. For many years attempts had been made to produce a milk mixture that emulated human milk. It is known that human milk has approximately five times more LA than cows milk, which seemed to suggest that LA is essential for infants. The infants who received the fat free diets or the diets low in LA developed dry scaly skin which tended to thicken. The infants on the fat free diets or low LA diets had high total fatty acids in the serum. Of specific interest were the diene fatty acid levels in the serum, as they directly reflected the LA content in the diet. Analysis of these levels indicated that the two kinds of diets were deficient in LA. It was noted that the deficiency symptoms were reversed when LA was given in the milk mixture. One deficiency symptom which was found and that had not been noted in rats was the susceptibility to bacterial skin infections. This study proved that LA was an essential nutrient for infants. Again it does not seem as though any follow up study was carried out or published concerning the future growth and welfare of these infants.

From these studies it can be seen that between the 1920's and the 1960's EFAs were a popular research topic and that their essentiality and metabolism was elucidated during this period.

1.3.1.3 The Function of EFAs

1.3.1.3.1 The Role of EFAs in Membranes

Biological membranes consist of lipid bilayers which are composed primarily of phospholipids and cholesterol. The membrane bilayers tend to exist at the transition point between gel and liquid crystal forms with the result that solid-like and fluid domains exist in the membrane. These domains have different fatty acid compositions with EFAs being an integral part of phospholipids. The β -position of the phospholipids is preferentially esterified with EFAs with the result that they play a structural role in membranes. Between the domains of the bilayer there are interfacial areas which may contain enzyme binding sites or transport proteins. Hydrophobic forces are the main forces binding these proteins to the lipid bilayer. In the bilayer, the phospholipids and the proteins affect the physical properties of each other due to the lipid-protein interactions. If there are increased PUFAs in the membrane phospholipids, this results in increased membrane fluidity which results in increased activity of membrane bound enzymes. Membrane integrity thus plays a role in the activity of membrane bound enzymes (69-84).

Membrane phospholipids also influence transmembrane transport. Within a membrane there are various channels which allow the passage of molecules and transport proteins which will transport molecules across the membrane. Both these channels and transport proteins are influenced by EFAs in the membrane. Control is required to ensure that sufficient nutrients enter the cell and also to ensure that certain products and hormones are allowed to leave the cell in a controlled manner (69,70,73,74,76-80).

EFAs, as constituents of phospholipids, can affect membrane function in four ways; they affect membrane fluidity, they can interact with membrane proteins, they can alter the membrane thickness and they can alter the composition of the membrane phase. All of these functions are vitally important to the normal functioning and integrity of the cells (60,70,73,74,76-80).

The alteration of membrane function as a result of a deficiency of EFAs has resulted in various diseases, some of which are discussed later. A classic symptom of EFA deficiency is scaly skin. The scaly skin results in an increased permeability of the skin membranes to water. The problem is completely reversed and cured by either the topical application of LA, GLA or AA or by the increased intake of these EFAs (50,53,69,85).

Both wound healing and burn healing have been shown to be greatly enhanced if EFA is added to the diet. In both cases it has been

shown that there is an EFA deficiency in the skin and surrounding tissues if the wounds or the burns are particularly severe. It is now possible to apply a LA cream to severe burns with very encouraging results. These results are thought to be due partially to the role of EFAs in the control of membrane integrity (86).

1.3.1.3.2 The Role of EFAs in the Immune System

EFAs are suggested to influence the mitogenic response of the T-cells of the immune system and consequently affect the immune system. For this reason EFAs are thought to play a role in infection. EFAs are also considered to aid in the defence against foreign cells. An EFA deficient diet results in increased bacterial infections and when LA is supplemented in the diet it has been shown to help protect against this infection (86-89).

1.3.1.3.3 Prostaglandin Synthesis

LA is the precursor for the synthesis of the three different series of PGs which in turn have many varied roles. It has been found that PG synthesis can be modified by varying the EFA intake. PGs have a very limited biological half life and as such are usually only synthesised when required. PGs have many varied physiological functions, for example PGE₁ has cytoprotective, anti-aggregatory, vasodilatory and anti-inflammatory functions and PGE₂ has aggregatory and inflammatory functions. PGs are thought to be involved in various cancers as most

human cancers have elevated amounts of the 2 series PGs. The significance of this will be discussed later. It is also thought that PG synthesis plays a role in membranes as the PG precursors are present in the membranes, mainly in the form of phospholipids (90-96).

PGs are present in a high concentration in seminal fluid and research has shown that PGs play an important role in sexual functioning. An EFA deficiency results in reproductive problems in both males and females and it is thought that these problems are a result of reduced PG synthesis. PGs are also known to mobilise or influence the mobilisation of free fatty acids. In this way PGs also play a role in the control of EFA synthesis (97,98).

1.3.1.3.4 The Role of EFAs in Cell Proliferation

As Fig. 1 shows, EFAs are thought to influence cell proliferation via PG and cAMP synthesis. There is generally an inverse relationship between cellular cAMP levels and cell proliferation. It has consequently been suggested that if there was reduced PGE₁ synthesis, due to an EFA deficiency, the synthesis of cAMP would be greatly reduced and thus cell proliferation would increase. This is one of the ways in which EFAs are thought to play a role in cancer, i e by influencing the rate of cell proliferation. This may also be a reason why these compounds are also important in burn healing and wound

healing, i e by increasing cell proliferation. Cell division may also be affected by the membrane status and this is another way in which EFAs can influence cell proliferation (5,22-30).

1.3.1.4 EFAs and Various Diseases

There are various reports that relate multiple sclerosis to a dietary deficiency of EFA. The one problem with these reports is that EFA deficiencies seem to be slightly inconsistent in that EFA quantities in the blood and tissues differ from case to case. It is thought that an EFA deficiency results in faulty myelination and as multiple sclerosis involves the loss of the myelin sheath around nerve cells, it is possible that this may be the result of a deficiency of EFAs (86,99-101). It is suggested that the ω -3 EFAs also play a role in multiple sclerosis as the ALA content in the blood and tissues is very low (100). The initial reason that the lipid nutrition was focused upon in relation to this disease was that 60% of the solid matter of the brain and 70% of the myelin sheath consists of lipid, and as such, it plays a vital role in nerve function. It has been known for some time that EFA deficiency retards brain development and alters synaptic function. A shift in the proportion of saturated and unsaturated fatty acids has been recorded in the brains of multiple sclerosis patients. At this stage however more evidence is required concerning the interaction of EFAs and multiple sclerosis (86,99-101).

It has been found that babies that have died from sudden infant death syndrome (cot death) have a lower level of DGLA compared to babies of a similar age. At present, however, it is not known how DGLA or EFAs in general play a role in this syndrome although DGLA is the precursor for the 1 series PG's which may play a role in this disease (102).

EFA metabolism appears to be abnormal in diabetics. Experimental data suggests that diabetes may result in partial EFA deficiency, although it is not known exactly how this plays a role in diabetes or whether it is just an effect of diabetes (86).

LA concentrations have been found to be lower in cystic fibrosis patients while the total PUFAs do not seem to be abnormal. The patterns of fatty acids occurring in the serum are different, but it is not known if this is a direct result of EFA function (86,103).

Acquired Immunodeficiency Syndrome (AIDS) has recently been hypothesised to be influenced by a dietary deficiency of certain EFAs, e g GLA. It is hypothesised that these fatty acids play a role in preventing the viral spread of AIDS. There is also a high incidence of sarcomas and other cancers in AIDS patients and it is well documented that EFAs play a role in cancer prevention (104).

EFAs have been implicated in numerous diseases, but as has been shown, it is not totally clear exactly what the relationship or the significance of EFAs are in these diseases.

1.3.2 The Role of Zinc in Nutrition and Metabolism

Zinc is an essential trace element and is the second most abundant of the transition and group II elements present in the body i.e. in a human adult of 70 kg there is 3g of zinc. Of the trace elements only iron is found more abundantly in humans (105).

1.3.2.1 The History of Zinc

In 1869 Raulin showed that zinc was essential for the growth of Aspergillus niger (cited in 106,107). In the ensuing fifty years it was discovered that zinc played an essential role in all of the biological kingdoms i.e. monera, protists, fungi, plants and animals (105-108). It was only in 1961 that it was first hypothesised that zinc deficiency could occur in humans, and was subsequently confirmed in 1963. The first studies carried out showed that zinc deficiency resulted in dwarfism, severe anaemia, hypogonadism, hepatosplenomegaly, rough and dry skin and mental lethargy. It was later discovered that the anaemia was a result of iron deficiency. It has since been shown that iron deficiency is quite commonly found together with zinc deficiency. If zinc was supplemented in the diet the symptoms were alleviated and normal growth re-established (cited in 108). Zinc, therefore, plays an important role in the development, growth and differentiation of humans and other animals. It is known that certain dietary factors inhibit the absorption or uptake of zinc

e g phytic acid (found in fibre), a low protein diet, calcium and copper (108,109).

Zinc deficiency in humans can be exhibited as a mild, moderate or severe form. Mild zinc deficiency is characterised by a decreased serum testosterone level and oligospermia in males, decreased lean body mass, hyperammonemia, neurosensory changes, anergy, decreased serum thymulin activity and decreased interleukin activity. Growth retardation and male hypogonadism in puberty, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions and abnormal neurosensory changes are found in moderate zinc deficiency. Severe zinc deficiency is indicated by bullous pustular dermatitis, alopecia, diarrhoea, emotional disorder, weight loss, intercurrent infections due to cell-mediated immune dysfunctions, hypogonadism in males, neurosensory disorders, problems with ulcer healing, and death in certain cases (110-113).

Zinc deficiency has been shown to occur all over the world and throughout entire populations (110,112,114). The recommended daily allowance of zinc for humans is 3mg for infants up to six months, 10mg for children, 15mg for adults and 25mg for lactating females (109,113).

The biochemical role of zinc was first demonstrated in 1940 when Keilin and Mann identified the first zinc metalloenzyme, carbonic anhydrase. This showed that zinc played an important role in the

catalytic functions and structural functions of organisms (cited in 105). This area of research has greatly expanded since 1940 and zinc is now known to be involved with over 200 metalloenzymes or zinc dependant enzymes and cofactors in many different species. These enzymes encompass a broad area of biological reactions and there are enzymes associated with zinc in each of the six IUB categories of enzymes. This is in fact the only metal that is involved with enzymes in all of the six categories (105,109). (See Table 1 on page 30).

Due to the association of zinc with a large number of enzymes, it is involved in nearly all types of metabolic processes, including the synthesis and degradation of carbohydrates, proteins, lipids and nucleic acids, and it can influence many important biological functions. Some of these, particularly those of interest to this study, will be discussed below (113-115).

1.3.2.2 The Functions of Zinc

1.3.2.2.1 The Role of Zinc in Gene Expression

Recently there has been much interest shown in the role that zinc plays in gene expression. This role involves both a structural and enzymatic role. It has been hypothesised that zinc promotes the transition of the B-form of DNA (right-handed helix) to the Z-form of DNA (left-handed helix). Z-DNA is thought to have regulatory functions related to gene expression (cited in 109). The involvement of zinc in this process requires further elucidation.

TABLE 1: ZINC METALLOENZYMES (105).

Name	Source	Role ^a
Class I - Oxidoreductases		
Alcohol dehydrogenase	Yeast	A
Alcohol dehydrogenase	Vertebrates, plants	A, D
D-Lactate dehydrogenase	Barnacle, bacteria	?
D-Lactate cytochrome reductase	Yeast	?
Superoxide dismutase	Vertebrates, plants, fungi, bacteria	(A) ^b D
Class II - Transferases		
Transcarboxylase	<i>Propionibacterium shermanii</i>	?
Aspartate transcarbamylase	<i>Escherichia coli</i>	B
Phosphoglucomutase	Yeast	?
RNA polymerase	Yeast, protists, wheat germ, bacteria	A
Reverse transcriptase	Oncogenic viruses	A
Nuclear poly(A) polymerase	Rat liver, virus	A
Terminal dNT transferase	Calf thymus	A
Mercaptopyruvate sulfur transferase	<i>E. coli</i>	?
Class III - Hydrolases		
Alkaline phosphatase	Mammals, bacteria	A, (C) ^b D
5' Nucleotidase	Bacteria, lymphoblast plasma	?
Fructose-1,6-bisphosphatase	Mammals	C
Phosphodiesterase (exonuclease)	Snake venom	A
Phospholipase C	<i>Bacillus cereus</i>	A
Cyclic nucleotide phosphodiesterase	Yeast	?
Nuclease	Microbes	?
-Amylase	<i>Bacillus subtilis</i>	B
-D-Mannosidase	Mammals, plants	?
Aminopeptidase	Mammals, fungi, bacteria	A, C
Aminotripeptidase	Rabbit intestine	A
Dipeptidase	Mammals, bacteria	A
Angiotensin-converting enzyme	Mammals	A
Procarboxypeptidase A	Pancreas	A
Procarboxypeptidase B	Pancreas	A
Carboxypeptidase A	Vertebrates, crustacea	A
Carboxypeptidase B	Mammals, crustacea	A
Carboxypeptidase (other)	Mammals, crustacea, plants, bacteria	A
DD-Carboxypeptidase	<i>Staphylococcus albus</i>	A
Elastase	<i>Pseudomonas aeruginosa</i>	?
Neutral protease	Vertebrates, fungi, bacteria	A(B) ^b
Collagenase	Mammals, bacteria	A
Aminocyclase	Pig kidney, microbes	?
Dihydropyrimidine aminohydrolase	Bovine liver	?
Dihydrocrotase	<i>Clostridium oroticum</i>	?
-Lactamase II	<i>B. cereus</i>	A
Creatininase	<i>Pseudomonas putida</i>	?
AMP deaminase	Rabbit muscle	?
Inorganic pyrophosphatase	Yeast	?
Nucleotide pyrophosphatase	Yeast	A
Class IV - Lyases		
Fructose-1,6-bisphosphate aldolase	Yeast, bacteria	A
Inhamulose-1-phosphate aldolase	<i>E. coli</i>	A
Carbonic anhydrase	Animals, plants	A
-Aminolevulinic acid dehydratase	Mammalian liver, erythrocytes	A
Glyoxalase I	Mammals, yeast	A
Class V - Isomerases		
Phosphomannose isomerase	Yeast	?
Class VI - Ligases		
tRNA synthetase	<i>E. coli</i> , <i>B. stearothermophilus</i>	A
Pyruvate carboxylase	Yeast, bacteria	?

A denotes a catalytic, B a structural, C a regulatory and D an undefined role, while ? indicates that available information is insufficient to make an assignment.

Letters in parentheses refer to roles fulfilled by metals other than zinc.

1.3.2.2.2 The Role of Zinc in Cell Proliferation

Since zinc deficiency results in reduced growth in nearly all organisms, including man, it was thought that zinc may influence cell proliferation through an association with the enzymes involved in the proliferation process. It was found that zinc influenced certain DNA synthesis enzymes, for example, DNA polymerase and thymidine kinase. Zinc was suggested to be a vital component of DNA polymerase, although there is now contradictory evidence indicating that zinc does not play a role in the DNA polymerase of Escherichia coli. If DNA polymerase is a zinc metalloenzyme, this could explain how zinc deficiency results in reduced growth. Zinc may also influence DNA synthesis via thymidine kinase (the regulatory enzyme in the DNA salvage pathway, previously discussed), but again no definite evidence that thymidine kinase is a zinc metalloenzyme or a zinc dependant enzyme is available (105,106,108,109,111,112,116-118). It has now been shown that zinc is essential for certain stages of the DNA synthetic cycle, and zinc definitely can influence these stages (119).

Numerous workers have shown that there is reduced protein synthesis, as expected, with the reduced DNA synthesis resulting from zinc deficiency (112,120-123).

Zinc deficiency has been shown to have a teratogenic effect on the fetuses of hens, mice and rats. These abnormalities occur in every organ system in the fetuses. It is not known conclusively if zinc deficiency is teratogenic for humans, but there is much circumstantial evidence to support this hypothesis. Geographical areas that have the highest incidence of malformations of the nervous system have been shown to broadly include those areas in which it is known that children and adults suffer from zinc deficiency. Alcohol is known to enhance zinc deficiency and alcoholic human mothers often give birth to infants with malformations similar to those observed in studies on rats (105,108,111,112,114,116,123-127).

1.3.2.2.3 Zinc and Various Diseases

Zinc deficiency in rats also results in a troublesome pregnancy. There is excessive bleeding during parturition and the mothers seem to be unable to look after their young, as they do not clean them immediately after birth and they do not consume the afterbirth. Many mothers die at parturition and most of the young do not survive (105,108,111,112,114,116,123,124-127).

Alcohol induces zinc deficiency. Livers which are cirrhotic are zinc deficient and patients show lymphocytopenia and increased infections. These symptoms are alleviated by the addition of zinc. Infants born to alcoholic mothers show the same zinc deficiency symptoms as rat infants born to zinc deficient mothers. These infants show what is termed "foetal alcohol syndrome". Most of the symptoms of this

syndrome are alleviated by the supplementation of zinc (108,114).

As has been previously stated zinc deficiency results in lymphocytopaenia (a reduction in lymphocyte production) and an increased susceptibility to infections. This increased susceptibility to infection is expected as the immune system has a reduced function as a result of zinc deficiency. Zinc is generally required for cell-mediated immune functions and as such plays a vital role in the immune response (108,110,112,114,123,128).

Acrodermatitis enteropathica is a genetic disease which results in malabsorption of zinc, with zinc deficiency as a resultant symptom. When zinc is supplemented in the diet the disease is alleviated. Before it was discovered that this disease was due to a lack of zinc, it was usually terminal (cited in 110,112,129).

If zinc is given to patients suffering from sickle cell anaemia there is a resultant increase in weight, growth of pubic hair, increased serum testosterone level, increased plasma zinc and increased neutrophils and a general alleviation of symptoms of the disease (108,112).

Zinc plays a protective role in maintaining integral cellular and organelle membranes via protection against PUFA and lipid peroxidation and by influencing enzymes associated with the membranes (some of

which control the structure and function of the membrane) e g ATPases. Zinc also influences membrane transport systems. This may also be one of the ways in which zinc influences cell proliferation (108,109,123,124,130,131).

1.3.2.2.4 Zinc Toxicity

Little is known about zinc toxicity and the effect it may have on cells. It is known that if zinc oxide fumes are inhaled the result is a mild to severe pulmonary inflammatory response with fever, chills and leukocytosis. Symptoms of zinc toxicity in humans include dehydration, electrolytic imbalance, abdominal pain, nausea, vomiting, lethargy, dizziness and muscular incoordination but these are with very high intakes of zinc. Zinc is also known to be non-accumulative. In general it would appear, therefore, that with the level of zinc supplementation normally used (two to ten times the recommended daily allowance (RDA)) there is unlikely to be any toxic effects (105,108,114).

1.3.3 The Relationship between Zinc and EFAs

The deficiencies of zinc and EFAs show very similar symptoms particularly with regard to skin lesions, alopecia, reproductive failure and growth. This evidence suggested a possible interaction between zinc and EFAs which is supported by other findings such as: the characteristics of either zinc or EFA deficiency are exaggerated when the other factor is also deficient; when pregnant rats are tested

with drugs which block EFA conversion to PGs, the symptoms are similar to those of zinc deficiency in pregnant rats and treatment of zinc deficient rats and humans with EFAs alleviates some of the symptoms of zinc deficiency (cited in 132-134).

It was subsequently proposed that zinc deficiency may mimic many of the clinical features of EFA deficiency as it may be required in the linoleoyl-CoA desaturase step of EFA synthesis. It has been shown experimentally that zinc could be a cofactor of this enzyme (133,135). Zinc deficiency has more recently been shown to reduce linoleoyl-CoA desaturase activity in many tissues except mammary tissue (132,133,136-143).

Kramer has suggested that linoleoyl-CoA desaturase was not affected by zinc directly, but instead it was effected by a protein energy deficiency, which could result from a zinc deficiency. However, a definite effect of zinc on linoleoyl-CoA desaturase activity has been found by other researchers (136,144).

Zinc effects the incorporation of EFAs into membrane phospholipids and zinc deficiency produces a number of abnormalities in lipid metabolism. For example, the capacity of the testes to synthesise PGs from EFAs is altered by zinc deficiency. In the parenchyma of the testes there is increased synthesis of PGE₂ and 6-keto PGF_{1α} and in the tunica of the testes there is reduced synthesis of PGE₂ and

6-keto $\text{PGF}_{1\alpha}$ (138,141,145). Zinc is also required for the mobilisation of DGLA from phospholipids and DGLA is the precursor for the synthesis of the 1 series PGs. Zinc may in addition be important in the conversion of DGLA to AA (146-149) and zinc may also be required for the release of EFA-containing free phospholipids from the membrane bound stores by phospholipase A_2 (148). It can, therefore, be seen that there is much evidence to support the hypothesis of an interaction between zinc and EFAs.

1.4 The Relationship between Cancer and Diet

As has been indicated earlier diet has been implicated as an important factor in cancer. Most agents in the diet which play a role in cancer are considered to be promoters of tumour development rather than initiators (2,4). As the promotion step of cancer is reversible, this explains why the relationship between diet and cancer has become of such major interest in recent times.

In 1809 William Lambe published a thesis on diet and cancer in which he implied that foods originating from animals and water were the principle causes of cancer. Lambe's diet for the prevention of cancer was that of a strict vegetarian, and drinking only distilled water (cited in 9). In 1954 McCormick advanced the hypothesis that cancer was secondary to a degenerative change in the connective

tissue, and that this change could in turn be secondary to a deficiency of vitamin C (cited in 150). In 1959 McCormick published another paper in support of this hypothesis (150). Although this has been contradicted by recent cancer research, it does show that diet may be important in cancer. A substantial part of cancer research undertaken recently, has involved the role of diet in the initiation and promotion of cancer. The influences of dietary lipid, fibre, vitamin, mineral and cholesterol intake have been the subject of intense investigation (cited in 2, cited in 3, 4, 5, cited in 6, cited in 8, 10, 150-158). Factors in the diet are estimated to be significantly involved in 30-40% of cancers found in men and in approximately 60% of cancers found in women. Factors in the diet are, however, not only promoters of cancer since many have been found to play a curative role in cancer and it is these factors which have created a great amount of interest in recent years (2-8, 11).

Diet can influence the initiation and promotion of cancer in a number of ways. Carcinogens and procarcinogens may be ingested directly, and once ingested, the diet of the subject may affect the ways these agents are transported and/or metabolised. It is also possible that the diet may provide substrates for carcinogen formation, such as nitrates, which are used in the preservation of most tinned meats for example and which upon contact with stomach acids and subsequent metabolism are converted to nitrosamines which are known carcinogens.

A diet may in addition alter cellular metabolism in such a way that it can increase the receptivity of agents for carcinogenic action. The bulk of the diet, in the form of calories or energy, may enhance the promotion or initiation of cancer. Dietary deficiencies, either as macro- or micro- deficiencies, may also result in the initiation or promotion of cancer (3,4,6,8,10,11,150-158).

There are many dietary factors such as lipids, proteins, carbohydrates, fibre, vitamins, minerals, alcohol and mutagens which influence the development of cancer. Of interest to this study, as previously indicated, is the influence of lipids and minerals, and they will now be discussed in more detail.

1.4.1 The Relationship between Lipids and Cancer.

In 1941 Smedley-Maclean and co-workers (151,152) showed that total fat and PUFAs had an influence on tumour growth. If rats were fat- and PUFA-starved and Walker tumours were implanted into these animals, it was found that the tumours grew faster than implanted tumours in rats on a fat- and PUFA-sufficient diet (151,152). In 1942 Tannenbaum (153) tested the effect of a high-fat diet on the growth of various tumours. He showed that there was a definite increase in the incidence and decrease in the growth time of spontaneous breast tumours in humans on high fat diets. Induced epithelial tumours also showed an increased incidence of tumours when high-fat diets were fed,

but the increase was not as great as with spontaneous breast tumours. High-fat diets had no significant effect on the growth of primary lung tumours or induced sarcomas in mice (153). Thus diverse effects of high-fat diets were noted by Tannenbaum.

In 1945 Tannenbaum (154) showed that tumour growth was dependant on the composition of an experimental diet and the degree of caloric restriction. Dunning et al (155) also found that restricting the caloric intake either reduced the formation of mammary tumours or increased the latency period of the tumours. They showed that high-fat diets tended to result in reduced latency periods and greater tumour formation than low-fat diets. When the caloric intake was restricted the high-fat diet showed reduced tumour formation as was also demonstrated by Tannenbaum (154,155). Silverstone and Tannenbaum in 1950 (156) and Engel and Copeland in 1951 (157) concurred with the results of Dunning et al regarding high-fat diets and increased mammary carcinoma growth. These results clarified certain contradictory reports that had appeared up to this time.

Engel and Copeland (156) showed that the effectiveness of a carcinogen, 2-acetylaminofluorene, at a particular site in the body, could be greatly influenced by the type of diet i e high- or low-fat diets and also by caloric restriction. For example, a low-fat diet and caloric restriction resulted in a reduced number of ear duct

tumours and a high-fat diet resulted in an increased number of mammary tumours. It was also shown that fibroadenomas exhibited increased growth with a high-fat diet (158). It has, therefore, been appreciated for some time that a relationship exists between dietary fat and cancer.

More recently there have been numerous papers published concerning the relationship between fat and cancer. Mammary cancer has been shown to be one of the cancers that are directly correlated with dietary fat. Experiments have shown that as dietary fat is increased so the incidence of mammary tumours has concurrently increased (2,3,159, cited in 160, cited in 161,162,cited in 163-167,168,cited in 169,cited in 170,171,172). Initially this increase in tumour incidence was thought to be related to the increase of total fat intake, but recently it has been shown that the increase in the tumour incidence correlates better to the increase of PUFAs in the diet (3,cited in 159-161,162,cited in 163,cited in 164,165,cited in 166,cited in 167,168,cited in 169,cited in 170,171,172). The mammary tumours that have shown an increased incidence as a result of a high-fat diet include a wide range of tumours, viz spontaneous, carcinogen-induced, X-irradiation- induced and transplantable mammary tumours. Apart from an increased incidence of these tumours there was also a reduced latency period (cited in 159,cited in 161,cited in 169,cited in 171).

An increased incidence of prostate cancer has also been correlated with a high fat diet. Again, when the fat is in the form of PUFA, instead of saturated fat, there is a higher incidence of tumour formation (3,4,159,165,173). Cancer of the testes, the corpus uteri and the ovaries has also been shown to have an increased incidence with high-fat diets. In these cases the experimental evidence points to the total dietary fat present in the diet as being the important factor (3,4,165).

There is not an abundance of evidence to indicate that stomach cancer is influenced by dietary fat, but the evidence that is available does point to some correlation between a high-fat diet and increased incidence of stomach cancer (3,166). There is an increased incidence of pancreatic cancer as a result of a high level of dietary PUFAs (3,163,165,166,172). Cancer of the colon also has a highest incidence in the presence of dietary PUFAs (3,159,163,165,172-174).

On a high fat diet obesity acts as an extra promotor of certain cancers, such as cancers of the endometrium, gallbladder, cervix, colon, pancreas and breasts (3,4,10).

In man the normal form of a dietary PUFA is the cis form. This is the form of PUFAs that most enzymes are capable of dealing with. One factor that has recently come to the fore is that during this century there has been an increase in the consumption of partially hydrogenated vegetable fats in the form of margerines, vegetable oils

and vegetable shortenings. In partially hydrogenating vegetable fats, significant quantities of the cis PUFAs are chemically altered to become trans PUFAs. These trans PUFAs are unnatural and are believed to play a role in the promotion of various cancers. This is an area of research which at the present time is still controversial as Institutes such as the Institute of Shortening and Edible Oils, Inc are questioning the validity of these studies (159,169,175-182).

1.4.1.1 The Relationship Between EFAs and Cancer

In considering the role of specific PUFAs, viz EFAs in cancer, it should be noted that many of the studies on the relationship between EFAs and cancer have been cell culture studies and while it is not always possible to directly extrapolate in vitro findings to in vivo situations, cell culture studies do provide valuable insight into the functioning of compounds such as EFAs in tumour cells (183-185).

At present contradictory data has been published concerning the role that EFAs and PGs play in cancer, as some reports state that EFAs and PGs promote cancer and others state that the same EFAs and PGs inhibit cancer growth. One of the reasons for this may be that the cell lines or experimental animals utilised in the experiments invariably differ. In general, however, EFAs and PGs are known, to affect cancer cells and their proliferation and it is suggested that this

occurs through a number of mechanisms e g control of cAMP synthesis, membrane fluidity and structure, regulation of the immune system and possibly lipid peroxidation (5,22,23,25-30,86-88,186-189).

In contrast to the above finding, many cell lines have been shown not to require EFAs in their media for continued growth. This is thought to be a result of the loss of the activity of linoleoyl-CoA desaturase (as cancer cells either have reduced or no linoleoyl-CoA desaturase activity). It has also been suggested that individual cells may not require EFAs as they may play an integral role in maintaining tissue activities and signalling between cells. Although EFAs may not be essential for the continued growth of some cell lines, these cell lines are still influenced by EFA addition to the growth media (190-193).

LA has been shown to enhance chemically induced mammary tumours in rats when it is present at a level of 3% in the diet (194). It has also been shown to result in an increased incidence of mammary adenocarcinomas in mice and rats (195-200). In cell culture, when LA was added to the media there was enhanced growth of a mammary tumour cell line (201,202). This seems to indicate that LA does play a role in the promotion of mammary cancer. A study carried out to determine the effect of a high LA diet on spontaneous tumours, showed that LA may play a role in the promotion of spontaneous tumours (196).

It is hypothesised that EFA induced increased tumour growth is mediated through a process which in some way involves PGs. PGs are thought to influence cancer by immunosuppression, which could be a result of tumour cell membrane changes. It is possible that the mechanism by which LA promotes tumour growth could be multifactorial, and that PGs play only a partial role. The role of PGs in this process becomes more difficult to define if it is considered that cancer cells have a reduced activity of linoleoyl-CoA desaturase as has been proposed. It should then follow that there would be reduced synthesis of PGs and, therefore, the presence of low and not elevated PG levels would be correlated with increased cancer growth (185,195,198,203-207).

Of the ω -6- EFAs, LA has been shown to be the EFA that is linked most often to cancer promotion (197-200,205-207). GLA on the other hand is suggested to inhibit cancer growth and not to influence the growth of normal cells. GLA is found in a large quantity in the evening primrose (Oenothera biennis) seed oil i.e. 9% of the oil (208-210).

GLA and AA have been both shown to kill cancer cells (human lung and breast cancer cells) preferentially when normal and cancer cells are mixed in cell culture in the presence of these EFAs (211). This was a very important study as normally cell culture studies are carried out using pure cultures of cancer or normal cells and in a host cancer

and normal cells are in contact and will compete for nutrients. GLA has also been shown to inhibit the in vitro growth of human prostatic adenocarcinoma, osteogenic sarcoma, laryngeal carcinoma, human neuroblastoma, murine melanomas, human mammary carcinomas, human hepatomas, human lung carcinomas and human oesophageal carcinomas (212-223). In addition, GLA has been shown to inhibit the in vivo growth of mammary tumours and murine leukemias (224-227).

GLA has been found to completely prevent or reverse genetic damage due to the presence of a chemical carcinogen. This is unusual as most dietary factors are thought to promote or inhibit the promotion stage of cancer, but GLA was shown here to inhibit the initiation stage of cancer. This could have important therapeutic implications (225).

AA has also been shown to be growth inhibitory to some tumour cells and LA has recently been found to be inhibitory to Ehrlich ascite cells. Further research is required with regard to this latter finding, as previously most of the data shows that LA had a promotory role in tumour growth (211,228).

A possible mechanism by which EFAs may inhibit tumour growth is suggested to be lipid peroxidation. Lipid peroxidation plays a role in controlling cell proliferation and as such has a very important biological function, although it can also cause cell injury. PUFAs

undergo lipid peroxidation both while in their free form and when esterified e g in phospholipids. When this occurs a primary reactive free radical interacts with a PUFA to initiate a complex series of reactions, which results in numerous degradatory products. A reduction in PUFA intake will result in a reduction in lipid peroxidation, which in turn may result in reduced control of cell proliferation (186-189,229,230).

Cancer cells and solid tumours display reduced amounts of lipid peroxidation in comparison to normal cells. This is hypothesised to be one of the reasons for loss of control of cell proliferation. The lipid peroxidation activity appears to be inversely related to tumour proliferation. Tumour membranes also seem to be less susceptible to lipid peroxidation which could be a result of alterations in membrane composition. Novikoff tumours, which are rapidly proliferating tumours, have reduced PUFA levels and consequently reduced lipid peroxidation levels. A slow growing tumour viz Morris 9618A hepatoma showed reduced lipid peroxidation, but this reduction was not as large as that of the rapidly proliferating Novikoff tumour cell (186-189,229-232). Therefore, increased EFA levels with resultant increased lipid peroxidation may bring about reduced cell proliferation.

EFAs, thus, appear to play an important role in the metabolism of cancer cells. Their effects can result in either inhibition or

promotion of cancer growth which is probably the result of a difference in metabolic effects of EFAs in different cells as outlined above.

1.4.2 The Relationship between Minerals and Cancer

There have been numerous studies conducted on laboratory animals to determine the relationship between dietary minerals and cancer. Unfortunately, a large number of these experiments have been conducted using differing and sometimes inappropriate methodology, particularly the older studies. These problems were mainly related to a lack of adequate controls. Early studies by Schroeder and his associates (cited in 3, cited in 233, cited in 234) were on the trace elements fluorine, titanium, vanadium, chromium, nickel, gallium, germanium, arsenic, selenium, yttrium, zirconium, niobium, rhodium, palladium, cadmium, indium, tin, antimony, tellurium and lead. The number of experimental animals used were small which could have resulted in incorrect conclusions being drawn. Their results showed that only rhodium and palladium produced minimal signs of carcinogenicity. Later studies on some of these trace elements and other elements will be discussed in greater detail in the ensuing paragraphs.

Selenium was first considered to play a role in cancer therapy when Dalbert suggested its use in 1912 (cited in 235). Dietary selenium is suggested to reduce the risk of cancer and this has been validated by

various animal experimental studies. There is some contradictory evidence concerning selenium in that two studies have shown that it was an inducer of hepatomas, but more recent studies have refuted this (cited in 4, cited in 160, cited in 161, cited in 234-236). Selenium has subsequently been found to significantly reduce the incidence of mammary carcinomas and high levels of dietary selenium suppressed both the initiation and promotion of chemically induced mammary carcinomas (cited in 161). In a recent review (3), it was stated that there have been no human or animal studies that have shown selenium to act as a carcinogen and that the conclusions drawn from the two earlier experiments which implicated selenium as an inducer of hepatomas has been invalidated.

As at this present time, there is still insufficient evidence to draw a firm conclusion about the relationship between iron and cancer. The evidence that is available seems to show that an iron deficiency results in an increased incidence of cancers of the upper alimentary tract, including the oesophagus and stomach. There is some evidence which suggests that the inhalation of large quantities of iron results in an increased risk of cancer promotion. As of yet there is no data indicating that a high dietary level of iron increases the risk of cancer (3,4).

As with iron, at this present time the evidence available concerning the relationship between dietary copper and cancer is insufficient to

permit any conclusions to be drawn. It has been found that there is a weak association with copper and increased incidences of cancers of the intestine, breast, lung and thyroid (cited in 3). Copper is an essential nutrient that is widely distributed amongst the foods and this leads to the problem that the influences noted may not be solely due to copper but may be due to an interaction between copper and some other compound. It has been shown in laboratory experiments that high levels of copper salts present in diets result in various degrees of protection against chemically induced liver tumours. On the other hand it has been suggested that the inhalation of copper, which can occur when certain fungicides are sprayed, can result in an increased risk of occurrence of cancers associated with the respiratory tract and lungs (4,6,236).

In general while numerous studies have investigated the relationship between dietary essential elements and cancer, there is still a great deal of research required to fully elucidate the importance of these nutrients to the disease. An element of particular interest to this study is zinc and will be discussed in greater detail.

1.4.2.1 The Relationship between Zinc and Cancer

Zinc is an essential constituent of more than two hundred enzymes (105) and it is an essential trace element. Zinc is known not to be

mutagenic and it has been shown to only induce tumours upon local application (3,4,105,233,235,237).

It has been found that certain cancers have lower than normal zinc levels in the tumour tissues, for example, oesophageal carcinoma, primary hepatic carcinoma, renal cell carcinoma, prostate carcinoma and leukaemic lymphocytes (cited in 238).

Zinc deficiency, on the other hand, has been shown to reduce tumour growth in Ehrlich ascites tumours (239,240), Walker 256 carcinosarcomas (6,241-243), Lewis lung sarcomas (243), L1210 mouse leukaemias (244), 350 Hepatomas (245) and murine melanoma tumours (BL6) (246). In reducing the tumour growth, zinc deficiency has been shown not to harm the host. If zinc deficiency was induced in an experimental animal, it was shown to prevent the initiation of Walker 256 carcinosarcomas (6,241-243), Lewis lung sarcomas (243) and L1210 mouse leukaemias (244). Mills et al (247) have established that mammary adenocarcinoma growth can be retarded solely by dietary zinc deficiency. Zinc deficiency has been shown to result in reduced growth of both normal and malignant cells (238).

Dietary zinc deficiency is thought to reduce the growth of certain tumours by reducing DNA synthesis. This could be as the result of lower activities of DNA polymerase and thymidine kinase (109,112,116,118,122,248-250). Zinc deficiency may also result in increased lipid peroxidation, which in turn would lead to reduced cell

proliferation (251). Of particular interest to this study is the possible interaction of zinc with linoleoyl-CoA desaturase which may result in reduced tumour growth and this will be discussed in the next section.

One of the very few cancers that has been shown to have an increased growth when a zinc deficient diet is fed, is oesophageal cancer. This is true for both spontaneous and chemically induced oesophageal cancers(cited in 160,238,252). It has been shown that the countries (such as South Africa) which have very high oesophageal cancer levels tend to have a zinc deficiency in the soils and, thus, the foods produced. In South Africa, the Transkei region has an oesophageal cancer level approaching 50% in males(cited in 238,cited in 251). Thus cancer has been very well correlated with zinc deficiency.

In contrast to the above studies a zinc supplemented (excess) diet has been shown to reduce the growth of a hamster cheek pouch carcinoma (244). It has also been shown to reduce the growth of L1210 mouse leukaemia cancers. This shows that both zinc deficiency and excess reduce the growth of L1210 mouse leukaemias (252). Excess zinc has been shown to reduce B16 melanoma growth in culture but when these melanoma cells were injected into mice and fed a zinc excess diet, the melanomas displayed increased growth (253,254). Two reports disagree with this finding and have suggested that excess zinc reduces the growth of B16 melanoma cells, both in vitro and in vivo (255,256).



Zinc excess may also result in reduced cell proliferation via reduced DNA synthesis as a result of lower activities of DNA polymerase and thymidine kinase (248-250). Zinc supplementation may also result in re-activation or increased activity of linoleoyl-CoA desaturase as it has been suggested that zinc is a possible cofactor of linoleoyl-CoA desaturase (133,135). So it can be seen that zinc could have an important role in cancer.

1.4.3 The Relationship Between Zinc, EFAs and Cancer

As has been outlined in the preceeding sections both zinc and EFAs play a role in the regulation of cell proliferation and hence in tumour growth. The possibility of an interaction between these two nutrients has also been discussed. It is therefore not unrealistic that zinc and EFAs may influence each other in the mechanism by which they inhibit cell and tumour proliferation. One possible mechanism could be through an association of zinc with the regulatory enzyme in EFA synthesis, linoleoyl-CoA desaturase. The possibility of such an involvement by zinc in EFA synthesis has already been eluded to in section 1.3.6 and as Fig. 2 outlines, this could lead to an effect on cellular cAMP levels in the cells and a subsequent effect on the rate of cell proliferation. A zinc deficiency could consequently result in a reduction in the activity of linoleoyl-CoA desaturase and a resultant decrease in cAMP synthesis which in turn could lead to an increased rate of cell proliferation (5,22-30,138,140,143). Skeef and Duncan (245,246) have shown that zinc does influence cAMP levels

i.e. added zinc results in an increased synthesis of cAMP and a reduction in the rate of cell proliferation, which may be the result of increased activity of linoleoyl-CoA desaturase.

The aim of this thesis was to study the effects of zinc, and the EFA, GLA on tumour growth and the effects of zinc and GLA supplementation on the enzyme complex linoleoyl-CoA desaturase activity in normal and malignant cells in vitro. The normal cell line used was a monkey kidney cell line (LLCMK) and the malignant cell line used was a murine melanoma cell line (BL6). The effect of GLA supplementation was studied as it is the product of one of the reactions that linoleoyl-CoA desaturase catalyses (35) and the effect of zinc supplementation on linoleoyl-CoA desaturase was studied as it has been suggested that zinc was a cofactor of linoleoyl-CoA desaturase (133,135). The isolation of the terminal enzyme in the linoleoyl-CoA desaturase complex, Δ^6 -desaturase was one of the primary aims of this study and it was decided that this would be approached by isolating the enzyme complex and then enzymatically removing the other two components of the complex i.e. cytochrome b₅ and cytochrome b₅ reductase with a pure Δ^6 -desaturase resulting. In order to enzymatically remove the two components from the linoleoyl-CoA desaturase complex it was decided initially to attempt to isolate and purify these components using enzymatic solubilisation from microsomes in order to determine if this was possible. Once this had been achieved then the isolation of Δ^6 -desaturase would be attempted.

CHAPTER 2

ISOLATION AND PURIFICATION OF CYTOCHROME b_5

2.1 Introduction

Linoleoyl-CoA desaturase is an enzyme complex which consists of cytochrome b_5 , cytochrome b_5 reductase and Δ^6 -desaturase (cited in 44). As can be seen in Fig. 4 NADH passes electrons to cytochrome b_5 reductase which in turn passes the electrons onto two cytochrome b_5 molecules and they are then passed onto two Δ^6 -desaturase molecules. The passage of electrons results in the conversion of cis-LA into GLA.

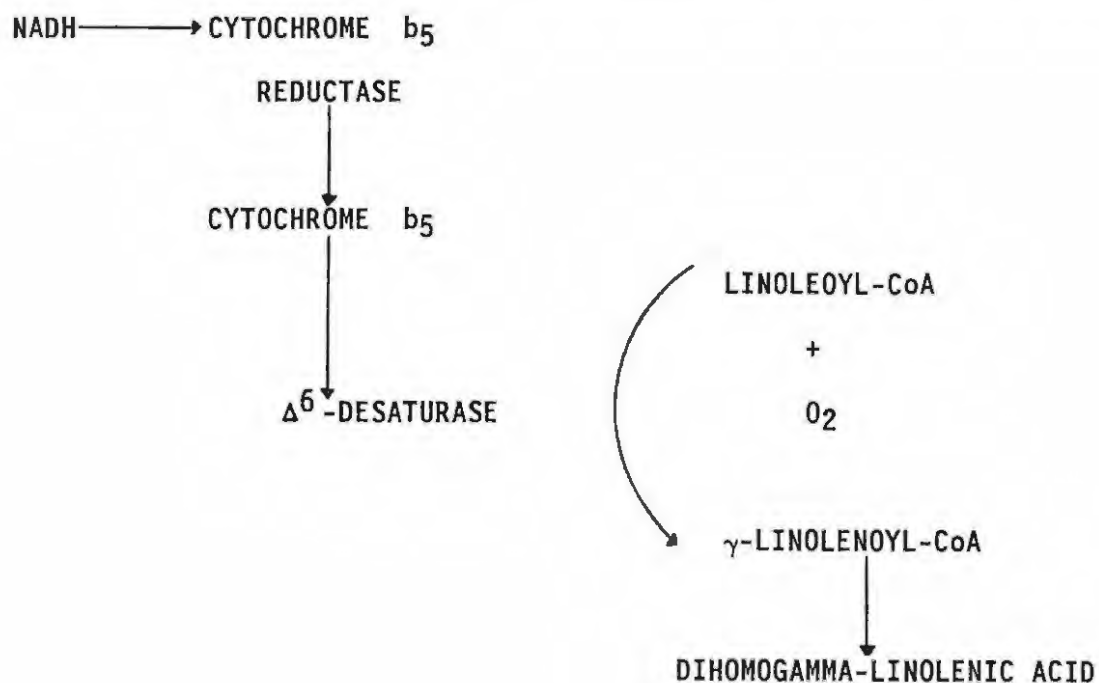


Figure 4. The electron flow through the linoleoyl-CoA desaturase (44)

One of the aims of this thesis was to isolate and purify each component of linoleoyl-CoA desaturase in an attempt to understand the functioning of the enzyme complex better. The first component isolated was cytochrome b₅ as most was known about this component.

Cytochrome b₅ was first isolated from rabbit liver microsomes by lipase action and classified as a haeme protein in 1956 by Strittmatter and Velick (257). Up to this time cytochrome b₅ had also been referred to as cytochrome b₁ and cytochrome m (cited in 257). Strittmatter and Velick noted that two very similar forms of cytochrome b₅ were isolated with molecular weights (MW) of approximately 17 000. Experimental results indicated that there was one haeme molecule and no non-haeme iron in cytochrome b₅ (257). By 1966 cytochrome b₅ was the generally accepted name for this haeme protein. Strittmatter and Ozols (258) showed that the two major forms of cytochrome b₅ that had been isolated differed in that the one form contained an extra peptide sequence but both forms were catalytically active. This sequence was shown to be the carboxyl terminal sequence. Tryptic digestion of the larger form resulted in the formation of the second and smaller form. The smaller, or core, form of cytochrome b₅ was shown to consist of 81 amino acids. In 1968 cytochrome b₅ was first solubilised from rabbit liver microsomes using the detergents Triton-X 100 and sodium deoxycholate. The detergent solubilised cytochrome b₅ had a MW of approximately 25 000 and whereas the lipase or protease solubilised cytochrome b₅ molecules

were present in solution as monomers, the detergent solubilised cytochrome b₅ was present in solution as an oligomer (259).

Ito and Sato (259) put forward the proposal that the catalytic activity of cytochrome b₅ was to be found in the haeme portion of the molecule and that the carboxyl terminal sequence was hydrophobic and could possibly have the role of anchoring cytochrome b₅ to membranes.

In 1968 Kajihara and Hagihara (260) crystallised cytochrome b₅ after solubilising it from rabbit liver microsomes using crystalline trypsin. This cytochrome b₅ had a MW of 11 500. As with the lipase action trypsin solubilisation resulted in two very similar forms of cytochrome b₅ being isolated. In 1969 several workers showed that cytochrome b₅ was susceptible to proteolytic action as a result of its positioning on the outside surface of membranes (261,262). Fig. 5 shows the present proposed structure of cytochrome b₅ and the two proposed sites of tryptic proteolysis (263).

Cytochrome b₅ is now known to be an amphipathic molecule which consists of a hydrophilic catalytic domain which is linked via a short flexible sequence to a hydrophobic domain (see Fig. 5). This hydrophobic domain anchors the molecule within membranes (264,265,cited in 266). Cytochrome b₅ is an integral protein viz. it is strongly bound to a membrane and interacts with the lipids of

the membrane. Cytochrome b₅, once synthesised, is spontaneously inserted into the intracellular membranes of cells (267-272).

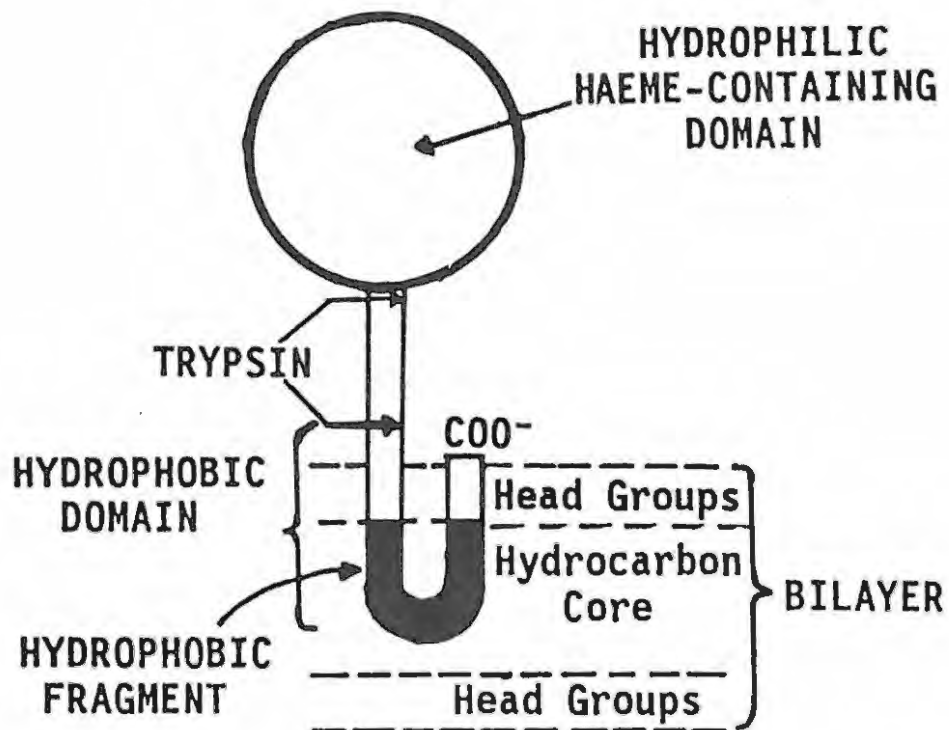


Figure 5. Schematic representation of cytochrome b₅ in a phospholipid bilayer, based on data derived from limited proteolysis and from dimensional studies in detergent solutions. The COOH-terminus of the protein must lie outside the hydrocarbon core of the bilayer, but there is no direct evidence that it emerges from the same side as the inter-domain link as shown in the figure (263).

Further research has shown that the cytochrome b₅ found in rabbit liver consists of 141 amino acids and that the catalytically active domain consists of 97 amino acids. This would mean that the detergent solubilised cytochrome b₅ would be 141 amino acids in length and the trypsin solubilised cytochrome b₅ would be 97 amino acids in length. The molecular weight of the intact cytochrome b₅ was shown to be approximately 16 700 (259,264,269).

Cytochrome b₅ is normally a 6-coordinate low spin haeme protein with histidine-39 and histidine-63 as the axial ligands (273,274). Mathews (cited in 275) used X-ray diffraction techniques at a resolution of 2Å to indicate that the haeme portion of cytochrome b₅ was inserted into a tight non-polar crevice within the compact globular molecule of cytochrome b₅. Most of the polar residues within the molecule were found to be on the outer surface of cytochrome b₅.

Studies of eight different species, i.e. man, monkey, pig, chicken, bovine, rabbit, murine and rat have shown that cytochrome b₅ from all of these eight species demonstrates sequence homology among amino acids 42-72. These include the two axial ligands. Sequence variability is located in the amino acids of the hydrophobic membrane binding region of cytochrome b₅ (276,277).

Cytochrome b₅ is found in membranes as a monomer which is consistent with its function as an electron carrier (275,278). Microsomal electron transport chain components are known to undergo movement within the membrane and integral proteins are thought to undergo rapid rotational and translational movement within the membrane bilayer (279,280). Cytochrome b₅ is known to be randomly distributed on the surface of membranes and for it to receive and pass on electrons it must be able to diffuse throughout the membrane (263,268,281). It has been shown that the rate of electron transfer from cytochrome b₅ reductase to cytochrome b₅ is determined by the lateral diffusion within the membrane of cytochrome b₅. So cytochrome b₅ is a mobile electron transport protein (275,cited in 263,278).

As cytochrome b₅ has been proven to be a component of linoleoyl-CoA desaturase via enzymatic and immunological methods it was decided to attempt to isolate and purify cytochrome b₅ from rat liver using a simplified methodology with a hopefully increased yield (282,283).

Cytochrome b₅ has been shown to be present in high quantities in the endoplasmic reticulum of mammalian liver cells and for this reason cytochrome b₅ was isolated and purified from rat liver microsomes (260,268,269,276,281).

2.2 Materials and Methods

2.2.1 Reagents

Unless stipulated, all buffer and other dilutions, in this thesis, were made with deionised water.

Homogenisation buffer. The homogenisation buffer consisted of 0.25 M sucrose, 0.15 M potassium chloride, 0.005 M magnesium chloride, 0.001M ethylenediamine tetra-acetic acid (EDTA) in 0.1 M Tris-HCl pH 7.4.

Protein solution A. Protein solution A consisted of 2.0 g of potassium sodium tartrate and 100 g of sodium carbonate (Na_2CO_3) dissolved in 500 cm^3 of 1.0 N sodium hydroxide (NaOH). The volume was then made up to 1000 cm^3 with deionised water in a volumetric flask.

Protein solution B. Protein solution B consisted of 2.0 g of potassium sodium tartrate and 1.0 g copper sulphate hydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) dissolved in 90 cm^3 of deionised water and the solution was then made up to 100 cm^3 with 1.0 N NaOH in a volumetric flask.

Protein solution C. Protein solution C consisted of Folin and Ciocalteu's reagent (2.0 N) (Saarchem, RSA) diluted 1:10.

Protein Standard solution. The protein standard solution consisted of bovine serum albumin (BSA) at $150 \mu\text{g}/\text{cm}^3$ and the concentration of the standards were 25; 50; 100 and $150 \mu\text{g}/\text{cm}^3$. The standards contained the same volume of buffer as the samples and each time a different buffer was utilised a different standard curve was prepared.

Carboxymethyl-cellulose (CM-cellulose). CM-cellulose (Bio-Rad Laboratories, USA) was activated as specified in Clark and Switzer (284).

Diethylaminoethyl-cellulose (DEAE-cellulose). DEAE-cellulose was purchased as the chemical Cellex-D (Bio-Rad Laboratories, USA) and was activated as specified in Clark and Switzer (284).

Diethylaminoethyl-Sephadex (DEAE-Sephadex). DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Sweden) was swollen for 24 hrs in the appropriate buffer (1 g swelled to a final volume of $12\text{-}15 \text{ cm}^3$) and the "dead" beads were removed from the surface.

2.2.2 Animals

The rats utilised were Long-Evans female rats obtained from the University of Port Elizabeth animal unit, RSA. They were fed normal chow pellets.

2.2.3 Methods

Unless stipulated all procedures were conducted at room temperature.

Microsome Preparation. The procedure for the preparation of rat liver microsomes was a modification of the methods of Larsson and Brimmer, Mahfouz and De Schriver and Privett (285-287). Ten rats were anaesthetised and the livers were rapidly removed and immediately washed in homogenisation buffer and placed on ice.

The livers were then homogenised with a dounce homogeniser (20 x and 20 x with the loose and the tight plungers respectively) in 3 volumes (v/w) (100 g/420 cm³) of homogenisation buffer. The homogenate was centrifuged (Beckman J2-21 centrifuge and a JA 21 rotor) at 10 000 g for 20 min at 4°C (to pellet the cellular debris and mitochondrial fractions). The supernatant was centrifuged (Beckman LS 3801 ultracentrifuge and a 70.1 Ti rotor) at 110 000 g for 90 min at 4°C. The pellet was resuspended in homogenisation buffer at 4°C for 30 min to provide microsomes equivalent to 1 g of original tissue per cm³ of homogenisation buffer. The volume of the microsome solution was 150 cm³. The protein concentration was determined utilising the Hartree modification of the Lowry method (288,289). The preparations were diluted appropriately (see Appendix 1 for the protein standard curve).

The assay for the presence of cytochrome b_5 utilised an increase in the major Soret peak of cytochrome b_5 at 423 nm in the presence of 0.2 mM reduced nicotinamide adenine dinucleotide (NADH). The assay utilised the wavelengths 419-430 nm. A NADH reduced minus oxidised cytochrome b_5 difference spectrum (between 400-610 nm) was run to ensure that cytochrome b_5 was present, as this is a spectrum characteristic of cytochrome b_5 . This spectrum also gave an indication that the major Soret peak of cytochrome b_5 was present and this peak is utilised in the cytochrome b_5 assay (290,291). The assays and spectrum were run on a Bausch and Lomb Spectronic 1001 using a multiwavelength programme. Cytochrome b_5 was assayed for at each step in the isolation and purification procedure.

Solubilisation of Cytochrome b_5 . From this stage the general methodology followed was that of Kajihara and Hagihara (260) and any modifications made to their methodology will be discussed later in this chapter. Crystalline trypsin (Sigma Chemical Company, USA) was added at 0.005 mg per mg of microsomal protein (total microsomal protein was 1050 mg) and consequently 5.25 mg of trypsin was added. The solution was stirred for 5 hours at 37°C to solubilise the catalytic domain of cytochrome b_5 . Streptomycin and Penicillin (100 international units (IU)/cm³ and 100 µg/cm³ respectively) were added at 1.0 cm³ per 100 cm³ of microsomal solution, which had a final volume of 151.5 cm³.

Ammonium Sulphate Fractionation of Cytochrome b₅. Ammonium sulphate was added to the microsomal solution until 50% saturation was reached (291 mg of ammonium sulphate/cm³). The solution was left at 4°C for 1 hour and was then filtered through 4 sheets of Whatman No 1 filter paper and Celite 545 on a Buchner funnel. 100 cm³ of 50% ammonium sulphate solution, pH 7.4, was then washed through the residue on the filter paper. The washing and filtrate were combined and brought to 75% saturation by the further addition of ammonium sulphate. The cytochrome b₅ solution was filtered through 4 sheets of Whatman No 1 filter paper and Celite 545 again and it was then dialysed against 0.02 M potassium phosphate buffer pH 6.5 for 24 hours with frequent changing of the buffer. The final volume was 545 cm³.

CM-cellulose and DEAE-cellulose chromatography. The dialysate was passed through a CM-cellulose column (1.5 x 30 cm) at a rate of approximately 12 cm³/hr. The column had previously been equilibrated with 0.02 M potassium phosphate buffer pH 6.5. The eluent was collected and then placed directly onto a DEAE-cellulose column (2.5 x 38 cm) with a flow rate of approximately 60 cm³/hr. Haemoglobin and other proteins were bound to the CM-cellulose column but not the cytochrome b₅. When passed through the DEAE-cellulose column the cytochrome b₅ was absorbed onto the top of the column. This column was washed with 1 l of 0.03 M potassium phosphate buffer pH 7.4 to

move the reddish-brown cytochrome b_5 band into the column. The cytochrome b_5 was eluted from the DEAE-cellulose column using a 0.25 M potassium phosphate buffer pH 7.4. One hundred fractions of approximately 3 cm³ were collected. These fractions were assayed for the presence of cytochrome b_5 and protein. Fractions 49-60 (the combined volume was 46 cm³) were kept as they were found to contain the cytochrome b_5 . These fractions had a reddish-brown colouration.

The combined fractions were diluted 8.33 times so that the solution had the same molarity as the column and the solution was then passed through a second DEAE-cellulose column (2.5 x 25 cm³) at a flow rate of approximately 66 cm³/hr. The column had previously been equilibrated with 0.03 M potassium phosphate buffer pH 7.4. The cytochrome b_5 was absorbed onto the top of the column as a reddish-brown band. The cytochrome b_5 was eluted from the column with 0.25 M potassium phosphate buffer pH 7.4. Fractions (approximately 7 cm³) were collected from this column. Fractions 19-28 were found to contain cytochrome b_5 and these fractions were slightly coloured. The combined volume of the fractions was 59 cm³.

DEAE-Sephadex A-50 chromatography. The solution was diluted 1.92 times to adjust the buffer concentration to 0.13 M and the solution passed through a DEAE-Sephadex A-50 column (2.5 x 35 cm³) at a flow rate of approximately 20 cm³/hr. The column had previously been

equilibrated with 0.13 M potassium phosphate buffer pH 7.4. The cytochrome b_5 was eluted from the column using 0.3 M potassium phosphate buffer pH 7.4 and fractions of approximately 6 cm³ were collected. Fractions 76-89 were found to contain the cytochrome b_5 . The combined volume of the fractions was 72 cm³.

Freeze Drying. The cytochrome b_5 solution was then freeze dried (Virtis Freezemobile 6) and the resulting powder was assayed for protein and cytochrome b_5 content and stored frozen.

Disc gel electrophoresis. Disc gel electrophoresis was performed on the freeze dried cytochrome b_5 , at room temperature and according to the methodology of Clark and Switzer (284). After electrophoresis the gels were fixed in methanol-acetic acid (1:1) for 1 hr. The gels were then stained in 0.25% Coomassie Brilliant Blue R-250 (Merck, West Germany) in methanol-acetic acid-water (5:1:5) for 1 hr. The gels were destained with 5% trichloroacetic acid.

2.3 Results

The NADH reduced minus oxidised cytochrome b_5 difference spectrum (as shown in Fig. 6) correlated well with a previous report (291) in that the major Soret was shown to exist at 425 nm and the literature results showed the peak to exist at 423 or 424 nm.

Fig. 7 shows the elution profile of the cytochrome b_5 solution from the first DEAE-cellulose column. The cytochrome b_5 was found to be eluted in a relatively broad peak.

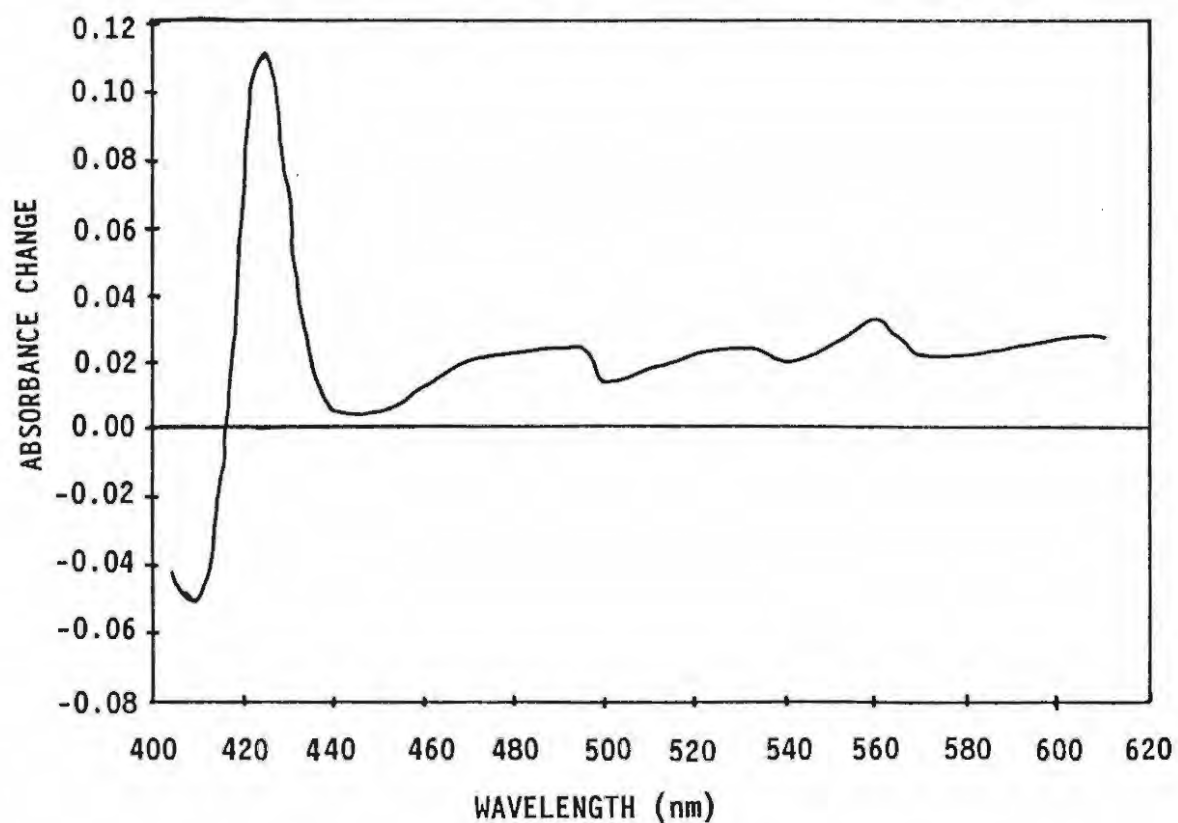


Figure 6. The NADH-reduced minus oxidised difference spectrum of the microsome solution in the isolation and purification of cytochrome b_5 .

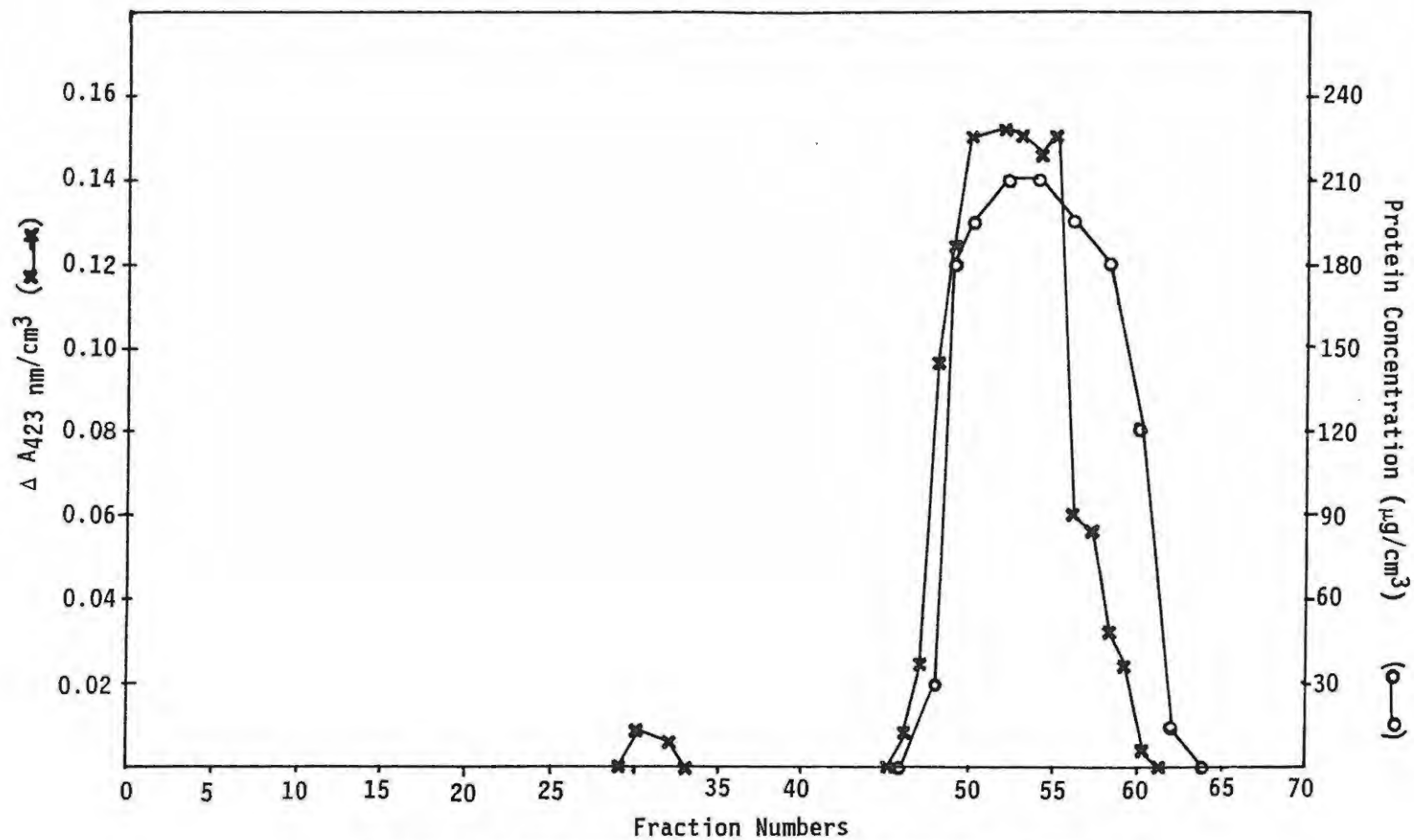


Figure 7. The elution profile of cytochrome b_5 from the first DEAE-cellulose column.

Fig. 8 shows the elution profile of cytochrome b_5 from the second DEAE-cellulose column. It was expected that the cytochrome b_5 band would separate into two bands. This was not shown to occur although a small protein peak was located in fraction 39. No cytochrome b_5 was identified in this peak.

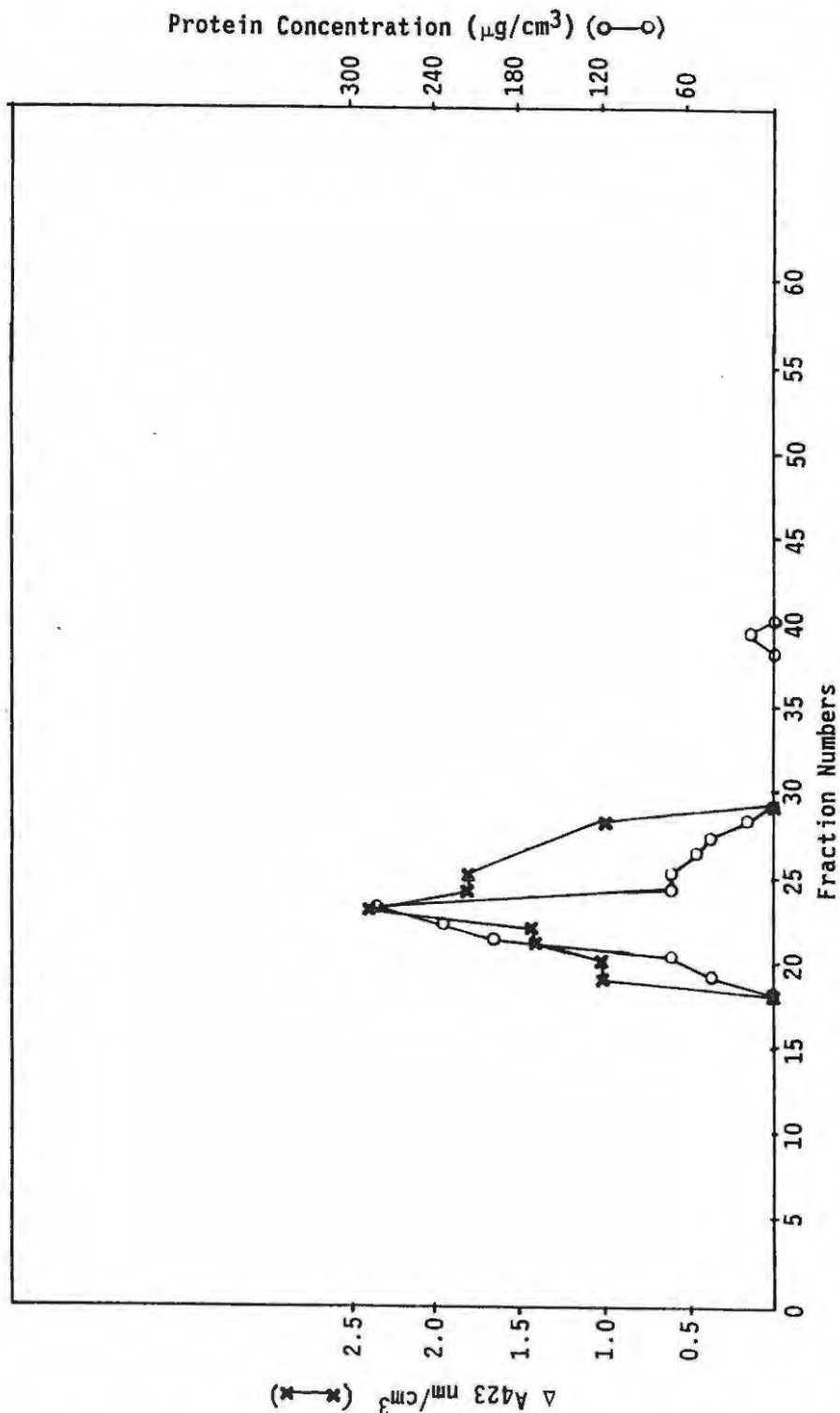


Figure 8. The elution profile of cytochrome b_5 from the second DEAE-cellulose column.

Fig. 9 shows the elution profile of cytochrome b_5 from the DEAE-Sephadex A-50 column. As with the previous column it was expected that the cytochrome b_5 band would separate into two distinct bands. Again this was not found.

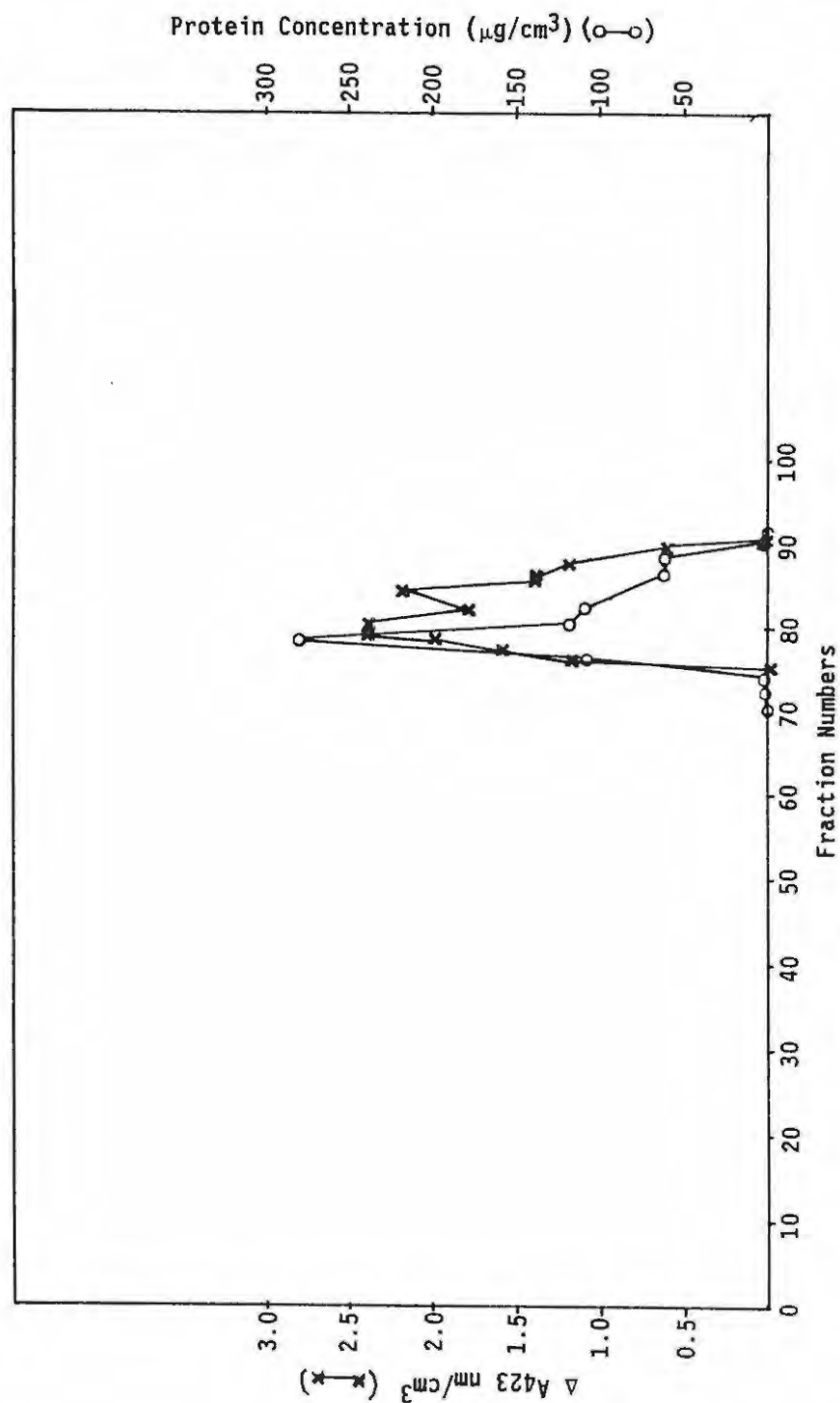


Figure 9. The elution profile of cytochrome b_5 from the DEAE-Sephadex A-50 column.

Table 2 is the purification table of cytochrome b_5 . The table shows that 11 mg of cytochrome b_5 was isolated from 100 g of starting rat liver. This is a three to four times increase in yield of that cited in literature (260,264). Previous studies have also tended to use, as the enzyme source, kgs of liver instead of the gram quantities used in this study. The ammonium sulphate fractionation step showed a great increase in specific activity indicating that other contaminating proteins had been precipitated out. All the chromatography steps utilised showed increased specific activity and were thus of value in the isolation procedure.

TABLE 2: PURIFICATION OF CYTOCHROME b_5

Step	Total Protein (mg)	Total activity (unit)	Specific activity		Yield (%)
			units/mg	ratio	
Microsome Solution	1050	2917	2.78	1.00	100
Trypsin Solubilisation	1098	2183	1.99	0.72	75
Ammonium Sulphate Fractionation	242	1847	7.63	2.74	63
Dialysis	123	1384	11.25	4.05	47
1st DEAE-Cellulose Column	22	260	11.82	4.25	9
2nd DEAE-Cellulose Column	16	200	12.50	4.50	7
DEAE Sephadex A-50 Column	13	244	18.77	6.75	8
Freeze Drying	11	236	21.45	7.72	8

Disc gel electrophoresis showed one discrete band in the gels indicating that cytochrome b₅ had been isolated in one pure form. No photographs have been included in this thesis of the gels from this and ensuing chapters as a result of the bands not being visible in the photographs. Both colour and monochrome photographs were attempted with various filters and lenses. The bands were clearly visible to the naked eye within the gels and photographs of the gels have been included in a previous publication (292) even though the bands were not visible in the photographic plates. The cytochrome b₅ band had a R_f value of approximately 0.90 - 0.95. Carlsen et al utilised immunoblotting to enable a photograph with a visual cytochrome b₅ band to be published (293). This article was published after this study had been concluded and, thus, immunoblotting was not attempted.

2.4 Discussion

There are several points worthy of noting in the isolation and purification procedure used in this study. As indicated previously smaller starting quantities of rat liver were used i.e. only ten rats were used in contrast to at least one hundred cited in literature. This smaller quantity did not in any way affect the efficiency of the procedure. Also, working with a smaller number of rats and consequently a smaller volume of cytochrome b₅ solution greatly simplified the procedure. As stated previously the aim was to achieve a simplified but successful methodology for the isolation and

purification of catalytically active cytochrome b₅ and consequently all the stages in the isolation and purification procedures cited in literature were scrutinised in an attempt to simplify the methodology.

When trypsin had previously been used to solubilise cytochrome b₅ from microsomes this had been conducted at 0°C for approximately fifteen hours (260). Trypsin [EC 3.4.21.4] is classified as a peptidyl peptide hydrolase and it hydrolyses peptides, amides and esters at lysine and arginine carboxyl bonds. Trypsin is found to be most active at 37°C and as such it seemed slightly illogical from the enzyme action point of view for the microsome solution to be incubated at 0°C (12,294,295). Incubation with trypsin was consequently conducted at 37°C in this study.

In order to prevent any microbial or bacterial contamination antibiotics were added to the microsome solution with the trypsin. The solution was constantly stirred in an attempt to maximise the cytochrome b₅ solubilisation from the microsomes. As Fig. 5 shows, trypsin has two points at which it can hydrolyse cytochrome b₅. Another reason for incubating the solution at 37°C was an attempt to prevent two forms of cytochrome b₅ from being solubilised as had been cited in literature (260,264). It was hoped that the increased enzymatic activity of trypsin would result in the smallest form of cytochrome b₅ being solubilised i.e. the trypsin would hydrolyse the solubilised cytochrome b₅ at both the possible hydrolysis points.

The results showed that this modified methodology achieved the increased solubilisation of only one form of cytochrome b₅ as hypothesised.

Literature reports had indicated that all the stages of the isolation and purification procedure had to be conducted at approximately 4°C (260,264). This can be problematic as it would mean that the entire procedure would have to be conducted within a cold room and it was decided to attempt the isolation and purification procedures at room temperature, except for a few critical stages, which were indicated in the methodology section. It was shown that the use of room temperature did not result in loss of cytochrome b₅. The isolation and purification procedure was thus greatly simplified by being able to work mainly at room temperature.

In general, the flow rates utilised by Kajihara and Hagihara (260) seemed to be excessively fast and all the flow rates utilised in this study were greatly reduced. The reduced flow rates were expected to result in greater binding of the cytochrome b₅ to the ion exchange columns.

The CM-cellulose step was efficient in binding the haemoglobin and other protein impurities and allowing the elution of the cytochrome b₅ through the column without binding to it. Kajihara and Hagihara

(260) found that the cytochrome b₅ band separated into two distinct bands on both the second DEAE-cellulose and the DEAE-sephadex A-50 columns. As stated in the results, in this study the cytochrome b₅ band was not found to separate into two distinct bands using the modified methodology. As there was no second cytochrome b₅ band it was unnecessary to utilise the additional chromatography steps that Kajihara and Hagihara had used. This also resulted in a simplified procedure.

As Kajihara and Hagihara (260) utilised 2 kgs of liver, their resulting volumes of cytochrome b₅ solution were consequently much larger than obtained in this study, and as such it was unnecessary to carry out the concentration stage that Kajihara and Hagihara had conducted. It has been noted in literature that cytochrome b₅ can be unusually difficult to crystallise (260,264). Initially it was attempted to crystallise the cytochrome b₅, but when the methodologies cited by Kajihara and Hagihara and normal organic methods for crystallisation were unsuccessful, it was decided to attempt freeze drying as a viable alternative. It was found that freeze drying in no way altered the catalytic activity of cytochrome b₅, and this provided a quick and convenient method of producing a storable form of cytochrome b₅. The freeze dried powder was found to be very hygroscopic and thus, had to be stored in a tightly sealed container.

The cytochrome b_5 could be stored in a freezer in this form for in excess of two years without appreciable loss of its ability to transfer electrons.

The fold purification, 7.72, achieved using this modified methodology showed a large increase compared to that achieved in a previous literature report of 1.36 (260). The overall yield achieved in this study was greater than that in a previous literature report (260). In this study there was increased purification at each step except that of trypsin solubilisation but this was compensated for with the ammonium sulphate fractionation step. There was a small increase in fold purification with the first DEAE-cellulose chromatography step but this step did result in the removal of some of the contaminating protein and as such was extremely useful. The second DEAE-cellulose chromatography step resulted in further purification of the cytochrome b_5 . Both the DEAE-Sephadex A-50 and freeze drying resulted in greater fold purification and as such were important steps in the isolation and purification of cytochrome b_5 .

From these results it can be seen that a catalytically active and pure form of cytochrome b_5 could be easily and reproducibly isolated using a simplified procedure requiring less starting material and less rigorous conditions. This methodology was repeated several times in order to ensure that it was easily reproducible and could thus be readily utilised for the isolation of Δ^6 -desaturase from the linoleoyl-CoA desaturase complex.

CHAPTER 3

ISOLATION AND PURIFICATION OF CYTOCHROME b₅ REDUCTASE

3.1 Introduction

Cytochrome b₅ reductase [EC 1.6.2.2] is the first component in the linoleoyl-CoA desaturase complex and transfers electrons to cytochrome b₅ (see Fig. 4) (290,296). Cytochrome b₅ reductase was first isolated by Strittmatter and Velick in 1956 and 1957 (297,298) by solubilising the enzyme from microsomes using cobra venom. Takesue and Omura (cited in 299,300) reported that this solubilisation of cytochrome b₅ reductase was not due to the cobra venom but in fact was due to lysosomal contamination of the microsomes. This work was confirmed by St Louis *et al* (299) and they identified the active component of the lysosomes as an acid proteinase. Lysosomal solubilisation resulted in the isolation of a catalytically active cytochrome b₅ reductase with a MW of approximately 33 000 (299,301,302).

Cytochrome b₅ reductase has also been isolated when solubilised utilising detergents, i e Triton-X 100 and sodium deoxycholate. Detergent solubilised cytochrome b₅ reductase had a MW of approximately 45 000 (265,302-304). As with cytochrome b₅, cytochrome b₅ reductase can therefore be isolated in two forms and the smaller form is still catalically active.

Cytochrome b₅ reductase is a NADH: ferricytochrome b₅ oxidoreductase (305). It was discovered that the structure of cytochrome b₅ reductase was similar to that of cytochrome b₅ in that it is an amphipathic integral protein which is anchored to membranes via a short hydrophobic section with a large catalytic domain attached to the membrane binding section via a short flexible neck (cited in 269). Detergent solubilisation resulted in the isolation of the intact form of cytochrome b₅ reductase and lysosomal solubilisation resulted in the isolation of a smaller form of cytochrome b₅ reductase that still contained the flavin prosthetic group and was catalytically active (304). Cytochrome b₅ reductase was found to contain one molecule of flavine adenine dinucleotide (FAD) per enzyme molecule (303).

Cytochrome b₅ reductase has been isolated from various species i.e. human, pig, bovine, rat and rabbit (44,265,302,306-318). In humans cytochrome b₅ reductase has been isolated from erythrocytes in which both soluble and membrane bound enzymes have been found (306,312,315); and from placenta, leucocytes, muscle, liver, fibroblasts (313,cited in 313). All of these human cytochrome b₅ reductases were found to exhibit great immunological similarities (cited in 313). Yubisui et al (315) determined that cytochrome b₅ reductase isolated from human erythrocytes consisted of 275 amino acids. Tamura et al (308) compared the structures of bovine erythrocyte, brain and liver cytochrome b₅ reductases and found that the enzymes have identical structures

except for the amino-terminal end of the enzymes. They determined that the cytochrome b₅ reductases isolated from both erythrocytes and the brain consisted of 273 amino acids whereas those isolated from the liver consisted of 274 amino acids (308).

As with cytochrome b₅, cytochrome b₅ reductase is not fixed within the membrane structure and it is able to undergo rapid rotational and translational movement within the lipid bilayer (279,281,319). Cytochrome b₅ reductase has been shown to be present mainly in the endoplasmic reticulum of cells. It has also been shown that cytochrome b₅ reductase is randomly distributed within the endoplasmic reticulum (281,320,321).

It was discovered by Ozols et al (322) that the amino-terminal end of cytochrome b₅ reductase, isolated from bovine livers, was blocked with n-tetradecanoyl acid (myristic acid) and it was proposed that this could selectively stabilise the structure and membrane orientation of the enzyme so that there is maximal electron transfer between it and cytochrome b₅. Cytochrome b₅ reductase accepts two electrons from NADH resulting in the formation of a NAD⁺-reduced flavoprotein charge-transfer complex. This complex will then transfer a single electron to a molecule of cytochrome b₅. It has been suggested that one molecule of cytochrome b₅ reductase concomittantly transfers two electrons onto two molecules of cytochrome b₅ (one electron per cytochrome b₅) (323,324,cited in 324). Dailey and Strittmatter (325)

have shown that the interaction between cytochrome b₅ reductase and cytochrome b₅ occurs via complementary charge pair interactions involving carboxyl groups of cytochrome b₅. So it can be seen that the interactions between cytochrome b₅ reductase and cytochrome b₅ are vitally important for the functioning of the linoleoyl-CoA complex.

As with cytochrome b₅ it was decided in this study to attempt to isolate and purify cytochrome b₅ reductase using a simplified method with a hopefully increased yield and which could be utilised in the final isolation of Δ^6 -desaturase.

3.2 Materials and Methods

3.2.1 Reagents

Protein solution A. As in section 2.2.1.

Protein solution B. As in section 2.2.1.

Protein solution C. As in section 2.2.1.

Protein Standard solution. As in section 2.2.1.

Sephadex G-100. Sephadex G-100 (Pharmacia Fine Chemicals, Sweden) was swollen for 24 hrs in the appropriate buffer and the "dead" beads were removed from the surface.

DEAE-cellulose. As in section 2.2.1.

CM-Sephadex C-50. CM-Sephadex C-50 (Pharmacia Fine Chemicals, Sweden) was activated as specified in Clark and Switzer (284).

3.2.2 Animals

As in section 2.2.2.

3.2.3 Methods

Unless stipulated all procedures were conducted at room temperature.

Lysosome-Microsome Fraction Preparation. The methodology followed was a modification of that of Takesue and Omura (311). Rat livers (100 g), from freshly killed rats, were blended in a Moulinex Type 241 liquidiser, with four volumes of 0.25 M sucrose containing 1 mM EDTA pH 7.5. The homogenate was then centrifuged at 7000 g for 10 min (Beckman J2-21 centrifuge and a JA 21 rotor). The pellet and "fluffy" layer were discarded. The supernatant was centrifuged at 78 000 g for 90 min at 4°C (Beckman LS 3801 ultracentrifuge and a 70.1 Ti rotor). This sedimented the lysosomes and microsomes. The pellet was washed by resuspending it in half of the original volume of 0.15 M potassium chloride containing 1 mM EDTA pH 7.5. To resuspend the

pellet it was constantly stirred at 4°C for 30 min. The resuspended pellet was then centrifuged at 78 000 g for 60 min at 4°C and the resulting pellet was again resuspended with constant stirring at 4°C for 30 min in 100 cm³ of 0.1 M Tris-HCl buffer pH 5.7 containing 1 mM EDTA. This solution represented the lysosomal-microsome fraction and the final volume was 206 cm³. The protein concentration was determined throughout utilising the Hartree modification of the Lowry method (288,289).

The assay for the presence of cytochrome b₅ reductase was as described by Mihara and Sato (303) with the exception that a Bausch and Lomb Spectronic 1001 was used in its kinetic mode. This assay utilised the increased reduction of 1 mM of potassium ferricyanide in the presence of 0.1 mM of NADH and cytochrome b₅ reductase. 1 unit of enzyme activity (cytochrome b₅ reductase activity) is defined as the reduction of 1 mol of potassium ferricyanide per min at 420 nm. At each stage in the isolation and purification cytochrome b₅ activity was assayed for.

Solubilisation of Cytochrome b₅ Reductase. Penicillin and Streptomycin were added to the lysosome-microsome fraction at 100 IU/cm³ and 100 µg/cm³ respectively. This solution was incubated in a 37°C water bath with occasional stirring. The solution was then centrifuged at 78 000 g for 90 min at 4°C.

The solubilised cytochrome b_5 reductase was located in the supernatant and this solution was neutralised (to pH 7.0) with 1.0 M sodium hydroxide. If necessary the solution can be frozen at this stage for several weeks without significant loss of enzyme activity. The final volume of this solution was 92 cm³.

Ammonium Sulphate Fractionation of Cytochrome b_5 Reductase. Ammonium sulphate was added to the solution until 50% saturation was achieved (291 mg of ammonium sulphate/cm³). The solution was left to stand at 4°C for 1 hr to allow for some protein precipitation to occur. The solution was centrifuged at 8000 g for 15 min to remove any precipitate that may have formed. The supernatant was then brought to 75% saturation and was again left to stand at 4°C for 1 hr. The solution was centrifuged at 8000 g for 15 min. The pellet was dissolved in 20 cm³ of 20 mM Tris-HCl buffer pH 8.5.

Sephadex G-100 chromatography. The solution was passed through a Sephadex G-100 column (3 x 70 cm) at a rate of approximately 30 cm³/hr. The column had been previously packed and equilibrated with 20 mM Tris-HCl buffer pH 8.5. The enzyme solution was eluted from the column utilising the same buffer. Fractions of 10 cm³ were collected and fractions 21-29 were found to contain high cytochrome b_5 reductase activity and were combined giving a final volume of 90 cm³.

DEAE-cellulose chromatography. The eluent was passed through a DEAE-cellulose column (2.5 x 16 cm) which had previously been equilibrated with 20 mM Tris-HCl buffer pH 8.5. The flow rate of the column was approximately 60 cm³/hr. Once the eluent had been applied to the column, it was washed with 20 cm³ of 50 mM Tris-HCl buffer pH 8.5 and then eluted with an increasing linear concentration gradient of potassium chloride. The gradient consisted of 150 cm³ of 50 mM Tris-HCl buffer pH 8.5 and 150 cm³ of 50 mM Tris-HCl pH 8.5 with 0.1 M potassium chloride. The eluent was collected in 10 cm³ fractions and fractions 15-21 were combined as they were found to contain the cytochrome b₅ reductase and had a final volume of 80 cm³.

CM-Sephadex C-50 chromatography. Mihara and Sato (303) suggested that after the DEAE-cellulose column a CM-Sephadex C-50 column should be utilised, as they had shown that this resulted in greater purification. A CM-Sephadex C-50 column (1 x 15 cm) was prepared and equilibrated with 10 mM potassium phosphate buffer pH 6.5. The cytochrome b₅ reductase solution was diluted 10-fold with deionised water and was then applied to the column. The column was eluted at a flow rate of approximately 60 cm³/hr and 10 cm³ fractions were collected. An increasing linear concentration gradient of phosphate was set up using 100 cm³ of 10 mM potassium phosphate buffer pH 6.5 and 100 cm³ of 100 mM potassium phosphate pH 6.5. The cytochrome b₅ reductase did not bind to the column and thus the eluent (the

volume eluted through the column before the fractions were collected) and fractions 1-6 were combined as they contained the cytochrome b₅ reductase activity.

Dialysis and Freeze Drying. The cytochrome b₅ reductase solution was dialysed against 50 mM potassium phosphate buffer pH 7.5 with 1 mM EDTA at 4°C for 20 hrs. The dialysate had a final volume of 112 cm³. It was necessary to store the enzyme for a long period of time and it had been reported (303,311) that the dialysate lost activity after several months. Thus, in contrast to the other studies, the dialysate was freeze dried. The resulting powder was redissolved in 5 cm³ of 50 mM potassium phosphate buffer pH 7.5 with 1 mM EDTA and was freeze dried once more to yield a crystalline form of cytochrome b₅ reductase.

Disc gel electrophoresis. Disc gel electrophoresis was performed on the freeze dried cytochrome b₅ reductase, at room temperature and according to the methodology of Clark and Switzer (284). The fixing, staining and destaining were carried out as in section 2.2.3.

3.3 Results

Fig. 10 shows the elution profile of cytochrome b₅ reductase from the Sephadex G-100 gel filtration column. The cytochrome b₅ reductase

was eluted in a relatively narrow peak. Relatively large amounts of protein, were also eluted from the column in other fractions.

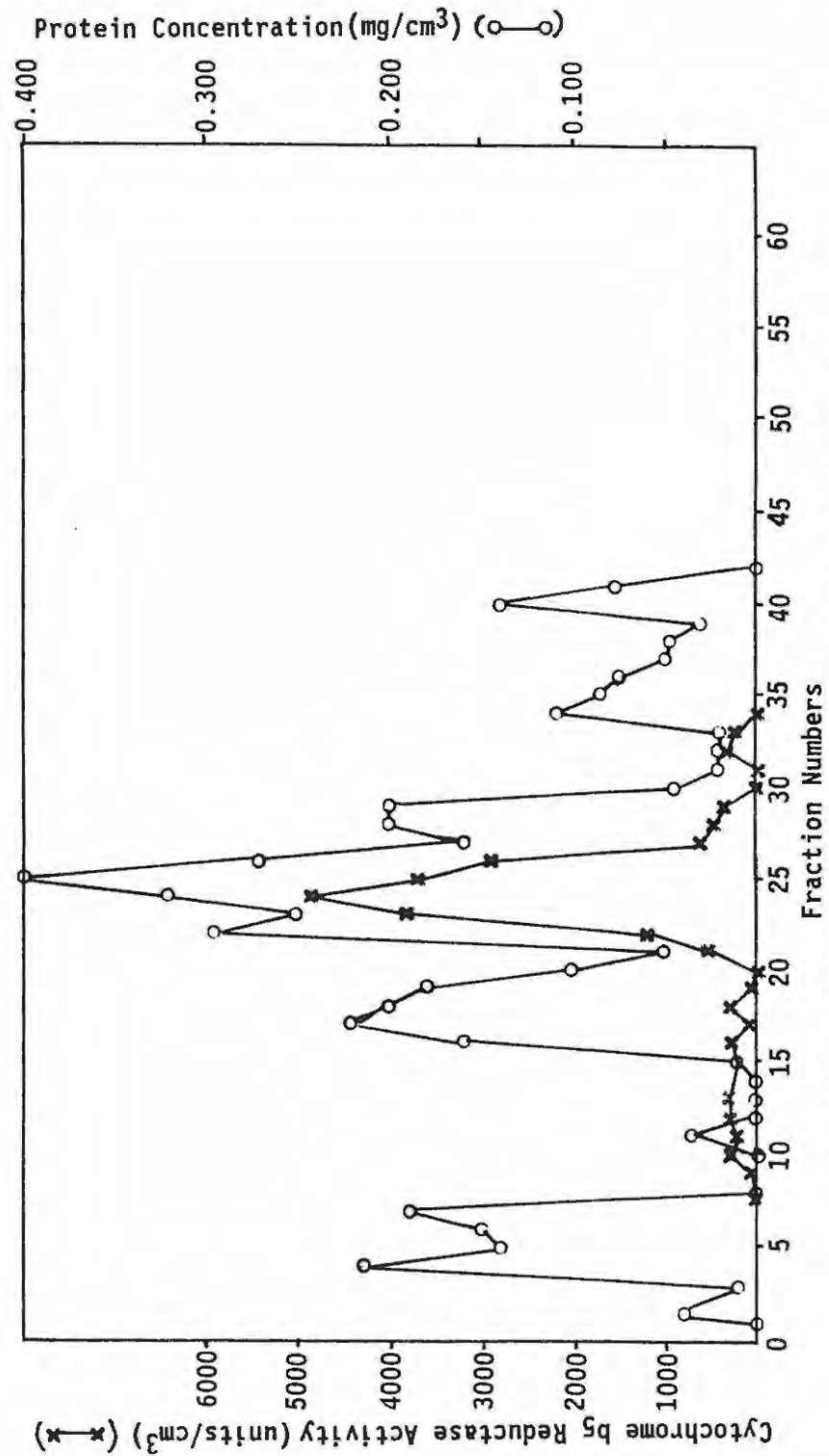


Figure 10. The elution profile of Cytochrome b₅ Reductase from the Sephadex G-100 Column.

Fig. 11 shows the elution profile of cytochrome b₅ reductase from the DEAE-cellulose column. The enzyme was eluted at 0.061 M potassium chloride. Again the cytochrome b₅ reductase was eluted in a relatively narrow peak.

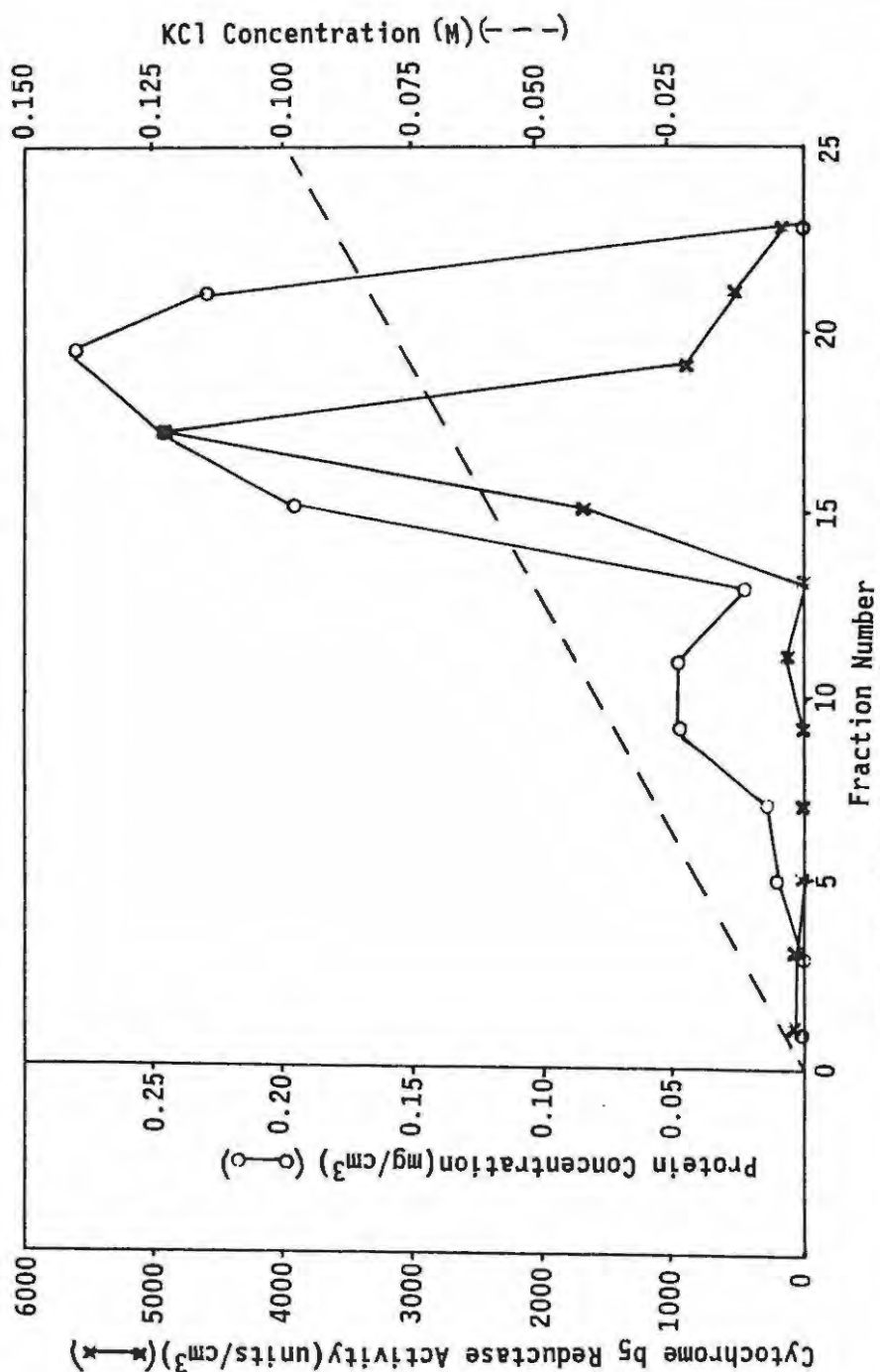


Figure 11. The elution profile of cytochrome b₅ reductase from the DEAE-Cellulose column.

Fig. 12 shows the elution profile of cytochrome b_5 reductase from the CM-Sephadex C-50 column. It was expected that the cytochrome b_5 reductase would bind to the column, but instead the enzyme activity was located in the eluent (fractions were only collected once the entire enzyme solution had been applied to the column) and the first six fractions.

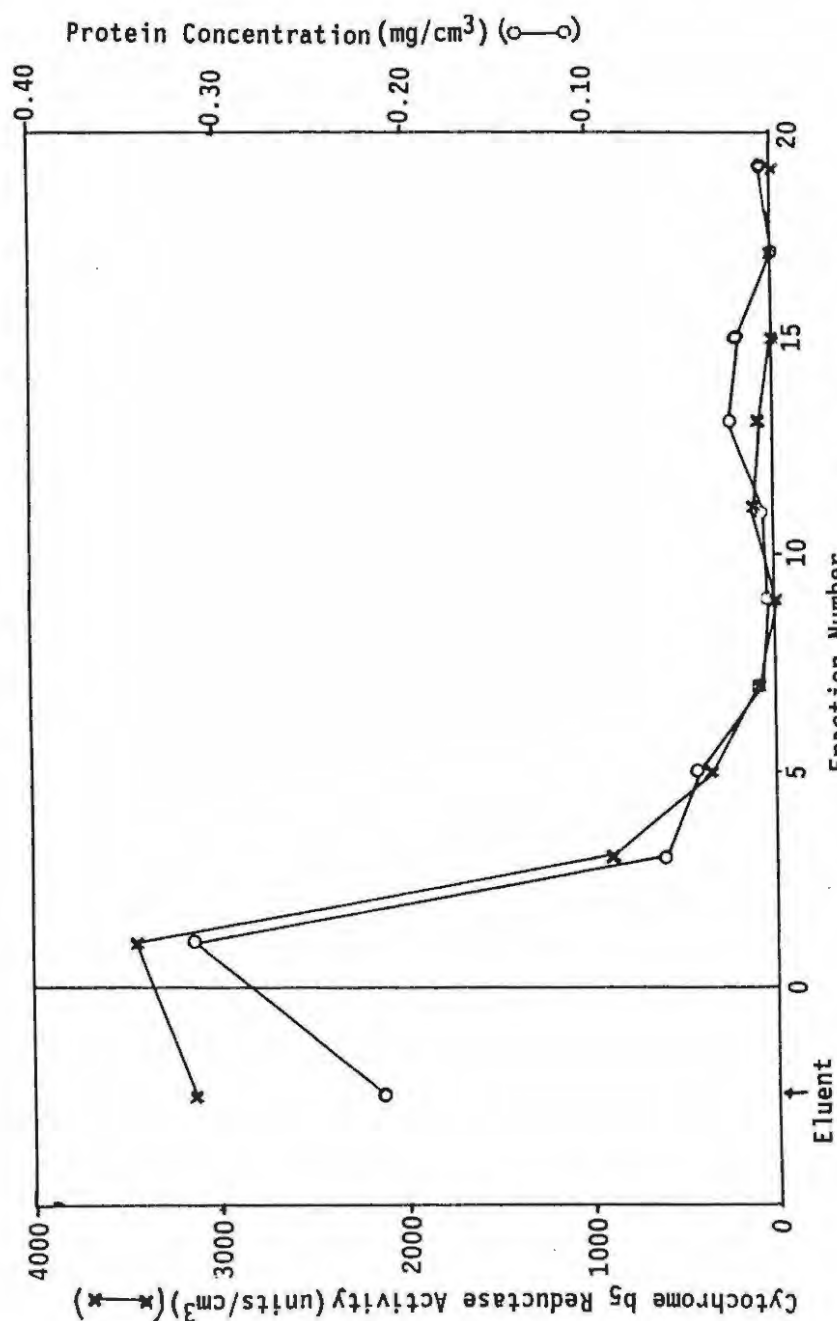


Figure 12. The elution profile of cytochrome b_5 reductase from the CM-Sephadex C-50 column.

Table 3 is the purification table of cytochrome b_5 reductase. This table shows that 9.8 mg of cytochrome b_5 reductase was isolated from 100 g of starting rat liver. This is a 9-fold increase in yield of that cited in literature (303). Literature reports have also cited, as with cytochrome b_5 , that starting quantities of rat liver were usually in the kgs and not grams (303,311). Unlike the cytochrome b_5 isolation and purification, the largest increase in specific activity came from the utilisation of the gel filtration step i.e. most of the contaminating proteins were removed at this stage. Of the column chromatography steps only the CM-Sephadex C-50 did not seem to be really necessary to this isolation and purification procedure.

TABLE 3: PURIFICATION OF CYTOCHROME b_5 REDUCTASE

Step	Total protein (mg)	Total activity (units x 10^3)	Specific activity		Yield (%)
			units/mg	ratio	
Lysosome-microsome	1747	851	487	1.00	100
Digested supernatant	226	133	588	1.21	16
Ammonium sulphate fraction	171	111	649	1.33	13
Sephadex G-100 gel filtration	37.8	95.2	2519	5.17	11
DEAE-cellulose chromatography	25.6	69.2	2703	5.55	8
CM-Sephadex C-50 chromatography	24.6	92.3	3752	7.70	11
Dialysis	10.3	48.5	4708	9.67	6
Freeze drying	9.8	47.2	4816	9.89	6

Disc gel electrophoresis showed one discrete band in the gels indicating that cytochrome b₅ reductase had been isolated in a pure form. As explained in Chapter 2, no photographs have been included in this thesis although they have been included in a previous publication (292) even though the bands were only visible to the naked eye. The R_f value of the cytochrome b₅ reductase band was approximately 0.80 - 0.85. The disc gel electrophoresis was conducted several times with very similar R_f being shown.

3.4 Discussion

The potassium ferricyanide assay had been chosen to assay for the presence of cytochrome b₅ reductase in preference to the cytochrome b₅ assay. The difference between the two assays is that in the cytochrome b₅ assay, cytochrome b₅ reductase transfers its electrons to cytochrome b₅ and the reduction of cytochrome b₅ is followed at 423 nm in the presence of NADH to assay for the activity of cytochrome b₅ reductase whereas in the potassium ferricyanide assay, cytochrome b₅ reductase transfers its electrons to potassium ferricyanide, and the reduction of the potassium ferricyanide, in the presence of NADH, is followed at 420 nm to indicate the activity of cytochrome b₅ reductase. The potassium ferricyanide assay was chosen in preference to the cytochrome b₅ assay as this latter assay is known to vary according to the amount of free and bound cytochrome b₅ reductase

present in a solution and also it assumes that at no time is cytochrome b₅ rate controlling and this may not always be true (317,318).

There are several points worthy of noting in this isolation and purification procedure. As mentioned previously smaller starting quantities of rat liver were used i.e. only 100 g in contrast to at least 1 kg cited in literature (303,311). This involved the killing of only 10 rats in comparison to at least 100 rats in the experiments cited in literature. The smaller quantity did not in any way affect the procedure. Instead it actually simplified and shortened the procedure. As with the cytochrome b₅ isolation, the aim was to achieve a simplified but successful methodology for the isolation and purification of a catalytically active cytochrome b₅ reductase.

During the preparation of the lysosome-microsome fraction, the pellets were resuspended at 4°C for 30 min to ensure that they were completely resuspended. It was found that if the majority of the lysosomes and microsomes were not in solution, the resulting yield of cytochrome b₅ reductase was very low. Although previous studies have indicated that the entire isolation and purification procedure should be conducted at 4°C, this was found to be unnecessary and only the ultracentrifugation spins, the two resuspension stages and the dialysis stage were conducted at 4°C. This greatly simplified the

methodology and the isolated cytochrome b₅ reductase retained a high level of activity even when the chromatography was conducted at room temperature.

Takesue and Omura (311) found that the cytochrome b₅ reductase was eluted from the DEAE-cellulose column at approximately 0.05 M potassium chloride, but in this study it was found that the enzyme eluted at 0.061 M potassium chloride. As stated in the results, although Mihara and Sato (303) found that a CM-Sephadex C-50 column resulted in much greater purification, in this study it was found that there was very little or no binding of cytochrome b₅ reductase to the column. Thus, it is suggested that in future isolations this step be omitted.

Due to the problems experienced in this study with the attempted crystallisation of cytochrome b₅ and the lack of reports of attempts to crystallise cytochrome b₅ reductase, it was decided to freeze dry the cytochrome b₅ reductase solution. There were no contaminating proteins present (as shown by disc gel electrophoresis) and thus freeze drying would not result in any contamination of the powder by other proteins. This also allowed for the enzyme to be stored for a long period without appreciable loss of activity. As noted in the results, in the dialysate solution loss of the enzyme activity was found if it was stored for more than a few months.

The disc gel electrophoresis of the freeze dried cytochrome b₅ reductase indicated the presence of one band and thus that the enzyme had been isolated in a pure form. This procedure was repeated several times and no contaminating compounds were found. Takesue and Omura (311) had found that two bands were produced in the gels of cytochrome b₅ reductase they had isolated. They discovered that this second band was a modified cytochrome b₅ reductase which still retained the characteristics of the original enzyme. As they had incubated microsomes with continual shaking for 6 hrs, it was decided in this study, in an attempt to isolate one form of cytochrome b₅ reductase, to only incubate the lysosome-microsome fraction for 30 min with constant shaking. This did result in the isolation of one pure form of cytochrome b₅ reductase.

In general the fold purification achieved by Takesue and Omura (311) was generally much greater than that achieved in this study but this could be a result of the very low activities noted by Takesue and Omura (311) in the first step i e the lysosome-microsome step. In this study there was more activity found in the first step than in a previous report (311). The greatest fold purification achieved by Takesue and Omura (311) was that from the lysosome-microsome to the digested supernatant step i e from 1.0 to 7.9 fold purification whereas this study showed only a 1.0 to 1.21 fold purification at this stage and the greatest fold purification found in this study was from the ammonium fractionation step to the Sephadex G-100 step where the fold purification increased from 1.33 to 5.17. The final fold

purification achieved by Takesue and Omura (311) was 723.0 and in this study it was 9.89. The percentage yield in the study by Takesue and Omura (311) was 41% and in this study it was 6%. The purification table in this study shows an anomaly as the percentage yield decreases from 11% to 8% in going from Sephadex G-100 to the DEAE-cellulose chromatography and then it increases back to 11% after the CM-Sephadex C-50 step. This procedure was repeated several times and each time the same result was found. There seems to be no clear explanation for this occurrence. Although the fold purifications and the final percentage yield were lower than those achieved in the study of Takesue and Omura (311) the final enzyme yield achieved in this study was approximately 9 times greater than that achieved previously.

From these results, it can be seen that a catalytically active and pure form of cytochrome b₅ reductase could be easily isolated. Although the yield of enzyme was less than that reported previously, the total enzyme recovery relative to the quantity of starting material utilised was far greater. This methodology was conducted several times with very similar results being obtained and thus this methodology can be utilised in the isolation of Δ^6 -desaturase from the linoleoyl-CoA desaturase complex.

CHAPTER 4

THE ISOLATION AND PURIFICATION OF LINOLEOYL-CoA DESATURASE

4.1 Introduction

Linoleoyl-CoA desaturase [EC 1.14.99.25] is a multicomponent enzyme system which consists of cytochrome b₅ reductase, cytochrome b₅, and the terminal enzyme Δ^6 -desaturase (see Fig. 13). Linoleoyl-CoA desaturase is classified as a mixed function oxidase and the reaction of interest in which it plays a role is the conversion of cis-LA into GLA (when they are in the CoA form). This is the rate limiting step in the metabolism of the ω -6-EFAs (45,326-329).

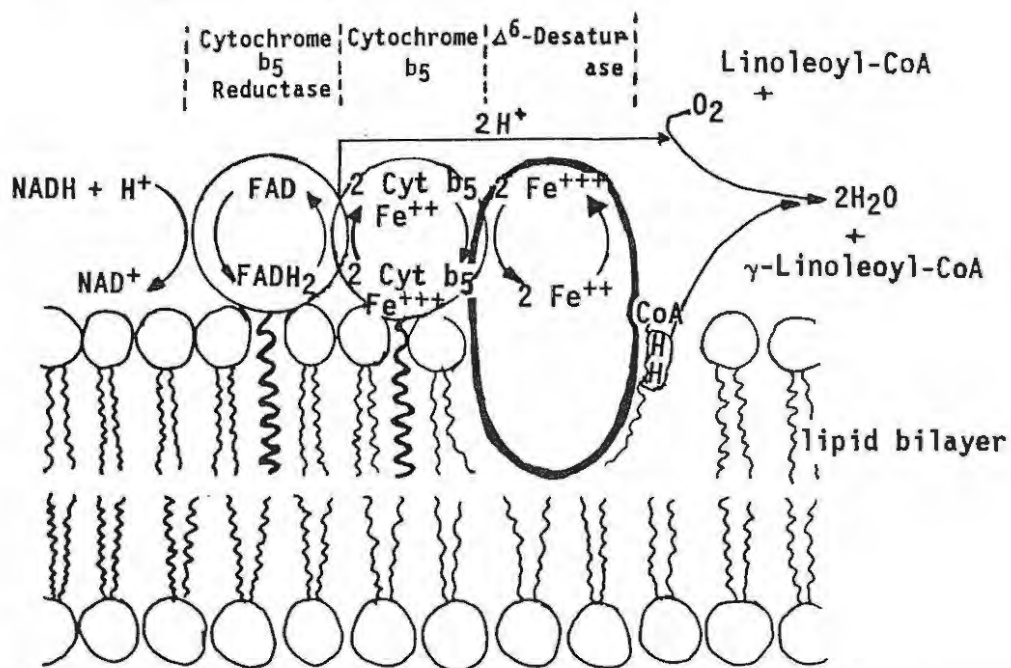


Figure 13. The hypothetical structure of the components of the Linoleoyl-CoA Desaturase complex bound to the microsomal lipid bilayer (modified from 326).

In contrast to cytochrome b_5 and cytochrome b_5 reductase little is known about this enzyme complex. Linoleoyl-CoA desaturase was first solubilised in 1979 by Okayasu et al (330) when they utilised various detergents i.e Triton X-100, sodium cholate and sodium deoxycholate to solubilise the enzyme complex from microsomes. They took this work further in 1981 when they isolated linoleoyl-CoA desaturase. They reported that linoleoyl-CoA desaturase is a nonpolar single polypeptide with a MW of approximately 66 000 (328). Until the present no further work on the isolation and purification of linoleoyl-CoA desaturase has been reported.

It has been reported (326,329,331) that linoleoyl-CoA desaturase is bound to the endoplasmic reticulum on the cytoplasmic surface and that this enzyme complex is not present in a latent state in the endoplasmic reticulum but is constantly in an active form. This report verifies what was expected as cytochrome b_5 reductase and cytochrome b_5 transfer their electrons to Δ^6 -desaturase via their catalytic segments and it is known that both cytochrome b_5 reductase and cytochrome b_5 have their catalytic segments orientated on the cytoplasmic surface of the endoplasmic reticulum (326,328-331).

It has been suggested that since linoleoyl-CoA desaturase is unable to desaturate LA unless it is in the CoA form, that CoA plays a role in the binding of the substrate to the enzyme complex (cited in 331,332).

Brenner (333) also suggested that when the substrate is bound to the enzyme the 6-7 carbons of the substrate are located in front of the active centre of the enzyme, so that the double bond can be inserted. This hypothesis has been supported by Castuma et al (331).

Detergents were utilised to solubilise linoleoyl-CoA desaturase because detergents are typical amphipathic compounds like phospholipids and as such are extremely useful in solubilising enzymes from membranes and maintaining them within a simulated membrane environment i.e. a narrow band of hydrocarbon bounded by water soluble polar groups that allows them to remain catalytically active (263,267,334). The structure and localisation of linoleoyl-CoA desaturase (discussed previously) show the necessity for maintaining the entire enzyme complex in as natural an environment as possible and enzymatic solubilisation would in all probability only result in partial solubilisation of the enzyme complex.

It was decided to attempt to isolate and purify the enzyme complex, linoleoyl-CoA desaturase in order to utilise the complex for the isolation of Δ^6 -desaturase via the enzymatic removal of cytochrome b₅ and cytochrome b₅ reductase from the complex. Detergent solubilisation of the complex from microsomes was to be attempted and hopefully a simplified methodology and increased yield of linoleoyl-CoA desaturase would result.

4.2 Materials and Methods

4.2.1 Reagents

Homogenisation buffer. As in section 2.2.1.

Protein solution A. As in section 2.2.1.

Protein solution B. As in section 2.2.1.

Protein solution C. As in section 2.2.1.

Protein standard solution. As in section 2.2.1.

DEAE-cellulose. DEAE-cellulose was purchased as the chemical Cellex-D (Bio-Rad Laboratories, USA) and was activated as specified in Clark and Switzer (284).

CM-Sephadex C-50. CM-Sephadex C-50 (Pharmacia Fine Chemicals, Sweden) was activated as specified in Clark and Switzer (284).

4.2.2 Animals

As in section 2.2.2.

4.2.3 Methods

Unless stipulated all procedures were conducted at room temperature.

Microsome preparation. The procedure for the preparation of microsomes was the same as in section 2.2.3. Three rats were killed and the starting mass of rat liver used was 25 g. The final volume of microsome solution was 45 cm³.

The assay for linoleoyl-CoA desaturase activity was a cytochrome b₅ reoxidation assay. The difference between the time for NADH (3 μM per assay) oxidation in the presence and absence of linoleoyl-CoA desaturase, indicated by the onset of cytochrome b₅ reoxidation, was used to calculate linoleoyl-CoA desaturase activity. This assay was followed utilising a Bausch and Lomb spectronic 1001, using the dual wavelength programme at 423nm (the Soret peak of reduced cytochrome b₅) and 410nm. Rotenone (2 μM per assay) was added to the assay mixtures to prevent any mitochondrial contamination i.e. any extra reduction of NADH. Assays were conducted as pairs, with the one assay mixture (containing sample, Rotenone and buffer -0.1 M Tris-HCl pH 7.4) being incubated for 4 min at room temperature and then the NADH was added but no substrate i.e. linoleoyl-CoA and the second assay mixture was also incubated at room temperature and then both NADH and

substrate (20 μ M per assay) were added. The assays were followed for 5 min with readings being taken every 6 seconds. The difference in the rates of cytochrome b₅ reoxidation was utilised to calculate the linoleoyl-CoA desaturase activity which had the units of μ mol of product formed/min (275,290,335).

At each stage in the isolation and purification procedure protein assays were conducted utilising the Hartree modification of the Lowry method of protein estimation (288,289) and cytochrome b₅ reoxidation assays were conducted utilising the above assay procedure.

Solubilisation of linoleoyl-CoA desaturase. From this stage the isolation and purification of linoleoyl-CoA desaturase is a modification of the methodology utilised by Okayasu *et al* (328). Ten percent (w/v) Triton X-100 was added to the microsome solution until a final concentration of 2% Triton X-100 was achieved. This solution was then stirred on ice for 30 min to solubilise linoleoyl-CoA desaturase. The volume of this mixture was 56 cm³. The microsome-2% Triton X-100 solution was then diluted with 20 mM Tris-HCl buffer pH 7.5 to lower the Triton X-100 concentration to 0.5%. This solution was then centrifuged at 77 000 g for 90 min at 4°C. The volume of the supernatant after the centrifugation was 196 cm³. All buffers used in further steps in this procedure, unless specified, contained 0.5% Triton X-100 and 10% glycerol as stabilisers.

DEAE-cellulose chromatography. The supernatant was passed through a DEAE-cellulose column (2.5 x 30 cm) at a rate of approximately 30 cm³/hr. The column had previously been equilibrated with 20 mM Tris-HCl buffer pH 7.5. The linoleoyl-CoA desaturase did not bind to the column and it was eluted from the column utilising the same buffer with which the column had been equilibrated. Fractions of approximately 4.5 cm³ were collected and fractions 31-37 were found to contain the linoleoyl-CoA desaturase and were combined to yield a final volume of 31.8 cm³.

CM-Sephadex C-50 chromatography. The linoleoyl-CoA desaturase solution was adjusted to pH 7.3 with 0.3 N HCl. This solution was then passed through a CM-Sephadex C-50 column (2.5 x 25 cm) which had been equilibrated with 20 mM Tris-HCl buffer pH 7.2. The flow rate of the column was approximately 30 cm³/hr and fractions of approximately 3.5 cm³ were collected (except fraction 7 which was 11.2 cm³). The column was eluted with the same buffer that it had been equilibrated with and the linoleoyl-CoA desaturase was eluted in the void volume of the column in fractions 6 and 7 with a final combined volume of 14.7 cm³.

Dialysis and Freeze Drying. As the buffers up to this stage had contained glycerol it was necessary to dialyse the glycerol out of the linoleoyl-CoA solution as it reduces the efficiency of freeze drying. The linoleoyl-CoA desaturase solution was dialysed at 4°C for

three days with frequent changes of the buffer (0.1 M Tris-HCl buffer pH 7.5). The resulting dialysate was then freeze dried to yield a crystalline form linoleoyl-CoA desaturase.

Disc gel electrophoresis. Disc gel electrophoresis was performed on the linoleoyl-CoA solution after its elution from the CM-Sephadex C-50 column and on the freeze dried linoleoyl-CoA desaturase. The electrophoresis was carried out at room temperature and according to the methodology of Clark and Switzer (284). The fixing, staining and destaining were conducted as in section 2.2.3.

4.3. Results

Fig. 14 shows the elution profile of linoleoyl-CoA desaturase from the DEAE-cellulose column. The linoleoyl-CoA desaturase was eluted from the column in a fairly narrow peak.

Fig. 15 shows the elution profile of linoleoyl-CoA desaturase from the CM-Sephadex C-50 column. As with the previous column the linoleoyl-CoA desaturase was eluted from the column in a fairly narrow peak.

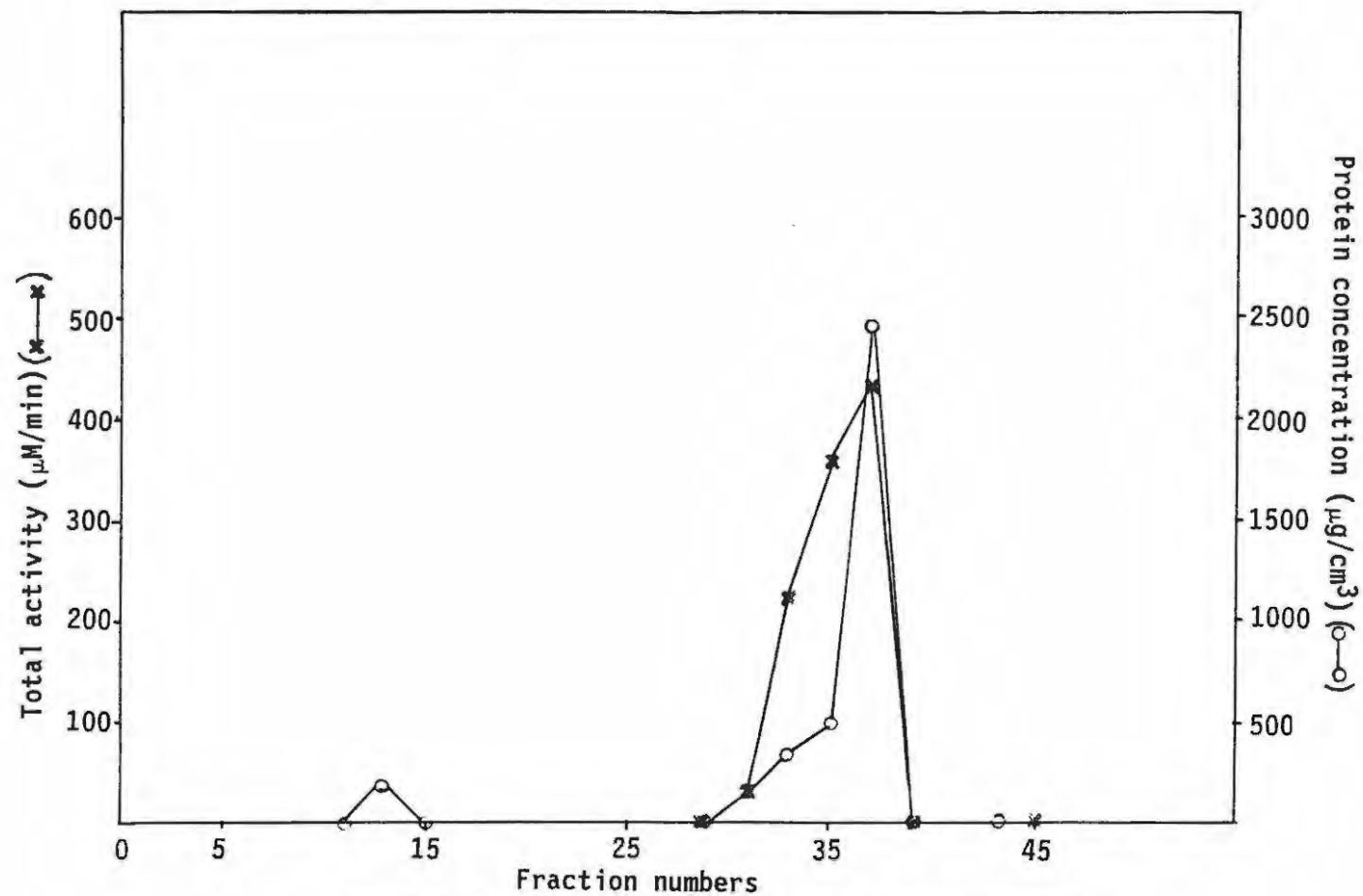


Figure 14. The elution profile of Linoleoyl-CoA Desaturase from the DEAE-cellulose column.

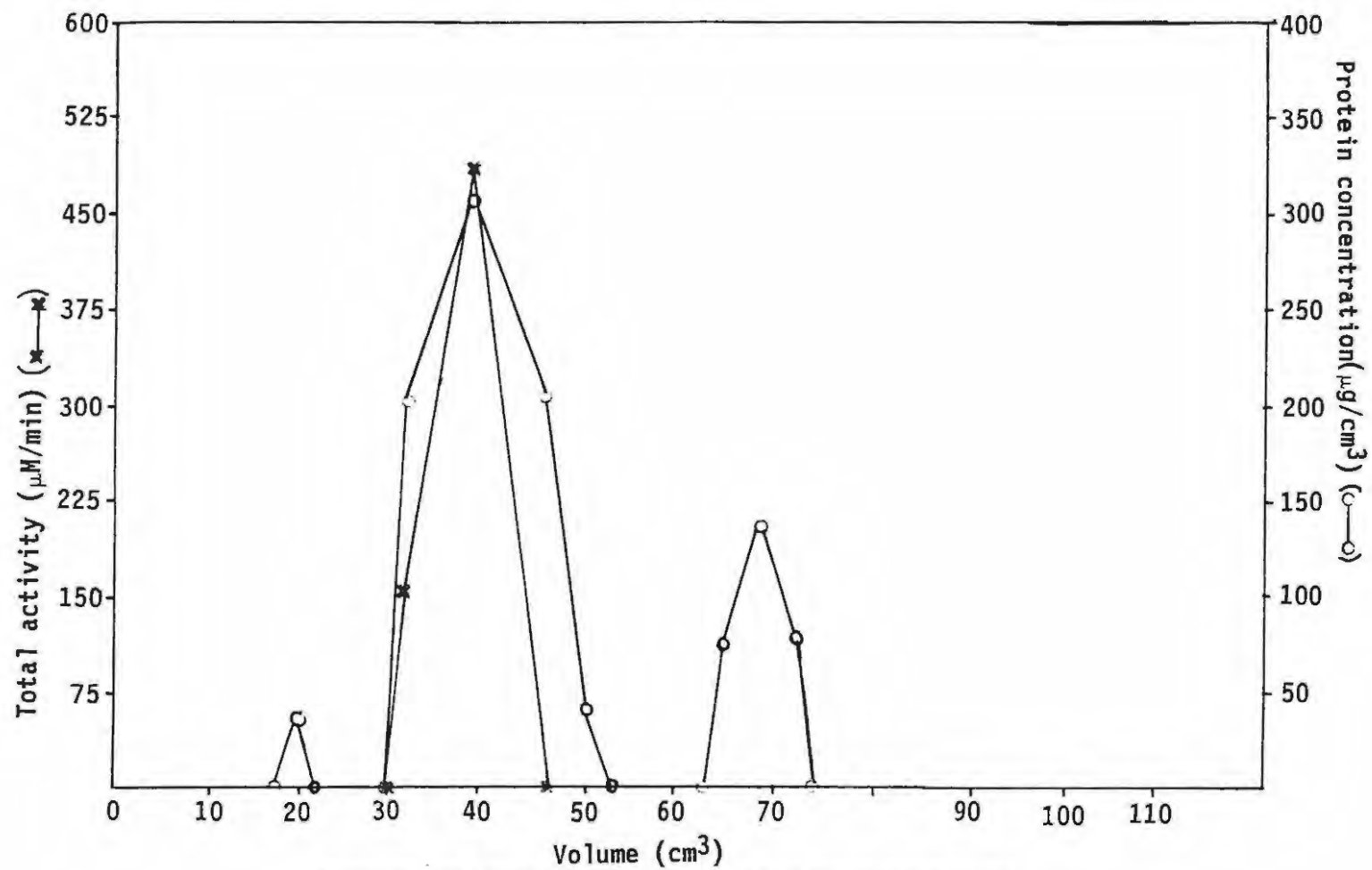


Figure 15. The elution profile of Linoleoyl-CoA Desaturase from the CM-Sephadex C-50 column.

Table 4 is the purification table of linoleoyl-CoA desaturase. The final yield of linoleoyl-CoA desaturase was 3.05 mg from 25 g of starting rat liver. This is a three- to four-fold increase in yield of that cited in literature (328). The largest increase in specific activity came from the combined Triton X-100 steps as the result of pelleting of extraneous protein. The linoleoyl-CoA desaturase did not bind to either of the columns but contaminating proteins did, as after both column steps great reductions in the total protein were found, especially after the CM-Sephadex C-50 column. It was found that a percentage yield of greater than 100% was achieved in certain steps. The possible reasons for this will be discussed later in the chapter.

TABLE 4: PURIFICATION TABLE OF LINOLEOYL-CoA DESATURASE

Step	Total Protein	Total Activity ($\mu\text{mol/min}$)	Specific Activity ($\mu\text{mol/min/mg}$) (ratio)		Yield (%)
Microsomes	1817.1	643	0.35	(1.00)	100
Triton X-100 (2%)	1271.3	1688	1.33	(3.80)	263
Triton X-100 (0.5%)	974.1	6533	6.71	(19.17)	1016
DEAE-Cellulose	263.9	3150	12.05	(34.43)	495
CM-Sephadex C-50	3.47	480	138.3	(395.10)	75
Freeze-dried	3.05	455	149.2	(426.30)	71

Disc gel electrophoresis of both the combined fractions after the CM-Sephadex C-50 column and the freeze dried powder of the linoleoyl-CoA desaturase isolation showed three discrete bands in the gels. Two of the bands were found to be characteristic, i.e. had the characteristic R_f values, of cytochrome b_5 and cytochrome b_5 reductase and the third band was assumed to be that of Δ^6 -desaturase. The third band had a R_f value of approximately 7.0 - 7.5. This seemed to indicate that the enzyme complex, linoleoyl-CoA desaturase separated into its components upon electrophoresis. If this was true then linoleoyl-CoA desaturase had been isolated in a pure form although it was not possible to confirm this because of the apparent instability of the complex during electrophoresis. As stated in Chapter 2 no photographs of the gels are included in this thesis. Photographs were included in a previous report (336) even though the bands were not clearly visible in the photographs. The bands in the gels were again, however, clearly visible to the naked eye.

4.4 Discussion

There are several points worthy of noting in the isolation and purification procedure utilised in this study. In order to prevent sacrificing an excess number of rats a smaller starting quantity of rat liver was used in this study as it was anticipated that the enzyme complex would be very quickly disrupted at room temperature conditions which were utilised at certain steps in the procedure. This was

expected due to previous literature reports (328,330) which had cited linoleoyl-CoA desaturase as being extremely labile and difficult to work with. It was decided to attempt to use room temperature at certain steps in the procedure, instead of 4°C as previously used (328,330), as room temperature had been used very successfully in this study in the isolation and purification procedures of cytochrome b₅ and cytochrome b₅ reductase despite the fact that in all previous studies the isolation of these enzymes was conducted at 4°C. As the results showed the enzyme complex was not in fact labile when left at room temperature and the complex in general was found to be as stable at room temperature as was found with the cytochrome b₅ and cytochrome b₅ reductase. As stated in Chapter 2, it was preferable to work at room temperature where possible in order to simplify the procedure. Although a smaller starting quantity of liver was utilised it in no way hindered the isolation and purification procedure.

This was the first study to utilise the cytochrome b₅ reoxidation assay as an indication of the activity of linoleoyl-CoA desaturase. Previously reported (328,330) assays were time consuming since they involved thin layer chromatography of saponified and methylated EFAs in order to assay the quantity of radioactive GLA formed. As this procedure involved attempting to isolate and purify a supposedly labile enzyme complex it was deemed necessary to utilise an assay which was quicker and thus a procedure which could be concluded in a shorter time period. Cytochrome b₅ reoxidation has been used to

assay the activity of both stearyl-CoA desaturase and malonyl-CoA desaturase (275,290,335). In a literature report a comparison was made between the thin layer assay and the cytochrome b₅ reoxidation assay for stearyl-CoA desaturase and it was noted that both assays gave very similar results (275). The cytochrome b₅ reoxidation assay is very specific for linoleoyl-CoA desaturase as the substrate for this enzyme complex is added to the assay, i.e. linoleoyl-CoA, and only when it has been converted into γ -linolenoyl-CoA will the cytochrome b₅ molecules become reoxidised. If linoleoyl-CoA desaturase is inactive then the cytochrome b₅ molecules are unable to pass their electrons to Δ^6 -desaturase and thus are unable to become reoxidised. The possibility of any external NADH or cytochrome b₅ reoxidation involvement is removed by the addition of rotenone, which blocks any mitochondrial oxidation of NADH. Thus this assay is quick, easily reproducible and utilises less equipment and chemicals.

Triton X-100 was utilised as the detergent to solubilise linoleoyl-CoA desaturase as it is a non-ionic detergent and some literature results have stated that ionic detergents can denature proteins (263,334). Sodium deoxycholate was utilised as the solubilising detergent in one attempted isolation procedure but this resulted in total loss of linoleoyl-CoA desaturase activity and it was presumed that this was either the result of total protein denaturation or of the breakage of ionic bonds which could possibly be involved within the enzyme

complex. A previous report (330) has stated that sodium deoxycholate can be utilised to solubilise linoleoyl-CoA desaturase but results from this study did not agree with this finding. Triton X-100 is known to interfere with protein assays and an attempt to overcome this was made by assaying small volumes of samples to reduce the Triton X-100 concentration and by ensuring that the protein standards contained the same quantity of Triton X-100 (263). However, some interference from Triton X-100 was in fact found. The presence of Triton X-100 is probably the reason why certain percentage yields were greater than 100% i.e. it interfered with the protein assays despite attempts made to reduce the interference. It is possible to remove Triton X-100 from samples but the methodologies involved are time consuming and expensive and tend to only be useful for large sample volumes. It would only be necessary in this study to remove the Triton X-100 from those samples being assayed for protein and enzyme activity and thus the volumes would be very small and the number of samples assayed was large. It would also not be advantageous to remove the Triton X-100 from the entire samples obtained as the main function of this detergent is to act a stabiliser of the enzyme complex. The methods available for the removal of Triton X-100 utilise activated charcoal (337), dialysis, density gradient centrifugation, gel filtration (cited in 337) and Bio-Beads (338). None of these methods lend themselves easily to use in this study.

The Triton X-100 solubilisation of linoleoyl-CoA desaturase resulted in an increase in fold purification of 3.80. When the solution was diluted (to reduce the percentage of detergent) and centrifuged (to remove extraneous proteins) there was a larger increase in fold purification of 19.17. Okayasu et al (328) also noted that Triton X-100 solubilisation resulted in an increase in purification but they noted a smaller increase, 1.47, than was found in this study. Triton X-100 thus had the dual role of solubilising linoleoyl-CoA desaturase and precipitating some extraneous protein.

The DEAE-cellulose chromatography resulted in an 80% increase in fold purification i.e. from 19.17 to 34.43 as a result of binding contaminating proteins and eluting the linoleoyl-CoA desaturase through the column without any binding. A similar increase was also noted by Okayasu et al i.e. from 1.47 to 4.07 (328).

In this study the greatest increase in fold purification was achieved with the CM-Sephadex C-50 step as the fold purification increased to 395.1. This step also resulted in the largest reduction in total protein i.e. from 263.9 mg to 3.47 mg. Okayasu et al (328) accounted for this by suggesting that although the linoleoyl-CoA desaturase did not bind to the column all the contaminating cytochrome P-450 did. Okayasu et al (328) did not find as great a reduction in total protein

i.e. from 321 mg to 116 mg or as great an increase in the fold purification i.e. from 4.07 to 8.0 as was noted by this study. From these results it can be seen that both the columns utilised in this study were very effective in further purifying linoleoyl-CoA desaturase.

The final yield in this study was 3.05 mg of linoleoyl-CoA desaturase from 25 g of starting rat liver, this was three to four times greater than that achieved by Okayasu et al (328). This was a percentage yield of 71% in comparison to the 12% achieved by Okayasu et al (328). They also utilised affinity chromatography in their study (328) i.e. cytochrome b₅-sepharose. It was at this stage that Okayasu et al (328) found the greatest fold purification (from 8.0 to 229.33). In this study the linoleoyl-CoA solution was freeze dried once it had eluted from the CM-Sephadex C-50 column and had been dialysed.

Disc gel electrophoresis was performed on fractions of both the eluent from the CM-Sephadex C-50 column and the freeze dried powder. Both fractions showed the same three bands in the gels and as two of the bands were characteristic of those of cytochrome b₅ and cytochrome b₅ reductase (showed characteristic R_f values) it was presumed that the third band was probably that of Δ^6 -desaturase. It was decided that it was unnecessary to conduct the cytochrome b₅-sepharose chromatography as in this study greater purification had already been achieved without this step than Okayasu et al (328) had

achieved with the inclusion of this step. The freeze dried linoleoyl-CoA desaturase had a final fold purification of 426.3. Okayasu et al (328) did not freeze dry their final linoleoyl-CoA solution but instead froze it under nitrogen at -70°C. The freeze dried powder was more stable than a frozen solution as Okayasu et al (328) had reported an instability of the enzyme complex and the loss of 80% of the enzyme complex activity with repeated freezing and thawing. The freeze dried powder retained most of the linoleoyl-CoA desaturase activity for the duration of this study. As this enzyme complex was to be utilised in the attempted isolation and purification of Δ^6 -desaturase it was necessary for the enzyme complex to be in as stable a form as possible.

From these results it can be seen that a catalytically active and pure form of linoleoyl-CoA desaturase had been isolated using a simplified methodology and with a greater yield and percentage purification to that cited in the only other report on the isolation of this enzyme complex (328).

CHAPTER 5

THE ATTEMPTED ISOLATION AND PURIFICATION OF Δ^6 -DESATURASE

5.1 Introduction

Δ^6 -desaturase is the terminal enzyme in the linoleoyl-CoA desaturase enzyme complex and it is the enzyme ultimately responsible for the enzymatic conversion of cis-LA into GLA (328,331). Of all the components of linoleoyl-CoA desaturase the least is known about Δ^6 -desaturase. There is some confusion concerning the isolation and purification of this enzyme as most reports that cite work with Δ^6 -desaturase are really referring to the entire enzyme complex of linoleoyl-CoA desaturase, e g Okayasu et al (328) refer to the isolation and purification of linoleoyl-CoA desaturase in their report but in the discussion of the results they cite facts concerning Δ^6 -desaturase when these really relate to the enzyme complex. Others such as Fujiwara et al (329) have made the same error.

It is known that Δ^6 -desaturase is membrane bound, as are the other components of linoleoyl-CoA desaturase (329,331). Due to the positioning of cytochrome b₅ and cytochrome b₅ reductase within the endoplasmic reticulum, exposing their catalytic sites at the cytoplasmic side of the endoplasmic reticulum, it was assumed that

Δ^6 -desaturase had similar positioning. This assumption was made as it is essential for the activity of linoleoyl-CoA desaturase that the cytochrome b_5 molecules can pass their electrons to Δ^6 -desaturase to initiate the conversion of cis-LA into GLA (328,329).

It was decided that as so little was known about Δ^6 -desaturase the best method to utilise in the isolation of this enzyme would involve initially the isolation and purification of linoleoyl-CoA desaturase and then an attempt would be made to enzymatically remove the cytochrome b_5 and cytochrome b_5 reductase molecules from the linoleoyl-CoA desaturase complexes in the hope that a pure Δ^6 -desaturase would be the end result. As trypsin had already been utilised in the isolation and purification of cytochrome b_5 , it was decided that in this study it would again be utilised to attempt to remove the cytochrome b_5 from the linoleoyl-CoA desaturase complex. It is known that trypsin will only solubilise cytochrome b_5 and not cytochrome b_5 reductase (260,261). When cytochrome b_5 reductase was solubilised using lysosomes, one of the active enzymes within the lysosomes was cathepsin D [EC 3.4.4.23]. Cathepsin D only solubilises cytochrome b_5 reductase and not cytochrome b_5 (298,317). It was consequently decided to utilise this enzyme to solubilise cytochrome b_5 reductase from the linoleoyl-CoA desaturase complex. It was hoped that this methodology would result in the isolation of Δ^6 -desaturase.

5.2 Materials and Methods

5.2.1 Reagents

Protein solution A. As in section 2.2.1.

Protein solution B. As in section 2.2.1.

Protein solution C. As in section 2.2.1.

Protein standard solution. As in section 2.2.1.

Sephadex G-75. Sephadex G-75 (Pharmacia Fine Chemicals, Sweden) was swollen for 24 hrs in the appropriate buffer and the "dead" beads were removed from the buffer surface.

5.2.2 Methods

Unless stipulated all procedures were conducted at room temperature.

Isolation and Purification of Linoleoyl-CoA desaturase. As in Chapter 4.

Cathepsin D solubilisation of Cytochrome b₅ Reductase. The methodology utilised for the solubilisation of cytochrome b₅ reductase via cathepsin D (Sigma Chemical Company, USA) enzymatic action was a modification of the methodology of Ito (279). The cathepsin D solution (50 µg of crystalline cathepsin D was dissolved in 200 µl of 0.1 M Tris-HCl buffer pH 7.5) was added to the linoleoyl-CoA desaturase solution (0.5 mg of purified linoleoyl-CoA desaturase freeze dried powder was dissolved in 0.5 cm³ of 0.1 M Tris-HCl buffer pH 7.5). The enzyme solution was incubated at 37°C for 4 hrs with occasional shaking.

Sephadex G-75 chromatography. The enzyme solution was then passed through a Sephadex G-75 column (1.5 x 23 cm) at a rate of approximately 10 cm³/hr. The column had previously been equilibrated with 0.1 M Tris-HCl buffer pH 7.5 and it was also eluted with this buffer. Fractions of approximately 5.2 cm³ were collected.

Each fraction was assayed for the presence of protein, cytochrome b₅ reductase, cytochrome b₅ (as an indication of the location of Δ^6 -desaturase, as there is no assay for Δ^6 -desaturase that does not involve the entire linoleoyl-CoA desaturase complex) and cathepsin D. The protein assays were conducted utilising the Hartree modification of the Lowry method of protein estimation (288,289). The assay for the presence of cytochrome b₅ reductase was the same as that described

in section 3.2.3. The assay for the presence of cytochrome b_5 was the same as that described in section 2.2.3. The assay for the presence of cathepsin D was a modification of the method used by Boethling (339) in which 1 cm³ of diluted sample, 1 cm³ of haemoglobin (10 mg/cm³ dissolved in 0.5 M Tris-HCl buffer pH 4.5) and 1 cm³ of deionised water were incubated at 37°C for 5 min separately and were then mixed and incubated for a further 15 min at 37°C. After 15 min 2cm³ of 10% trichloroacetic acid (TCA) was added and the sample was placed on ice for an hour for precipitation to occur. The solution was then filtered through Whatman Number 1 filter paper and the filtrate was assayed for the presence of peptides as previously described. The presence of increased peptides in fractions is an indication of the presence of cathepsin D in those fractions. Cytochrome b_5 reductase was located in fractions 11-15 and 27. Cytochrome b_5 was located in fractions 11-15 and 19-20. Cathepsin D was located in fractions 4-6, 14-15 and 20. Fractions 11-15 were combined, with a final volume of 25.8 cm³, and were freeze dried for utilisation in the the next stage of Δ^6 -desaturase isolation. The freeze dried powder retained the enzyme activities that were present in the fractions.

Trypsin solubilisation of Cytochrome b_5 . The methodology utilised for the solubilisation of cytochrome b_5 was the same as that utilised in Chapter 2, i e a modification of that used by Kajihara and

Hagihara (260). The freeze dried powder of fractions 11-15 was dissolved in 3 cm³ of 0.1 M Tris-HCl buffer pH 7.5. Crystalline trypsin was added at 0.5% (v/v). The solution was then incubated at 37°C for approximately 4 hrs with occasional shaking.

Sephadex G-75 chromatography. The enzyme solution was passed through a Sephadex G-75 column (1.5 x 23 cm) at a flow rate of approximately 6 cm³/hr. The column had previously been equilibrated with 0.1 M Tris-HCl buffer pH 7.5. Fractions of approximately 3 cm³ were collected and they were assayed for the presence of protein, cytochrome b₅ reductase, cytochrome b₅ and trypsin. The assays for protein, cytochrome b₅ reductase and cytochrome b₅ were conducted as previously described. The assay for the presence of trypsin was the same as that for cathepsin D with the exceptions that as trypsin is an alkaline protease, unlike cathepsin D which is an acid protease, the substrate in the assay was casein (10 mg/cm³ dissolved in 0.5 M Tris-HCl buffer pH 7.5) and the assay was conducted under alkaline conditions.

Cytochrome b₅ was located in fractions 4-5, 8-9 and 41-42. Cytochrome b₅ reductase was located in fractions 7-9 and 39-41. Trypsin was located in fractions 4-7. From these results it was not known exactly where the Δ^6 -desaturase was located and fractions 8-9 were combined and freeze dried as were fractions 39-42.

5.3 Results

Fig. 16 shows the elution profile of the Sephadex G-75 column after cathepsin D solubilisation of the linoleoyl-CoA desaturase solution. The cathepsin D was eluted mainly in fractions 4-6 with small quantities being eluted in fractions 14-15 and 20. Cytochrome b₅ reductase was eluted mainly in fraction 27 and a much smaller quantity was eluted in fractions 11-15. Cathepsin D also solubilised some cytochrome b₅ and this was eluted in fractions 19-20. The major portion of cytochrome b₅ was eluted in fractions 11-15. In the fractions 11-15 it was found that there was still some cytochrome b₅ reductase present with the majority of the cytochrome b₅ present. This indicates that the solubilisation of cytochrome b₅ reductase was not totally successful. There was a relatively large quantity of protein present in these fractions as well as a small quantity of cathepsin D. It was assumed that Δ^6 -desaturase was present in these fractions because of the high concentration of cytochrome b₅ and these fractions were combined and freeze dried.

Fig. 17 shows the elution profile of the Sephadex G-75 column after trypsin solubilisation of the freeze dried fractions 11-15 from the previous stage. The trypsin was eluted in fractions 4-7. Cytochrome b₅ was eluted in fractions 4-5 on its own, in fractions 8-9 with cytochrome b₅ reductase and trypsin and in fractions 41-42 only

with cytochrome b₅ reductase. Cytochrome b₅ reductase was eluted in fractions 7-9 with cytochrome b₅ and trypsin and in fractions 39-41 where it was only found with cytochrome b₅. This indicates that the trypsin solubilisation of cytochrome b₅ was also not totally successful.

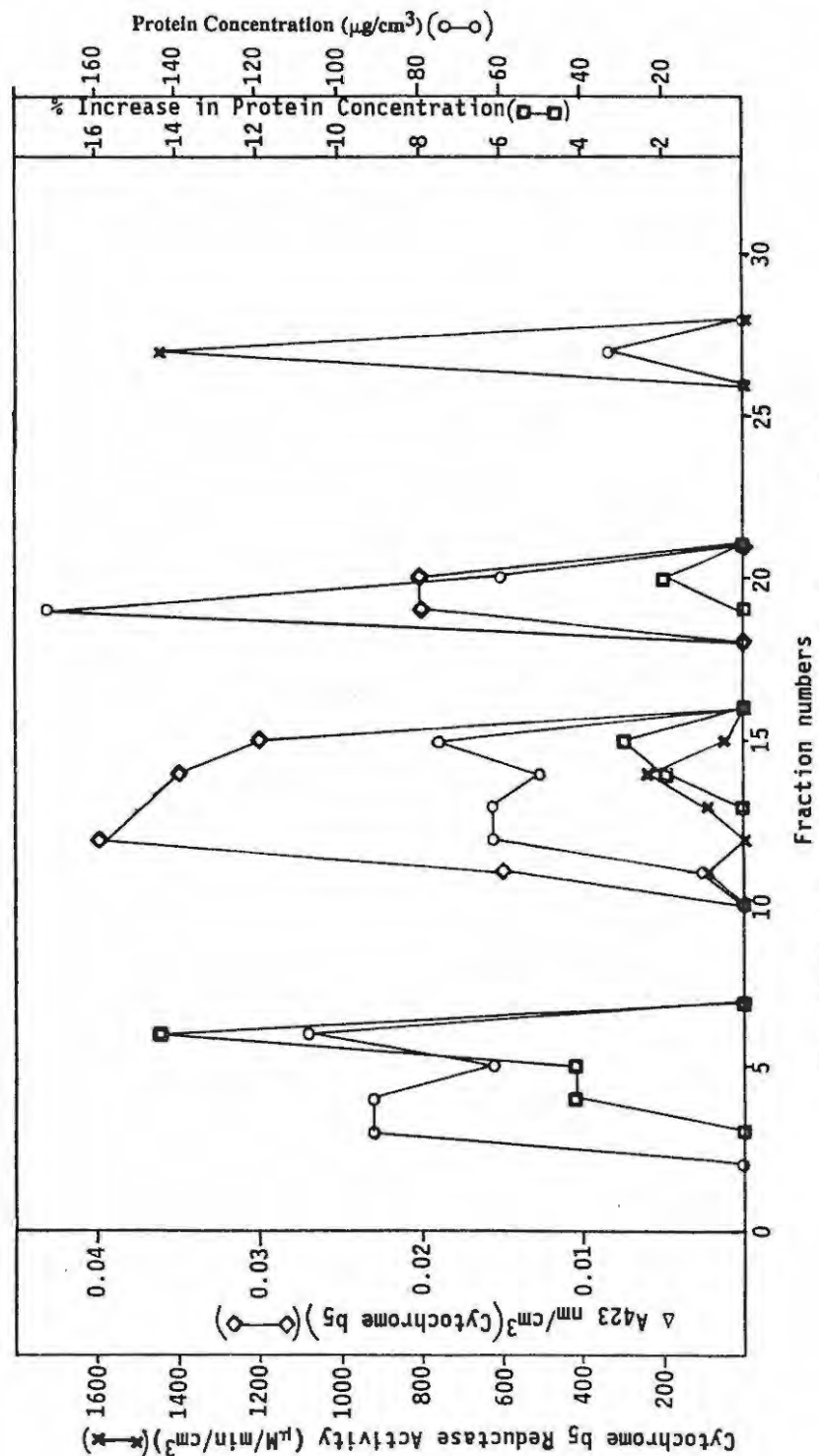


Figure 16. The elution profile of the Linoleoyl-CoA Desaturase Complex after the first Cathepsin D solubilisation.

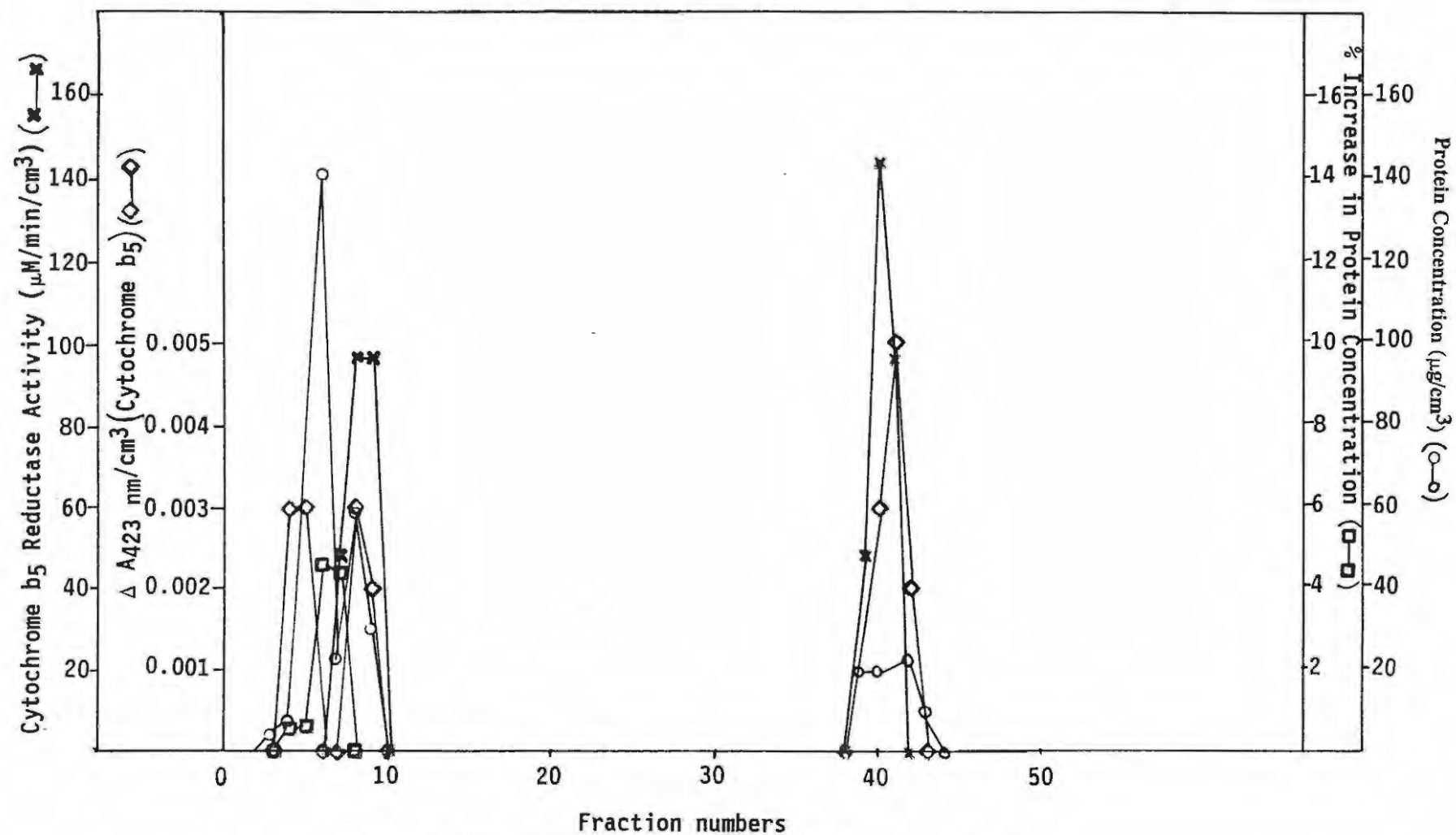


Figure 17. The elution profile of the Linoleoyl-CoA Desaturase Complex after the first Trypsin solubilisation.

Fractions 8-9 and 39-42 were freeze dried and second cathepsin D and trypsin solubilisations were conducted on both the freeze dried powders. The second solubilisations resulted in greatly reduced quantities of both cytochrome b_5 and cytochrome b_5 reductase after the chromatography steps with the result that cytochrome b_5 reductase was shown not to be present in combination with cytochrome b_5 and after the second cytochrome b_5 solubilisation with trypsin, cytochrome b_5 reductase was not found to be present at all which was partially one of the aims of this study. Cytochrome b_5 was found to be present in more than one peak even after the second solubilisation of cytochrome b_5 . This is shown in Fig. 18.

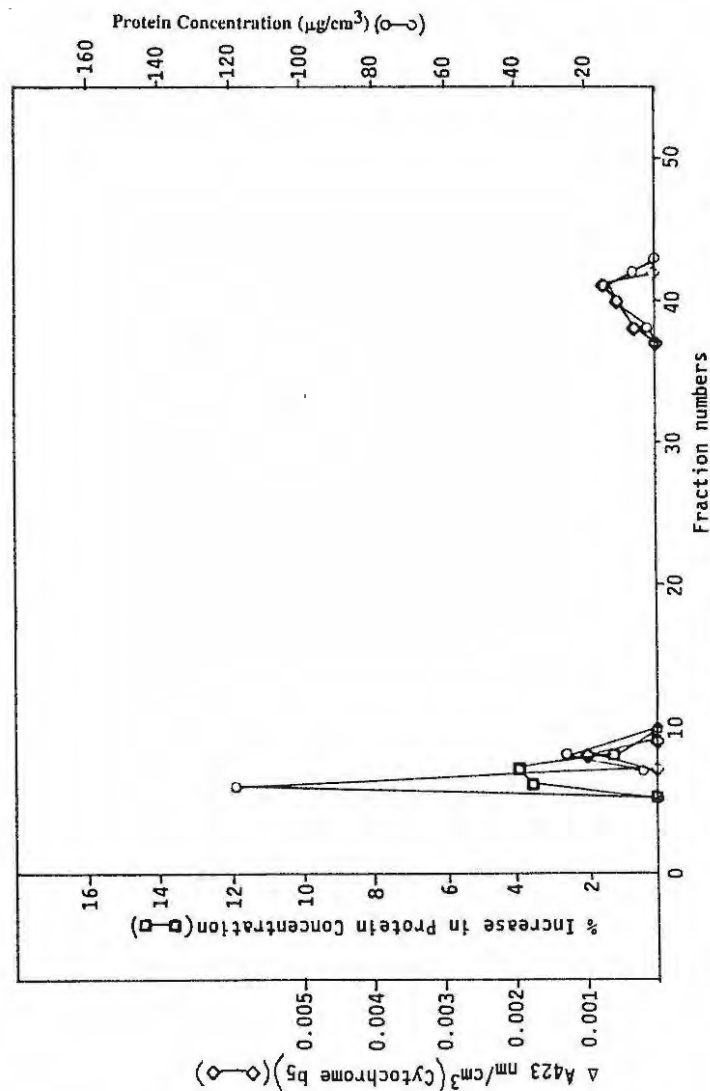


Figure 18. The elution profile of the Linoleoyl-CoA Desaturase Complex after the second Cathepsin D and Trypsin solubilisations.

5.4 Discussion

These results have shown that although Δ^6 -desaturase was not isolated in a pure form it is possible to utilise enzymatic solubilisation of the components of linoleoyl-CoA desaturase to attempt to isolate a purified form of Δ^6 -desaturase. Cytochrome b₅ reductase seems to be relatively easily removed from the linoleoyl-CoA desaturase complex after a second cathepsin D solubilisation. This is not totally unexpected as it is the first component of the linoleoyl-CoA desaturase complex and as such should be the most accessible of all the components. In the linoleoyl-CoA desaturase complex it is assumed that for every molecule of cytochrome b₅ reductase (which carries two electrons) there are two molecules of cytochrome b₅ (each of which carries one electron) and as such there is less cytochrome b₅ reductase to solubilise from the complex (see Fig. 13). Cytochrome b₅ seems to be relatively more difficult to totally remove from the linoleoyl-CoA desaturase complex and this could be due to the extra cytochrome b₅ (in comparison to cytochrome b₅ reductase) present in the linoleoyl-CoA desaturase complex.

The major problem with this isolation is the small quantities of enzyme which are present and which could result in relatively large errors in enzyme activity and protein assays. Future studies could perhaps utilise increased quantities of starting material. Disc gel

electrophoresis was run on the freeze dried powders and no bands were detected. This was repeated several times. This is probably the result of the very low concentration of the enzyme used.

The aim of this section (Chapters 2 - 5) of the thesis was to isolate the linoleoyl-CoA desaturase complex and from the complex to isolate the terminal enzyme, Δ^6 -desaturase by enzymatically solubilising the other two components of linoleoyl-CoA desaturase i.e cytochrome b₅ and cytochrome b₅ reductase. Interest was shown in this enzyme and the complex, as has previously been discussed, as it is believed to play a role in the control of cell proliferation and because it appears to have reduced activity in some cancer cells and it has been suggested that this could be one of the reasons for the loss of control of cell proliferation in cancer cells (5,22-24).

In order to enzymatically solubilise cytochrome b₅ and cytochrome b₅ reductase from the linoleoyl-CoA desaturase complex it was first necessary to isolate and purify these two components individually from rat liver microsomes. Both of these components were isolated in a purified form utilising simplified and easily reproducible methodologies and with increased yields in comparison to those reported in earlier studies. Once this had been achieved it was then possible to attempt to isolate and purify the linoleoyl-CoA desaturase complex. The complex was also isolated utilising a simplified methodology and an increased yield. Unfortunately the purification

of the Δ^6 -desaturase enzyme was not entirely successful for the reasons previously discussed.

The isolation and purification procedures were mainly conducted at room temperature with no detrimental effects. Disc gel electrophoresis showed the purity of the isolated compounds.

The final stage of this section was the attempted isolation of Δ^6 -desaturase from the linoleoyl-CoA desaturase complex. This was not totally achieved in that the presence of some cytochrome b_5 was still detected with the Δ^6 -desaturase. It was realised that the enzymatic solubilisation of cytochrome b_5 and cytochrome b_5 reductase would result in a portion of both components remaining in the membrane bilayer with the Δ^6 -desaturase but if they had been solubilised utilising detergents it was felt that this would probably result in the total disruption of the membrane bilayer and thus the complex with the resultant loss of all enzymatic activity, as the membrane bilayer is essential for enzyme activity.

This section of the thesis shows that it is possible to utilise enzymatic solubilisation of cytochrome b_5 and cytochrome b_5 reductase for the isolation of Δ^6 -desaturase.

CHAPTER 6

THE EFFECT OF GLA AND ZINC SUPPLEMENTATION ON THE GROWTH OF NORMAL AND TUMOUR CELLS IN VITRO

6.1 Introduction

One of the aims of this thesis was to study the effect of GLA, zinc and a GLA/zinc combination on the growth of normal and tumour cells in culture to determine whether the linoleoyl-CoA desaturase enzyme complex was a site of action of zinc inhibited tumour growth as it had been suggested in other studies (133,135). Cell culture was used for this purpose as it allows the utilisation of a homogeneous cell population (with a virtually conserved genetic complement) which grows in an easily manipulated environment (340). The manipulation of the environment allows for the study of the different effects nutrient supplementations have on the cell population. It is this characteristic of cell culture that is utilised in this study. Cell culture also has the advantage of allowing numerous experiments to be conducted without the ethical problems that working with live animals involve (340), although it is not always possible to directly extrapolate results achieved in in vitro experiments to those that will be achieved in in vivo situations.

GLA was utilised in this study as it is the product from the reaction when LA is converted into GLA by linoleoyl-CoA desaturase and this enzyme complex is of primary interest to this study as it has been found to have reduced activity in certain tumour cell lines (5,22-24). If there is reduced activity of the linoleoyl-CoA desaturase complex then correspondingly there will be reduced GLA produced in the tumour cells and it is hypothesised that with the supplementation of GLA, tumour growth may be inhibited (5). GLA has in fact been found to inhibit the growth of human mammary carcinomas, prostatic adenocarcinomas, lung carcinomas, neuroblastomas, hepatomas, oesophageal carcinomas and murine melanomas (211-223). GLA has also been shown to preferentially inhibit the growth of mammary carcinoma cells when placed together with normal lung cells and as such is not toxic to normal cells (211).

Zinc deficiency is well known for its inhibitory effect on the growth of tumour cells while not effecting the growth of normal cells. It has been shown to inhibit the growth of Ehrlich ascites tumours, Walker 256 carcinosarcomas, Lewis lung sarcomas, L1210 murine leukaemias, 350 hepatomas and murine melanomas (BL6) (6,239-246). Zinc supplementation (excess) has also been shown to inhibit the growth of tumour cells e g it inhibits the growth of hamster cheek pouch carcinoma, L1210 murine leukaemias, BL6 murine melanomas (244,252,253,256). Zinc is thought to be a cofactor of the linoleoyl-CoA desaturase enzyme complex (133,135) and it is possible that zinc supplementation results in the inhibition of tumour cell

growth as a result of the reactivation of linoleoyl-CoA desaturase. In this study it was decided to utilise zinc supplementation and not zinc deficiency as this is more easily controlled in culture and is of more relevance with regard to the possible therapeutic value of zinc in cancer treatment.

As stated in Chapter 1, it is possible that GLA and zinc may influence each other in the mechanism/s by which they inhibit the growth of tumour cells in culture. It has been hypothesised that a zinc deficiency could result in a reduction in the activity of linoleoyl-CoA desaturase and that this could in turn result in a lowering in the synthesis of GLA and consequently a lowering in the synthesis of cAMP which could result in an increased rate of cell proliferation (5,22-30,138,140,143). Although several reports have indicated that there is a possible relationship between zinc and EFAs, specifically GLA (133,134,137,139,141-143,146-149) there are no published reports elucidating the relationship between GLA and zinc in cell culture. This study attempted to determine if any relationship was apparent in cell culture experiments between GLA and zinc, when both were concurrently supplemented to cells and subsequently (in Chapter 7) whether this may be through an association with the linoleoyl-CoA desaturase enzyme complex.

6.2 Materials and Methods

6.2.1 Reagents

Antibiotic Solution. The antibiotic solution was prepared by dissolving one vial of sodium benzylpenicillin (10^6 IU) (Novo Industries, RSA) and one vial of streptomycin sulphate (10^6 μ g) (Novo Industries, RSA) in 100 cm³ of deionised water.

Cell Culture Media. The cell culture medium utilised was Eagle's Basal Medium (Modified) (Flow Laboratories, Scotland). Medium was prepared in batches of 10 l by dissolving the powdered medium in Milli-Q water and adding ascorbic acid (0.5g), serine (0.1g), glycine (0.06g), NaHCO₃ (7.5g) and antibiotic solution (50 cm³). The pH was checked to ensure that it was 7.2-7.4 and if necessary 1 M HCl or NaOH was added to yield the desired pH and the volume was made up to 10 l with Milli-Q water. The medium was filtered through a millipore filtration unit (Millipore Corporation, USA) utilising a Sartorius prefilter, a filter with pore size of 0.45 μ m and finally a filter with pore size of 0.22 μ m into autoclaved media bottles of volumes 100 cm³, 250 cm³ and 500cm³. This ensured that the medium should be sterile. The media bottles were then incubated for one week at 37°C to ensure no contamination had occurred and they were consequently stored at 4°C until used.

Growth medium (GM) was prepared by sterile filtering, through a 0.45 μm Millipore filter using a Swinnex-25 holder (Millipore Corporation, USA), foetal calf serum (FCS) (State Vaccine Institute, RSA) into the Eagle's Basal Medium until it contained 10 % (v/v) FCS. The GM was incubated at 37°C for approximately 48 hrs before use to ensure that no contamination of the medium had occurred. Maintenance medium (MM) was prepared by sterile filtering FCS into Eagle's Basal Medium until it contained 5 % (v/v) FCS. The MM was incubated under the same conditions as the GM. Shelf Culture medium (SCM) was prepared by sterile filtering FCS into Eagle's Basal Medium until it contained 2 % (v/v) FCS. The SCM was incubated under the same conditions as the GM. Freezing medium (FM) was prepared by adding 10 % (v/v) dimethyl sulphoxide (DMSO) and the medium was frozen until required and then it was sterile filtered in the same method as the FCS.

The GLA-supplemented media was prepared by sterile filtering the appropriate volume of a GLA solution (10 mg/cm^3 diluted in 0.1 M NaCO_3) (Sigma Chemical Corporation, USA) into 100 cm^3 bottles of GM to yield solutions containing 0 (control), 0.5, 2, 4, 6, 8 and 10 $\mu\text{g}/\text{cm}^3$ of GLA.

The zinc-supplemented media was prepared by sterile filtering the appropriated volume of a zinc standard atomic absorption (AA) spectroscopy standard solution (SMM Chemicals, USA) of 1000 $\mu\text{g}/\text{cm}^3$ into 100 cm^3 bottles of GM to yield solutions containing 0 (control), 1, 2, 3, 4, 5 and 10 $\mu\text{g}/\text{cm}^3$ of zinc.

The GLA- plus zinc-supplemented media was prepared as above to yield solutions that had the following concentrations of GLA ($\mu\text{g}/\text{cm}^3$) : zinc ($\mu\text{g}/\text{cm}^3$), 0:0, 4:2, 4:3, 4:4, 6:2, 6:3, 6:4, 8:2, 8:3, and 8:4. These concentrations were chosen as they had been shown from previous experiments to be the most probable concentrations that could be used therapeutically in the treatment of cancer.

Trypsin Subculturing Solution. The trypsin subculturing solution contained NaCl (8.0 g), KCl (0.4 g), D-glucose (1.0 g), NaHCO_3 (0.58 g), EDTA (0.2 g), phenol red (0.02 g), trypsin (5×10^5 units) (Sigma Chemical Company, USA) and antibiotic solution (10 cm^3) in 1 l.

Dulbecco's Phosphate Buffered Saline without calcium or magnesium.(Phosphate buffered saline - PBS) The PBS contained KCl (0.2 g), NaCl (8.0 g), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (1.15 g) and KH_2PO_4 (0.2 g) in 1 l.

AA Zinc Standard solution. The zinc standard solution was purchased (SMM Chemicals, USA) at 1000 parts per million (ppm) ($1 \text{ ppm} = 1 \mu\text{g}/\text{cm}^3$) and the standards were 0.1, 0.2, 0.5 and $1.0 \mu\text{g}/\text{cm}^3$.

Phenol-Cresol mixture. The phenol-cresol mixture was prepared by dissolving 500 g of detached phenol crystals and 0.5 g of 8-hydroxy-quinolone in 70 cm^3 of meta-cresol (no ortho-cresol must be present and 55 cm^3 of deionised water. The phenol-cresol mixture must be stored in an amber bottle.

DNA Standard solution. The DNA standard solution consisted of crystalline DNA (Sigma Chemical Company, USA) at $300 \mu\text{g}/\text{cm}^3$ and the standards contained 50, 100, 150, 200, 250 and $300 \mu\text{g}/\text{cm}^3$ of DNA.

Diphenylamine solution. The diphenylamine solution was prepared by dissolving 1.5 g of analytical grade diphenylamine in 100 cm^3 of glacial acetic acid and 1.5 cm^3 of concentrated sulphuric acid. This solution is stored in an amber bottle and in the dark and immediately prior to use 0.1 cm^3 of aqueous acetaldehyde (1.6 %) was added to every 20 cm^3 of diphenylamine solution used.

6.2.2 Cell Lines

The two continuous cell lines utilised in this study were the B16F10BL6 mouse melanoma cell line (BL-6) and the Lilly Laboratory Continuous Monkey Kidney cell line (LLCMK). The cell lines were obtained from the Department of Physiology, Medunsa, RSA.

6.2.3 Methods

Routine Cell Culture Procedures. As sterility was vital for successful cell culture experiments all work was conducted on a laminar flow bench which had previously been sterilised by ultra-violet (UV) light, all equipment was purchased sterile or was autoclaved, solutions were sterile filtered and incubated at 37°C for

a minimum of 48 hrs, prior to use, to ensure no contamination had occurred and all equipment was swabbed with 95 % alcohol prior to use.

Cells not being utilised for experiments were grown in Cell-Cult large cell culture flasks (75cm²) (Sterilin, UK) with MM. The medium was changed frequently and the cell lines were trypsinised and passaged just before they reached confluency within a flask. The flasks were incubated at 37°C. To maintain the cell line, some cells were frozen in FM using liquid air, and several flasks of cells were maintained at room temperature utilising SCM for 4 weeks at a time. This ensured that if any mass contamination was to occur the cell lines could easily be regrown.

Experimental Cell Culture Procedure. When experiments were set up five duplicates of each point were prepared and the flasks utilised were 25 cm² Cell-Cult flasks. Large cell culture flasks with nearly confluent cell populations were trypsinised with 10 cm³ of trypsin subculturing solution and were incubated at 37°C until the cells lifted from the flask surfaces where upon the solutions were transferred to sterile centrifuge tubes (Sterilin, UK) and centrifuged to pellet the cells. The trypsin solution was then decanted and 2 cm³ of GM was added to the centrifuge tubes and the cells were dispersed with a sterile pasteur pipette. A drop of the cell solution was then placed on a haemocytometer (Neabauer counting chamber) and covered with a glass cover slip. The cells were counted and the total number of cells in the entire suspension was calculated

in order to pipette a set volume of the cell suspension into each flask, so that each flask had 350 000 cells at the start of an experiment. The experimental media i.e the GLA-, zinc- and GLA- plus zinc-supplemented media was then added to the flasks (10 cm³ per flask) and controls were always set-up (i.e. had no supplementations added).

The flasks were incubated at 37°C and the media was changed if necessary during the experiment. Twelve hours before the cells were to be harvested a radio-active label was added to the cells. Methyl-³H thymidine (Amersham International, UK) was the radio-active label and 5 µCurries (µCi) was added per flask. The label was in PBS. The cells were harvested (trypsinised) under non-sterile conditions and once the cells had been spun and pelleted they were suspended in 10 cm³ of PBS and the cells were counted on a haemocytometer.

DNA Isolation and ³H-Thymidine Uptake. The methodology utilised was that of Baker (341). Once the cells had been counted, the cell suspensions were once again centrifuged to pellet the cells. The PBS was decanted and 2cm³ of 6 % sodium-4-amino salicylate, 2 cm³ of 1 % NaCl and 2 cm³ of the phenol-cresol mixture was added to each sample. The cells were homogenised in a dounce homogeniser (10 strokes with the loose plunger and 10 strokes with the tight plunger). The homogenate was shaken for 20 mins at room temperature and then centrifuged at 500 g for 30 min. The aqueous phase was removed and to this mixture was added 3.0 g of NaCl per 100 cm³ of sample and 0.5

volume of phenol-cresol mixture. The resulting solution was allowed to stand for 10 mins at room temperature and was then centrifuged at 7000 g for 20 mins. The aqueous phase was removed and mixed with 2 volumes of an ethanol-meta-cresol mixture (9:1 v/v). The solution was left to stand at 4°C for 30-60 mins. The solution could be left overnight if necessary.

The solution was then centrifuged at 18 000 g for 15 mins and the precipitate was extracted twice with 6 cm³ of cold 3.0 M sodium acetate (pH 6.0) and then centrifuged at 4000 g for 10 mins to remove the remaining precipitate. The washings were combined and mixed with 10 cm³ of 2-ethoxy-ethanol. If no precipitation occurred, then 2-ethoxy-ethanol was added until precipitation occurred. The solution was centrifuged at 18 000 g for 15 mins to pellet the precipitate and this precipitate was suspended in 5.0 cm³ of 0.5 M NaCl and 3.0 M sodium acetate (1:1 v/v).

The samples were analysed for ³H-thymidine in a scintillation counter (Beckman LS 3801 scintillation counter) by adding 10.0 cm³ of scintillation cocktail (Ready-Solv EP, Beckman, Ireland) to 1.0 cm³ of sample. Duplicates were prepared as were vials containing scintillation cocktail only for background reading.

The samples were also analysed for DNA content. The methodology utilised for this was that of Burton (342). Duplicates of 1.0 cm³ of the samples were added to 4.0 cm³ of diphenylamine solution and were

thoroughly mixed. The samples were heated in a boiling water bath for 10 mins and then cooled, where upon the absorbances of the samples at 600 nm were read on a Spectronic 1001 (Bausch and Lomb). The DNA content was determined from a DNA standard curve (see Appendix 2).

Atomic Absorption Spectroscopy. All the glassware utilised for AA assays was acid washed. AA spectroscopy was utilised in order to determine the zinc content of growth medium. A Varian Techtron Model 1000 AA Spectrophotometer with an air/acetylene flame, a slit width of 0.5 nm, a wavelength of 213.9 nm and a lamp current of 5 mA was utilised. A zinc standard curve was determined (see Appendix 3). The GM was diluted with deionised water to obtain readings that were on the standard curve.

Statistical Analysis. The statistical significance of differences between curves was tested utilising two-way analysis of variance and the Student Newman Keuls test. The means and standard errors of the mean (SEM) five replicates are present and were checked by hand calculations.

6.3 Results

AA spectroscopy showed that the GM contained $0.3 \mu\text{g}/\text{cm}^3$ of zinc. The effect of added zinc on the growth of BL-6 and LLCMK cells is shown in Figures 19 and 20, utilising cell counts (Figure 19) and ^3H -thymidine incorporation into DNA (cpm/mg of DNA) (Figure 20) as indications of growth.

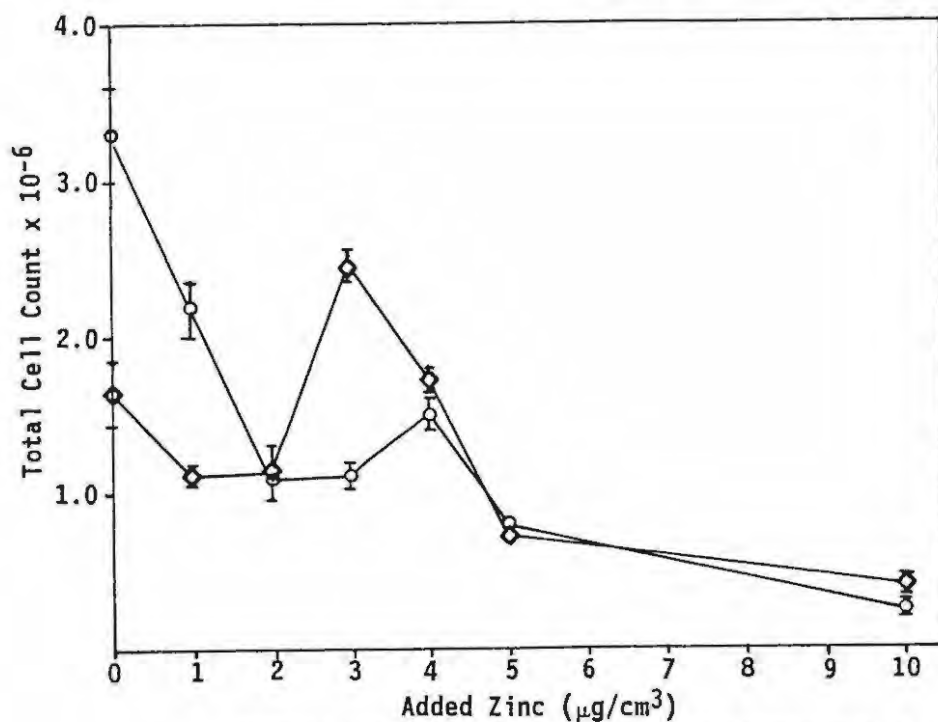


Figure 19. The effects of added zinc on the growth (cell counts) of LLCMK and BL-6 cells.

○ = BL-6 cells
◇ = LLCMK cells

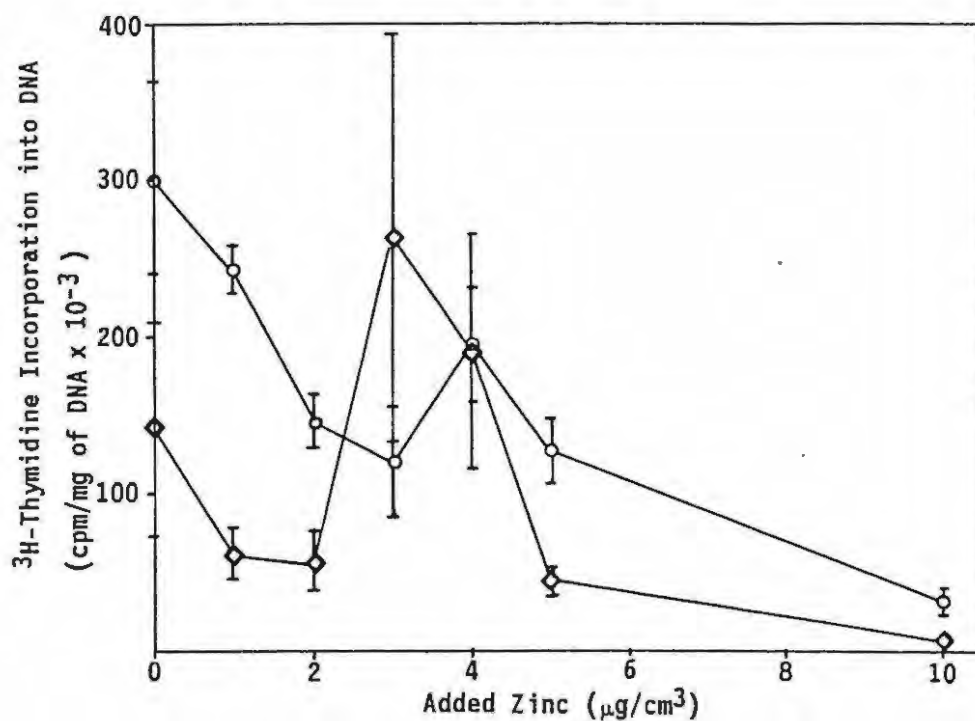


Figure 20. The effects of added zinc on the growth (cpm/mg of DNA values) of LLCMK and BL-6 cells.

○ = BL-6 cells
◇ = LLCMK cells

Table 5 shows that statistically there was a significant decrease in the cell count of the BL-6 cells upon the addition of zinc as compared to the control (when no zinc was added). When $4 \mu\text{g}/\text{cm}^3$ of zinc was added to the BL-6 cells there was a small increase in the cell count of the BL-6 cells. The cell counts of the LLCMK cells were significantly decreased upon the addition of 1, 2, 5 and $10 \mu\text{g}/\text{cm}^3$ of zinc, with the latter two additions showing a greater statistically significant decrease than the former two additions. When $3 \mu\text{g}/\text{cm}^3$ of zinc was added to the LLCMK cells there was a significant increase in the cell count.

TABLE 5: THE EFFECT OF ADDED ZINC ON THE GROWTH OF BL-6 AND LLCMK CELLS WHEN EXPRESSED AS CELL COUNTS

[Zn] ($\mu\text{g}/\text{cm}^3$)	BL-6 (cell counts) $\bar{x} \pm \text{SEM} \times 10^{-6}$	LLCMK (cell counts) $\bar{x} \pm \text{SEM} \times 10^{-6}$
0	3.29 ± 0.30	1.65 ± 0.21
1	$2.19 \pm 0.17^{**}$	$1.13 \pm 0.05^*$
2	$1.09 \pm 0.07^{**}$	$1.14 \pm 0.18^*$
3	$1.13 \pm 0.07^{**}$	$2.46 \pm 0.10^{**}$
4	$1.51 \pm 0.11^{**}$	1.73 ± 0.07
5	$0.78 \pm 0.05^{**}$	$0.72 \pm 0.08^{**}$
10	$0.26 \pm 0.05^{**}$	$0.40 \pm 0.07^{**}$

* $p > 0.01$

** $p > 0.001$

Table 6 shows that when zinc was added there was a significant decrease in the ^3H -thymidine incorporation into the DNA of the BL-6 cells except when 1 and 4 $\mu\text{g}/\text{cm}^3$ of zinc was added when compared to the control. Only when 10 $\mu\text{g}/\text{cm}^3$ of zinc was added, was there a statistically significant decrease in the ^3H -thymidine incorporation into the DNA of the LLCMK cells.

TABLE 6: THE EFFECT OF ADDED ZINC ON THE GROWTH OF BL-6 AND LLCMK CELLS WHEN EXPRESSED AS ^3H -THYMIDINE INCORPORATION INTO DNA

[Zn] ($\mu\text{g}/\text{cm}^3$)	BL-6 (cpm/mg DNA) $\bar{x} \pm \text{SEM} \times 10^{-3}$	LLCMK (cpm/mg DNA) $\bar{x} \pm \text{SEM} \times 10^{-3}$
0	310.52 $\pm$ 48.60	138.98 $\pm$ 52.28
1	243.62 $\pm$ 11.90	62.68 $\pm$ 15.99
2	147.24 $\pm$ 12.92**	62.96 $\pm$ 15.22
3	120.70 $\pm$ 35.34***	264.82 $\pm$ 130.00
4	195.78 $\pm$ 37.06	196.98 $\pm$ 74.90
5	127.94 $\pm$ 21.31***	46.36 $\pm$ 7.36
10	32.78 $\pm$ 7.07****	5.37 $\pm$ 0.69*

* $p > 0.05$

** $p > 0.025$

*** $p > 0.01$

**** $p > 0.001$

The effect of added GLA on the growth of BL-6 and LLCMK cells is shown in Figures 21 and 22, utilising cell counts (Figure 21) and ^3H -thymidine incorporation into DNA (Figure 22) as indications of growth. Figure 21 shows that the addition of GLA tended to result in a general decrease in both cell count and ^3H -thymidine incorporation into DNA in the BL-6 cells while in general not really affecting the growth of the LLCMK cells.

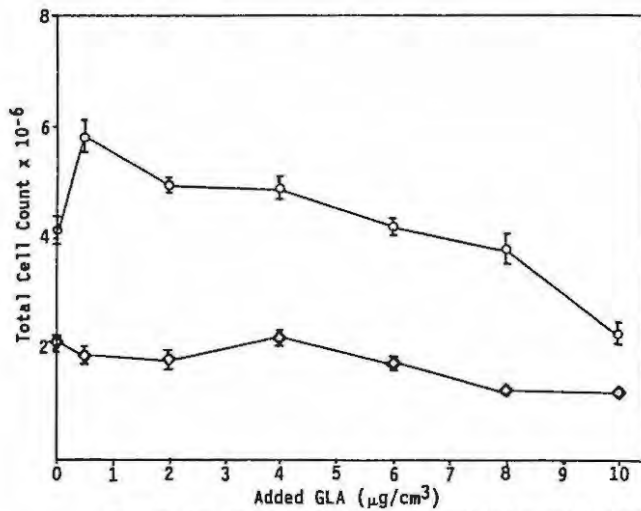


Figure 21. The effects of added GLA on the growth (cell counts) of LLCMK and BL-6 cells.

○ = BL-6 cells
◇ = LLCMK cells

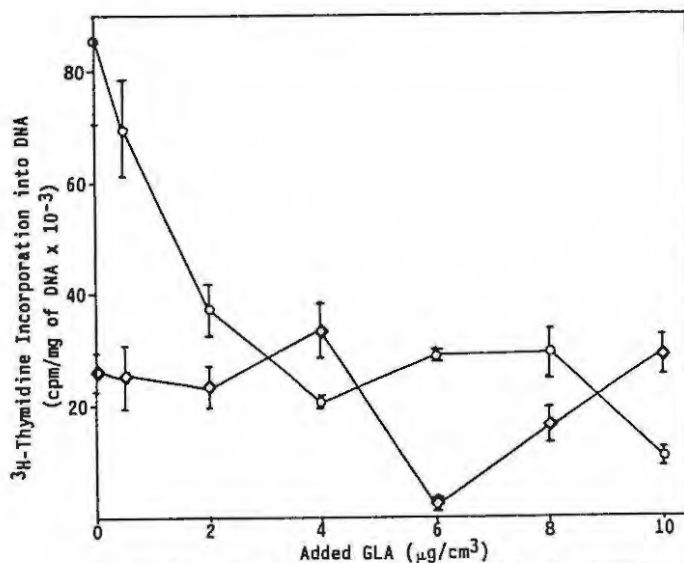


Figure 22. The effects of added GLA on the growth (cpm/mg of DNA values) of LLCMK and BL-6 cells.

○ = BL-6 cells
◇ = LLCMK cells

Table 7 shows that statistically the difference in cell counts in the BL-6 cells was significantly higher than the control when 0.5 - 4.0 $\mu\text{g}/\text{cm}^3$ of GLA was added, while there was a significant decrease in cell counts when 10 $\mu\text{g}/\text{cm}^3$ of GLA was added. The only affect of GLA on the LLCMK cell counts was a significant decrease when 8.0 and 10.0 $\mu\text{g}/\text{cm}^3$ of GLA was added. In general, the addition of GLA had little effect on the cell counts of the LLCMK cells.

TABLE 7: THE EFFECT OF ADDED GLA ON THE GROWTH OF BL-6 AND LLCMK CELLS WHEN EXPRESSED AS CELL COUNTS

[GLA] ($\mu\text{g}/\text{cm}^3$)	BL-6 (cell counts) $\bar{x} \pm \text{SEM} \times 10^{-6}$	LLCMK (cell counts) $\bar{x} \pm \text{SEM} \times 10^{-6}$
0	4.17 $\pm$ 0.25	2.11 $\pm$ 0.16
0.5	5.84 $\pm$ 0.29**	1.94 $\pm$ 0.09
2.0	4.98 $\pm$ 0.16*	1.87 $\pm$ 0.13
4.0	4.90 $\pm$ 0.21*	2.22 $\pm$ 0.12
6.0	4.22 $\pm$ 0.17	1.70 $\pm$ 0.08
8.0	3.80 $\pm$ 0.25	1.25 $\pm$ 0.09**
10.0	2.29 $\pm$ 0.15**	1.21 $\pm$ 0.09**

* $p > 0.005$

** $p > 0.001$

The effect of GLA addition on the ^3H -thymidine incorporation into DNA in the BL-6 and LLCMK cells is shown in Table 8. The addition of GLA resulted in a significant decrease in the ^3H -thymidine incorporation into DNA at all concentrations of added GLA, with the most significant decrease being with the addition of $2.0 \mu\text{g}/\text{cm}^3$ or greater. The LLCMK cells ^3H -thymidine incorporation into DNA only showed a statistically significant decrease when $6.0 \mu\text{g}/\text{cm}^3$ of GLA was added. The addition of GLA at other concentration had very little effect on the ^3H -thymidine incorporation into DNA in the LLCMK cells.

TABLE 8: THE EFFECT OF ADDED GLA ON THE GROWTH OF BL-6 AND LLCMK CELLS
WHEN EXPRESSED AS ^3H -THYMIDINE INCORPORATION INTO DNA

[GLA] ($\mu\text{g}/\text{cm}^3$)	BL-6 (cpm/mg DNA) $\bar{x} \pm \text{SEM} \times 10^{-3}$	LLCMK (cpm/mg DNA) $\bar{x} \pm \text{SEM} \times 10^{-3}$
0	85.16 $\pm$ 14.74	25.91 $\pm$ 3.29
0.5	66.25 $\pm$ 7.53*	25.35 $\pm$ 5.59
2.0	37.22 $\pm$ 4.69***	22.43 $\pm$ 2.95
4.0	20.59 $\pm$ 0.86***	33.85 $\pm$ 2.70
6.0	20.95 $\pm$ 1.00***	2.25 $\pm$ 0.28**
8.0	30.34 $\pm$ 3.75***	14.79 $\pm$ 2.05
10.0	10.69 $\pm$ 1.50***	28.86 $\pm$ 3.37

* $p > 0.025$

** $p > 0.005$

*** $p > 0.001$

Figures 23-26 and Tables 9 and 10 are data obtained from studies on the effects of added zinc together with added GLA on tumour and normal growth in the BL-6 and LLCMK cells. In these tables and figures the effect of added GLA at different levels of added zinc are considered. Controls were cultures with no added GLA at the different concentrations of added zinc. Figure 23 and Table 9 show the combined effect of added zinc and added GLA on the cell counts of the BL-6 cells. The addition of zinc and GLA together resulted in the general decrease in cell counts of the BL-6 cells. When no zinc was added, the addition of 4 $\mu\text{g}/\text{cm}^3$ of GLA resulted in a significant increase in the cell count and the addition of 8 $\mu\text{g}/\text{cm}^3$ resulted in a significant decrease in the cell count compared to the control with no added GLA in this group. When 2 $\mu\text{g}/\text{cm}^3$ of zinc was added, the addition of 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA resulted in a significant decrease in the cell count of the BL-6 cells. In the 3 or 4 $\mu\text{g}/\text{cm}^3$ of added zinc group, the further addition of 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA resulted in statistically significant decreases in the cell count. In Figures 23-26 where necessary at the start of the curves, numbers have been placed next to the curve such that 0 represents 0 $\mu\text{g}/\text{cm}^3$ of added zinc. In Figures 27-31 where necessary at the start of the curves, numbers have been placed next to the curve such that 0 represents 0 $\mu\text{g}/\text{cm}^3$ of added GLA.

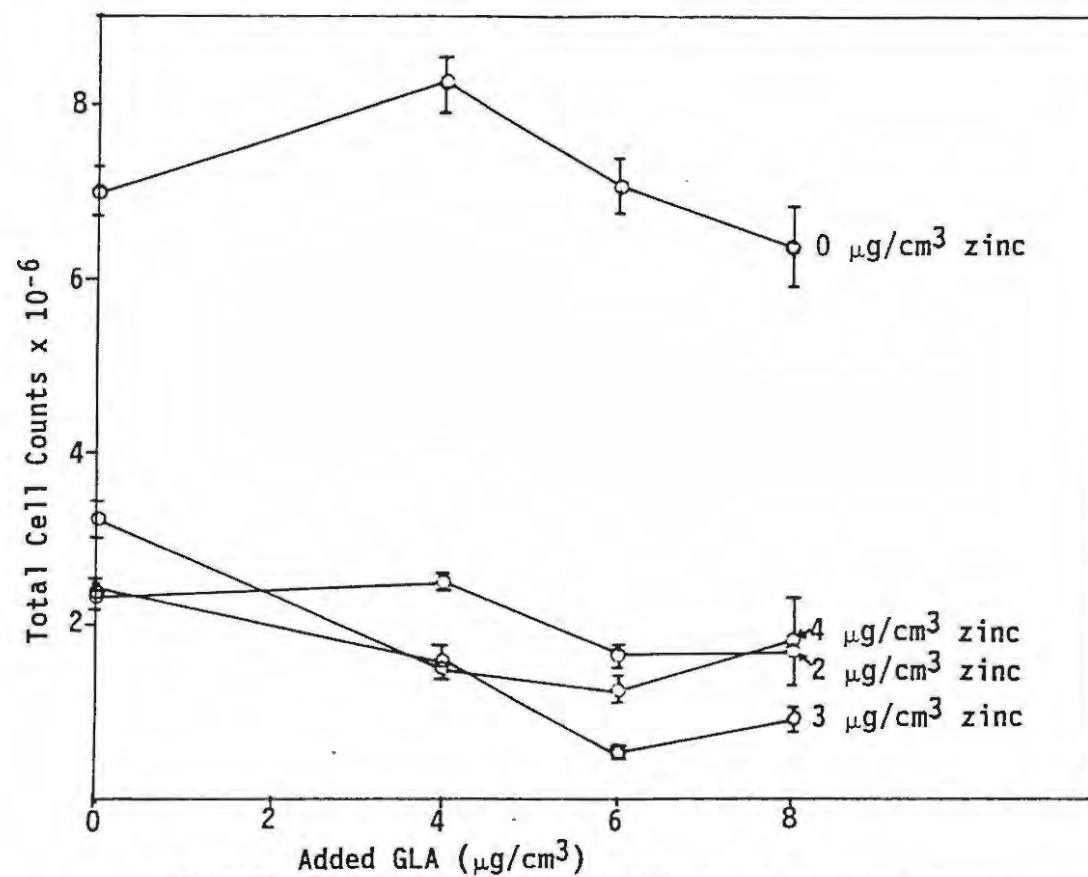


Figure 23. The effects of added zinc and GLA on the growth (cell counts) of BL-6 cells.

Figure 24 and Table 9 show the combined effect of added zinc and added GLA on ^3H -thymidine incorporation into DNA in the BL-6 cells. There was a general decrease in the ^3H -thymidine incorporation into DNA upon the addition of zinc and GLA. Using cultures with no added GLA, when no zinc was added, the addition of 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA to the BL-6 cells resulted in significant decreases in the ^3H -thymidine incorporation into DNA. When 2 and 4 $\mu\text{g}/\text{cm}^3$ of zinc were added, the addition of 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA significantly decreased the ^3H -thymidine incorporation into DNA in the BL-6 cells. In the 3 $\mu\text{g}/\text{cm}^3$ of added zinc group, only when 4 and 6 $\mu\text{g}/\text{cm}^3$ of GLA were added, was there a statistically significant decrease in the ^3H -thymidine incorporation into DNA.

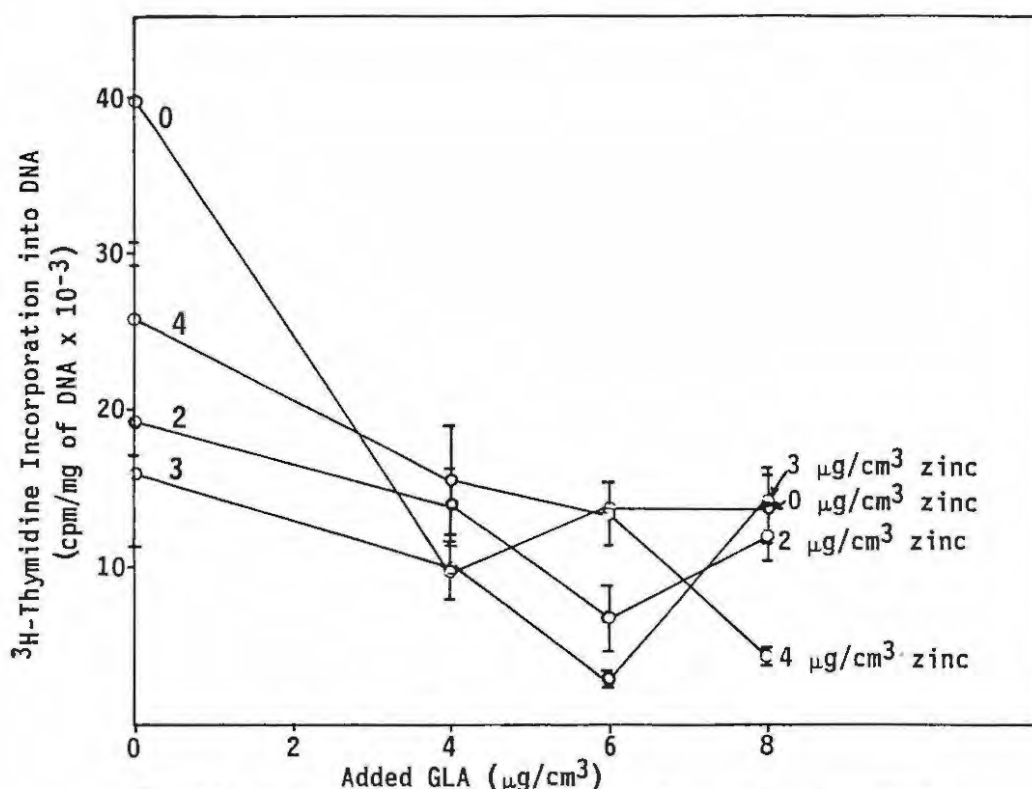


Figure 24. The effects of added zinc and GLA on the growth (cpm/mg of DNA values) of BL-6 cells.

TABLE 9: THE EFFECT OF ADDED ZINC AND GLA (ZINC KEPT CONSTANT) ON THE GROWTH OF BL-6 CELLS

[Zn : GLA] ($\mu\text{g}/\text{cm}^3$)	Cell Counts $\bar{x} \pm \text{SEM} \times 10^{-6}$	cpm/mg DNA $\bar{x} \pm \text{SEM} \times 10^{-3}$
0 : 0	7.02 $\pm$ 0.29	39.70 $\pm$ 9.01
0 : 4	8.31 $\pm$ 0.42***	9.75 $\pm$ 0.50***
0 : 6	7.08 $\pm$ 0.28	13.50 $\pm$ 0.49***
0 : 8	6.39 $\pm$ 0.45***	13.75 $\pm$ 2.51***
2 : 0	2.34 $\pm$ 0.14	19.45 $\pm$ 1.64
2 : 4	2.50 $\pm$ 0.11	13.91 $\pm$ 2.36*
2 : 6	1.69 $\pm$ 0.11 ***	6.64 $\pm$ 2.09***
2 : 8	1.72 $\pm$ 0.07***	11.79 $\pm$ 1.46**
3 : 0	2.41 $\pm$ 0.10	16.01 $\pm$ 1.10
3 : 4	1.61 $\pm$ 0.18***	9.95 $\pm$ 2.04*
3 : 6	0.58 $\pm$ 0.06***	2.80 $\pm$ 0.48***
3 : 8	0.73 $\pm$ 0.17***	13.89 $\pm$ 2.35
4 : 0	3.21 $\pm$ 0.21	25.75 $\pm$ 3.30
4 : 4	1.51 $\pm$ 0.11***	16.18 $\pm$ 1.65***
4 : 6	1.28 $\pm$ 0.15***	13.35 $\pm$ 2.03***
4 : 8	1.84 $\pm$ 0.50***	4.15 $\pm$ 0.68***

* $p > 0.025$

** $p > 0.005$

*** $p > 0.001$

Figure 25 and Table 10 show the combined effect of added zinc and added GLA on the cell counts of LLCMK cells. There is generally less effect of added zinc and GLA on the cell counts of the LLCMK cells than that found in the BL-6 cells. When no zinc and 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA were added there were statistically significant decreases in the cell counts when compared to cultures with no added GLA. When 3 $\mu\text{g}/\text{cm}^3$ of zinc was added, the addition of 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA significantly decreased the cell counts. Statistically significant increases in the LLCMK cell counts were however found in the group to which 4 $\mu\text{g}/\text{cm}^3$ of zinc was added, when 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA were added.

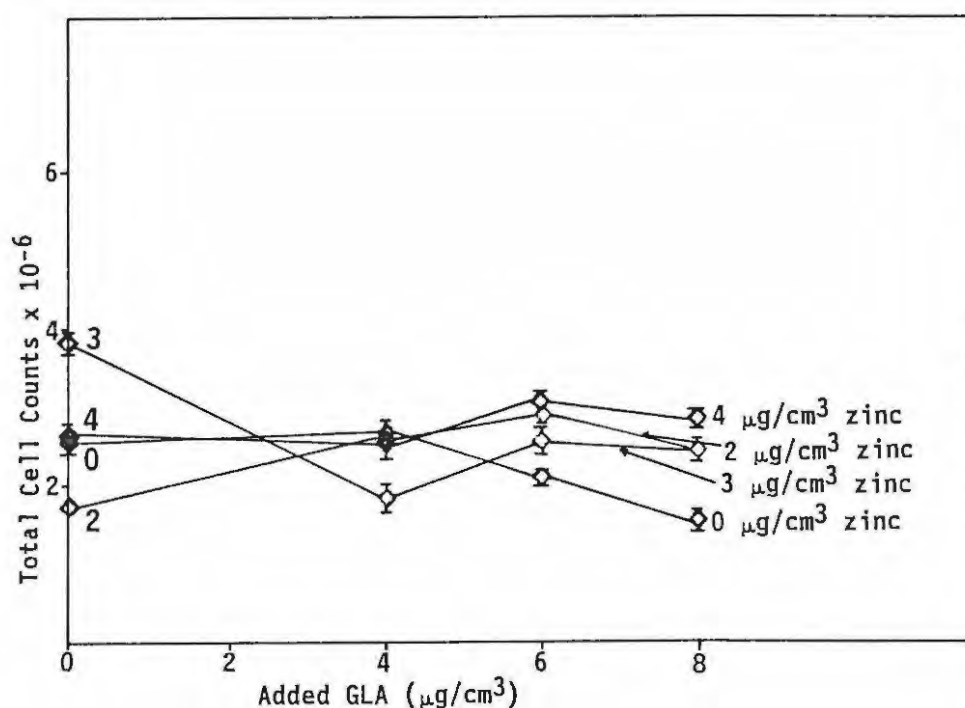


Figure 25. The effects of added zinc and GLA on the growth (cell counts) of LLCMK cells.

Figure 26 and Table 10 show the combined effect of zinc and GLA addition on ^3H -thymidine incorporation into DNA of LLCMK cells. In general, there is also less effect than that found in the BL-6 cells. When no zinc was added, 6 $\mu\text{g}/\text{cm}^3$ of added GLA resulted in a significant decrease. When 2 $\mu\text{g}/\text{cm}^3$ of zinc was added, the addition of 4, and 8 $\mu\text{g}/\text{cm}^3$ of GLA resulted in significant increases in the ^3H -thymidine incorporation into DNA of LLCMK cells, while 6 $\mu\text{g}/\text{cm}^3$ of added GLA caused a significant decrease in the ^3H -thymidine incorporation into DNA of LLCMK cells. When 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc were added, 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of added GLA resulted in significantly decreased ^3H -thymidine incorporation into DNA.

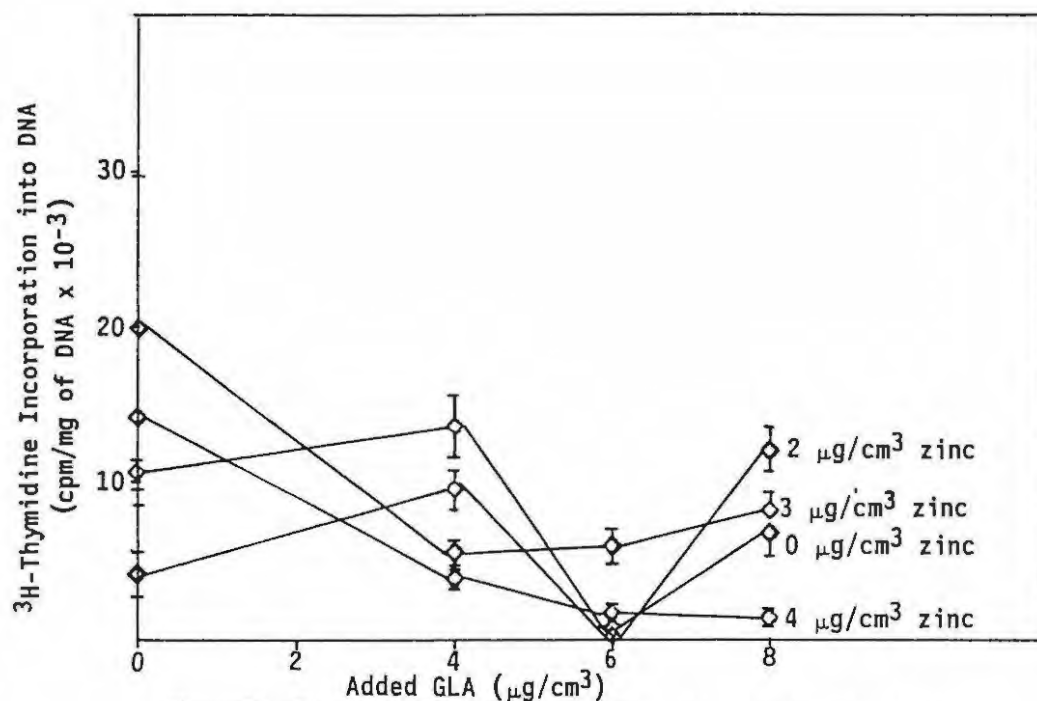


Figure 26. The effects of added zinc and GLA on the growth (cpm/mg of DNA values) of LLCMK cells.

TABLE 10: THE EFFECT OF ADDED ZINC AND GLA (ZINC KEPT CONSTANT) ON THE GROWTH OF LLCMK CELLS

[Zn : GLA] ($\mu\text{g}/\text{cm}^3$)	Cell Counts $\bar{x} \pm \text{SEM} \times 10^{-6}$	cpm/mg DNA $\bar{x} \pm \text{SEM} \times 10^{-3}$
0 : 0	2.57 $\pm$ 0.15	10.64 $\pm$ 1.30
0 : 4	2.70 $\pm$ 0.13	13.65 $\pm$ 2.10
0 : 6	2.10 $\pm$ 0.10***	0.85 $\pm$ 0.01***
0 : 8	1.51 $\pm$ 0.11***	6.80 $\pm$ 1.22
2 : 0	2.68 $\pm$ 0.12	4.30 $\pm$ 1.30
2 : 4	2.58 $\pm$ 0.18	9.87 $\pm$ 1.09*
2 : 6	2.92 $\pm$ 0.18	0.23 $\pm$ 0.10***
2 : 8	2.45 $\pm$ 0.15	12.29 $\pm$ 1.34**
3 : 0	3.82 $\pm$ 0.18	20.10 $\pm$ 9.78
3 : 4	1.85 $\pm$ 0.18***	5.65 $\pm$ 0.77***
3 : 6	2.56 $\pm$ 0.17***	5.98 $\pm$ 0.75***
3 : 8	2.45 $\pm$ 0.17***	8.10 $\pm$ 1.38***
4 : 0	1.78 $\pm$ 0.05	14.45 $\pm$ 5.64
4 : 4	2.54 $\pm$ 0.19***	4.14 $\pm$ 0.45***
4 : 6	3.08 $\pm$ 0.12***	1.90 $\pm$ 0.37***
4 : 8	2.82 $\pm$ 0.12***	1.51 $\pm$ 0.28***

* $p > 0.05$

** $p > 0.005$

*** $p > 0.001$

In Figures 27-30 and Tables 11 and 12 the same data as shown in Figures 23-26 and Tables 9 and 10 were used but in this case the effect of added zinc on cell growth is considered at different levels of added GLA. Controls were the cultures with no added zinc at the different levels of added GLA. Figure 27 and Table 11 show the combined effects of GLA and zinc on the cell counts of the BL-6 cells. The general trend is a decrease in the cell counts of the BL6-cells. Table 11 shows that when no GLA was added and 2, 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc were added, there was statistically significant decreases in the cell counts compared to the counts with no added zinc. The same trend occurred at all levels of added GLA.

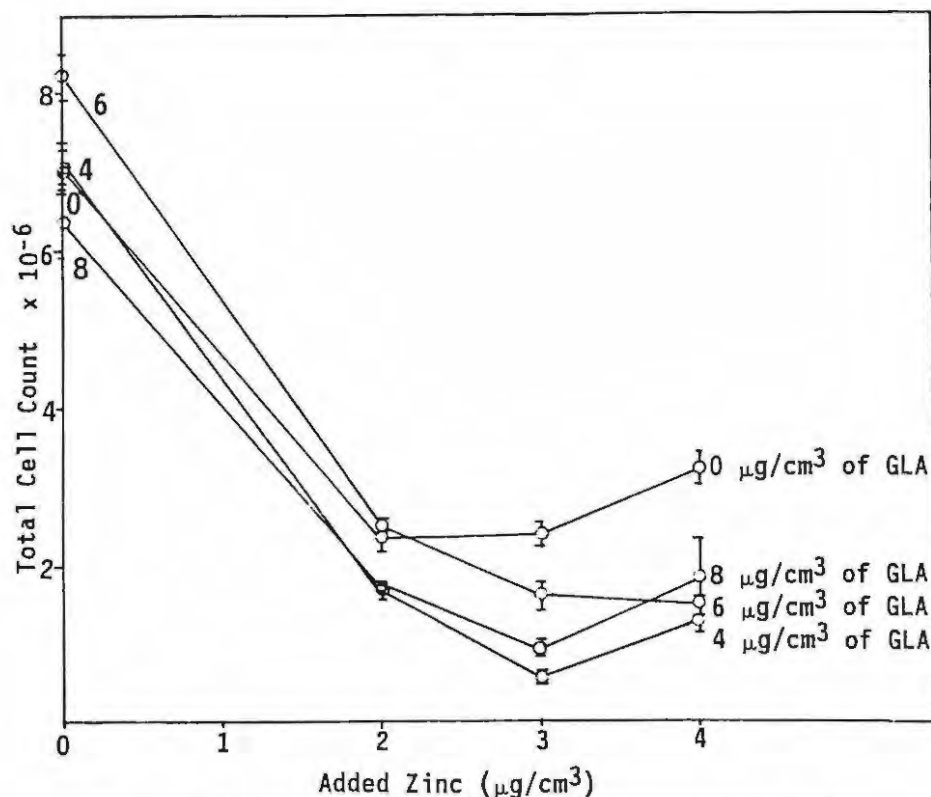


Figure 27. The effects of added GLA and zinc on the growth (cell counts) of BL-6 cells.

Figure 28 and Table 11 show the combined effect of added GLA and zinc on ^3H -thymidine incorporation into DNA in the BL-6 cells. With the addition of zinc and GLA there is again a general trend of decreased ^3H -thymidine incorporation into DNA. When no GLA was added, the addition of 2, 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc resulted in a significant decrease in the ^3H -thymidine incorporation into DNA in the BL-6 cells. When 4 $\mu\text{g}/\text{cm}^3$ of GLA was added there were no statistical significance differences noted when zinc was added. When 6 $\mu\text{g}/\text{cm}^3$ of GLA was added, at 2 and 3 $\mu\text{g}/\text{cm}^3$ of added zinc significant decreases occurred. The only statistically significant decrease in ^3H -thymidine incorporation that occurred when 8 $\mu\text{g}/\text{cm}^3$ of GLA was added, was when 4 $\mu\text{g}/\text{cm}^3$ of zinc was added compared to the control with no added zinc.

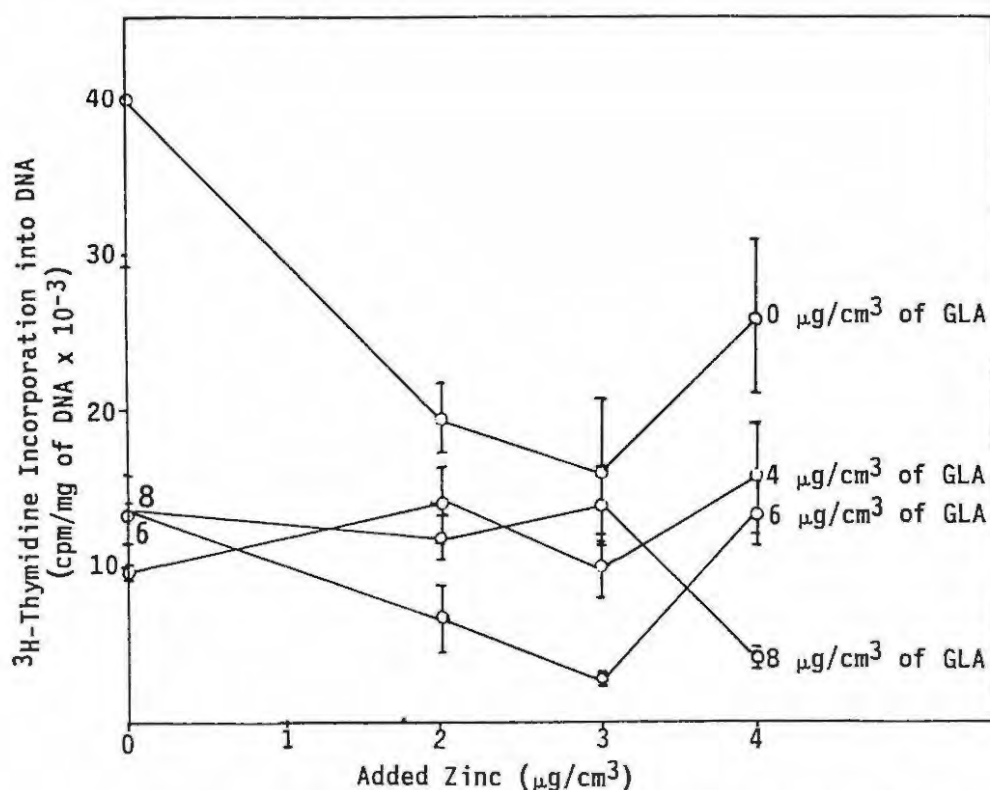


Figure 28. The effects of added GLA and zinc on the growth (cpm/mg of DNA values) of BL-6 cells.

TABLE 11: THE EFFECT OF ADDED GLA AND ZINC (GLA KEPT CONSTANT) ON THE GROWTH OF BL-6 CELLS

[GLA : Zn] ($\mu\text{g}/\text{cm}^3$)	Cell Counts $\bar{x} \pm \text{SEM} \times 10^{-6}$	cpm/mg DNA $\bar{x} \pm \text{SEM} \times 10^{-3}$
0 : 0	7.02 $\pm$ 0.23	39.88 $\pm$ 10.63
0 : 2	2.37 $\pm$ 0.14***	19.30 $\pm$ 2.61***
0 : 3	2.40 $\pm$ 0.13***	15.95 $\pm$ 4.70***
0 : 4	3.22 $\pm$ 0.24***	25.75 $\pm$ 5.10***
4 : 0	8.35 $\pm$ 0.44	9.55 $\pm$ 0.58
4 : 2	2.50 $\pm$ 0.11***	13.91 $\pm$ 2.36
4 : 3	1.61 $\pm$ 0.18***	9.95 $\pm$ 2.04
4 : 4	1.51 $\pm$ 0.07***	16.18 $\pm$ 1.65
6 : 0	7.08 $\pm$ 0.28	13.50 $\pm$ 0.65
6 : 2	1.69 $\pm$ 0.11***	6.64 $\pm$ 2.09*
6 : 3	0.58 $\pm$ 0.06***	2.80 $\pm$ 0.48***
6 : 4	1.28 $\pm$ 0.15***	13.35 $\pm$ 2.03
8 : 0	6.38 $\pm$ 0.45	13.51 $\pm$ 1.80
8 : 2	1.72 $\pm$ 0.07***	11.79 $\pm$ 1.46
8 : 3	0.73 $\pm$ 0.17***	13.89 $\pm$ 2.35
8 : 4	1.84 $\pm$ 0.51***	4.15 $\pm$ 0.68**

* $p > 0.025$

** $p > 0.005$

*** $p > 0.001$

Figure 29 and Table 12 show the combined effects of added GLA and zinc on the cell counts of LLCMK cells. In general there is little effect on the distinct trend in the cell counts of the LLCMK cells upon these additions. When no GLA was added and 2 $\mu\text{g}/\text{cm}^3$ of zinc was added there was a significant decrease in the cell count, but when no GLA and 3 $\mu\text{g}/\text{cm}^3$ of zinc were added there was a significant increase in the cell count. The only statistically significant difference in the group with 4 $\mu\text{g}/\text{cm}^3$ of added GLA was found when 3 $\mu\text{g}/\text{cm}^3$ of zinc was added and this resulted in a decrease in the cell count. When 6 $\mu\text{g}/\text{cm}^3$ of GLA was added, the addition of 2, 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc resulted in a significantly increased cell count. This significantly increased cell count was also found in the 8 $\mu\text{g}/\text{cm}^3$ of added GLA group, when 2, 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc were added.

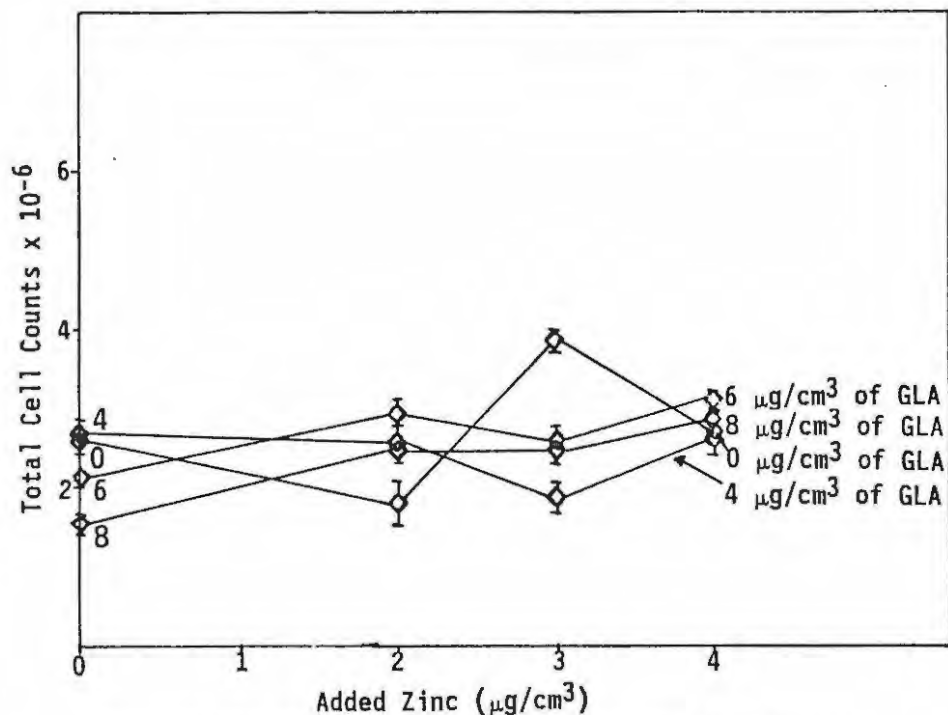


Figure 29. The effects of added GLA and zinc on the growth (cell counts) of LLCMK cells.

Figure 30 and Table 12 show the combined effects of added GLA and zinc on ^3H -thymidine incorporation into DNA in the LLCMK cells. Generally there was less effect than that found when using cell counts as an indication of cell growth. When no or $6\ \mu\text{g}/\text{cm}^3$ of GLA were added, the addition of $2\ \mu\text{g}/\text{cm}^3$ of zinc resulted in a significant decrease in the ^3H -thymidine incorporation into DNA, while the addition of $3\ \mu\text{g}/\text{cm}^3$ of zinc caused a significant increase. When $4\ \mu\text{g}/\text{cm}^3$ of GLA was added and 3 and $4\ \mu\text{g}/\text{cm}^3$ of zinc were added there was a significant decrease in the ^3H -thymidine incorporation into DNA in the LLCMK cells. In the $8\ \mu\text{g}/\text{cm}^3$ of added GLA group, $2\ \mu\text{g}/\text{cm}^3$ of added zinc caused a significant increase while $4\ \mu\text{g}/\text{cm}^3$ of added zinc, resulted in a significant decrease in the ^3H -thymidine incorporation into DNA in the LLCMK cells.

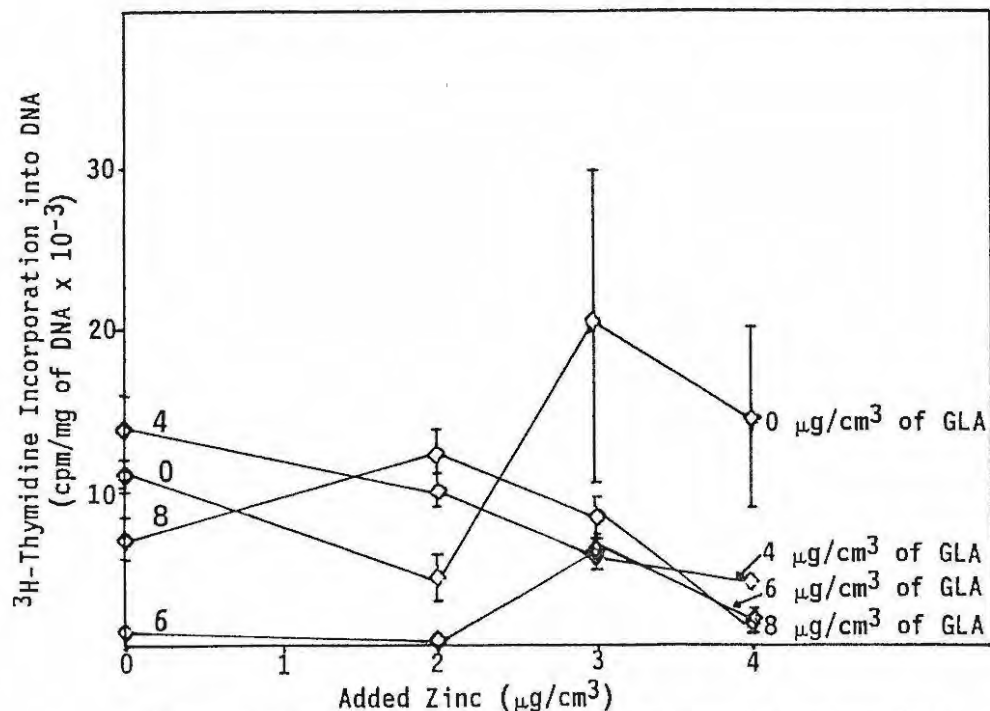


Figure 30. The effects of added GLA and zinc on the growth (cpm/mg of DNA values) of LLCMK cells.

TABLE 12: THE EFFECT OF ADDED GLA AND ZINC (GLA KEPT CONSTANT) ON THE GROWTH OF LLCMK CELLS

[GLA : Zn] ($\mu\text{g}/\text{cm}^3$)	Cell Counts $\bar{x} \pm \text{SEM} \times 10^{-6}$	cpm/mg DNA $\bar{x} \pm \text{SEM} \times 10^{-3}$
0 : 0	2.58 $\pm$ 0.16	10.71 $\pm$ 0.72
0 : 2	1.78 $\pm$ 0.28****	4.35 $\pm$ 1.31**
0 : 3	3.82 $\pm$ 0.16****	20.55 $\pm$ 9.38****
0 : 4	2.68 $\pm$ 0.10	14.41 $\pm$ 5.73
4 : 0	2.68 $\pm$ 0.14	13.62 $\pm$ 3.09
4 : 2	2.58 $\pm$ 0.18	9.87 $\pm$ 1.09
4 : 3	1.85 $\pm$ 0.18****	5.65 $\pm$ 0.77***
4 : 4	2.54 $\pm$ 0.19	4.14 $\pm$ 0.45****
6 : 0	2.12 $\pm$ 0.11	0.85 $\pm$ 0.05
6 : 2	2.92 $\pm$ 0.18****	0.23 $\pm$ 0.10**
6 : 3	2.56 $\pm$ 0.17***	5.98 $\pm$ 0.75****
6 : 4	3.08 $\pm$ 0.12****	1.90 $\pm$ 0.37
8 : 0	1.55 $\pm$ 0.08	6.58 $\pm$ 1.07
8 : 2	2.45 $\pm$ 0.15****	12.29 $\pm$ 1.34*
8 : 3	2.45 $\pm$ 0.17****	8.09 $\pm$ 1.38
8 : 4	2.82 $\pm$ 0.12****	1.51 $\pm$ 0.28*

* $p > 0.05$

** $p > 0.025$

*** $p > 0.005$

**** $p > 0.001$

6.4 Discussion

This study showed that the general trend upon zinc addition BL-6 cells was a overall reduction in cell counts and ^3H -thymidine incorporation into the DNA of these cells. This general inhibition of tumour cell growth was found to be similar to that found in previous reports (244,252,253,256). This effect was not as apparent in the LLCMK cells in which the addition of zinc at $3\text{ }\mu\text{g}/\text{cm}^3$, in fact, caused an increase in the cell count in these cells. Although this trend seems unusual and cannot be explained, this experiment was conducted several times and concurred with results previously achieved within this laboratory. The addition of zinc at all other concentrations resulted in decreased cell counts in the LLCMK cultures, although these decreases were not as marked as those in the BL-6 cells. The addition of zinc also resulted in the general decrease in the ^3H -thymidine incorporation into DNA in the LLCMK cells, except when 3 and $4\text{ }\mu\text{g}/\text{cm}^3$ of zinc was added but none of these groups with the exception of that to which $10\text{ }\mu\text{g}/\text{cm}^3$ of zinc had been added, were significantly different to the control group.

When GLA was added to the GM there was a general increase in the cell counts of the BL-6 cells at low concentrations of added GLA and a decrease at higher concentrations. There was little effect on the LLCMK cells except at high concentrations of added GLA, when a decrease in the cell count was noted. When the ^3H -thymidine incorporation into DNA was measured upon the addition of GLA there was a general and significant decrease in the BL-6 cells and little effect

in the LLCMK cells, except for a significant decrease in the ^3H -thymidine incorporation when $6.0 \mu\text{g}/\text{cm}^3$ of GLA was added. These results concur with those of previous literature reports (6,239-246).

It was subsequently decided to investigate the combined effects of zinc and GLA addition on the BL-6 and LLCMK cell growth. Initially the effect of varied added GLA concentrations was examined in groups to which different amounts of zinc had been added. In general, in the BL-6 cells, when zinc was added to the medium, the further addition of GLA resulted in a significant decrease in the cell growth when measured as cell counts or ^3H -thymidine incorporation. While the effect of added GLA was enhanced by the addition of zinc to the medium as well, the addition of more than $2 \mu\text{g}/\text{cm}^3$ of zinc did not increase this effect.

In the LLCMK cells, the cell counts and ^3H -thymidine incorporation was less effected by the combined addition of GLA and zinc than that found in the BL-6 cells. There was also no general trend to this effect with cell growth being found in certain cases and decreased growth in others.

The data on the combined effect of added zinc and GLA on the cell growth was also examined in a slightly different manner, by determining the effect of different concentrations of added zinc to groups of cell cultures which had been supplemented with increasing concentrations of GLA. In this case, in the BL-6 cells, this

resulted in increased inhibition of cell growth, as determined by cell counts, at all concentrations of added zinc in all the groups supplemented with GLA (including those to which no GLA was added). When cell growth was expressed as ^3H -thymidine incorporation into DNA this effect was not as apparent, although the same general trend was found. As found when examining this data by looking at the effect of increasing zinc in the different groups, the effect of added zinc on cell growth inhibition was enhanced by the presence of GLA in the medium, but GLA at concentrations above $4\text{ }\mu\text{g}/\text{cm}^3$ had no additional effect to that found at lower concentrations.

Examining this data in a similar manner in the LLCMK cells again showed that there was generally less effect of zinc and GLA in these cells and that the effect was variable.

One of the aims of this study was to investigate if it was possible to inhibit the growth of the cancer cell line i.e. the BL-6 cells, by the addition of zinc, GLA or a combination of the two while maintaining normal growth of the LLCMK cell line (the normal cells). This study showed that it was possible to manipulate the cell growth of both the LLCMK and BL-6 cell lines by varying the concentrations of added zinc and GLA but that there indeed appeared to be a greater effect on the BL-6 cells with an inhibition of growth of these cells being the most common result. The results of the combined effect of supplementary zinc and GLA clearly showed this inhibitory effect on the BL-6 cell growth,

while a less and variable effect on the LLCMK cells was found. It was also demonstrated that the presence of both zinc and GLA in the medium produced an enhanced effect than when only the one nutrient was supplemented in the medium. The effect was most evident when relatively high concentrations of the one nutrient was present together with a low concentration of the other. High concentrations of both nutrients did not further enhance the inhibition of cell growth.

CHAPTER 7

THE EFFECT OF GLA AND ZINC SUPPLEMENTATION ON LINOLEOYL-CoA DESATURASE ACTIVITY IN NORMAL AND TUMOUR CELLS IN VITRO

7.1 Introduction

Results from the previous chapter illustrated that both GLA and zinc supplementation resulted in a more significant inhibition of tumour cell growth than normal cell growth and at low concentrations the additions did not adversely affect the growth of normal cells while still significantly inhibiting that of the tumour cells. It was, therefore, decided to see if there was any correlation with the growth inhibition and the activity of linoleoyl-CoA desaturase. Several reports (147-149) suggest that zinc possibly plays a role in the desaturation of LA into GLA through an influence on the enzyme complex linoleoyl-CoA desaturase. Only two reports i.e. those of Cunnane and Wahle (139) and Ayala and Brenner (143) have attempted to correlate the influence of zinc with activity of linoleoyl-CoA desaturase. Cunnane and Wahle (139) demonstrated that zinc deficiency resulted in increased desaturation of LA but Ayala and Brenner (143) demonstrated that zinc deficiency resulted in a decrease in LA desaturation. This indicated that the mechanism by which zinc influences the activity of linoleoyl-CoA desaturase is unclear, if in

fact it influences the enzyme complex at all. This study attempts to more clearly define the relationship between zinc and this enzyme complex by examining the effect of zinc addition on the activity of the linoleoyl-CoA desaturase complex itself.

7.2 Materials and Methods

7.2.1 Reagents

Antibiotic solution. As in section 6.2.1.

Cell Culture Media. The GM, GLA-supplemented media, the zinc-supplemented media and the GLA- and zinc-supplemented media was prepared as in section 6.2.1.

Trypsin Subculturing solution. As in section 6.2.1.

7.2.2 Cell Lines

As in section 6.2.2.

7.2.3 Methods

Routine Cell Culture Procedures. As in section 6.2.3.

Experimental Cell Culture Procedures. As in section 6.2.3 except that no radio-active label was added and the harvested and pelleted cells were suspended in 2.0 cm^3 of 0.1 M Tris-HCl buffer pH 7.4 and the cells were then counted. Once the cells had been counted then the cell suspensions were homogenised in a dounce homogeniser (20 strokes with the loose plunger and 20 strokes with the tight plunger).

Cytochrome b_5 Reoxidation Assay. The cytochrome b_5 reoxidation assay was conducted on each of the sample homogenates as described in section 4.2.3. The units of enzyme activity were μmol of product/min/ 10^6 cells.

Statistical Analysis. As in section 6.2.3.

7.3 Results

The cell counts were not included as they were almost identical to those in Chapter 6. Figure 31 and Table 13 shows the effect of zinc addition on the activity of linoleoyl-CoA desaturase in the BL-6 and LLCMK cells.

The BL-6 cells generally showed lower linoleoyl-CoA desaturase activity than the LLCMK cells. The only statistically significant increase in the linoleoyl-CoA desaturase activity when zinc was added to the BL-6 cells, occurred when $10 \mu\text{g}/\text{cm}^3$ of zinc was added. The

addition of 1, 3 and 10 $\mu\text{g}/\text{cm}^3$ of zinc resulted in a small increase in the enzyme complex activity in the cells while the addition of 3, 4 and 5 $\mu\text{g}/\text{cm}^3$ of zinc led to decreases in the linoleoyl-CoA desaturase activity. The trend of the cell count of the BL-6 cells did not correlate with the linoleoyl-CoA desaturase activity since the addition of zinc resulted in a decrease in the cell counts (see Figure 19 and Table 5 in Chapter 6). These results show that the linoleoyl-CoA activity of the BL-6 cells showed a tendency to increase as the cell count decreased. This increase in activity was however only significant when the cell count was at its lowest i.e. when 10 $\mu\text{g}/\text{cm}^3$ of zinc was added.

In the LLCMK cells the trend shown was one of increasing enzyme activity upon the addition of zinc when compared to the control. These increases were statistically significant on all additions of zinc except when 2 $\mu\text{g}/\text{cm}^3$ of zinc was added. The greatest increase in enzyme activity occurred when 5 $\mu\text{g}/\text{cm}^3$ of zinc was added. The cell counts of the LLCMK cells showed increases when 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc were added (see Figure 19 and Table 5 in Chapter 6), and unlike the BL-6 cells, these cell count increases did not correlate with a decrease in linoleoyl-CoA desaturase activity. As with the BL-6 cells, the lowest LLCMK cell counts tended to have the higher enzyme complex activities. These trends, with both the BL-6 and LLCMK cells, seem to intimate that as the cell growth is inhibited with the addition of zinc, the linoleoyl-CoA desaturase enzyme complex is activated to higher levels.

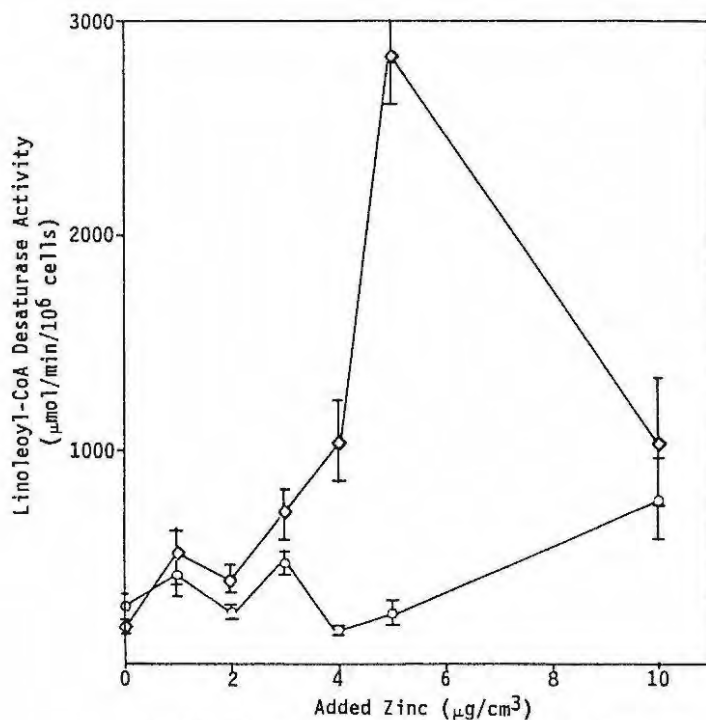


Figure 31. The effect of added zinc on the Linoleoyl-CoA Desaturase activity of BL-6 and LLCMK cells.

○ = BL-6 cells
◇ = LLCMK cells

TABLE 13: THE EFFECT OF ZINC ADDITION ON LINOLEOYL-CoA DESATURASE ACTIVITY IN BL-6 AND LLCMK CELLS

[Zinc] (μg/cm ³)	BL-6 Total Enzyme Activity (μmol/min/10 ⁶ cells) x ± SEM	LLCMK Total Enzyme Activity (μmol/min/10 ⁶ cells) x ± SEM
0	278.1 ± 57.8	170.0 ± 20.6
1	410.0 ± 93.0	515.8 ± 117.3*
2	247.7 ± 27.5	399.5 ± 65.7
3	478.6 ± 48.9	705.9 ± 118.7**
4	153.0 ± 12.8	1040.5 ± 197.7***
5	239.0 ± 60.0	2833.3 ± 204.1***
10	776.1 ± 183.4**	1037.0 ± 291.4***

* p > 0.05

** p > 0.005

*** p > 0.001

Figure 32 and Table 14 show the effects of added GLA on the linoleoyl-CoA desaturase enzyme complex activity of BL-6 and LLCMK cells. GLA addition has no statistically significant effect on the activity of the enzyme complex in the BL-6 cells when compared to the control. The effect of GLA addition on the cell counts of BL-6 cells was also not noticeable except when $10 \mu\text{g}/\text{cm}^3$ of GLA was added and this resulted in a decrease in the cell count (see Figure 21 and Table 7 in Chapter 6). As with the addition of zinc, the highest concentration of GLA addition did result in the lowest cell count and the most linoleoyl-CoA desaturase enzyme complex activity although this was not statistically significant. This again suggests that the inhibition of cell growth may be correlated with the activation of the enzyme complex.

The addition of GLA resulted in a general increase in linoleoyl-CoA desaturase activity in the LLCMK cells. Statistically significant increases in the enzyme activity occurred when 6 and $8 \mu\text{g}/\text{cm}^3$ of GLA were added to the BL-6 cells. As with the addition of zinc, the general trend emerging seemed to again suggest that as the cell growth was inhibited the activity of the linoleoyl-CoA desaturase enzyme complex was increased (see Figure 21 and Table 7 in Chapter 6).

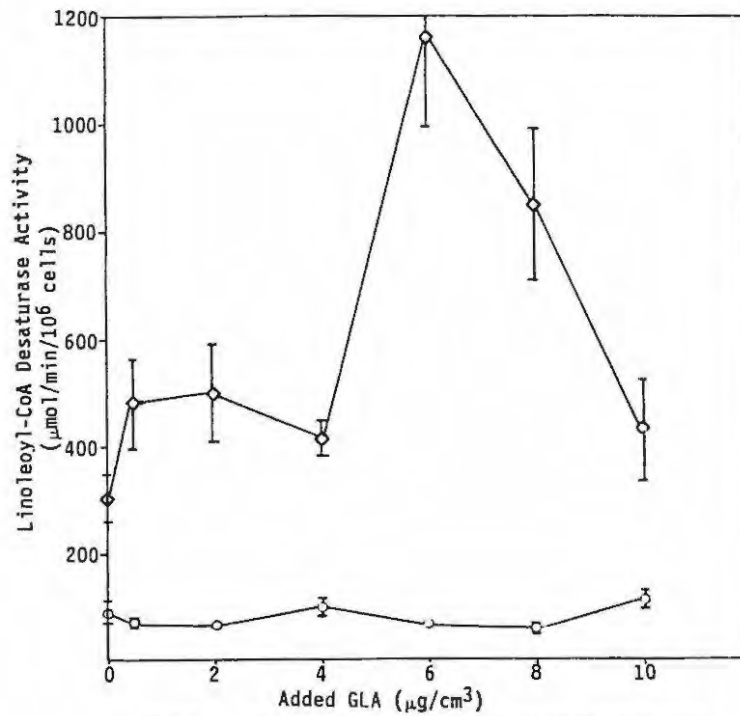


Figure 32. The effect of added GLA on the Linoleoyl-CoA Desaturase activity of BL-6 and LLCMK cells.

○ = BL-6 cells
◇ = LLCMK cells

TABLE 14: THE EFFECT OF GLA ADDITION ON LINOLEOYL-CoA DESATURASE ACTIVITY IN BL-6 AND LLCMK CELLS

[GLA] (μg/cm ³)	BL-6	LLCMK
	Total Enzyme Activity (μmol/min/10 ⁶ cells) x ± SEM	Total Enzyme Activity (μmol/min/10 ⁶ cells) x ± SEM
0	86.3 ± 24.4	302.4 ± 44.2
0.5	75.4 ± 11.2	482.5 ± 86.7
2.0	66.0 ± 3.1	500.5 ± 90.0
4.0	98.0 ± 17.9	416.2 ± 34.8
6.0	66.1 ± 3.5	1161.3 ± 186.6*
8.0	59.7 ± 11.6	846.9 ± 136.3*
10.0	116.3 ± 20.4	434.0 ± 87.8

* p > 0.001

The effects of the combined addition of zinc and GLA on the linoleoyl-CoA desaturase enzyme complex activity of the BL-6 cells, is shown in Figure 33 and Table 15. Controls in this case were cell cultures to which different concentrations of zinc but no GLA had been added. When no zinc but GLA was added to the BL-6 cells there was a general decrease in the linoleoyl-CoA desaturase enzyme activity as compared to the control. The cell counts showed little effect upon GLA addition (see Figure 23 and Table 9 in Chapter 6). When either 2, 3 or 4 $\mu\text{g}/\text{cm}^3$ of zinc were added and at all points of GLA addition there was a significant increase in enzyme activity in the BL-6 cells. The combined effects of zinc and GLA tended to show a greater increase in the enzyme complex activity than when zinc or GLA was supplemented on their own. The cell counts of the BL-6 cells upon the combined addition tended to reflect a greater inhibition of cell growth. Once again it was noted that as the cell counts decreased there was a general trend to increased enzyme activity.

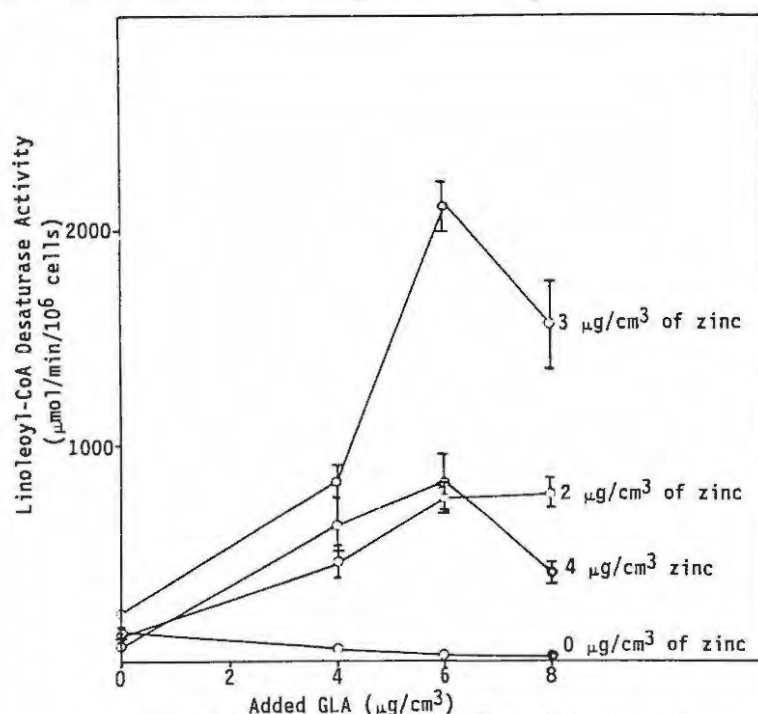


Figure 33. The effects of added zinc and GLA on the Linoleoyl-CoA Desaturase activity of BL-6 cells.

TABLE 15: THE EFFECTS OF ZINC AND GLA ADDITION (ZINC KEPT CONSTANT) ON THE
LINOLEOYL-CoA DESATURASE ACTIVITY OF BL-6 AND LLCMK CELLS

[Zn : GLA] ($\mu\text{g}/\text{cm}^3$)	BL-6 Total Enzyme Activity ($\mu\text{mol}/\text{min}/10^6$ cells) $\bar{x} \pm \text{SEM}$	LLCMK Total Enzyme Activity ($\mu\text{mol}/\text{min}/10^6$ cells) $\bar{x} \pm \text{SEM}$
0 : 0	138.2 $\pm$ 34.7	243.3 $\pm$ 20.0
0 : 4	57.5 $\pm$ 2.1	343.7 $\pm$ 6.5
0 : 6	39.0 $\pm$ 2.1	940.3 $\pm$ 48.1 ^{*****}
0 : 8	35.4 $\pm$ 2.3	690.6 $\pm$ 24.9 ^{*****}
2 : 0	116.4 $\pm$ 7.5	166.1 $\pm$ 8.8
2 : 4	462.8 $\pm$ 77.2 ^{***}	465.1 $\pm$ 0.0 ^{**}
2 : 6	754.1 $\pm$ 82.1 ^{*****}	220.2 $\pm$ 66.1
2 : 8	772.5 $\pm$ 51.9 ^{*****}	239.0 $\pm$ 75.8
3 : 0	227.1 $\pm$ 8.0	452.6 $\pm$ 11.4
3 : 4	838.9 $\pm$ 62.9 ^{*****}	711.7 $\pm$ 9.0 [*]
3 : 6	2112.2 $\pm$ 114.0 ^{*****}	410.2 $\pm$ 58.6
3 : 8	1563.9 $\pm$ 198.0 ^{*****}	326.5 $\pm$ 40.9
4 : 0	72.3 $\pm$ 1.4	1022.7 $\pm$ 295.2
4 : 4	635.8 $\pm$ 121.4 ^{*****}	547.2 $\pm$ 99.8 ^{*****}
4 : 6	834.9 $\pm$ 137.6 ^{*****}	357.0 $\pm$ 30.3 ^{*****}
4 : 8	411.4 $\pm$ 47.2 ^{****}	266.0 $\pm$ 30.7 ^{*****}

* $p > 0.05$

** $p > 0.025$

*** $p > 0.01$

**** $p > 0.005$

***** $p > 0.001$

Figure 34 and Table 15 are data on the combined effects of zinc and GLA addition on the linoleoyl-CoA desaturase enzyme complex activity of the LLCMK cells. When no zinc was added and 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA were added, increases were noted in the enzyme complex activity compared to controls in which no GLA was supplemented. These increases were statistically significant when 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA were supplemented. Unlike the BL-6 cells, the lowest cell count did not correlate to the greatest enzyme activity, although the general trend of a decreasing cell count correlating with an increasing enzyme complex activity was maintained (see Figure 25 and Table 10 in Chapter 6). In the group to which 2 $\mu\text{g}/\text{cm}^3$ of zinc was added, addition of GLA only resulted in a significant increase in enzyme activity when 4 $\mu\text{g}/\text{cm}^3$ of GLA was added, although the addition of GLA at higher concentrations resulted in increased enzyme activity. The cell count curve correlated with the trend of decreased cell counts with increased enzyme complex activities. When 3 $\mu\text{g}/\text{cm}^3$ of zinc was added and as with the addition of 2 $\mu\text{g}/\text{cm}^3$ of zinc, the only statistically significant increase in enzyme activity was noted when 4 $\mu\text{g}/\text{cm}^3$ of GLA was added to the LLCMK cells. Upon the addition of 8 $\mu\text{g}/\text{cm}^3$ of GLA it was noted for the first time that there was a decrease in the linoleoyl-CoA desaturase enzyme complex activity as compared to the control. The lowest cell count was again shown to correlate with the highest enzyme activity. The addition of 4 $\mu\text{g}/\text{cm}^3$ of zinc and 4, 6 and 8 $\mu\text{g}/\text{cm}^3$ of GLA resulted in statistically significant decreases in the enzyme complex activity upon all additions of GLA compared to the control group to which no GLA was added. The cell counts showed

increases at all levels of GLA addition compared to the control. The smallest decrease in the enzyme complex activity correlated with the smallest increase in the cell counts. Thus, as previously noted increases in cell counts corresponded with decreases in the activity of the linoleoyl-CoA desaturase enzyme complex.

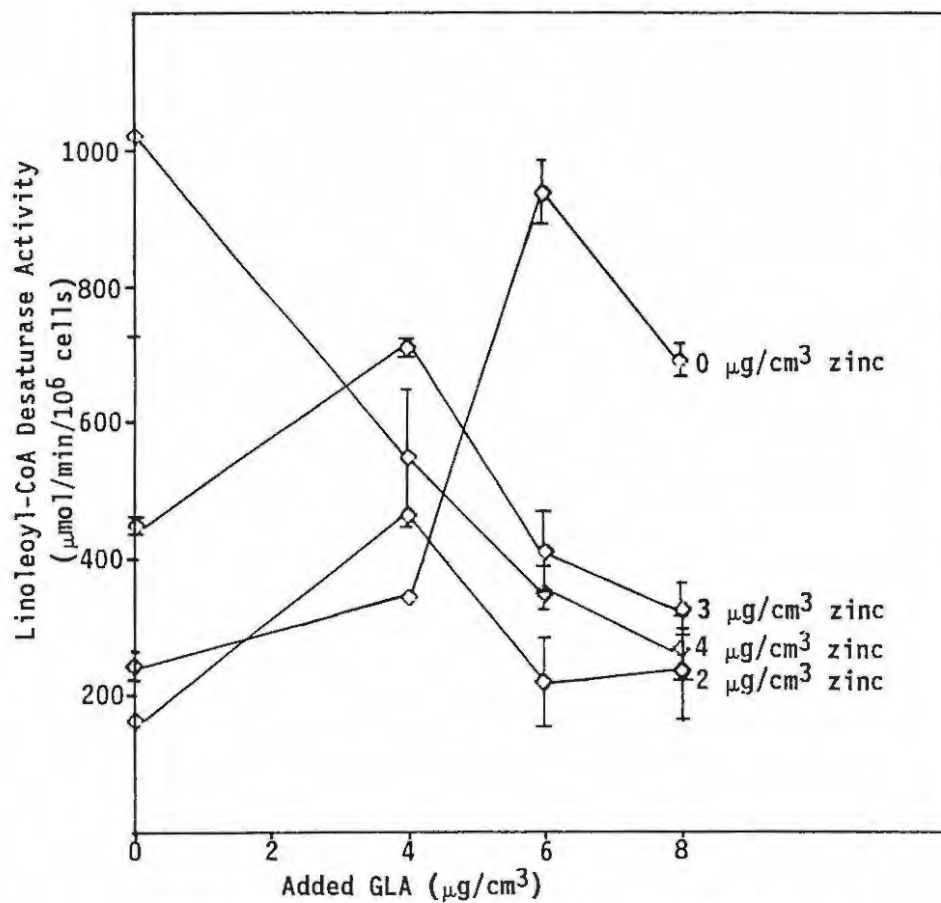


Figure 34. The effects of added zinc and GLA on the Linoleoyl-CoA Desaturase activity of LLCMK cells.

In Figures 35 and 36 and Table 16 the same data to that obtained above is shown but in this case the results are grouped into those with different concentrations of GLA addition and controls being those cell cultures in each group to which no zinc had been added.

As shown in Figure 35 and Table 16 in the group to which no GLA had been added there was no statistically significant effect of added zinc on the enzyme activity in the BL-6 cells. An increase in the cell count with the addition of $\mu\text{g}/\text{cm}^3$ of zinc did correlate with the largest decrease in enzyme activity found with this treatment (see Figure 27 and Table 11 in Chapter 6). To the group to which 4, 6, or 8 $\mu\text{g}/\text{cm}^3$ of GLA had been added there was a statistically significant increase in the activity of the linoleoyl-CoA desaturase enzyme complex upon the addition of zinc at all levels of zinc addition. The cell counts again tended to decrease as the enzyme activity increased with the lowest cell count being found to generally correlate with the highest enzyme activity.

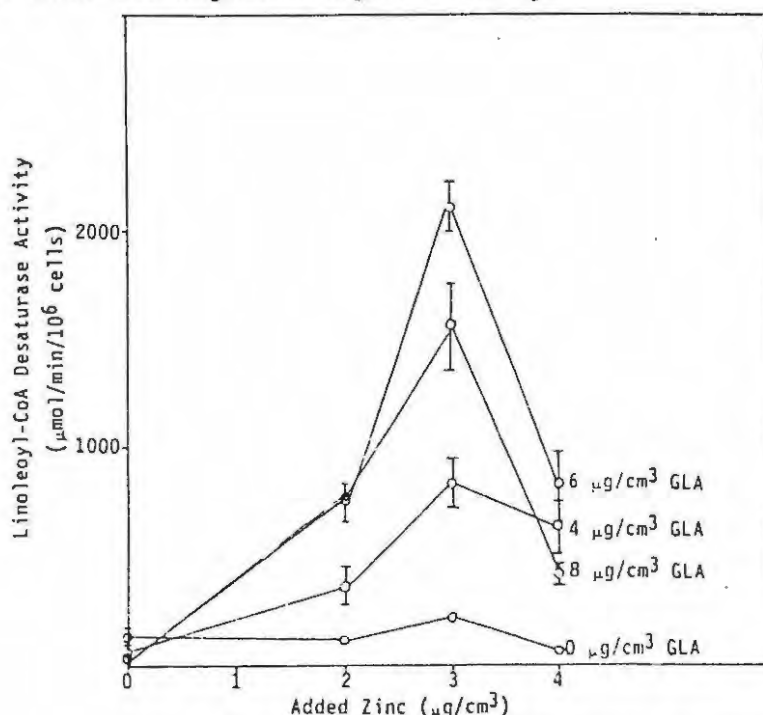


Figure 35. The effects of added GLA and zinc on the Linoleoyl-CoA Desaturase activity of BL-6 cells.

Figure 36 and Table 16 showed the combined effects of added GLA and zinc on the linoleoyl-CoA desaturase enzyme complex activities in the LLCMK cells.

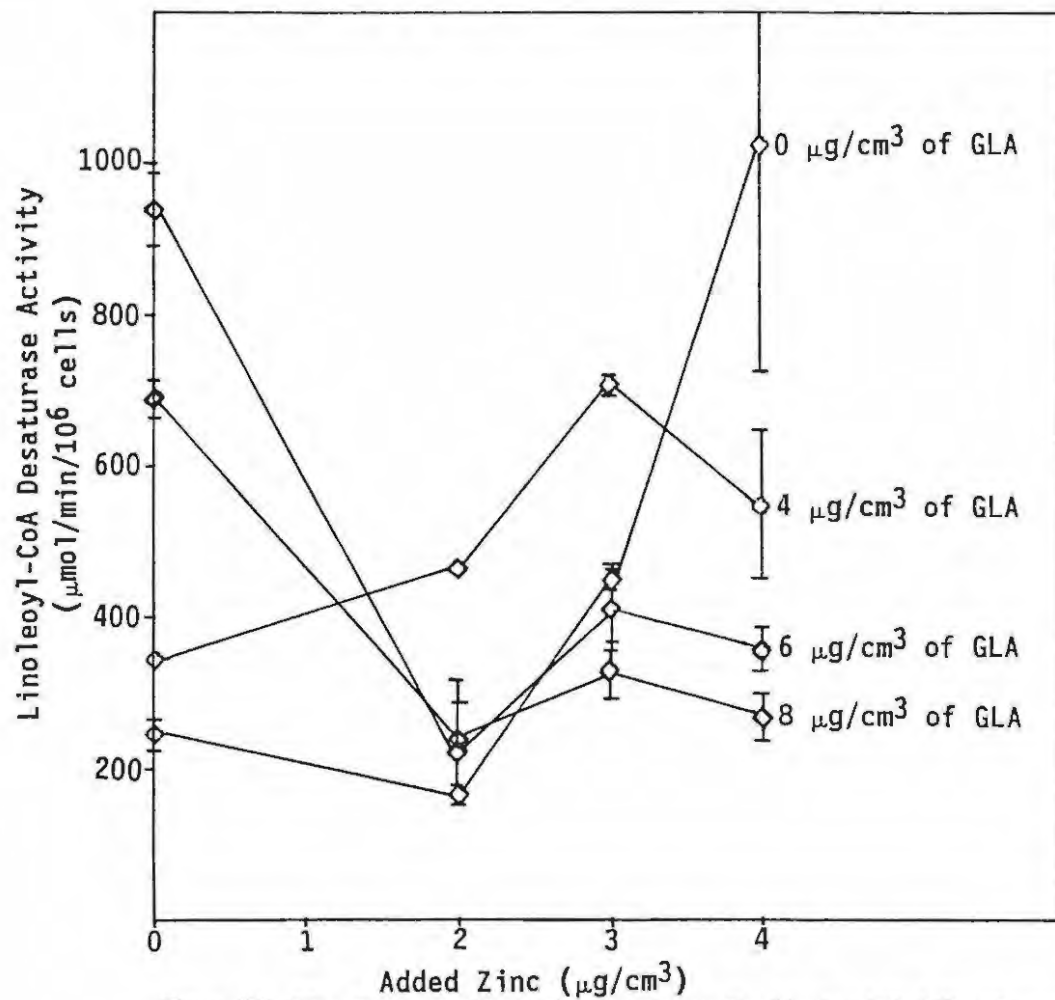


Figure 36. The effects of added GLA and zinc on the Linoleoyl-CoA Desaturase activity of LLCMK cells.

TABLE 16: THE EFFECTS OF GLA AND ZINC ADDITION (GLA KEPT CONSTANT) ON THE
LINOLEOYL-CoA DESATURASE ACTIVITY OF BL-6 AND LLCMK CELLS

[GLA : Zn] ($\mu\text{g}/\text{cm}^3$)	BL-6 Total Enzyme Activity ($\mu\text{mol}/\text{min}/10^6$ cells) $\bar{x} \pm \text{SEM}$	LLCMK Total Enzyme Activity ($\mu\text{mol}/\text{min}/10^6$ cells) $\bar{x} \pm \text{SEM}$
0 : 0	138.2 $\pm$ 34.7	243.2 $\pm$ 20.2
0 : 2	116.4 $\pm$ 7.5	166.1 $\pm$ 8.8
0 : 3	227.1 $\pm$ 8.0	452.6 $\pm$ 11.4
0 : 4	72.3 $\pm$ 1.4	1022.7 $\pm$ 295.2***
4 : 0	57.5 $\pm$ 2.1	343.7 $\pm$ 6.5
4 : 2	362.8 $\pm$ 90.8*	465.1 $\pm$ 0.0
4 : 3	838.9 $\pm$ 62.9***	711.7 $\pm$ 9.0**
4 : 4	635.8 $\pm$ 121.4***	547.2 $\pm$ 99.8
6 : 0	39.0 $\pm$ 2.1	940.5 $\pm$ 47.9
6 : 2	754.1 $\pm$ 82.1***	220.2 $\pm$ 66.1***
6 : 3	2112.2 $\pm$ 114.0***	410.2 $\pm$ 58.6***
6 : 4	834.9 $\pm$ 137.6***	357.0 $\pm$ 30.3***
8 : 0	35.4 $\pm$ 2.3	690.6 $\pm$ 24.9
8 : 2	772.5 $\pm$ 51.9***	239.0 $\pm$ 75.8***
8 : 3	1563.9 $\pm$ 198.0***	326.5 $\pm$ 40.8**
8 : 4	411.4 $\pm$ 47.2**	266.0 $\pm$ 30.7**

* $p > 0.025$

** $p > 0.005$

*** $p > 0.001$

**** $p > 0.001$

In general the effect on the enzyme activity of added zinc to the different GLA supplemented groups was variable. As can be seen in Table 16 in the 0 GLA supplemented group, a significant increase in enzyme activity was found in the cells supplemented with the highest concentrations of zinc. Similarly when 4 $\mu\text{g}/\text{cm}^3$ of GLA was added, the addition of higher concentrations of zinc resulted in increased enzyme activity. However when 6 or 8 $\mu\text{g}/\text{cm}^3$ of GLA was added, the further addition of zinc at all concentrations resulted in significant decreases in enzyme activity. In correlating the results with the cell counts in each group (see Figure 29 and Table 12 in Chapter 6) a general trend that had been found earlier was apparent i.e. the groups with the highest cell counts were those in which the lowest enzyme activity was found.

7.4 Discussion

The most significant trend that was found in the study of the effect of zinc and GLA on linoleoyl-CoA desaturase enzyme activity was that when the cell count was lowest, the linoleoyl-CoA desaturase enzyme complex activity was highest. This trend was more apparent upon the combined addition of zinc and GLA than when either was added separately. As was hypothesised in Chapter 1, the addition of zinc on its own and particularly zinc and GLA together did result in the increased activity of the linoleoyl-CoA desaturase enzyme complex. These results tend to support the hypothesis that zinc may be a

associated with the enzyme complex, linoleoyl-CoA desaturase. The increased effect on the activity of the enzyme complex with the combined addition of zinc and GLA may be the result of an increased activity of the rest of the ω -6 EFA pathway as a result of the addition of GLA to the cells. The addition of zinc may thus in turn activate the linoleoyl-CoA desaturase enzyme complex, resulting in a further stimulation of the pathway. This could result in an increased synthesis of PGE₁ (as outlined in Figure 2) which would in turn lead to an increased synthesis of cAMP. An increased synthesis of cAMP could result in regulation of cell proliferation in the tumour cells. Such an effect is more apparent in the tumour cells (BL-6) than the normal cells (LLCMK) indicating that the combined effect of GLA and zinc may be more important in the regulation of cell proliferation in tumour cells. This hypothesis is however very speculative and requires a great deal of further study.

CHAPTER 8

GENERAL DISCUSSION AND SUMMARY

The aim of the first section (Chapters 2 - 5) of this thesis was to isolate the enzyme complex, linoleoyl-CoA desaturase and the terminal enzyme in this complex, Δ^6 -desaturase. The interest in this enzyme complex was the result of the hypothesis that it played a role in the loss of regulation of cell proliferation in tumour cells as certain tumour cells had been found to have reduced or no linoleoyl-CoA desaturase activity, which may be due to a lack of the terminal component of the complex, Δ^6 -desaturase (5,22-24). In order to study this component of the complex in more detail it was necessary to isolate it from cells known to possess linoleoyl-CoA desaturase activity. Linoleoyl-CoA desaturase consists of cytochrome b₅, cytochrome b₅ reductase and Δ^6 -desaturase (326). It was decided that Δ^6 -desaturase could best be isolated from the linoleoyl-CoA desaturase complex and in order to achieve this it was necessary to first isolate the enzyme complex. It was further decided that once the complex had been isolated, the most probable chance of successfully isolating Δ^6 -desaturase would involve the enzymatic solubilisation of cytochrome b₅ and cytochrome b₅ reductase from the complex. It was therefore

necessary to first enzymatically solubilise, isolate and purify cytochrome b₅ and cytochrome b₅ reductase to optimise methodology for the purification of Δ^6 -desaturase.

Cytochrome b₅ was isolated from rat liver microsomes in a catalytically pure form. Cytochrome b₅ has a major Soret peak at 423 nm and this was utilised in the assay for the presence of cytochrome b₅. Smaller quantities of liver was used as starting material, requiring smaller number of rats to be sacrificed than in previous literature reports (260,264). It was however found that this did not adversely affect the isolation procedure, instead it simplified the procedure. Cytochrome b₅ was enzymatically solubilised from the rat liver microsomes using trypsin. In contrast to previous literature reports (260,264), the solubilisation procedure was conducted at 37°C instead of 4°C. The solubilisation procedure was conducted at this temperature as trypsin is most catalytically active at 37°C and it was hoped that this would result in increased cytochrome b₅ solubilisation (12,294,295). In order to prevent any microbial or bacterial contamination antibiotics were utilised during the solubilisation procedure. It was hoped that with the increased trypsin activity only one form of catalytically active cytochrome b₅ would be isolated, and this proved to be the case.

The isolation procedure used for cytochrome b_5 showed that it was not always necessary to conduct isolation experiments at 4°C as had previously been reported (260,264). Working at room temperature proved to be less problematic than working in a cold room. Working at room temperature did not result in any loss of cytochrome b_5 , although 4°C was utilised for a few critical stages in the isolation procedure.

It was found that during the column chromatography, slower flow rates than those cited in a literature report (260) resulted in the increased isolation and purification of cytochrome b_5 . The CM-cellulose step resulted in the removal of haemoglobin and some other protein impurities which bound to this column while the cytochrome b_5 was eluted without binding to the column. In this study it was noted that the cytochrome b_5 band did not separate into two distinct bands as previously reported (260,264). This gave an initial indication that the cytochrome b_5 had been solubilised in one catalytic form, as had been hoped.

Crystallisation of cytochrome b_5 was attempted, but as expected from literature reports (260,264), the methodology cited as well as normal organic methods were unsuccessful. It was decided to utilise freeze drying to overcome this problem and to facilitate the storage of cytochrome b_5 for long periods of time. It was found that the freeze

dried cytochrome b₅ was as catalytically active as the cytochrome b₅ solution prior to freeze drying. The freeze dried powder was very hygroscopic and, thus, had to be stored in a tightly sealed container.

Disc gel electrophoresis of the freeze dried cytochrome b₅ yielded one discrete band with a R_f value of 0.90 - 0.95. This procedure was repeated several times with similar results being obtained. This indicated that the cytochrome b₅ had been isolated in a single and pure form.

A fold purification of 7.72 was achieved utilising this modified methodology which was a larger increase in that achieved in a previous literature report - 1.36 (260). The overall yield in this study was three to four times greater than that previously cited (260). This methodology therefore resulted in the purification of a catalytically active single form of cytochrome b₅. The method was repeated several times with the same results which ensured the feasibility of using enzymatic solubilisation of cytochrome b₅ from the linoleoyl-CoA desaturase complex.

Cytochrome b₅ reductase [EC 1.6.2.2.] was isolated from rat liver microsomes utilising lysosomal solubilisation of the enzyme from the microsomes (311). The presence of cytochrome b₅ reductase was detected by utilising a potassium ferricyanide assay in preference to

a cytochrome b₅ assay as the former assay does not vary according to the quantity of free and bound cytochrome b₅ reductase, as the latter assay does, nor does it assume that cytochrome b₅ is at any time rate controlling (317,318).

As with the isolation and purification of cytochrome b₅, the quantity of starting material utilised in the isolation and purification of cytochrome b₅ reductase was less than that previously cited. The smaller quantity again did not detrimentally influence the procedure, but instead resulted in the simplification of the procedure.

Lysosomal solubilisation of the cytochrome b₅ reductase was utilised as this involved enzymatic solubilisation with the main enzyme being a cathepsin, which had been previously reported to successfully solubilise cytochrome b₅ reductase (311). It was noted that during the cytochrome b₅ reductase solubilisation procedure it was necessary that the entire microsome-lysosome pellet be solubilised for efficient enzyme solubilisation to occur. The solubilisation procedure was conducted at 4°C, but, as with the cytochrome b₅ isolation and purification, the majority of the isolation procedure was conducted at room temperature without any detrimental effects on the yield. Previous literature reports had cited the necessity of conducting the procedure at 4°C (303,311).

It was decided not to attempt to crystallise cytochrome b₅ reductase due to the problems experienced with the attempted crystallisation of cytochrome b₅ and the lack of literature reports concerning the successful crystallisation of cytochrome b₅ reductase. The purified enzyme solution was consequently freeze dried.

Disc gel electrophoresis of the freeze dried powder yielded one discrete band with a R_f value of 0.80 - 0.85. The disc gel electrophoresis was repeated several times with very similar results. The disc gel electrophoresis indicated that the cytochrome b₅ reductase had been isolated in a single and pure form. Unlike a previous literature report (311), only one band of cytochrome b₅ reductase was identified.

An earlier literature report (311) cited a much greater fold purification of this enzyme than was achieved in this study, probably due to the very low activities found in that study (311) after the solubilisation step of the procedure. The final enzyme yield achieved in this study was approximately nine times greater than previously achieved (303,311). This isolation and purification procedure was repeated several times with reproducible results being obtained. This methodology could, thus, be utilised for the enzymatic solubilisation of cytochrome b₅ reductase from the linoleoyl-CoA desaturase enzyme complex.

The isolation and purification of linoleoyl-CoA desaturase [EC 1.14.99.25.] was expected to be extremely problematic, as this had been suggested to be the case in two publications (328,330). A small number of rats were consequently sacrificed in order to avoid the possible unnecessary sacrificing of large numbers of animals. The procedure was not found to be as problematic as expected and the enzyme complex was not quickly and totally disrupted at room temperature as suggested by other workers (328,330). Room temperature was again utilised during certain steps of this isolation and purification procedure as it had proved to be extremely successful in simplifying the isolation and purification procedures of cytochrome b₅ and cytochrome b₅ reductase, despite previous literature reports to the contrary (260,264,303,311).

This was the first study to utilise the cytochrome b₅ reoxidation assay as an indication of the presence and activity of linoleoyl-CoA desaturase. This assay proved to be very efficient, as it required far less chemicals and was less time consuming. This assay was very specific for linoleoyl-CoA desaturase as the substrate for the enzyme complex is utilised in this assay and only when the substrate has been converted into the product, GLA, will the cytochrome b₅ molecules become reoxidised. Rotenone was added to the assay to prevent the possibility of any external involvement of NADH or cytochrome b₅ reoxidation (275,290,335).

Linoleoyl-CoA desaturase was solubilised from rat liver microsomes by Triton X-100 as it is a non-ionic detergent and it has been found that ionic detergents can denature proteins (263,334). Sodium deoxycholate, an ionic detergent, was utilised as the solubilising detergent in one attempted isolation procedure, but this resulted in total loss of enzyme activity. This loss of enzyme activity was presumed to either be the result of protein denaturation or the breakage of ionic bonds which could possibly be involved in holding the enzyme complex together. Although Triton X-100 was known to interfere with protein assays (263), because of the above problems with ionic detergents it was still utilised as the solubilising detergent. In order to reduce the Triton X-100 interference, very small sample volumes were utilised. However yields greater than 100 % were found, probably as a result of Triton X-100 interference with the protein assay. It was not feasible to remove the Triton X-100 from the samples not only because this would have been time consuming, but also because it was used as a stabiliser and, thus, its removal could have resulted in the total disruption of the enzyme complex (263,334,337,338).

The Triton X-100 solubilisation of linoleoyl-CoA desaturase resulted in a greatly increased fold purification (19.17) than that previously reported (328). The use of Triton X-100 also resulted in the precipitation of extraneous protein. The DEAE-cellulose

chromatography resulted in an 80 % increase in purification, as this column bound contaminating proteins and the linoleoyl-CoA desaturase was eluted through the column. The greatest fold purification increase achieved in this study was with the CM-Sephadex C-50 column. Both the columns utilised in the isolation and purification procedure therefore resulted in substantial increases in the purification of linoleoyl-CoA desaturase.

The final yield achieved in this study was three to four times greater than that cited previously in which study Triton X-100 was also utilised to solubilise the enzyme complex (328). An earlier study (328) had cited the necessity of utilising affinity chromatography in the isolation and purification procedure of this complex, but this was deemed unnecessary in this study. The linoleoyl-CoA desaturase solution was freeze dried and disc gel electrophoresis was performed on fractions from the final column chromatography step and on the freeze dried powder. Both of these samples yielded three discrete bands upon disc gel electrophoresis indicating that the enzyme complex had become disrupted as two of the bands were characteristic of cytochrome b₅ and cytochrome b₅ reductase (the bands had the characteristic R_f values reported earlier). The third band was assumed to be that of Δ^6 -desaturase.

The freeze dried powder retained most of the linoleoyl-CoA desaturase activity for the duration of this study. This was important as this purified complex was to be utilised in the next stage of this study, it was necessary that the complex be in as stable a form as possible. This methodology showed that linoleoyl-CoA desaturase could be isolated and purified from rat liver microsomes in a catalytically active form using a more simplified methodology than that cited previously (328).

As stated previously the linoleoyl-CoA desaturase enzyme complex was to be utilised for the isolation and purification of Δ^6 -desaturase by the enzymatic solubilisation of cytochrome b_5 and cytochrome b_5 reductase from the enzyme complex. The results from this study showed that although the Δ^6 -desaturase was not isolated in a totally pure form, it was possible to utilise enzymatic solubilisation to isolate the enzyme. Cytochrome b_5 reductase was enzymatically solubilised from the enzyme complex utilising cathepsin D (298,317). It was necessary to utilise two cathepsin D solubilisations before the cytochrome b_5 reductase was removed entirely from the enzyme complex. Cytochrome b_5 reductase was removed more easily than cytochrome b_5 , but this was not totally unexpected as it the first enzyme in the linoleoyl-CoA desaturase complex and also there is one molecule of cytochrome b_5 reductase for every two molecules of cytochrome b_5 . Even after two solubilisations of cytochrome b_5 utilising trypsin

there was still a small quantity of cytochrome b_5 present with the Δ^6 -desaturase (260,261).

It is suggested that future studies should utilise greater quantities of linoleoyl-CoA desaturase as the small quantity of starting material was one of the major problems experienced in attempting to purify Δ^6 -desaturase. Disc gel electrophoresis of the isolated Δ^6 -desaturase preparation was conducted several times, but no bands were detected. This again could be the result of the very small quantities of enzymes present.

The aim of the second section of this thesis (Chapters 6 and 7) was to study the effects of added zinc, GLA and the combined effects of added zinc and GLA on the activity of the enzyme complex linoleoyl-CoA desaturase, and on the in vitro cell growth of normal and tumour cells.

Initially the effects of added zinc, GLA and zinc and GLA on the cell growth of normal and tumour cell lines were studied. Zinc was utilised in this study as it had been hypothesised that it was a cofactor of linoleoyl-CoA desaturase (133,135) and GLA was utilised as it was the product of one of the reactions that linoleoyl-CoA desaturase catalyses (35). It has been hypothesised that the loss of linoleoyl-CoA desaturase activity in tumour cells could be a result of

a zinc deficiency and, thus, the addition of zinc could result in the activation of the enzyme complex and thus, the reactivation of the regulation of cell proliferation (132,133,136-143). GLA was also utilised in this study as it was hypothesised that the regulation of cell proliferation via the EFA pathway occurred through a control of cAMP synthesis by PGE₁ which is synthesised from one of the end products of the EFA pathway and it was thought that addition of GLA may result in increased synthesis of PGE₁ and hence reduced cell proliferation (25-30).

The normal cell line used in this study was LLCMK monkey kidney cells, and the tumour cell line was a murine melanoma i.e. BL-6 melanoma cells. AA spectroscopy showed that the GM contained 0.3 $\mu\text{g}/\text{cm}^3$ of zinc. Zinc supplementation was shown to stimulate the growth of LLCMK cells when 3 and 4 $\mu\text{g}/\text{cm}^3$ of zinc was added although the stimulation was only significant with the former addition. Significant inhibition of growth occurred in the LLCMK cells with the additions of 2, 5 and 10 $\mu\text{g}/\text{cm}^3$ of zinc. In BL-6 cells inhibition of growth occurred at all levels of zinc addition. Thus high concentrations of added zinc resulted in the inhibition of cell growth in both the cell lines whereas low concentrations only reduced cell growth in the tumour cells. These results agree with previous literature reports (244,252,253,256) and with other experiments conducted in this laboratory.

The addition of GLA did not dramatically reduce the cell counts of LLCMK cells, but the BL-6 cells did show a decrease although this was not as significant as with the addition of zinc. All the additions of GLA significantly reduced the ^3H -thymidine incorporation into DNA in the BL-6 cells indicating a reduced level of DNA replication in the BL-6 cells. This decrease was not noted in the LLCMK cells except when $6\text{ }\mu\text{g}/\text{cm}^3$ of GLA was added. GLA had previously been shown to inhibit the growth of tumour cells and thus the results achieved in this study correlated with previous findings (211-223).

The effects of the combined addition of zinc and GLA (the zinc concentration was kept constant and the GLA concentration was varied initially and then this was reversed) on the growth of the BL-6 and LLCMK cells was subsequently studied. When zinc and GLA were added in combination the BL-6 cells showed a significant reduction in the cell growth compared to that found when GLA was added alone (this occurred in both the cell counts and the ^3H -thymidine incorporation into DNA in the BL-6 cells). The growth of the LLCMK cells was not substantially affected by the combined addition and the effects were variable with no general trend emerging.

When the GLA concentration was held constant and the zinc concentration was varied it was noted that there was a significant decrease in the cell counts of the BL-6 cells in cell cultures

to which GLA was added except in that group to which $4 \mu\text{g}/\text{cm}^3$ of zinc had been added. The ^3H -thymidine incorporation into DNA generally followed the same trend except for the addition of $8 \mu\text{g}/\text{cm}^3$ of GLA, although this did vary with the concentration of zinc added. The effect of the combined addition on the cell growth of the LLCMK cells was not as noticeable as with the BL-6 cells. The addition of zinc and GLA was shown to result in generally increased cell counts of the LLCMK cells with the addition of 6 and $8 \mu\text{g}/\text{cm}^3$ of GLA and varying concentrations of zinc, when compared to the control. The effects on the values of the ^3H -thymidine incorporation into DNA were varied and both statistically significant increases and decreases were found.

This study showed that it could be possible to manipulate the cell growth of both the BL-6 and LLCMK cells by varying the concentrations of zinc and GLA added to the GM. It was also shown in this study that it was possible to inhibit the cell growth of the BL-6 cells while maintaining the normal growth of the LLCMK cells. The combined addition of zinc and GLA resulted in a general trend of increased cell growth inhibition in the BL-6 cells but not in the LLCMK cells. The cell growth of tumour cells in vitro could thus be manipulated by varying the supply of zinc and/or GLA.

A further study concerned the activity of linoleoyl-CoA desaturase in BL-6 and LLCMK cell lines and the effect of the addition of zinc, GLA and the combined addition of zinc and GLA on this activity. The

cytochrome b₅ reoxidation assay was utilised to assay for the presence and activity of the linoleoyl-CoA desaturase enzyme complex.

In general it was noted that as the cell count decreased the linoleoyl-CoA desaturase activity increased with the addition of zinc. Although the enzyme activity increased in the BL-6 cells it was always lower than that noted in the LLCMK cells except for the controls which were very similar. The only significant increase in enzyme activity in the BL-6 cells was found with the addition of $10 \mu\text{g}/\text{cm}^3$ of zinc. The addition of zinc resulted in statistically significant increases in enzyme activity of the linoleoyl-CoA desaturase enzyme complex in the LLCMK cells at all concentrations of added zinc except in the cells to which the addition of $2 \mu\text{g}/\text{cm}^3$ of zinc had been added. In general the linoleoyl-CoA desaturase activity was lower in the BL-6 cells than in the LLCMK cells when zinc was added and at various concentrations this difference was statistically significant.

With the addition of GLA the linoleoyl-CoA desaturase activity curve was almost identical to the cell count curve of the BL-6 cells. The addition of GLA resulted in no significant changes in the linoleoyl-CoA desaturase activity in the BL-6 cells. In the LLCMK cells however, the addition of GLA resulted in statistically significant increases in the enzyme activity when 6 and $8 \mu\text{g}/\text{cm}^3$ of GLA were added.

When both zinc and GLA were added (the zinc concentration was kept constant and the GLA concentration was varied initially and then this was reversed) it was noted that there was generally an inverse relationship in the BL-6 cells between cell count and linoleoyl-CoA desaturase activity i e the inhibition of cancer growth occurred simultaneously with the activation of the linoleoyl-CoA desaturase enzyme complex. Although it cannot be categorically stated that the activation of the enzyme complex resulted in the growth inhibition, the relationship is still very interesting. When only GLA was added the BL-6 cell count was much greater than when zinc and GLA were added together, while the linoleoyl-CoA desaturase activity was significantly lower when both the zinc and GLA were added. If the cell count curves (when zinc and GLA had been added together) were studied it was noted that they were the inverse of the linoleoyl-CoA desaturase activity curves of the BL-6 cells. It was noted that the addition of both zinc and GLA did not noticeably influence the activity of linoleoyl-CoA desaturase in the LLCMK cells except at the highest GLA concentration i e $8 \mu\text{g}/\text{cm}^3$ of GLA which resulted in significantly reduced linoleoyl-CoA desaturase activity in all groups when compared to the control.

When the GLA concentration was held constant and the zinc concentration was varied, it was again noted that when both GLA and zinc were added the cell count curves of the BL-6 cells were almost inverse curves of the linoleoyl-CoA desaturase activity curves of the

BL-6 cells. The combined addition of zinc and GLA resulted in significant increases in the linoleoyl-CoA desaturase activity in the BL-6 cells. This would again suggest that cancer cell growth inhibition occurs simultaneously with the activation of the linoleoyl-CoA desaturase enzyme complex. It was also noted that apart from when 0 and 8 $\mu\text{g}/\text{cm}^3$ of GLA were added and zinc was added, the LLCMK cell count curves were inverse curves of the linoleoyl-CoA desaturase activity curves of the LLCMK cells. The combined addition of GLA and zinc to the LLCMK cells resulted in significant increases in the linoleoyl-CoA desaturase activity in all groups to which zinc had been supplemented when 6 or 8 $\mu\text{g}/\text{cm}^3$ of GLA was also added.

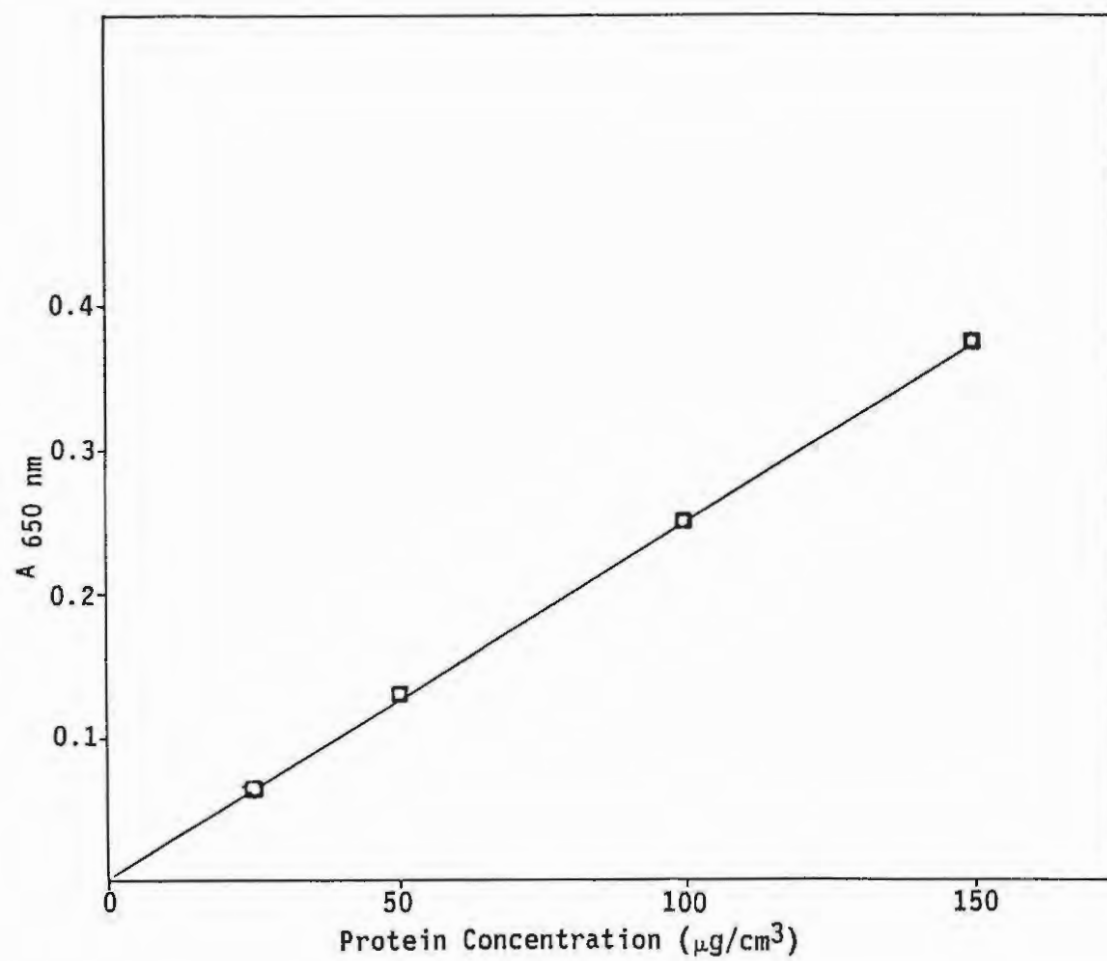
These results suggest an association between the addition of zinc and GLA and the inhibition of cancer cell growth. This may occur via the reactivation of the linoleoyl-CoA desaturase complex. These results also support the hypothesis that zinc is a cofactor of the linoleoyl-CoA desaturase enzyme complex (133,135). This fits in well with the hypothesis that the loss of regulation of cell proliferation in tumour cells may be related to the inhibition of linoleoyl-CoA desaturase activity and if this activity could be restored, it may be possible to restore the regulation of cell proliferation.

While some relationship between the effects of zinc and GLA addition on the growth of normal (LLCMK) cells appears to exist and that these

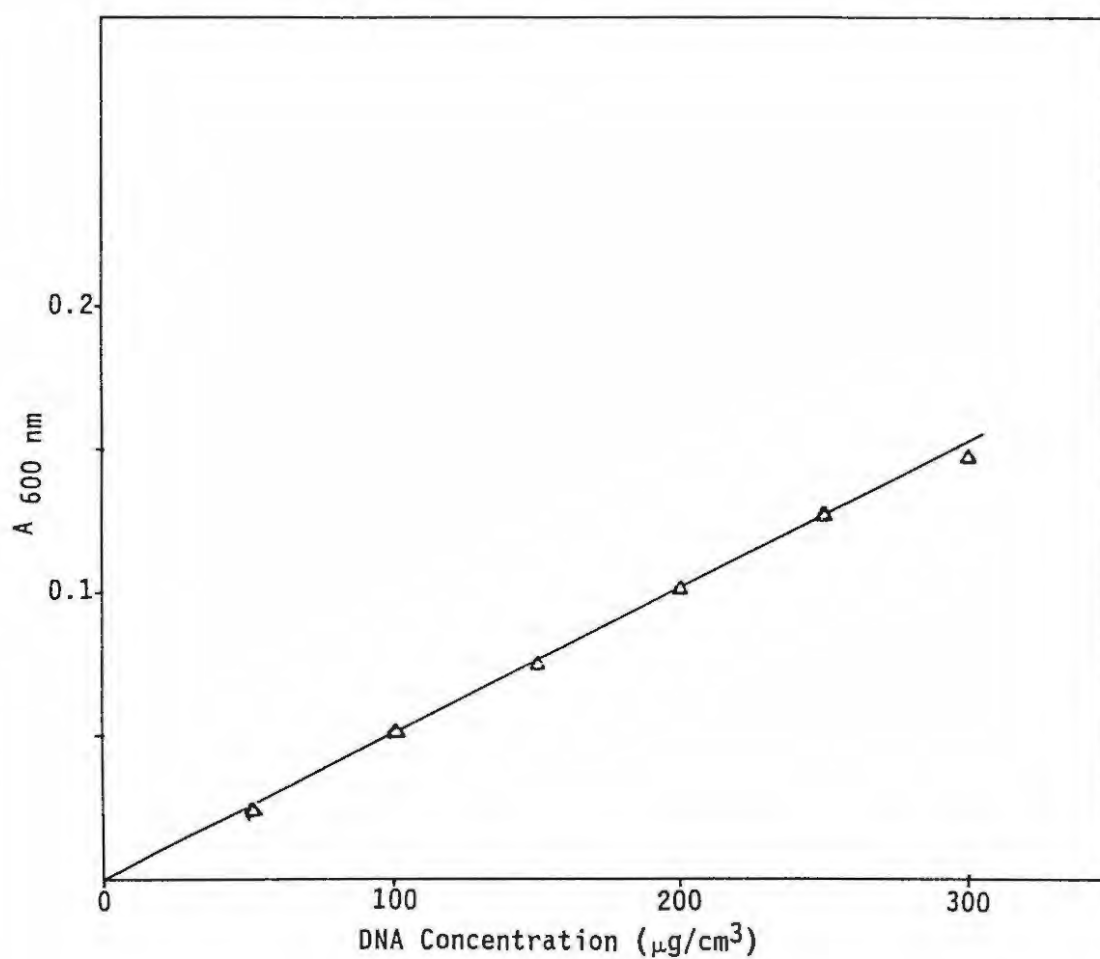
effects may be related to an association with the linoleoyl-CoA desaturase complex, this relationship is not as significant or as consistent as that found with the BL-6 cells.

The results of this study raise the possibility of a treatment of cancer, for certain tumours at least, by the use of dietary supplementation of both zinc and GLA. However, for such a possibility to be tested, a great deal of further research is required on the biochemical sites of action of the two nutrients. It is hoped that this study will further support the possible association of zinc with the linoleoyl-CoA desaturase enzyme complex as one site of biochemical action and that the methods described for the isolation of the complex and its components will provide a basis for more in depth studies on this enzyme complex.

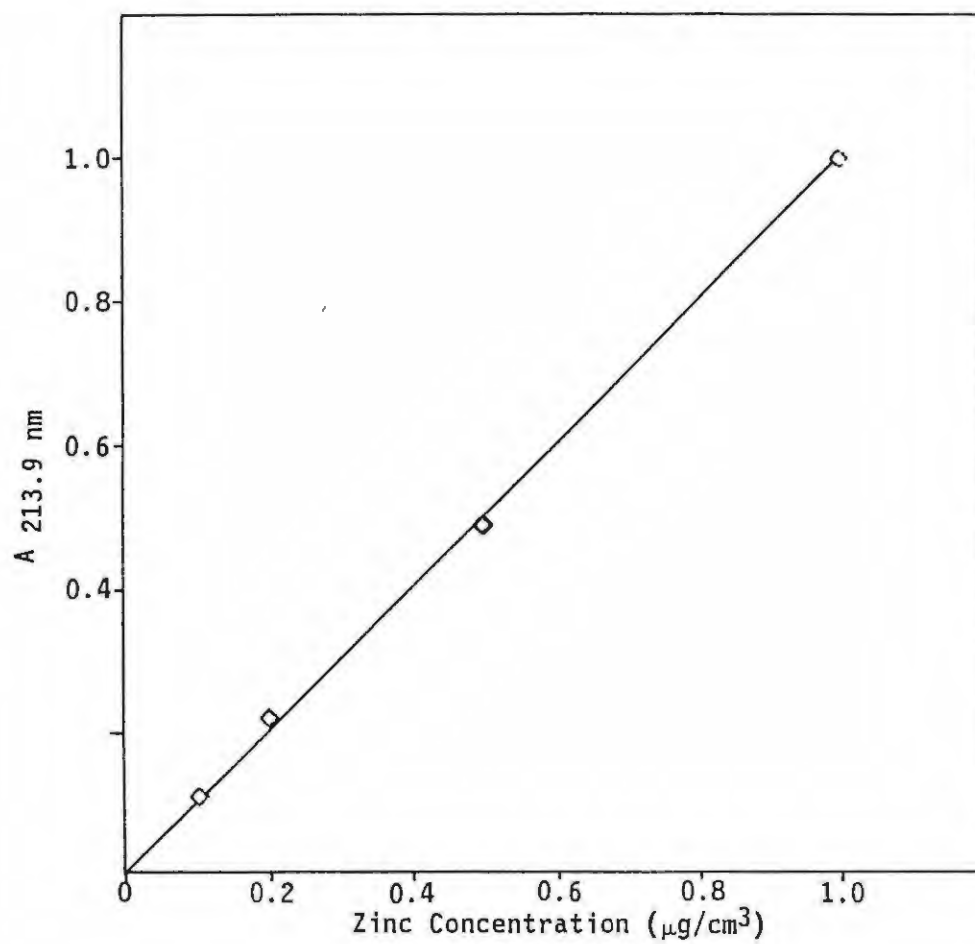
Appendix 1. Protein standard curve. The SEM values were not included as they were negligible.



Appendix 2. DNA standard curve. The SEM values were not included as they were negligible.



Appendix 3. Zinc standard curve. The SEM values were not included as they were negligible.



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