# THE MODULATION OF RAT LIVER MICROSOMAL CALCIUM ION-PUMPING ATPase BY DICOPHANE AND LOW PROTEIN INTAKE

*BY*

GBENGA ADEBOLA ADENUGA
M.Sc. Biochemistry (Kharkov)

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# ABSTRACT

The effects of the liver tumour promoter, dicophane, with those of low protein intake (LPI) on the functional expression of rat liver microsomal $Ca^{2+}$-ATPase were compared. The effects of dicophane and LPI on the activity of the microsomal enzyme after carcinogenic initiation by pretreatment with aflatoxin $B_1$ ($AFB_1$), a genotoxic liver carcinogen, were also compared. The status of membrane - bound $Ca^{2+}$-ATPase of erythrocytes of humans having primary liver cancer (PLC) and kwashiorkor was assessed.

The specific activity of membrane - bound microsomal $Ca^{2+}$-ATPase of the livers of untreated rats was 4.543 $\pm$ 0.857 µmole P/mg protein/hr. at pH 8.0 and was insensitive to calmodulin. The specific activity of the enzyme was significantly decreased ($P < 0.01$) following subcutaneous administration of a single dose of 75mg dicophane/kg body wt.; the affinity of the enzyme for $Ca^{2+}$ was however unaffected. Similarly, liver microsomal $Ca^{2+}$-ATPase activity was significantly diminished following the ingestion of low protein diet by rats for 12 weeks. The mean $Ca^{2+}$-ATPase activity of $AFB_1$-treated animals (in the absence of dicophane) was not significantly different ($P > 0.05$) from that of $AFB_1$-treated rats which subsequently received dicophane. In contrast, liver microsomal $Ca^{2+}$-ATPase activity of animals fed low protein diet prior to and after AFB ingestion was higher ($P < 0.05$) than that of animals which were on low protein diet only. Basal activity of erythrocyte $Ca^{2+}$-ATPase in paediatric controls and those having kwashiorkor (protein-energy-malnutrition) were similar ($P > 0.05$); similar observations were made between normal adults and those suffering from PLC. Erythrocyte $Ca^{2+}$-ATPase of either PLC or kwashiorkor patients was however, somewhat, less sensitive (15-40 %) to the stimulatory effect of calmodulin, an endogenous activator of the $Ca^{2+}$-pump.

These results suggest that liver microsomal $Ca^{2+}$-ATPase could be a useful biochemical marker to determine the onset or occurrence of tumour promotion in liver cells. Finally, chronic dietary protein malnutrition mimics the effect of chemical liver tumour promoters and could possibly enhance the development of human PLC particularly in those areas of the tropics where malnutrition is prevalent. Future confirmatory experiments are however required to fully justify this postulate.

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# DEDICATION

This work is dedicated to my father Mr. Emmanuel ADENUGA Senboyejo and the entire ADENUGA FAMILY.

# CERTIFICATION

I certify that this work was carried out by Mr. Gbenga Adebola ADENUGA at the Department of Biochemistry, University of Ibadan, Nigeria.

Professor E.A. Bababunmi Ph.D., D.Sc., FRCPath.
*SUPERVISOR.*

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# ABBREVIATIONS

DDT - Dicophane or Dicholorodiphenyltrichloroethane or 1,1,1, trichloro 2,2-bis (p-chlorophenyl) ethane

PB - Phenobarbital

EGM - Erythrocyte Ghost Membrane

LPI - Low Protein Intake

$AFB_1$ - Aflatoxin $B_1$

PLC - Primary Liver Cancer

$Ca^{2+}$-ATPase or ($Ca^{2+}$ + $Mg^{2+}$) - ATPase - $Mg^{2+}$-stimulated calcium-dependent Adenosine triphosphatase or ATP phosphohydrolase

GGT - Gamma Glutamyl Transpeptidase

SDS-PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

DNA - Deoxyribonucleic acid

PI - Isoelectric point

cAMP - Cyclic Adenosinetriphosphate

KDa - Kilodalton

FITC - Fluorescein Isothiocyanate

CaM - Calmodulin

hPMCA - Human Plasma Membrane $Ca^{2+}$-ATPase

rPMCA - Rat Plasma Membrane $Ca^{2+}$-ATPase

ATP - Adenosine Triphosphate

TFP - Trifluoperazine

$[Ca^{2+}_{sm}]$ - Submembrane Concentration of Calcium

PKC - Protein Kinase C

Bis-acrylamide - N, N'methylene-bis-acrylamide

$PIP_2$ - Phosphatidylinositol-4,5-bisphosphate

$IP_3$ - Inositoltrisphosphate

DAG - Diacylglycerol

GTP - Guanosinetriphosphate

[O] - Activated Oxygen (AO)

TPA or PMA - 12-0-Tetradecanoylphorbol-13-acetate orPhorbol-12-Myristate-13-acetate

PLC - Phospholipase C

PDGF - Platelet Derived Growth Factor

EGF - Epidermal Growth Factor

S6 - Small Ribosomal Subunit protein 6.

$AP_1$ - Transcription factor $AP_1$

ADPR - ADP-ribose or Adenosinediphosphate ribose

ER - Endoplasmic reticulum

Tris - Tris (hydroxymethyl) methylamine or 2-amino-2-(hydroxymethyl) propane-1,3-diol

EGTA - Ethyleneglycol-bis-( -aminoethyl ether) NNN'N'-tetraacetic acid

PMSF - PhenylmethylSulfonylfluoride

EDTA - Ethylenediaminetetraacetic acid

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonicacid

BSA - Bovine Serum albumen

AMC - 7-Amino-4-methyl-Coumarin

Ammediol-2-amino 2-methyl-1,3, propanediol

TEMED - N,N,N',N'-tetramethylethylenediamine

SR - Sarcoplasmic reticulum

HBV - Hepatitis B Virus

DMSO - DimethylSulfonyloxide

# CHAPTER ONE

## INTRODUCTION

### 1.1 Definitions and concepts of the multi-stage process of carcinogenesis

A tumour is a large aggregation of cancer cells, derived from a single "founder" cell. The single ancestor was once a normal cell, with a normal function in a particular tissue but somehow underwent a fundamental change. As a result of that change it began to divide and proliferate in response to some imperative of its own rather than in response to the external stimuli ordinarily required for cellular growth. (Weinberg, 1983). Tumours arise with great frequency especially in older animals and humans, but most pose little risk to their host because they are localized. On rare occasions, they become life-threatening because they spread throughout the body and become malignant (Fig. 1).

Benign tumours contain cells that closely resemble normal cells and may function as such. The forces that keep benign tumour cells (and normal cells) localized to appropriate tissues are not clearly understood. A fibrous capsule usually delineates the extent of a benign tumour and makes it an easy target for the surgeon. They become serious medical problems only if their sheer bulk interferes with normal functions or if they secrete excess amounts of biologically active substances like hormones. The major characteristics that differentiate malignant tumours from benign ones are their properties of invasiveness and spread. Malignant tumours do not remain localized and encapsulated: they invade surrounding tissues, get into the body's circulatory system, and set up areas of proliferation away from the site of their original appearance in a process known as metastasis (Darnell et al., 1986).

The abnormal behaviour of cancer cells is characterized by many distinctive traits the most obvious of which is uncontrolled growth. Cancer cells often exhibit a shape that is very different from that of their normal counterparts. They fail to respect the territorial rules that confine normal cells to particular tissues. Many of them import sugar molecules at an unusually high rate. They also rely to an unusual extent on anaerobic metabolism. The outer membrane of cancer cells is different from that of a normal cell and displays special tumour

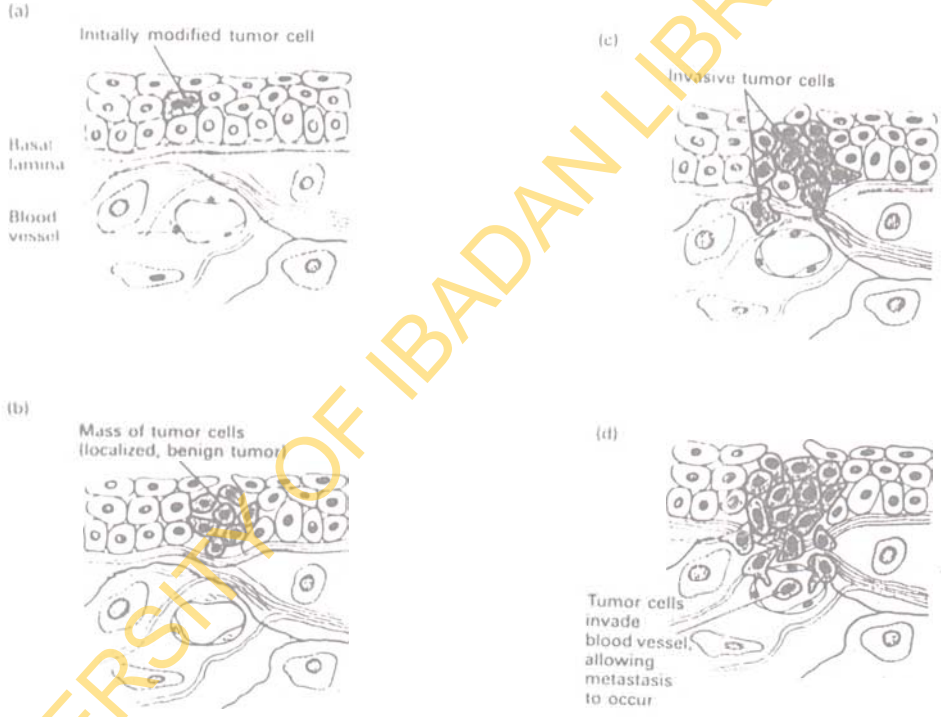



Figure 1. Stages in tumour growth and metastasis (Darnell et al., 1986)

antigens giving the cells distinctive immunological properties (Weinberg, 1983).

Malignant cells usually have enough of the hallmarks of the normal cell type from which they were derived, that it is possible to classify them by their relationship to normal tissue. Normal cells arise from one of three embryonic cell layers-endoderm, ectoderm or mesoderm. Malignant tumours derived from epithelial tissues such as the skin and the lining of the digestive or respiratory tract, are called carcinomas. Those derived from tissues of mesodermal origin, such as connective tissues, bones, or muscles are referred to as sarcomas. The leukemias are a subdivision of sarcoma (Klein, 1982; Darnell et al. 1986).

Neoplasm that arise in animals that have not been treated in anyway to cause the development of cancer are known as spontaneous while those that are produced purposely by a cancer-producing agent or carcinogen are termed induced neoplasm.

Carcinogens can be classified into three major groups viz physical, chemical and biological. Radiation (e.g UV radiation) chemical compounds (e.g polycyclic hydrocarbons and aromatic amines) biological factors (e.g hormones and the oncogenic viruses) are examples of physical, chemical and biological carcinogens respectively. The most widely studied and better understood of the three is chemical carcinogens. While some chemical carcinogens are genotoxic, others are not, and are referred to as epigenetic carcinogens (Table 1). Tumour promoters belong to the latter group (Weisburger and Horn, 1982). There are two broad categories of genotoxic chemical carcinogens, direct-acting and indirect-acting, with the latter requiring metabolic activation to become carcinogens (Fig. 2). The direct-acting carcinogens, of which there are only a few, are reactive electrophiles (compounds that seek out and react with negatively charged centers in other compounds). The metabolic activation process of indirect carcinogens makes ultimate carcinogens from the precursors by giving them electrophilic centers. The metabolic activation of carcinogens is carried out by drug metabolizing enzymes which are bound to the endoplasmic reticulum. The pathway involved in the metabolic activation of aflatoxin $B_1$ - an indirect-acting carcinogens - is illustrated in Fig. 3.

Chemical carcinogenesis is a multistep process. Rous and Kidd (1941) were among the first to provide experimental evidence suggesting a two-stage mechanism for carcinogenesis in skin. Within the last two decades, however, it has become apparent that the two-stage phenomenon in the development of neoplasm is not unique to the skin. According to this concept, there is a stage of initiation when a cell is affected by a carcinogen and the promotion stage when tumour growth occurs.

Table 1: CLASSIFICATION OF CARCINOGENIC CHEMICALS

| Type | Mode of action | Example |
|---|---|---|
| Genotoxic | | |
| 1. Direct-acting | Electrophile, organic compound genotoxic, interacts with DNA | Ethylene imine, bis (chloromethyl) ether |
| 2. Procarcinogen | Requires conversion through metabolic activation by host or in vitro to type 1 | Vinyl chloride, benzo (a) pyrene 2-naphthylamine, dimethylnitrosamine |
| 3. Inorganic carcinogen | Not directly genotoxic, leads to changes in DNA by selective alteration in fidelity of DNA replication | Nickel, chromium |
| Epigenetic | | |
| Solid-state carcinogen | Exact mechanism unknown: usually affects only mesenchymal cells and tissues: physical form vital | Polymer or metal foils, asbestos |
| Hormone | Usually not genotoxic: mainly alters endocrine system balance and differentiation: often acts as promoter | Estradiol, diethylstilbestrol |
| Immunosuppressor | Usually not genotoxic: mainly stimulates "virally induced", transplanted or metastatic neoplasms | Azathioprine, antilymphocytic serum |
| Cocarcinogen | Not genotoxic or carcinogenic but enhances effect of type 1 or type 2 agent when given at the same time. May modify conversion of type 3 to type 1 | Phorbol esters, catechol, ethanol n-dodecane, $SO_2$. |
| Promoter | Not genotoxic or carcinogenic, but enhances effect of type 1 or type 2 agent when given subsequently | Phorbol esters, phenol anthralin, bile acids,tryptophan metabolites, saccharin |

(Weisburger and Horn, 1982)

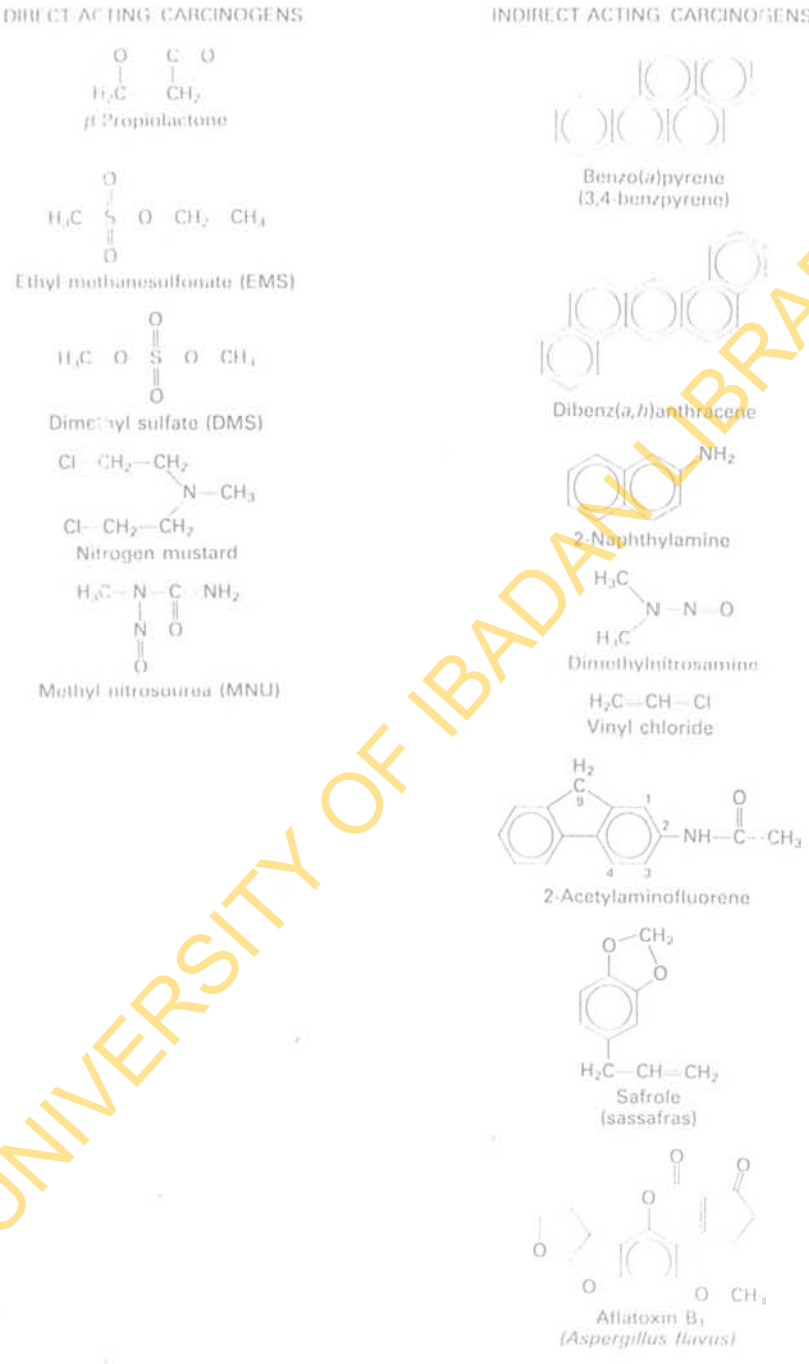





Fig. 2 Structures of selected chemical carcinogens (Darnell et al., 1986)

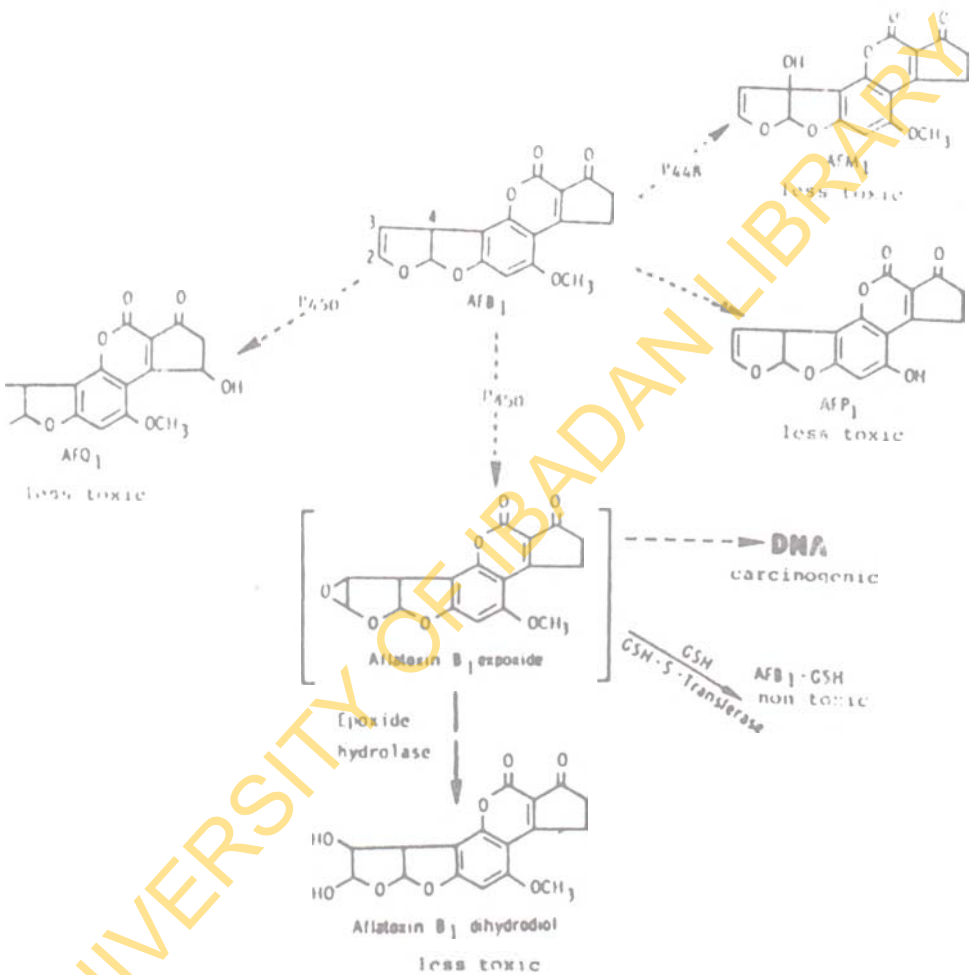



Fig. 3 Principal aflatoxin-B1 toxifying and detoxifying pathways (Mandel et al., 1987)

A more general concept which extends the two stage mechanism is that of tumour progression proposed by Foulds (1969). The importance of the progression stage was recently emphasized by Cerutti (1987) (Fig. 4). First, a carcinogenic initiator causes genetic damage but no visible tumour. There is then a promotion stage during which the controls of cell growth and differentiation are disturbed. The affected cells expand to a visible tumour at the expense of its neighbours. Finally, there can be a progression stage during which the tumour becomes malignant - it infiltrates surrounding tissue and metastasizes.

An initiating agent or initiator is a chemical, physical or biological agent which is capable of directly altering in an irreversible manner the native molecular structure of the genetic component (DNA) of the cell. Such alteration(s) maybe the result of a covalent reaction of DNA with the initiating agent itself or with one of its metabolites, but this alteration may also include a distortion of the structure of DNA without covalent binding to its components. Initiation is irreversible, and a cell once initiated, does not lose this induced property with time. The initiated cells and immediate progeny are not usually identifiable. Pure initiator (incomplete carcinogen) causes irreversible change but not neoplasm unless a promoter is applied. This process is dependent on cell cycle and for many chemicals on the metabolism of the cell (Pitot and Sirica, 1980).

A promoting agent or promoter is an agent that alters the expression of genetic information of the cell. Example of such agents include hormones, drugs, plant products etc., which in themselves do not directly react with the genetic material but rather affect its expression by a variety of mechanisms including their interaction with cell surface receptors or with cytoplasmic and nuclear protein receptors, or by an alteration of other cellular components and functions. The promoting stage is reversible, at least in early stages. Promoted neoplasm can be seen grossly. Promoting agents are not carcinogenic but may promote fortuitously initiated cells. Promotion is modulated by dietary, hormonal, environmental, and related factors (Pitot and Sirica, 1980).

Carcinogens may gain access to the body by several routes

(a) through the skin e.g. polycyclic hydrocarbons and products containing them such as tars and soot

(b) through the respiratory tract (by inhalation) e.g. soot, polycyclic hydrocarbons, asbestos, nickel, vinyl chlorides and naphthyl amine, smoke of cigarette and

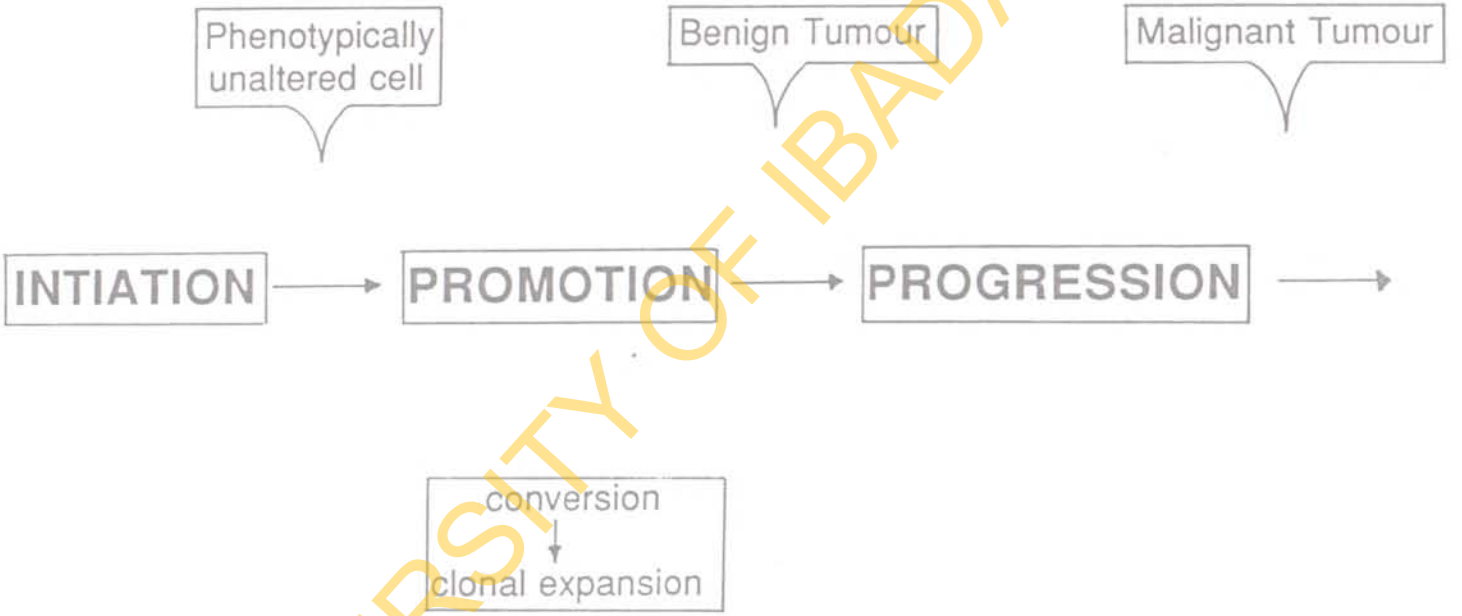



Fig. 4 Multistep carcinogenesis: Mechanistic model (Cerutti, 1987)

(c) through the digestive tract.

Carcinogens can originate in the digestive tract from several sources:

1. They can be natural constituents of the food or metabolites of fungal organisms contaminating it e.g. mycotoxins such as aflatoxin $B_1$.
2. They can be formed by interactions of foodstuffs e.g. formation of nitrosamines from secondary amines which can be present as components of fish products and nitrites which can be ingested with plant products or added to food products as preservative.
3. They can be formed during cooking e.g. imidazoquino line may be generated by the heating of a mixture of creatinine, glycine and glucose, and nitropyrenes are formed when meat is cooked over a smoky flame i.e. barbecued meat.
4. They can be formed by bacterial action on partially digested foods or on the bile acids (Thomas and Gillham, 1985).

Dose and frequency of application, synergism and promoters are some of the factors affecting the carcinogenicity of chemicals. The incidence of tumours produced by any chemical is dependent on the total dose and the frequency of application, although simple quantitative predictions are seldom possible. Two chemicals may frequently exhibit synergistic effects. Although it is possible that one of the chemicals may be acting as a promoter, this is by no means definite. Addition of promoter after initiator will always induce tumours, whereas, when promoter precedes initiator no tumours develop (Thomas and Gillham, 1985).

In a specific tissue, cancer cells are usually recognized by the characteristics of rapidly growing cells : a high nucleus-to-cytoplasm ratio, prominent nucleoli, many mitoses, and relatively little specialized structure. The presence of invading cells in an otherwise normal tissue section is the most diagnostic indication of a malignancy (Darnell et al., 1986). Cancer cells have abnormal and unstable numbers of chromosomes as well as many chromosomal abnormalities.

The study of cells in an animal is impractical because of the difficulty of identifying the relevant cells, manipulating their behaviour in a controlled manner, and separating the effects due to the intrinsic properties of the cells from the effects due to the interactions among the many cell types present in the organism. When cells growing in culture are exposed to a

carcinogen, these problems can be controlled. The environment of the cell can be controlled. The environment of the cell can be manipulated by the investigator, the target cell can be well defined, the changes in the cell following treatment can be examined, and the fate of the carcinogen can be determined. Furthermore, in culture, the cells can be quiescent to growing; they can have, in fact, precisely defined growth parameters. They can also be manipulated genetically. For these reasons, studies of normal cell growth as well as of cancer induction depend heavily on the use of cultured cells. Exposure of cells to carcinogens can dramatically change their growth properties in culture. Furthermore, these treatments can cause the cells to form tumours after they are injected into susceptible animals. Such changes in the growth properties of cells and their subsequent development of tumour forming capacity are collectively referred to as malignant transformation, or just transformation. Because transformation can be carried out entirely in culture, it is widely studied as an analog of cancer induction in animals, although the relationship between the two processes has not been established.

The transformation of adherent cells involves changes in a constellation of cellular properties. These include: Increased saturation density, decreased growth factor requirements, loss of capacity for growth arrest, loss of dependence on anchorage for growth, changed cell morphology and growth habits, loss of contact inhibition of movement, cell surface alterations, easier agglutination by lectins, increased glucose transport, reduced or absent surface fibronectin, loss of actin microfilaments, release of transforming growth factors, protease secretion, altered gene transcription and immortalization (Darnell et al., 1986).

An important step forward in the understanding of one way in which chemical carcinogens might cause transformation of cells was made when it was appreciated that some cells transformed in this way possess activated oncogenes i.e. genes that cause cancer. It is now recognized that two classes of oncogenes exist in nature: endogenous or cellular oncogenes, which are present in the germ line, and exogenous or viral oncogenes which may be found in several types of retrovirus or in the genomes of tumour cells. In fact, the term oncogene is not quite accurate as far as the endogenous genes are concerned since they are not normally oncogenic (although they may well be expressed as normal genes). The term proto-oncogene has, therefore, been introduced to indicate that they require some sort of alteration

or mutation (which may be induced by chemical carcinogens) for their oncogenic potential to be expressed (Bishop, 1982). A number of molecular mechanisms have been implicated in the conversion of proto-oncogenes to oncogenes. In the case of mammalian tumour genes they include point mutation, chromosomal rearrangement and gene amplification. In the case of retro-virus associated genes, the transduced gene may undergo mutation or may end up adjacent to a viral regulator that increases its level of expression (Weinberg, 1983).

It is now clear, from an evolutionary point of view, that proto-oncogenes have been greatly conserved. This observation has led to the conclusion that they play an important role in normal cell biology and that this in some way becomes perverted in transformed cells. A possible biological role for the proto-oncogenes being actively considered at present, and for which there is some evidence is that they are involved in development (Thomas and Gillham, 1985). Like other genes, oncogenes encode proteins. However, the proteins encoded by oncogenes function abnormally, and somehow induce the transformation of a normal cell into a cancer cell. Some known oncogenes, their protein products and their functions are shown in Table 2. Some are protein kinases, others are growth factors, GTP-binding proteins or DNA-binding proteins (Hunter, 1984).

Hepatocellular carcinoma, is one of the ten most frequent cancers worldwide accounting for 4% of the total. While relatively rare in Europe and the Americas, it is frequent in The People's Republic of China, where almost one-half of the new cases in the world (251, 200 cases) occur, and in Africa. The aetiology of this cancer has been associated with two major risk factors, persistent hepatitis B virus (HBV) infection and exposure to dietary aflatoxins, although other aetiological agents like smoking, and some occupational exposures, have also been implicated (Cova et al., 1990). A number of studies have demonstrated many similarities between the pathogenesis and morphological changes of experimental and human liver cancer. Evidence that specific environmental chemicals from industrial, medical and dietary sources are carcinogenic to the human has now become clear (Pilot and Sirica, 1980).
It is well established that during the administration of hepatocarcinogens to the rodent, several distinct focal and nodular hyperplastic lesions develop in the liver before the appearance of hepatocarcinoma. Some of the specific biochemical markers used to distinguish these preneoplastic lesions from normal liver parenchyma are shown in Table 3.

Table 2: Protein products of known Oncogenes

| NAME OF ONCOGENE | RETROVIRUS | TUMOR | ONCOGENIC PROTEIN | | |
|---|---|---|---|---|---|
| | | | CELLULAR LOCATION | FUNCTION | CLASS |
| src | CHICKEN SARCOMA | - | PLASMA MEMBRANE | TYROSINE-SPECIFIC PROTEIN KINASE | CLASS 1 (CYTOPLASMIC TYROSINE PROTEIN KINASE |
| yes | CHICKEN SARCOMA | - | PLASMA MEMBRANE(?) | | |
| fgr | CAT SARCOMA | - | (?) | | |
| abl | MOUSE LEUKEMIA | HUMAN LEUKEMIA | PLASMA MEMBRANE | | |
| fps | CHICKEN SARCOMA | - | CYTOPLASM (PLASMA MEMBRANE?) | | |
| fes | CAT SARCOME | - | CYTOPLASM (CYTOSKELETON?) | | |
| ros | CHICKEN SARCOMA | - | (?) | | |
| erb-B | CHICKEN LEUKEMIA | - | PLASMA AND CYTOPLASMIC MEMBRANES | EGF RECEPTOR'S CYTOPLASMIC TYRO- SPECIFIC KINASE | CLASS 1-RELATE (POTENTIAL PROTEIN KINASES) |
| fms | | - | PLASMA AND CYTOPLASMIC MEMBRANES | CYTOPLASMIC DOMAIN OF A GROWTH-FACTOR RECEPTOR (?) | |
| mil | CHICKEN CARCINOMA | - | CYTOPLASM | (?) | |
| raf | MOUSE SARCOME | - | CYTOPLASM | (?) | |
| mos | MOUSE SARCOMA | MOUSE LEUKEMIA | CYTOPLASM | (?) | |
| sis | MONKEY SARCOMA | - | SECRETED | PDGF-LIKE GROWTH FACTOR | CLASS 2 (GROWTH FACTOR |
| Ha-ras | RAT SARCOMA | HUMAN CARCINOMA RAT CARCINOMA | PLASMA MEMBRANE | GTP-BINDING | CLASS 3 (CYTOPLASMIC, GTP-BINDING) |
| Ki-ras | RAT SARCOMA | HUMAN CARCINOMA, LEUKEMIA AND SARCOMA | PLASMA MEMBRANE | | |
| N-ras | - | HUMAN LEUKEMIA AND CARCINOMA | PLASMA MEMBRANE | | |
| fos | MOUSE SARCOMA | - | NUCLEUS | (?) | CLASS 4 (NUCLEAR) |
| myc | CHICKEN LEUKEMIA | HUMAN LYMPHOMA | NUCLEUS | DNA-BINDING | |
| myb | CHICKEN LEUKEMIA | HUMAN LEUKEMIA | NUCLEUS | (?) | |
| B-lym | - | CHICKEN LYMPHOMA, HUMAN LYMPHOMA | NUCLEUS (?) | (?) | |
| ski | CHICKEN SARCOMA | - | NUCLEUS (?) | (?) | |
| rel | TURKEY LEUKEMIA | - | (?) | (?) | UNCLASSIFIED |
| erb-A | CHICKEN LEUKEMIA | - | (?) | (?) | |
| ets | CHICKEN LEUKEMIA | - | (?) | (?) | |

(Hunter, 1984)

Table 3: Some properties of enzyme-altered foci and hyperplastic nodules induced in the rat liver by chemical carcinogens.

| | |
|---|---|
| 1. | Show altered metabolism of glycogen |
| 2. | Deficient in the enzyme activities of: |
| | glucose-6-phosphatase |
| | adenosine triphosphatase (canalicular) |
| | β-glucuronidase |
| | Serine dehydratase |
| | acid phosphatase |
| 3. | Exhibit increased activity of the fetal hepatocyte enzyme γ-glutamyl transpeptidase. |
| 4. | Express preneoplastic antigen. |
| 5. | Show a deficiency to store iron. |
| 6. | Resistant to the cytotoxic action of hepatotoxins and carcinogens. |
| 7. | Exhibit an elevated DNA synthesis and mitosis. |
| 8. | Possess phenotypic heterogeneity with respect to above characteristics. |

(Pitot and Sirica, 1980).

1.2 Social and environmental factors in the development of cancer.

Since the energy and substance for the development of the first cancer cells are derived principally from the animal and since the new cell type increases in number by assimilating nutrients from the host; it might be expected that the social and the environmental conditions of the host influence the formation and growth of tumours. These factors include food contaminants (e.g. the mycotoxin aflatoxin $B_1$), environmental pollutants (e.g. the insecticide dicophane), naturally occurring flavonoids (e.g. the antioxidant quercetin), malnutrition (e.g. protein malnutrition) and drugs (e.g. phenobarbital).

Food contaminants

Perhaps one of the most widely distributed food contaminants in Tropical Africa is the mycotoxin aflatoxin $B_1$ (See Fig. 5 for structure). Aflatoxins are metabolic products of a few strains of Aspergillus flavus and A.parasiticus, which grow on virtually all types of food in warm humid environments. It is therefore not surprising that aflatoxins contaminate most foodstuffs in tropical and subtropical regions of the world where the warm and humid conditions exist that are necessary for the growth of the fungi which produce the toxins (Bababunmi et al., 1978; Wogan, 1966, 1969). This explains the widespread ingestion of these toxins with food in these areas by both old (Bababunmi et al., 1976) and young (Coulter et al., 1986; Hendrickse et al., 1982; De-Vries et al., 1987, 1989). Aflatoxin $B_1$ appears to be the most potent member of the aflatoxin series as regards its toxicity to animals (Wogan, 1969) and in naturally contaminated materials, the $B_1$ compound occurs predominantly (Feuell, 1969). The liver is the principal target for toxicity and pathological changes include fatty infiltration, biliary proliferation, acute toxic necrosis and portal fibrosis (Busby and Wogan, 1981).

Series of investigations have shown aflatoxin $B_1$ to have a potent hepatocarcinogenic, teratogenic and mutagenic activities in many species (Busby and Wogan, 1981). Moreover, it has been observed that young animals are more susceptible to the toxic effects of aflatoxin than older one (Patterson, 1973; Butler, 1964). Furthermore, female rats are less prone to the carcinogenic effect of aflatoxin than male rats (Ward et al., 1975; Butler, 1964).

Epidemiological studies from some developing countries suggest a relationship between ingestion of aflatoxin $B_1$ ($AFB_1$) - contaminated food and increased frequency of human liver cancer (Bababunmi, 1976; Alpert et al., 1971; Shank et al., 1972; Peers and Linsell, 1973).

Environmental pollutants

The problem of pesticides as environmental pollutants particularly as carcinogens for man has become a matter of special concern, especially because some of them (e.g. the chlorine-containing DDT) tend to accumulate in nature, as they are not destroyed by bacteria in the soil and water, or by plant or animal tissue. This raises other toxicological problems apart from carcinogenicity (Berenblum, 1974). DDT is a chemical widely spread in the environment, particularly in the developing countries, to which man is known to be exposed and for which experimental evidence for carcinogenicity exists (Shabad et al., 1972; Tarjan, 1969; Turusov et al., 1973; Berenblum, 1974). It has been proven that the carcinogenic effect of DDT on the liver is due to a promotion action since DDT is not genotoxic (Williams, 1980; 1983). DDT (See Fig.5 for structure) has therefore been classified as an epigenetic carcinogen functioning as liver tumour promoter (Williams, 1983).

DDT has been shown to enhance the effects of 2-acetyl-aminofluorene (Peraino et al., 1971), diethylnitrosamine (Nishizumi, 1979; Kitagawa and Sugano, 1978), 3-methyl-4-(dimethylamino)-azobenzene (Kitagawa and Sugano, 1978) and aflatoxin $B_1$ (Rojanapo et al., 1988) in inducing hepatocellular carcinoma in rats and mice. In addition, DDT has been shown to enhance the development of mammary gland tumours in male rats treated with 2-aminophenanthrene (Scribner and Mottet, 1981).

Naturally occurring flavonoids

Worldwide, quercetin – a flavonoid-occurs in conjugated or as free forms in many edible plant foods (Herrmann, 1976; Harborne and Williams, 1975; Wollenweber and Dietz, 1981; Mabry and Ulubelen, 1980). Numerous observations both in-vivo and in-vitro have demonstrated a wide variety of physiological and pharmacological effects for a number of flavonoids, many of which could be beneficial to health (Bohm, 1968; Brown, 1980; Kuhnau, 1970).

Quercetin (See Fig.5 for structure) has been reported very widely as a non-carcinogenic naturally occurring flavone (De Eds 1968; Sugimura, 1979; Wang et al., 1976). Some workers, however, have demonstrated the mutagenicity or carcinogenicity of Bracken Fern (Pteridium aquilinum) a component of which is quercetin. (Bryan and Pamukcu, 1979; Pamukcu et al., 1980). Quercetin has been shown to affect the growth of transplanted sarcoma (Bohm, 1968). Yoshida et al. (1990) have reported recently that quercetin markedly inhibits the

growth of human gastric cancer cells and blocks cells progression from the $G_1$ to the S phase. In addition, quercetin inhibits the activities of miscellaneous enzymes including the ion-motive ATPases (Lang and Racker, 1974; Suolinna et al., 1974; Graziani, 1977; Apps et al., 1982) and glutathione-s-transferase (Frohlich et al., 1989). It has been recognized that inhibitors of glutathione-s-transferase can be effective as cell antiproliferating agents (Sato et al., 1990). At very low concentrations, quercetin inhibits microsome-catalyzed adduct formation between the chemical carcinogen benzpyrene and DNA (Shah and Bhattacharya, 1986). Quercetin has also been shown to enhance the antiproliferative activity of Cis-diaminedichloroplatinum (II) both in vitro and in-vivo (Hofmann et al., 1988; Hofmann et al., 1990).

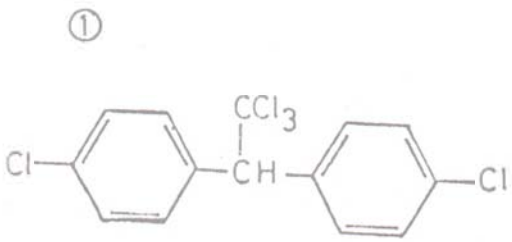
Quercetin is also an antioxidant. Its antioxidant property is due primarily to the presence of phenolic hydroxyl groups; the antioxidant effect increasing with the degree of hydroxylation of the rings (Brown, 1980). In carcinogenesis activated form of oxygen appears to play a role mostly in the promotion phase during which gene expression of initiated cells is modulated by affecting genes that regulate cell differentiation and growth (Freeman and Gapo, 1982).

Many tumour promoters are recognized as oxidant carcinogens which induce cellular pro-oxidant state (Cerutti, 1987). Furthermore, Cerutti (1985) has outlined evidence in support of the hypothesis that many anti-oxidants are anticarcinogens.

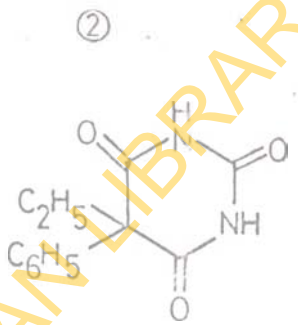
Malnutrition.

Malnutrition, particularly protein malnutrition is widespread and endemic in developing countries and is universally recognized as the single most important contributor to the high sickness and death rates in childhood in these countries (Hendrickse et al, 1982). It is probable, therefore, that protein-malnutrition acting in concert with environmental carcinogens which are also widespread in the developing countries might influence the incidence of hepatocellular carcinoma in Africa and Asia.

A number of studies have shown that the development of cancer can be modified either by changing the quality or the quantity of dietary protein. This has been demonstrated for tumours of the liver (Tannenbaum and Silverstone, 1953). A reduced incidence of spontaneously occurring benign hepatoma in mice fed 9 per cent casein diets ad libitum or isocalorically and also in experiments in which caloric intakes were controlled to maintain equivalent body weights has been reported (White et al., 1947; Silverstone and Tannenbaum, 1951).




DICOPHANE
(DDT)

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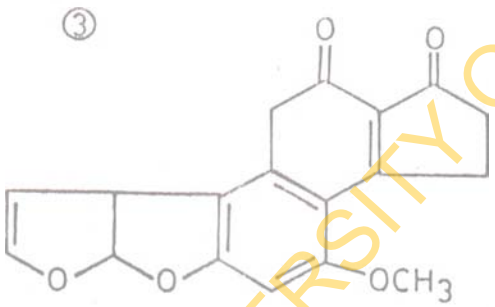
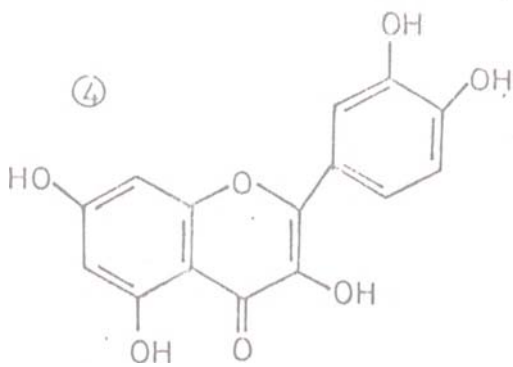



AFLATOXIN $B_1$




QUERCETIN



Fig.5 Structures of some chemicals relevant to tumour growth and development

In laboratory rats treated with aflatoxin $B_1$, low protein intake has been shown to inhibit the development of both liver tumours and putatively preneoplastic foci (Madhavan and Gopalan, 1968, Appleton and Campbell, 1982). Foci developments have been found to be associated with casein levels above 10-12% in the diet (Dunaif and Campbell, 1987). The effect of low protein intake it has been suggested is to decrease the enzymatic activation of aflatoxin $B_1$ to form covalent adducts with DNA, RNA and protein (Preston et al., 1976) thereby suppressing the formation of initiated cells. However, low protein intake has a more significant effect on development of foci after aflatoxin $B_1$ initiation is completed (Appleton and Campbell, 1983. Dunaif and Campbell, 1987). In addition, it has been shown that diets deficient in essential amino acids e.g. cystine (White et al., 1947) and lysine (Voegtlin and Thompson, 1936) suppressed the genesis of certain tumours.

The inhibition of the development of foci either by decreasing the quantity of protein intake and holding the quality of the protein constant or by decreasing the quality and holding the quantity constant as reported recently by Schulsinger et al. (1989), has again demonstrated the important role of dietary protein in the development of hepatocellular carcinoma. Furthermore, it has been shown that supplementing the diet of rats with dietary protein protects against the acute toxicity of aflatoxins but enhances the risk of carcinoma (Madhavan and Gopalan, 1968; Mirmomeni et al. (1979).

Drug

The drug phenobarbital has long been recognized as one of the agents that stimulate the proliferation of the smooth portion of the endoplasmic reticulum of the liver. Subsequently, it was demonstrated that phenobarbital, like DDT is a tumour promoter of the epigenetic class (Williams, 1983; Wantanabe and Williams, 1978; Shimada et al., 1981). It is recognized that this compound affects the hepatic activity of several membrane-associated enzymes (Ratanasavanh et al., 1979; Williams et al., 1980).

Phenobarbital (See Fig.5 for structure) has been shown to enhance the hepatocarcinogenicity of 2-acetylaminofluorene (Peraino et al., 1973 & 1975), diethylnitrosamine (Ito et al., 1980; Kitagawa and Sugano, 1978; Nishizumi, 1979) and 3-methyl-4-(dimethylamino) azobenzene (Kitagawa and Sugano, 1978) in animals.

## 1.3 Calcium ion, Calmodulin and Oncomodulin and the control of proliferation in normal and cancer cells.

A significant body of circumstantial evidence suggests that $Ca^{2+}$ and Calmodulin are important regulators of cell growth and malignant transformation (Veigl et al., 1984). The divalent ion calcium, like the cyclic nucleotides, is a major second messenger for regulation of numerous physiological processes, among them cell motility, egg activation, muscle contraction, neurotransmission, secretion and cell proliferation (Whitfield et al., 1979).

It has been established as a general phenomenon, in both fibroblast and epithelial cell types, that the proliferation of normal, non-neoplastic cells is tightly controlled by extracellular $Ca^{2+}$ concentration: proliferative quiescence sets in as the $Ca^{2+}$ level is reduced below 1mM (Durham and Walton, 1982). Neoplastic cells transformed either naturally in-vivo; or in-vitro by viruses, chemicals etc., can proliferate at much lower $Ca^{2+}$ concentrations (1 to 10 $\mu$M). These data suggest that $Ca^{2+}$ regulates some aspects of normal cell division that is aborted upon cell transformation. This inability of calcium to control tumour cell proliferation is one of the few (maybe the only) qualitative differences between normal and cancer cells (Whitfield et al., 1979).

There is a good correlation between loss of $Ca^{2+}$ control and progression towards tumourigenicity. As rodent cells become preneoplastic by repeated passage in culture, and then neoplastic by treatment with chemical carcinogens or transforming viruses, the level of $Ca^{2+}$ ions required for cell replication progressively decreases (Boynton et al., 1977; Boynton and Whitfield, 1978). In addition, Rixon and Whitfield (1976) observed that the cellular proliferation in rat liver following partial hepatectomy is prevented in the presence of hypocalcemia induced by prior parathyroidectomy. Furthermore, it has been shown that growth of tumours in-vivo in young animals can be inhibited by calcitonin treatment (Anghileri et al., 1980).

Numerous studies have shown that calcium can exert its second messenger function via interaction with a family of small acidic, structurally similar proteins, which bind calcium through helix-loop-helix structures. This family includes calmodulin, troponin C, parvalbumins and a new protein that has thus far only been detected in tumour tissue-oncomodulin. The most multifaceted member of this family is the protein calmodulin, which is present in all eukaryotes and activates a wide variety of target proteins (Veigl et al., 1984). Calmodulin is a single

polypeptide chain with a molecular weight of 16,700 dalton and 148 amino acid residues. Some 30 percent of its amino acids consists of aspartate and glutamate - accounting for the isoelectric point of 4.3. Calmodulin contains four $Ca^{2+}$ - binding sites. This protein itself is not active; the active form is the calmodulin - calcium complex (Cheung, 1980).

A number of laboratories over the past several years have reported that calmodulin levels are higher in transformed and rapidly dividing normal cells and tissues than in their quiescent normal counterparts (Watterson et al., 1976; LaPorte et al., 1979; Uenishi et al, 1980; MacManus et al., 1981; Veigl et al., 1982; Van Eldik and Burgess, 1983; and Nakajo et al., 1983). This observation suggests that calmodulin levels regulate cell division. Furthermore, investigation of synchronized cells in culture indicates that calmodulin content of cells increase at a specific time in the cell cycle. Indeed, it has been shown that calmodulin is synthesized at the $G_1$/S interface (Veigl et al., 1982; Chafouleas et al; 1982). The best correlative in-vitro change in cells with in-vivo tumour formation appears to be acquisition of anchorage-independence-reflected in the ability of transformed cells to grow in a semi-solid medium. The ability of transformed cells to grow without attachment to a substratum contrasts with normal cells, which can grow only on a surface that will support a flattened cell morphology (Folkman and Moscona, 1978). Calmodulin has been shown to regulate a wide variety of Cellular activities including cell morphology, motility, communication with other cells and interaction with the growth environment - all processes that change upon cell transformation (Veigl et al., 1984).

Recent work in a number of laboratories suggests that calmodulin is a major regulator of cyto-skeletal function through a variety of mechanisms, including regulation of filament-based motility systems in both muscle and non-muscle tissues of eukaryotes. The role of calmodulin as a $Ca^{2+}$ dependent activator of myosin light-chain kinase in these tissues is now well established. Calmodulin and specific phosphatases provides for reversible, specific activation of the actomyosin ATPase activity responsible for contraction (Adelstein, et al., 1980; Gorecka et al., 1976). Interestingly, cAMP - dependent phosphorylation of myosin light chain kinase appears to inhibit the activation by $Ca^{2+}$ calmodulin. This may allow coupling between regulatory signals, thus providing a rational regulatory cycle in smooth and non-muscle tissues (Veigl et al., 1984). In addition to the regulation of ATP hydrolysis in these motility

systems, calmodulin may play an important role in regulating the polymerization of the associated filament systems. Calmodulin has been shown to become associated during mitosis with the polar regions of the spindle fiber apparatus believed to contain micro-tubule organizing centers (Willingham et al., 1983; Welsh et al., 1978, 1979) and moves together with tubulin, actin and myosin during axonal transport (Erickson et al., 1980).

In 1979, a novel calcium-binding protein was detected in lysates of Morris hepatoma 5123tc. The protein was subsequently named "oncomodulin" in recognition of its frequent expression in neoplastic tissues and its ability to stimulate cyclic nucleotide phosphodiesterase in a calmodulin-like manner (Henzl and Birnhaum, 1988). Oncomodulin is an oncodevelopmental calcium-binding protein. Its expression is restricted to tumour cells and the placental cytotrophoblasts of rodents and man (MacManus et al., 1983; Gillen et al., 1987). Oncomodulin strongly resembles another calcium-binding protein called parvalbumin, and shows a very high degree of sequence homology. Oncomodulin isolated from rat tumour contains 108 amino acid residues; parvalbumin from skeletal muscle from the same animal contains 109 residues. The reported sequences are identical at 55 positions. Both proteins possess two high affinity calcium-binding domains which are referred to as the CD (residues 51-63) and EF (residues 90-102) sites. In the rat, the EF sites of oncomodulin and parvalbumin are nearly identical (11 of 12), while the CD site exhibits somewhat less homology (7 of 12 residues). However, despite the pronounced similarity between the two proteins, it is becoming increasingly clear that oncomodulin is not merely another parvalbumin (Henzl and Birnbaum, 1988).

Although it has been recognized that chemically transformed rat fibroblast cell line expresses high levels of oncomodulin (Sommer et al., 1989), the role of oncomodulin as well as its possible function within the tumour cell remains unclear. Furthermore, it has been shown that the expression of oncomodulin does not lead to the transformation or immortalization of mammalian cells in-vitro (Mes-Masson et al., 1989). It has been proposed that the active molecule could be a cysteine linked oncomodulin dimer (Mutus et al., 1988). The disulfide-linked dimer of oncomodulin appears to be more similar to calmodulin than oncomodulin since the dimer displayed "calmodulin-like" affinity for the amphiphilic peptide melittin. In addition, Oncomodulin dimer was shown to activate two calmodulin-dependent enzymes, cyclic nucle-

otide phosphodiesterase and calcineurin phosphatase. Nevertheless, recent studies indicate that oncomodulin can regulate the activity of glutathione reductase (Palmer et al., 1990). Furthermore, glutathione reductase was able to catalyze the reduction of the disulfide-linked dimer of oncomodulin.

While elevated levels of calcium, calmodulin and oncomodulin in tumour cells have been established, neither the underlying factors nor the significance of the elevation is fully understood at present.

Table 4 Examples of some ion motive ATPases discovered to date

| ATPase | Source | Membrane | ATPase class |
|---|---|---|---|
| $H^+$ | Lower eukaryotes (yeast, fungi) | Plasma | P |
| $H^+$ | Higher eukaryotes | | |
| | plants | Plasma | P |
| | animals | Plasma (bladder,tumor) | P |
| $K^+$ | E. coli, S. faecalis | Inner | P |
| $H^+/K^+$ | Higher eukaryotes (animals) | Plasma (intestine) | P |
| $Na^+/K^+$ | Higher eukaryotes (animals) | Plasma | P |
| $Ca^{2+}$ | Higher eukaryotes (animals) | Plasma | P |
| $Ca^{2+}$ | Higher eukaryotes (animals) | Sarcoplasmic reticulum | P |
| $Ca^{2+}$ | Higher eukaryotes (animals) | Lysosomes, Go'gi | P |
| $H^+$ | Lower eukaryotes (yeast, fungi) | Vacuoles | V |
| $H^+$ | Higher eukaryotes (plants) | Tonoplasts | V |
| $H^+$ | Higher eukaryotes (animals) | Lysosomes | V |
| $H^+$ | Higher eukaryotes (animals) | Endosomes | V |
| $H^+$ | Higher eukaryotes (animals) | Secretory granules | V |
| $H^+$ | Higher eukaryotes (animals) | Storage granules | V |
| $H^+$ | Higher eukaryotes (animals) | Clathrin coated vesicles | V |
| $H^+$ | Most bacteria | Inner | F |
| $H^+$ | Eukaryotes | | |
| | animals, plants | Mitochondrial inner | F |
| $H^+$ | plants | Chloroplast thylakoid | F |

(Pedersen and Carafoli, 1987)

## 1.4 The regulation of intracellular calcium

ATPases involved in ion translocation are present in a diverse variety of biological membranes. The ion-motive ATPases discovered to date can be grouped into three major categories designated as "P" (Phosphorylated), "V" (Vacuolar) and "F" ($F_0$-$F_1$) type (Pedersen and Carafoli, 1987) (Table 4).

Ion-motive ATPases of the "V" type are defined as those associated with membrane bound organelles other than the mitochondria and the endoplasmic or sarcoplasmic reticulums. They are found in the Vacuoles (hence the Symbol "V") of Neurospora and yeasts and in tonoplasts of plants, as well as in lysosomes, endosomes, clathrin coated vesicles, hormone storage granules, secretory granules and Golgi vesicles. This may be the largest of the three major classes of ion-motive ATPases.

Ion-motive ATPases of the "F" category are defined as those of the $F_o F_1$ type found in bacteria, chloroplasts and mitochondria. These are the most complicated of the ion-motive ATPases, consisting of a water soluble $F_1$ moiety involved in catalytic activity (i.e either ATP synthesis or ATP hydrolysis) and an $F_o$ moiety involved in $H^+$ translocation (Pedersen and Carafoli, 1987).

ATPases of the "P" class are defined as those which form a covalent phosphorylated intermediate (see Fig.6) as part of their reaction cycle (hence the symbol "P"). Examples are the $Na^+/K^+$, $Ca^{2+}$, and $H^+$ transporting ATPases of the plasma membranes of eukaryotic cells and the $Ca^{2+}$ transporting ATPase of the plasma membranes of eukaryotic cells and the $Ca^{2+}$ transporting ATPase of the sarcoplasmic and Endoplasmic reticulum. The K+ transporting ATPases of E. coli and Streptococcus faecalis are also of the 'p' type. The common categorization of this class of ATPases as $E_1$-$E_2$ is inappropriate (Pedersen and Carafoli, 1987). Such ATPases are inhibited by vanadate, a transition state analog of phosphate and an inhibitor which also distinguishes many of the ATPases of the P-type from those of the V and F types. Structurally, these ATPases all consists of a peptide ( $\alpha$ ) of 70-100 KDa which contains the phosphorylation and ATP binding sites. The $Na^+$+$K^+$ - ATPase, unlike other known P-type ATPases, contains in addition, $\beta$ peptide of approximately 55 KDa; its function is, however, unknown. Several of the P-type ATPases have now been sequenced, and it has been observed that significant sequence homology exists among them suggesting that they may have been derived from a common ancestor. Further more, comparison of the $\alpha$-subunit

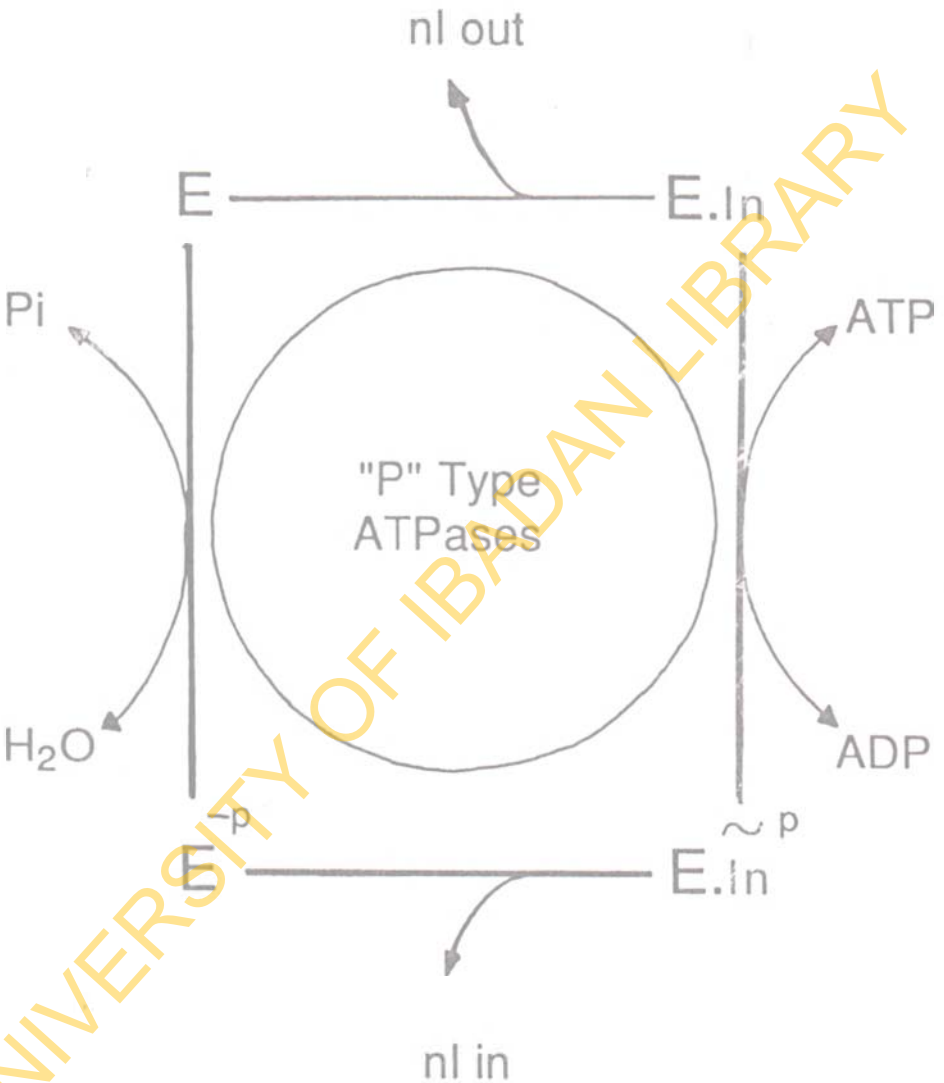





Fig.6 Simplified reaction pathway for P-type ATPases (Pedersen and Carafoli, 1987)

of the Kidney $Na^+/K^+$-ATPase with that of the $Ca^{2+}$-ATPase of cardiac muscle shows that the two proteins are very similar in size and virtually identical in the location of their phosphorylation and ATP binding sites. Thus it seems likely that all ATPases of the P-type will be found to have many reaction mechanisms in common.

Despite the striking similarities among the P-type ATPases, there are critically important differences which reflect their specific "ion-motive" properties; these include

(1) the amino acid sequence of the ion binding domains (or channels)

(2) inhibitor sensitivities and

(3) effect of some natural protein activators.

Finally, it seems likely that some ATPases may exhibit some subtle but very important differences even when they translocate the same ion (Pedersen and Carafoli, 1987).

The role of $Ca^{2+}$-ATPase in the regulation of intracellular calcium

The messenger role of $Ca^{2+}$ requires its maintenance within cells at a very low (submicromolar) ionic concentration, and systems to modulate it in the different cell compartments in keeping with the requirements of the messenger function. The control of cellular $Ca^{2+}$ is based on the reversible complexation to specific proteins that are either soluble in the cytoplasm (e.g. calmodulin), intrinsic to membranes (e.g. ATPases, $Ca^{2+}$ exchangers), or organized in nonmembranous structures (e.g. troponin C). Two $Ca^{2+}$ pumps (i.e. ATPases) are responsible for a large portion of the $Ca^{2+}$ movements across membrane barriers. One is located in Sarco (Endo)-plasmic reticulum. The other is located in the plasma-membrane (Carafoli, 1988a). (Fig. 7).

Plasma membrane $Ca^{2+}$-ATPase

The $Ca^{2+}$-ATPase of the plasma membrane interacts with Ca with high affinity (Km, about 0.5 μM), but has low total transport capacity: In the heart, it corresponds to about 0.5 nmol/mg of membrane protein/s. Given its high $Ca^{2+}$ affinity, the enzyme is qualified to export $Ca^{2+}$ from cells continuously, not only when its concentration in the cytosol has increased. Thus, the $Ca^{2+}$-ATPase plays the most important role in maintaining the $10^4$ fold gradient of $Ca^{2+}$ between cells and medium. The enzyme is an ATPase of the P-class, i.e. it forms an aspartyl phosphate during the reaction mechanism and is inhibited by vanadate. It is a target of calmodulin stimulation. The purified enzyme is a single polypeptide of about 138 KDa, and has been reconstituted in liposomes with optimal transporting efficiency (Carafoli, 1988b).

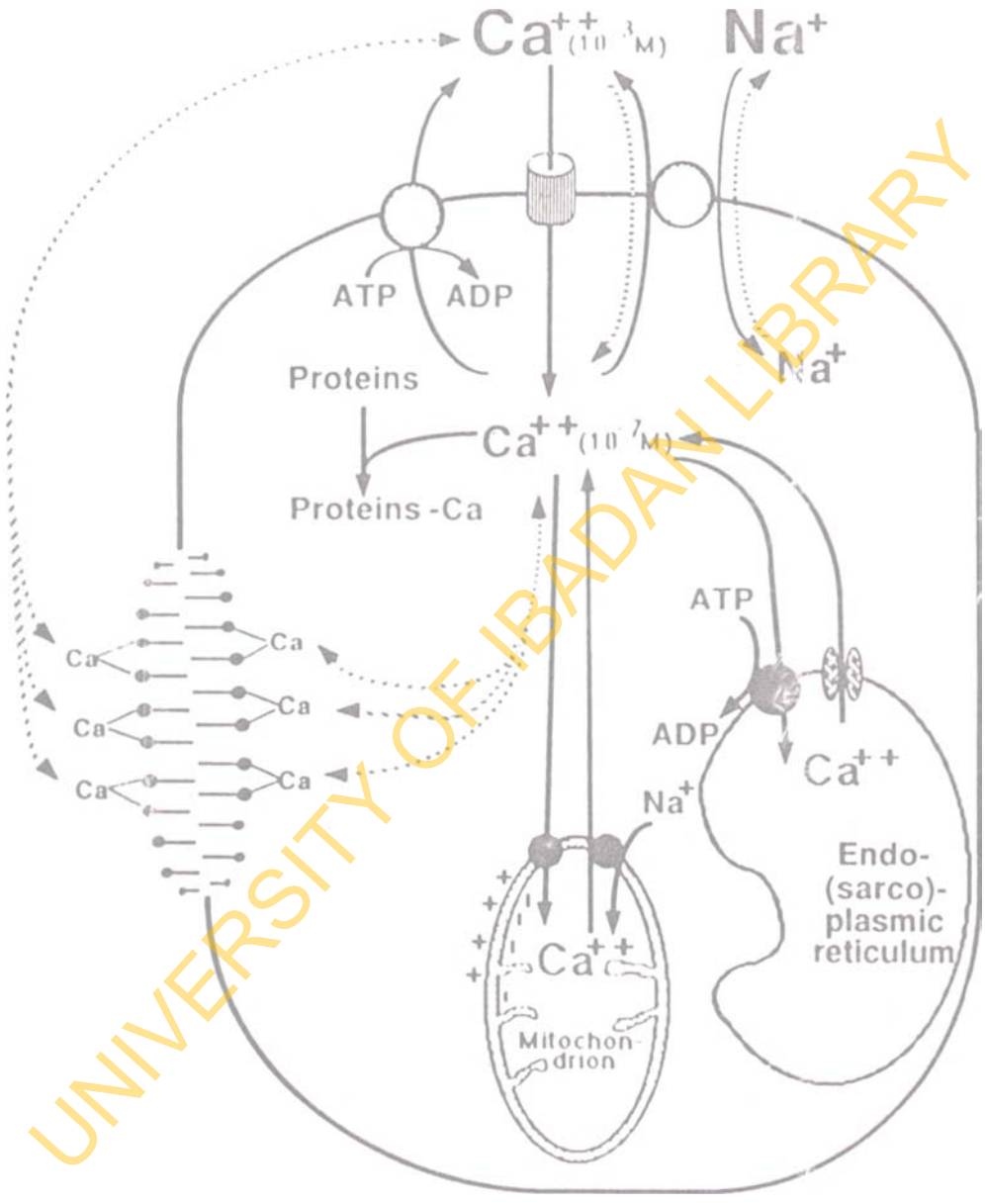



Fig.7 $Ca^{2+}$-transporting Systems in eukaryotic cells (Carafoli, 1988b)

The liposomal system shows that the ATPase transports $Ca^{2+}$ with a 1:1 stoichiometry to ATP hydrolysis, i.e it is less "efficient" than the analogous enzyme of sarcoplasmic reticulum. The enzyme is also stimulated by a cAMP-linked phosphorylation process. Work on the purified $Ca^{2+}$-ATPase has established that the enzyme can be stimulated by several treatments alternative to calmodulin, among them the exposure to acidic phospholipids and polyunsaturated fatty acids, and by a controlled proteolytic treatment with a number of proteases. Among the proteases that activate the purified ATPase, trypsin has been particularly useful, since it has permitted one to establish that the enzyme is degraded in sequence to products of 90-85-81-76 KDa, all of them acting as $Ca^{2+}$ stimulated ATPases. The first three have been reconstituted in liposomes and shown to be able to pump Ca. Although the 90 KDa component is fully reactive to calmodulin, the 85 KDa component binds calmodulin but does not respond to it (or responds negligibly), and the 81 and 76 KDa components do not bind calmodulin. The results have led to the suggestion that the calmodulin binding domain is contained in a 9 KDa fragment of the ATPase and contains a 4 KDa domain that binds calmodulin, and a 5 KDa domain that is required for the expression of calmodulin stimulation (Penniston, 1983, Carafoli 1984, Carafoli, 1987, Carafoli, 1988a).

It has been shown that protein Kinase C stimulates the activity of the plasma membrane $Ca^{2+}$-pump by a direct effect on the pump protein (Smallwood et al., 1988). The effect was a 5-7-fold increase of Vmax without a significant change in the apparent Km for $Ca^{2+}$. Furthermore, the effect of protein Kinase C and Calmodulin on $Ca^{2+}$ uptake were nearly additive and the stimulation by protein kinase C is reversible by treatment with alkaline phosphatase (Smallwood, et al., 1988).

The amino acid sequence of the plasma membrane $Ca^{2+}$ pump has been established by protein chemistry and DNA cloning techniques using a human teratoma $\lambda$gt 10 library (Verma et al., 1988). The isoform of the enzyme present in human teratoma cells contain 1220 amino acids, corresponding to a $M_r$ of 134,683. Asp 475 forms the acyl phosphate during the reaction cycle, and Lys 601 binds the ATP antagonist FITC. Asp 475 and Lys 601 are separated by a domain which is relatively conserved in the ion motive ATPases of the P-class and which may function as a hinge that permits the two residues to come close to each other in space during the reaction cycle. The calmodulin (CaM) binding domain has been identified next to the C-terminus (residues 1100-1127). Distinctive properties of this domain are the

predominance of basic residues, the propensity to form an amphiphilic helix and a tryptophan in the N-terminal portion of the domain (position 1107). It has been observed that if tryptophan 1107 is substituted with an alanine the affinity for calmodulin decreases several fold. The calmodulin binding domain interacts with a sequence adjacent to it on the N-terminal side which is very rich in Asp and Glu and which may play a role in the binding of $Ca^{2+}$ and in regulating the interaction of CaM with the pump. Although the Asp- and Glu- rich domain does not interact directly with calmodulin it apparently changes conformation when calmodulin interacts with the ATPase. The pump also contains, next to the N-terminus, two eleven amino acid stretches which resemble EF-hands (residues 22-33 and 310-321), and could thus also form $Ca^{2+}$-binding sites. Fig.8 shows a scheme of the architecture of the pump in the plasma membrane. Up to ten hydrophobic domains, presumably spanning the membrane, have been identified: 4 are located in the N-terminal portion of the pump, 6 in the C-terminal portion. The exact number of the membrane-spanning domains, however, is still uncertain. The mid portion of the pump (about 500 residues) contains no hydrophobic domains. Ser 1178, located on the C-terminal side of the CaM binding domain, is phosphorylated by the cAMP-dependent kinase, increasing the Ca-affinity of the pump. Studies on several isoforms of the pump from different tissues (e.g. muscle, brain, intestinal mucosa) show that they differ essentially in the calmodulin and cAMP-regulated domains.

The liver plasma membranes contain a high-affinity $Ca^{2+}$-ATPase (Kessler, et al., 1990) that is not sensitive to calmodulin but can be activated by another activator present in the cytosol (Lotersztajn et al., 1981). Furthermore, this ATPase is inactive in the absence of the protein activator. Lotersztajn and Pecker (1982) have shown the existence of a protein inhibitor of the purified $Ca^{2+}$-ATPase. The effect of this inhibitor is reversed by the addition of their activator, and the inhibitor's activity is dependent upon the presence of $Mg^{2+}$. The liver $Ca^{2+}$-ATPase in intact plasma membranes is not sensitive to concentrations of orthovanadate as high as 2mM (Iwasa et al., 1982).

The $Ca^{2+}$-ATPase has been shown to be a multigene family. Isoforms of the pump have been identified at least in humans. Four different products of this multigene family have been identified in rats and humans; and classified as h (for humans) and r (for rat) isoforms PMCA 1-4 (Carafoli, 1991; Greeb and Shull, 1989; Shull and Greeb, 1988; Strehler et al., 1989;

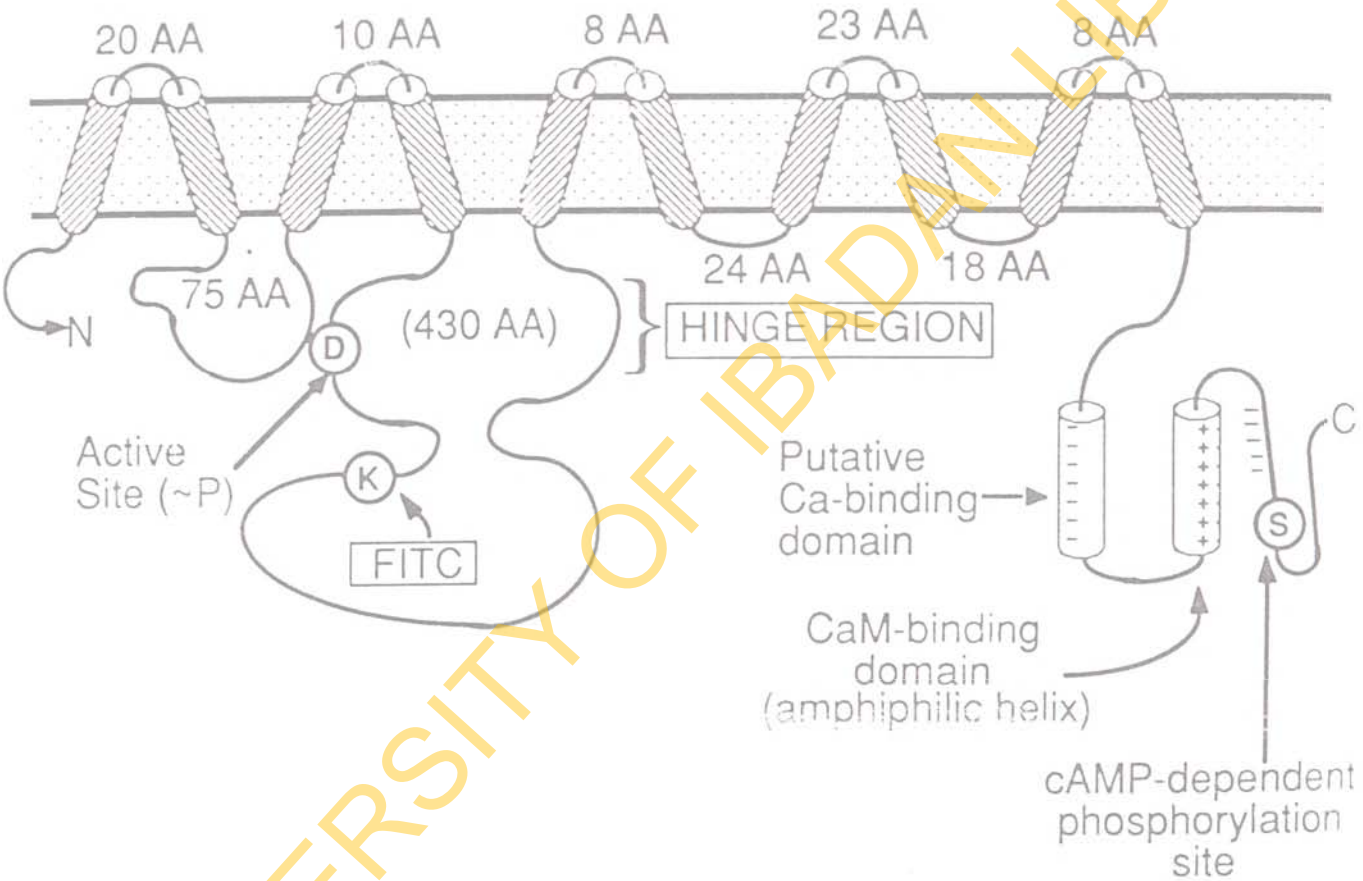



Fig.8 A Scheme of the molecular organisation of the $Ca^{2+}$-pump in the plasma membrane (Carafoli, 1988b)

Strehler et al., 1990). Each corresponds to a family of alternatively spliced isoforms. Furthermore, Isoform specific functional differences has been observed; for instance hPMCA 4 isoform has been shown to lack the cAMP-sensitive phosphorylation site (Khan and Grover, 1991).

The $Ca^{2+}$-ATPase of the Endo (sarco) plasmic reticulum:

The endo(sarco) plasmic reticulum is the membrane system responsible for the fine regulation of $Ca^{2+}$ in the cytosol. Most of the work on this membrane system has been performed on sarcoplasmic reticulum, essentially due to the easier availability of its key enzyme, the Ca-ATPase, with respect to endoplasmic reticulum. The latter organelle, however, has recently become prominent as a result of the discovery that its Ca pool is sensitive to inositol tris-phosphate (Carafoli 1988b). The sarcoplasmic reticulum $Ca^{2+}$-ATPase is of the same class as that of the plasma membrane, has a Km below 0.5 μM, and is present in very large amounts. As a result, the total $Ca^{2+}$ transporting capacity of the organelle is very high, particularly in fast skeletal muscles where it may reach 70 nmol/mg of membrane protein/s. The ATPase has been purified as a single polypeptide of about 100kDa. It forms an aspartyl phosphate, is inhibited by vanadate, and can be reconstituted into liposomes, where is transports $Ca^{2+}$ with a 2:1 stoichiometry to the hydrolyzed ATP. The primary structure of this ATPase has also been determined (MacLennan et al., 1985). It appears that the enzyme contains 10 transmembrane stretches, and a very large portion protruding from the cytosolic side of the membrane. The latter portion is postulated to consist of several domains, containing the ATP-binding sequence, the aspartic acid residue which becomes phosphorylated, the portion of the molecule that transduces the ATP energy to the region that translocates $Ca^{2+}$, and the $Ca^{2+}$-binding domain proper. The latter has been ascribed to a region of the molecule next to the hydrophobic intra-membrane α-helixes that contains an unusually high concentration of glutamic acid residues. Thus, the primary structure of the $Ca^{2+}$-ATPase of the fast skeletal muscles repeat the essential features of the P-type ATPases. The ATPase in heart, smooth, and slow (but not fast) skeletal muscles is modulated by an acidic proteolipid termed phospholamban. This proteolipid, which is a pentamer of five identical subunits of about 6 KDa, is phosphorylated by two kinases, one of which is cAMP dependent while the other is calmodulin dependent (Carafoli, 1988b). Phosphorylated phospholamban activates the

ATPase-linked transport of $Ca^{2+}$, thus transmitting to sarcoplasmic reticulum hormonal messages from the plasma membrane (Carafoli, 1988a). While the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of heart sarcoplasmic reticulum is under the control of a phosphorylation process mediated by cAMP and CaM, in skeletal muscle these regulatory systems are apparently absent. Much less, however, is known of the $Ca^{2+}$ transport systems in liver (Famulski and Carafoli, 1984). It has been demonstrated that the mycotoxin cyclopiazonic acid is a highly specific and potent inhibitor of the $Ca^{2+}$-ATPase of sarcoplasmic reticulum (Seidler, et al., 1989).

Furthermore, it has been argued that there are many more conformational changes in the cycle for calcium pumping than the single conformational change from $E_2$ to $E_1$ and back from $E_1$ - P.$Ca_1$ to $E_2$-p.$Ca_2$, that is postulated in the $E_1$-$E_2$ model (Jencks, 1989).

In liver, the endoplasmic reticulum seems to have a high affinity for $Ca^{2+}$, but a very small capacity for storage of $Ca^{2+}$. (Penniston, 1983). The hepatic $Ca^{2+}$-ATPase contains tightly bound calmodulin. Partial removal of this calmodulin by EGTA treatment results in an increased $^{45}Ca^{2+}$ uptake by added exogenous calmodulin and inhibition by trifluoperazine (TFP). The native microsomal enzyme is not activated by added calmodulin (Moore and Kraus-Friedmann 1983).

The stimulation of $Ca^{2+}$ transport is associated with the operation of a protein phosphorylation system dependent on $Ca^{2+}$ and calmodulin. Transport is inhibited by a protein phosphatase which is stimulated by $Ca^{2+}$ and calmodulin as well. This finding strongly supports the suggestion that the protein phosphorylation-dephosphorylation cycle is a regulatory device which controls the ($Ca^{2+}$ + $Mg^{2+}$-) ATPase in liver microsomes (Famulski and Carafoli, 1984).

Recently, it was demonstrated that acyl phosphatase is a soluble non-calmodulin activator of erythrocyte membrane $Ca^{2+}$-ATPase; and that Stimulation by acyl phosphatase was additive to that induced by calmodulin (Nassi et al, 1990).

## 1.5 Intracellular $Ca^{2+}$ signalling for transient and sustained cellular responses.

A common trigger precipitates biological events as diverse as the contraction of a muscle and the secretion of a hormone. The trigger is a minute flux of calcium ions. Calcium is one of the body's "second messengers": it relays electrical and chemical messages that arrive at a cell's surface membrane to the biochemical machinery within the cell. To control cellular processes effectively, calcium itself must be regulated. Thus cells have evolved an elaborate system of proteins that interact with the calcium ion, governing the transmission and reception of the intracellular message (See section 1.4). The mechanisms that regulate calcium in the cell do not operate in isolation. Networks rather than simple pathways typify cell physiology, and intracellular signalling systems are no exception. The modification by cyclic AMP of calcium channels and the calcium-pumping ATPase in the plasma membrane is one example of the interactions among messenger systems (Carafoli and Penniston, 1985). Thus, the old belief that $Ca^{2+}$ and cAMP are separate intracellular messengers and that these two messenger systems functioned separately - $Ca^{2+}$ in excitable cells and cAMP in non-excitable cells is no longer tenable.

It is now recognized that intracellular $Ca^{2+}$ signalling is more complex than previously thought for the following reasons:

(i) There is a synarchic relationship between $Ca^{2+}$ and cAMP (Rasmussen, 1970).

(ii) Intracellular free $Ca^{2+}$ ion concentration $[Ca^{2+}]i$ was transient even though the response was sustained (Grynkiewicz et al., 1985; Cobbold and Rink, 1987; Morgan and Morgan, 1984).

(iii) $Ca^{2+}$ cycling (i.e sustained increase in $Ca^{2+}$ influx without an increase in $[Ca^{2+}]i$ or in total cell calcium) alone without a $Ca^{2+}$ - sensitive transducer e.g. Protein Kinase C was not sufficient to induce a sustained cellular response (Rasmussen, 1989; 1990).

In several transient cellular responses, including the secretion of neurotransmitters by nerve cells and the contraction of skeletal and cardiac muscle cells, calcium serves as a simple on-off switch that conveys information from the cell surface to the cell interior. When a cell is stimulated by an extracellular signal, channels in its plasma membrane open and allow calcium ions to enter at from two to four times the normal rate. When the concentration

rises, calcium-binding proteins in the cytosol, such as the specific receptor calmodulin, attach to calcium ions; the calcium-protein complexes then interact with other proteins in the cell to alter their functions. When the calcium concentration in the cytosol falls again, the ions dissociate from the receptor proteins and the system turns off. In each case, the rise in calcium ion concentration in the cytosol initiates the response, and the fall in calcium ion concentration terminates it (Rasmussen, 1989).

The role of calcium in sustained cellular responses such as the secretion of insulin or the contraction of the smooth muscle surrounding the blood vessels, has historically been more elusive than its part in transient responses. It is now recognized that prolonged or sustained cellular response can be divided into two temporally distinct phases - a calmodulin branch which is active during the initial phase of the response and a protein Kinase C branch that operates in the second sustained phase (Rasmussen, 1989, 1990). Fig. 9 is a schematic summary of some of the $Ca^{2+}$-mediated processes relevant to toxicology. In the initial phase, the binding of an extracellular signal to its receptor prompts the break-down of the membrane component, $PIP_2$ into $IP_3$ and DAG. $IP_3$ causes the release of calcium ions from intracellular compartments called calcisomes or endoplasmic reticulum, resulting in a transient rise in cytosolic calcium ($Ca^{2+}$). The ions bind to calmodulin, and the calcium-calmodulin complex activates protein kinases i.e calmodulin dependent protein Kinases (enzymes that transfer phosphate groups to proteins). The phosphorylated proteins initiate the cellular response. The calcium ions released from the calcisomes and the increase in DAG also prompt the enzyme PKC to associate with the membrane. In the sustained phase, the signal increases calcium cycling across the membrane, and the resulting rise in the submembrane concentration of calcium [$Ca^{2+}$sm] activates the membrane-associated PKC. This activation brings about the phosphorylation of a different set of proteins that sustain the response (Rasmussen, 1989). In these two phases, however, the operation of the calcium-messenger system depends heavily on the activity of the cAMP-messenger system. Furthermore, one or more protein Kinase cascades function to convey information from cell surface to cell interior when the protein Kinase C branch of the $Ca^{2+}$-messenger system is activated (Rasmussen, 1990). Fig. 10 is a schematic expression of the initial and sustained phases of a prolonged cellular response.

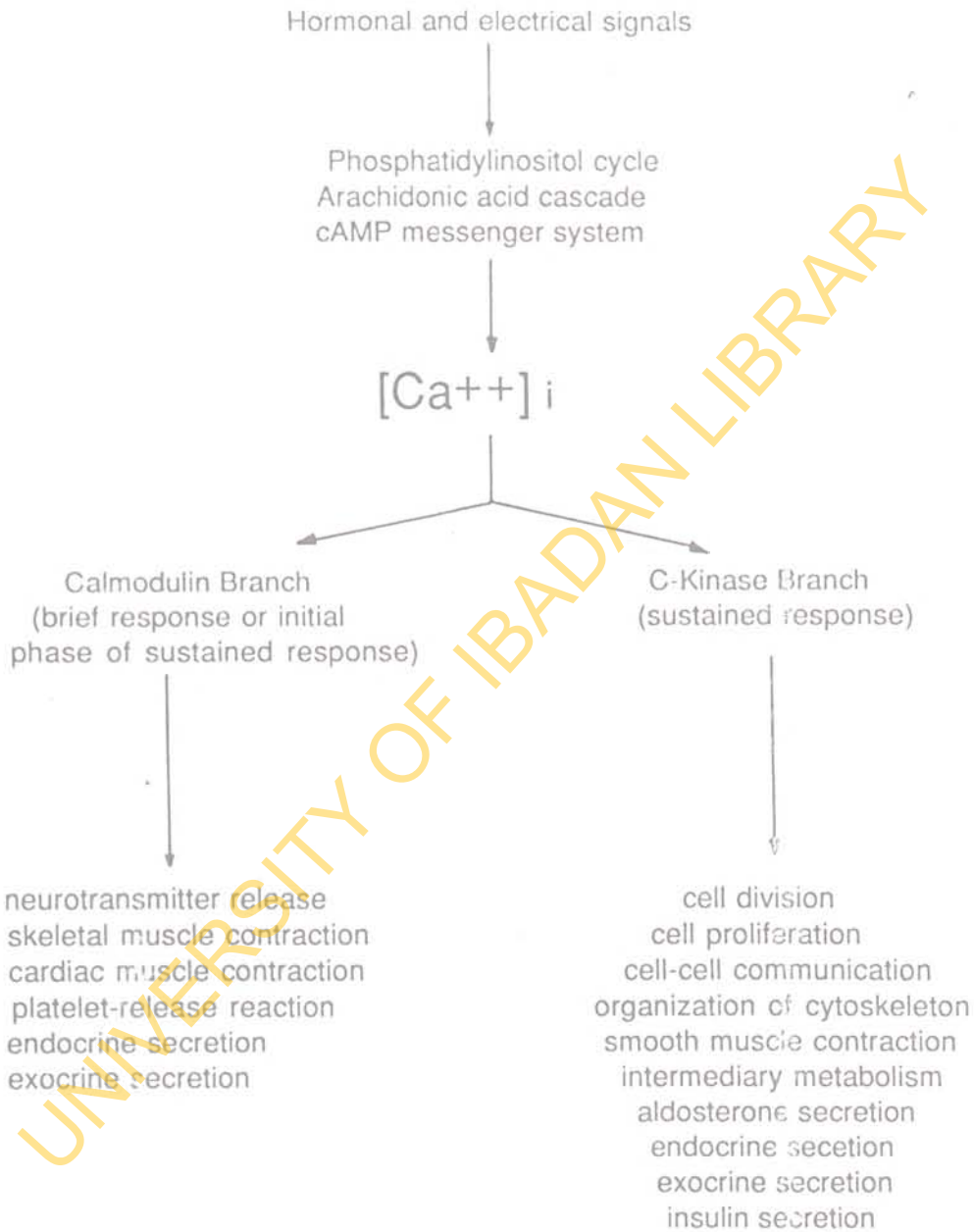





Fig. 9 Schematic summary of some of the $Ca^{2+}$-mediated processes relevant to toxicology (Pounds and Rosen, 1988)

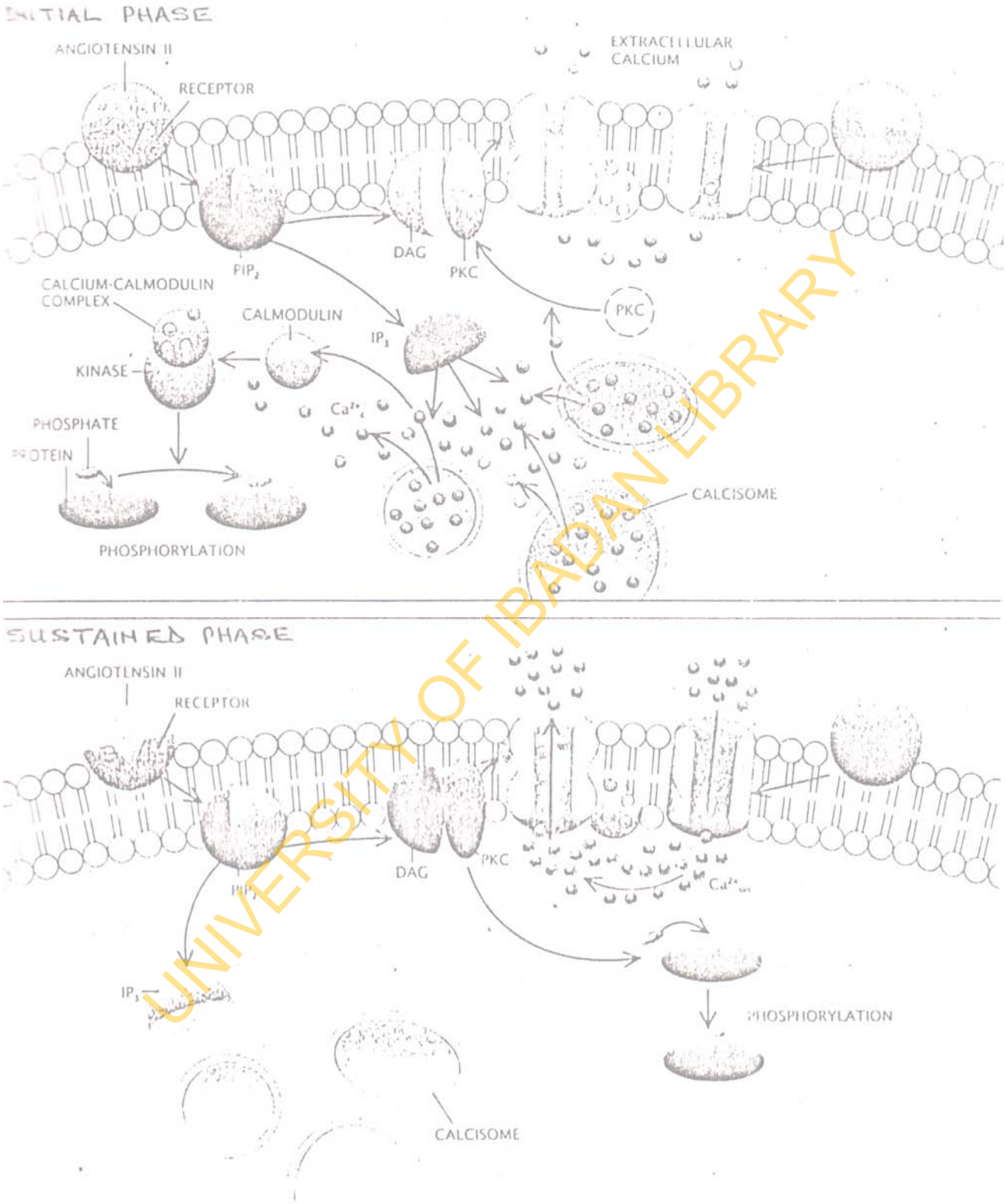





Fig. 10 A sechematic summary of the two temporally distinct phases of prolonged cellular response (Rasmussen, 1989).

## 1.6 Cellular $Ca^{2+}$ homeostasis and $Ca^{2+}$-mediated cellular processes as critical targets for toxicant action.

On the basis of the central role of the $Ca^{2+}$-messenger system in several aspects of cell function, it is logical to examine possible disturbances in $Ca^{2+}$ homeostasis and $Ca^{2+}$-mediated functions as underlying mechanisms of toxicant effects at different levels of biological organization (Pounds and Rosen, 1988). Perturbations in the $Ca^{2+}$ messenger system by toxicants may place the regulation of Cellular processes out of the normal range of physiological control. Hence, disturbances in intracellular $Ca^{2+}$ homeostasis and $Ca^{2+}$-mediated functions have become attractive and frequently postulated targets for numerous pathophysiological processes including neurosecretion, ischemic injury and toxicant-induced cell death, cell growth and transformation, tumour promotion, and hypertension (Dubovsky and Franks, 1983; Lock-Caruso and Trosko, 1985; Metcalfe et al., 1985; Starke et al., 1986; Trump and Berezesky, 1985; Viegl et al., 1984). Perturbations of $Ca^{2+}$ homeostasis and $Ca^{2+}$-mediated cell functions following toxicant exposure may each result from at least three general types of interactions : direct, indirect and secondary interactions (Pounds and Rosen, 1988).

### Toxicant Effects on $Ca^{2+}$ Cellular Homeostasis

Toxicants may directly alter $Ca^{2+}$ homeostasis by substituting for $Ca^{2+}$ at specific sites of $Ca^{2+}$ transport or storage. In this sense these interactions are competitive with $Ca^{2+}$ and generally reversible at the molecular level. Direct interactions are most commonly observed with other divalent metals, including lead, ruthenium, cadmium, and others, which directly compete with or displace $Ca^{2+}$ at transport sites in the plasma membrane (Atchison and Narahashi, 1984; Cooper et al., 1984; Simons, 1986). Other examples include the direct actions of the calcium channel blockers, nifedipine or verapamil which block calcium mobilization through membrane channels in myocardial and smooth muscle cells. It must be recognized that a direct interaction does not necessarily result in an inhibition of a $Ca^{2+}$-mediated function, but may also produce an exaggerated $Ca^{2+}$-mediated response if the clearance of $Ca^{2+}$ from the cytosol and other cellular compartments is impaired (Pounds, 1984).

Toxicants may indirectly act on specific $Ca^{2+}$-homeostatic processes, such as $Ca^{2+}$ pumps or gates, but at molecular sites that are independent of $Ca^{2+}$, i.e., presumably not at

the $Ca^{2+}$ transport or binding site. Thus the interaction is noncompetitive in that adding more calcium will not alter the biochemical lesion. This type of interaction is observed with many organic toxicants. The most fully characterized example of an indirect action is the inactivation of the $Ca^{2+}$ transporter in smooth endoplasmic reticulum by carbon tetrachloride leading eventually to cell death (Long and Moore, 1986, Recknagel, 1983). An indirect interaction may be reversible at the molecular level, but if the injury is sublethal, the cell and organism may be able to repair and recover, within this instance, the biosynthesis of new endoplasmic reticulum and the associated $Ca^{2+}$-pump.

Toxicants may secondarily alter $Ca^{2+}$ homeostasis through nonspecific effects on cell function that are biochemically and functionally remote from the processes of $Ca^{2+}$ transport and storage. For example, ethanol and other aliphatic alcohols and local anesthetics, including dibucaine or tetracaine, produce changes in nerve cell excitability and neurotransmitter release which are the result of physical-chemical changes in the cell membrane, including increased fatty chain motion within the membrane bilayer, expansion of membrane volume, and increased membrane fluidity resulting in altered $Ca^{2+}$ permeability and transport properties of the membrane (Michaelis and Michaelis, 1983). Furthermore, toxicants which dissipate $Na^{+}$ gradient will secondarily alter $Ca^{2+}$ homeostasis in cells where $Ca^{2+}/Na^{+}$ exchange is a component of $Ca^{2+}$ homeostasis.

Toxicant Effects on $Ca^{2+}$-mediated cell Functions.

Toxicants may directly act on $Ca^{2+}$-mediated cell functions, including $Ca^{2+}$ receptor proteins or substrates, without necessarily altering $Ca^{2+}$ homeostasis. A well characterized example is the inhibition of Ca-calmodulin-mediated processes by the phenothiazines which bind to the hydrophobic region of calmodulin and prevent the formation of a $Ca^{2+}$-calmodulin receptor enzyme complex, thereby inhibiting $Ca^{2+}$-mediated functions. It must be noted, however, that some calmodulin antagonists also alter membrane currents of potassium and sodium (Klockner and Isenber, 1987). Lead, aluminum, cadmium, and other divalent metals effectively compete with $Ca^{2+}$ for binding sites on a variety of $Ca^{2+}$-binding proteins including calmodulin (Fullmer et al., 1985). Replacement of $Ca^{2+}$ in $Ca^{2+}$ receptor proteins by other metals may alter $Ca^{2+}$-mediated function in unpredictable ways. However, due to autoregulation of $Ca^{2+}$ homeostasis by calmodulin, direct actions of toxicants on calmodulin and related

proteins may likely result in alterations in $Ca^{2+}$ homeostasis (an indirect effect on $Ca^{2+}$ homeostasis).

Toxicants may indirectly alter $Ca^{2+}$ mediated cell functions, independently of changes in $Ca^{2+}$ homeostasis and independently of direct interaction with the $Ca^{2+}$ receptor or effector proteins. The response to a $Ca^{2+}$ signal is a cascade of biochemical events which often include phosphorylation, and is subject to feed-back control by cyclic nucleotides, and to sensitivity and response modulation (Rasmussen, 1986). Because many cAMP-mediated responses are antagonistic to the $Ca^{2+}$-mediated response, toxicants which affect adenylate cyclase, phosphodiesterase, or other parts of the cAMP messenger system will likely alter $Ca^{2+}$-mediated cell functions. Examples of indirect effects include the inhibitory effects of Xanthines on phosphodiesterase leading to pharmacologic and toxic changes in muscle contraction.

Toxicants may secondarily perturb $Ca^{2+}$-dependent functions as a result of toxic events remote from Ca-dependent processes. Toxicants which grossly alter cell and organelle function may impair the ability of a cell to respond to a $Ca^{2+}$ signal, but as a result of toxic action which has no relationship to the $Ca^{2+}$ messenger system. For example, methyl mercury impairs neuronal migration and cell division, but the cellular target for these actions is the neurotubules and microtubules of the cells rather than the $Ca^{2+}$-mediated aspects of cell movement and division. Other toxicants which alter cellular energetics, protein synthesis, etc., will alter the ability of cells and tissues to maintain proper $Ca^{2+}$ homeostasis and function. However, these secondary effects on $Ca^{2+}$-mediated function are more an expression of toxicity than the cause of toxicity. (Pounds and Rosen, 1988).

1.7 $Ca^{2+}$ homeostasis with particular reference to the role of the endoplasmic reticulum in tumourigenesis by chemical agents.

The oncogenic transformation of cells from a normal to malignant phenotype is associated with a variety of experimentally discernible changes in the pattern of metabolism. Many of these alterations involve processes modulated by the intracellular level and distribution of calcium (Murphy and Fiskum, 1987). Pathological excess of intracellular calcium has been implicated as a final common pathway of cell death produced by a wide range of toxins including the well studied hepatotoxin carbon tetrachloride (Lowrey et al, 1981a). Coincidentally, many of the chemicals that induce hepatocellular carcinoma in experimental animals are also acutely hepatotoxic (Manson, 1983).

In terms of general cellular metabolism, mitochondrial $Ca^{2+}$ transport appears to be involved primarily in the physiological regulation of intramitochondrial levels of $Ca^{2+}$, whereas transport activities at the plasma membrane and endoplasmic reticulum are thought to play primary roles respectively in regulation of basal levels and transient changes in the cytosolic $Ca^{2+}$ concentration. However, mitochondrial $Ca^{2+}$ uptake and release may assume a more critical role in regulating cytosolic $Ca^{2+}$ under metabolically stressful conditions, such as during ischemia and reperfusion (Murphy and Fiskum, 1987).

The endoplasmic reticulum of the liver cell is the first cellular organelle disrupted by carbon tetrachloride poisoning. The disruption is accompanied by a destruction of Calcium pump activity in the organelle (Moore et al., 1976). The destruction of liver microsomal $Ca^{2+}$ pump activity by carbon tetrachloride and other hepatotoxins has been confirmed by other workers (Lowrey et al., 1981 a, b). Furthermore, carbon tetrachloride toxicity in the rat can be potentiated by pretreatment of animals with agents which induce the mixed function oxidase complex of the liver endoplasmic reticulum. These agents which include phenobarbital (Garner and Mclean, 1969) and 1,1,1,-trichloro-2,2,-bis (p-chlorophenyl) ethane (McLean and McLean, 1966) are well known liver tumour promoters. It is now known, however, that the trichloromethyl radical of carbon tetrachloride initiates lipid peroxidation. It is a potent hepatotoxin, hepatopromoter and a complete carcinogen (Cerutti, 1985).

Thapsigargin, a naturally occurring sesquiterpene lactone and tumour promoter has been found to be a highly selective and potent inhibitor of the endoplasmic reticulum $Ca^{2+}$-ATPase.

Thapsigargin induced ER-$Ca^{2+}$-ATPase inhibition abolishes the $Ca^{2+}$-releasing ability of both GTP and Ins-1,4,5-$P_3$ (Thastrup, 1990, Thastrup et al., 1990).

It has been observed that the rate of $Ca^{2+}$ uptake by AS-30D hepatoma microsomes is approximately twice as fast as that observed with rat liver microsomes. In addition, the AS-30D hepatoma microsomes is more sensitive to the $Ca^{2+}$-releasing action of inositol 1,4,5 trisphosphate ($IP_3$) than the rat liver microsomes (Murphy and Fiskum, 1987).

Whether alterations in the transport or effects of calcium are the primary mechanism by which malignant cells dictate their high growth rate or whether they are the manifestation of some other transforming characteristic is unknown at this time.

1.8 Tumour promoter and biochemical mechanism of action

Tumour promotion encompasses the modulation of the expression of genes related to growth and differentiation of initiated cells (Cerutti, 1985). A tumour promoter is an agent that facilitates formation of neoplasms from altered cells that otherwise would remain dormant. Tumour promoters are usually identified by their enhancement of the tumour yield resulting from a previously administered carcinogen, called an initiating agent in the terminology adopted for multi-stage carcinogenesis. They can also be conceived to promote tumour formation by cells altered through effects other than experimentally induced initiation, such as cells with an inherited genetic abnormality or a spontaneous mutation (Williams, 1983).

While there is a wealth of information on genotoxic carcinogens, the process of tumour promotion is still poorly understood. Nevertheless, some progress have been made in this regard. Indeed, several biochemical effects of tumour promoters have been identified and these include:

(i) Generation of free radicals (Troll and Weisner, 1985; Albano et al., 1988; Connors, 1988; Das et al., 1990; Kozumbo et al., 1985; Nakamura et al., 1985; and O'Connel et al., 1986.

(ii) Impairment of antioxidant system (Perchellet et al., 1986; Perchellet et al., 1987; Kaplan and Groses, 1972; Peskin et al., 1978; Corrocher et al., 1986).

(iii) Inhibition of intercellular communication (Williams et al., 1981; Klaunig et al., 1990).

(iv) Stimulation of arachidonic acid metabolism (Levine, 1984 and 1988) and

(v) Modulation of the activities of several enzymes including

(1) Ornithine decarboxylase (Raunio and Pelkonen, 1983; O'Brien, 1976; Yanagi et al., 1981; and Kitchin and Brown, 1987)

(2) Protein Kinase C (Castagna et al., 1982)

(3) Protein phosphatases (Bialojan and Takai, 1988) and

(4) $Ca^{2+}$-ATPases (Parola et al., 1990; Lowrey et al., 1981 a; Lowrey et al., 1981b; Thastrup et al., 1990).

One major obstacle in synthesizing the multifarious effects of tumour promoters to give a comprehensive picture is the difficulty in differentiating a primary effect from a secondary effect. To date, the most acceptable and comprehensive hypothesis proposed to explain the

mechanism of action of tumour promoters appears to be that of oxidant carcinogenesis proposed by Cerutti and co-workers (1989). According to this hypothesis, promoters and progressors are oxidants and agents which induce cellular pro-oxidant states. The authors proposed that oxidants induce series of biochemical reactions that result in carcinogenic tumour promotion (Fig.11). According to the authors, many mitogens including tumour promoters act either indirectly by producing free radicals like activated oxygen or directly by binding to an agonist-specific receptor or both. The activated oxygen induces the mitochondrion to release its calcium pool, inhibits the plasma membrane $Ca^{2+}$-ATPase and induces the release of the membrane-bound protein Kinase C. The binding to the agonist-specific receptor is followed by hydrolysis of polyphosphoinositides, resulting in formation of inositol phosphates ($IP_3$) and diacylglycerols. These respectively increase intracellular calcium concentrations (via $IP_3$-induced calcium release from the endoplasmic reticulum) and activate protein kinase C with concomitant stimulation of protein phosphorylation and calcium/calmodulin-dependent processes (Nishizuka, 1984; Berridge, 1984; Graff et al. 1989; Cerutti et al., 1989). Furthermore, the multistage process of cancer development is known to involve both mutagenic and non-mutagenic mechanisms. These result in the induction of multiple direct and indirect genetic changes at target oncogenes or tumour suppressor genes as well as alterations in signal transduction pathways involved in growth control (Perera, 1990).

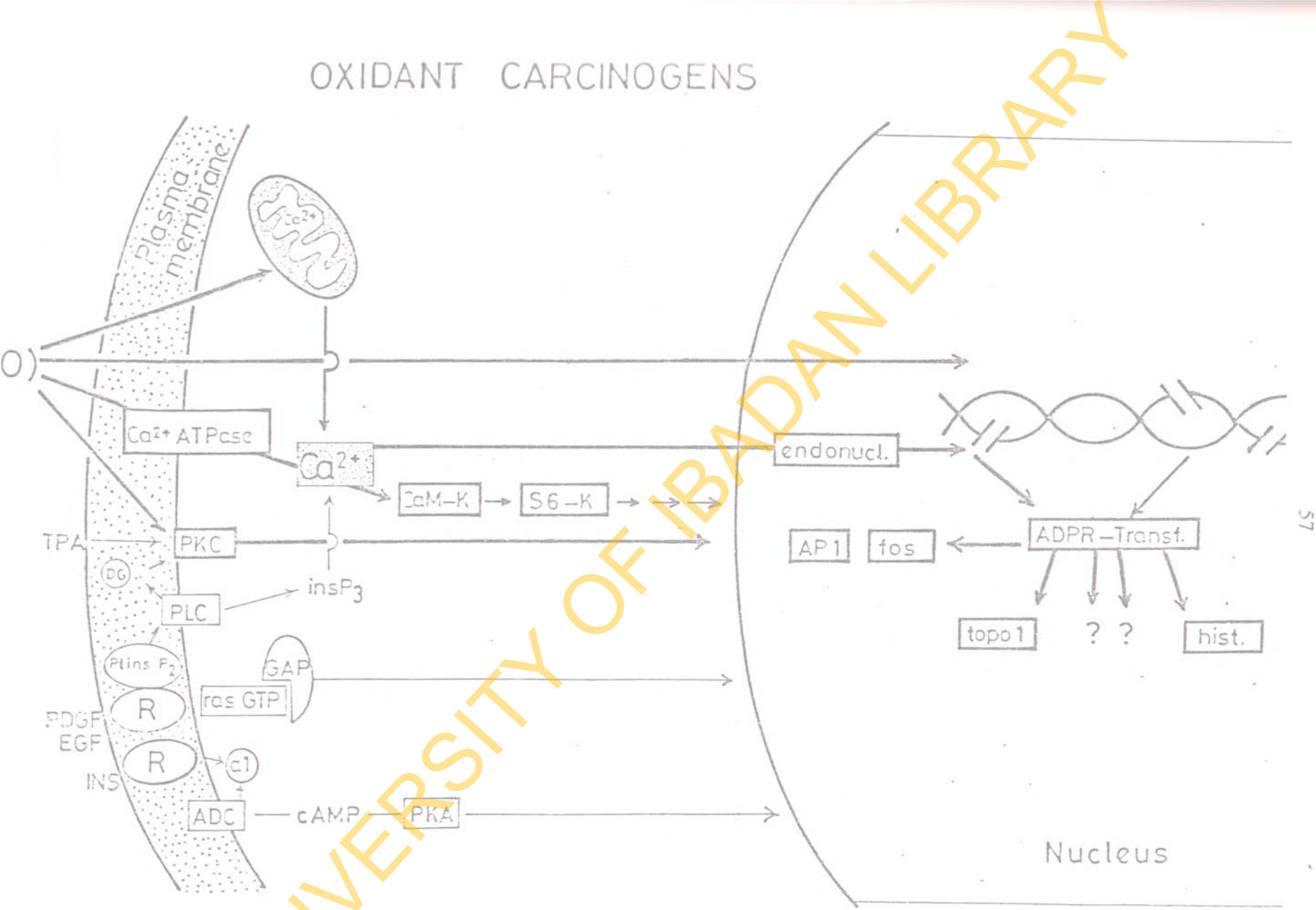



Fig. 11 Mechanisms of oxidant carcinogenesis (Cerutti et al., 1989)

1.9 Aims of the study

Protein Kinase C, the $Ca^{2+}$ and phospholipid dependent kinase, has been identified as the target of tumour promoters (Castagna et al., 1982; Ashendel et al., 1983 a,b). This enzyme has been well characterized (Inoue, 1977). It has been shown that diacylglycerol (a product of phosphatidylinositolbisphosphate breakdown) is the endogenous activator of protein Kinase C under physiological conditions (Kikkawa et al., 1982). The addition of diacylglycerol (μM range) increased the enzyme's affinity to both $Ca^{2+}$ and phospholipid (Takai et al., 1979). A number of agents, including β – adrenergic stimulators, growth factors (e.g. EGF), and other tumour promoters, mitogens, peptide hormones, and neurotransmitters are able to stimulate protein kinase C activity and to accelerate phosphatidyl inositol turnover (Castagna et al., 1982; Michell, 1979).

Usually, intracellular events are signalled by a transient rise in the concentration of $Ca^{2+}$ in the cytosol (Carafoli, 1987). Indeed, Berridge (1987) has concluded that a rapid and transient increase in cytosolic free $Ca^{2+}$ is part of the first response of cells to growth factors and that the release of $Ca^{2+}$ from the endoplasmic reticulum (ER) is often stimulated by agents that cause cell proliferation. Inositol 1,4,5-trisphosphate ($IP_3$) another product of phosphatidylinositolbisphosphate breakdown is the mediator in the release of intracellular stores of $Ca^{2+}$ from the ER into the cytosol (Suresh et al., 1984; Streb et al., 1983).

The mechanism by which liver tumour promoters exert their physiological effects is poorly understood.

The well-studied liver tumour promoter and complete carcinogen carbon tetrachloride ($CCl_4$) has been shown to:

(a) induce an inhibition of the plasma membrane $Ca^{2+}$-ATPase (Parola et al., 1990),

(b) induce the inhibition of the microsomal $Ca^{2+}$-pump (Recknagel et al., 1982; Long and Moore, 1986) and

(c) decrease the $Ca^{2+}$ and phospholipid dependent protein Kinase C content (Poli et al., 1988).

It has been demonstrated that the activity of the plasma membrane $Ca^{2+}$-ATPase is regulated by protein Kinase C (Smallwood et al., 1988). However, it is recognized that tumour promoters induce cellular prooxidant state (Cerutti, 1985) and that activated oxygen inhibits plasma

membrane $Ca^{2+}$-ATPase activity (Hebbel et al., 1986). However, the inhibition of the plasma membrane $Ca^{2+}$-ATPase even with the $IP_3$-induced $Ca^{2+}$ release from the ER, seems not sufficient to account for the very high level of $Ca^{2+}$ in the cancer cells; assuming that the ER $Ca^{2+}$-ATPase functions normally. It might be speculated therefore, that the effects of tumour promoters on the ER involve not only the $IP_3$-induced $Ca^{2+}$ release from the ER, but also an inhibition of the ER $Ca^{2+}$-ATPase.

Since carbon tetrachloride is a complete carcinogen, results obtained by the use of the chemical can not be taken as a true reflection of tumour promotion.

This thesis was designed to evaluate the role of the endoplasmic reticulum $Ca^{2+}$-ATPase in the process of liver tumour promotion. The specific liver tumour promoter and environmental contaminant, DDT as well as protein-malnutrition which is widely used and common respectively in Africa and Asia were used as models. It has been suggested that these factors might be associated with the preponderance of PLC in Africa and Asia (Rizvi et al., 1987; Rojanapo et al., 1988). The aims of this study are:

(1) To find out the effects of the tumour promoter dicophane on the membrane-bound rat liver microsomal $Ca^{2+}$-ATPase with the ultimate goal of using the enzyme as a marker of tumour promotion.

(2) To determine the effect of low protein intake on the liver ER $Ca^{2+}$-ATPase and

(3) To assess the possibility of using the erythrocyte ghost membrane $Ca^{2+}$-ATPase for the diagnosis of cancer in humans.

# CHAPTER TWO

## MATERIALS AND METHODS

### 2.1 Materials

(i) Chemicals: All chemicals were purchased either from Sigma Chemical Co. (U.S.A.) or from BDH Chemicals Ltd., (U.K.) Poole.

(ii) Animals: Male Wistar rats (150-200 g) or male Fisher F344 rats (weanlings or 8 weeks old) obtained from Bantin and Kingman Ltd., Aldbrough, Hull, England were used for the study.

(iii) Animal feeds: All animals except those on low protein diet were fed MRC 41B diet obtained from Pilsbury's Ltd., Birmingham, England (See appendix I for composition). Low protein diet (5 % protein) was obtained from Special Diets Services Ltd., Essex, England (See appendix II for composition).

### 2.2 Preparation of membranes

#### 2.2.1 Preparation of the light microsomal fraction.

The conventional microsomal fraction obtainable from the post-mitochondrial supernatant is known to originate from the endoplasmic reticulum. However, recent studies show that the post-mitochondrial supernatant of rat liver contains two vesicular fractions which transport $Ca^{2+}$ actively: a heavier fraction, enriched in plasma membrane markers and a lighter fraction, enriched in endoplasmic reticulum markers (Famulski and Carafoli, 1982, 1984, Gill et al, 1989). The heavy fraction is probably a contaminant. In the present study, the light microsomal fraction was used as microsome (endoplasmic reticulum) preparation.

Reagents

(A) Stock Solutions

(i) 100 mM Tris; 12.114 g Tris (hydroxymethyl) methylamine (BDH chemicals Ltd., England) was dissolved in 1 litre volumetric flask and the volume made up to the

1 litre mark with distilled water.

(ii) 40 mM EGTA: 1.5216 g of ethylene glycolbis-(p-aminoethylether) NN'N'N'-tetraacetic acid (Sigma chemicals Co. USA) was suspended in about 80 ml distilled water and 1M NaOH added in drops until the suspension became clear. The pH was adjusted to 7.4 and the volume made up to 100 ml in a volumetric flask.

(iii) 500 mM PMSF: 0.0871 g of phenylmethyl sulfonyl fluoride (PMSF)(Sigma chemicals Co. USA) was dissolved in 1ml dimethyl formamide (BDH chemicals Ltd., England).

(iv) β -Mercaptoethanol: β-mercaptoethanol (BDH chemicals Ltd., England) was obtained in commercial form.

(B) Working Solutions

(i) Isolation buffer : 250 mM Sucrose, 5 mM Tris,1 mM mercaptoethanol, 0.5 mM PMSF, pH 7.4

The buffer was prepared by dissolving 86.58 g of Sucrose (BDH chemicals Ltd., U.K.) in about 500 ml of distilled water. To the solution was added 50 ml of 100 mM Tris, 70.5 µl of β -mercaptoethanol and 1 ml of 500 mM PMSF in 1 litre beaker and distilled water added to 900 ml mark. The pH was adjusted to 7.4 and the solution transferred into a litre volumetric flask. The buffer solution was made up to the 1 litre mark with distilled water.

(ii) Storage buffer : 250 mM Sucrose, 5 mM Tris, 1 mM mercaptoethanol, 0.5 mM PMSF, 1 mM EGTA, pH 7.4

21.394 g of Sucrose was dissolved in about 100 ml distilled water, and to the solution was added 12.5 ml of 100 mM Tris, 17.6 µl of β-mercaptoethanol, 250 µl of 500 mM PMSF, 6.25 ml of 40 mM EGTA in 250 ml beaker and water adder to the 200 ml mark. The pH was adjusted to 7.4 and the volume made up to 250 ml in a volumetric flask with distilled water.

Procedure

Rats were $CO_2$ exsanguinated and the liver quickly removed. Rat liver light microsomal fraction (endoplasmic reticulum) was prepared as described by Famulski and Carafoli (1982). Rat liver (maintained on ice-cold isolation buffer) was carefully weighed and homogenized in 4 times ice-cold isolation buffer using a Potter-Elvehjem homogenizer. The homogenate was

filtered through 4 layers of cheese cloth and centrifuged at 3,000 x g for 6 min. The supernatant was centrifuged at 10000 x g for 10 min. and the post-mitochondrial supernatant was centrifuged at 17,500 x g for 20 min. to remove the heavy microsomal fraction. The obtained supernatant was centrifuged at 100,000 x g for 1 hr. The pellet (light microsomal fraction) was suspended in a small volume of storage buffer and kept frozen in aliquots at -20°c until required for use.

2.2.2 Preparation of erythrocyte ghost membranes

Erythrocyte ghost membrane (EGM) is the most studied plasma membrane chiefly because it is devoid of any contaminating intracellular membranes and also because it is relatively easier to prepare. Furthermore it can be obtained in large quantities. The red blood cell like most cell types can be lysed by using hypotonic buffer solutions. This is the principle on which the EGM preparation is based.

Reagents

(A) Stock Solution:

(i) 100 mM Tris: 12.114 g Tris (hydroxymethyl) methylamine (BDH Chemicals Ltd., England) was dissolved in 1 litre volumetric flask and the volume made up to the 1 litre mark with distilled water.

(ii) 3.25 M KCl: 121.23 g of potassium chloride (BDH Chemicals Ltd., England) was dissolved in 1 litre distilled water using a volumetric flask.

(iii) 100 mM EDTA : 0.093 g of disodium salt of ethylenediaminetetraacetic acid (EDTA) (BDH Chemicals Ltd., England) was dissolved in 250 ml distilled water using a volumetric flask.

(iv) 1 M HEPES: 23.830 g of HEPES (N-2-hydroxy ethylpiperazine-$N^1$-2 ethanesulfonic acid) (Sigma Chemicals Co., USA) was dissolved in 100 ml distilled water using a volumetric flask.

(v) 100 mM $MgCl_2$ : 2.3805 g of anhydrous magnesium chloride (BDH Chemicals Ltd., England) was dissolved in 250 ml distilled water using a volumetric flask.

(vi) 40 mM $CaCl_2$ : 1.4792 g of calcium chloride 2-hydrate (BDH Chemicals Ltd.,England) was dissolved in 250 ml distilled water using a volumetric flask.

(vii) 500 mM PMSF: 0.0871 g of phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemicals

Co., USA) was dissolved in 1 ml dimethylformamide (BDH Chemicals Ltd.,England).

(B) Working Solutions

(i) Isotonic buffer : 130 mM KCl, 20 mM Tris, pH 7.4

The buffer was prepared by mixing 80 ml of 3.25 M KCl with 400 ml of 100 mM Tris, and distilled water added to 1900 ml in a beaker. The pH was adjusted to 7.4 and the solution made up to 2 litres in a volumetric flask.

(ii) Hypotonic (Lysing) buffer : 10 mM Tris,1 mM EDTA, pH 7.4

The buffer was prepared by mixing 200 ml of 100 mM Tris with 20 ml of 100 mM EDTA and distilled water to 1900 ml in a beaker. The pH was adjusted to 7.4 and the solution made up to 2 litres in a volumetric flask. To every 500 ml of the buffer was added 0.2 ml of PMSF just before use.

(iii) Washing buffer : 10 mM HEPES pH 7.4

About 1700 ml of distilled water was added to 20 ml of 1 M HEPES in a beaker and the pH adjusted to 7.4. The solution was made up to the 2 litres mark with distilled water in a volumetric flask. To every 500 ml of the buffer was added 0.2 ml of PMSF just before use.

(iv) Storage (resealing) buffer : 130 mM KCl, 20 mM HEPES, 500 µM $MgCl_2$ and 50 µM $CaCl_2$ pH 7.4

The buffer was prepared by mixing 80 ml of 3.25 M KCl with 40 ml of 1M HEPES 10 ml of 100 mM $MgCl_2$, 2.5 ml of 40 mM $CaCl_2$ and about 1800 ml of distilled water in a beaker. The pH was adjusted to 7.4 and the solution made up to 2 litres in a volumetric flask. To every 50 ml of the buffer was added 20 µl of PMSF just before use.

Procedure

Haemoglobin-free ghost deficient in calmodulin were prepared by the procedure of Niggli et al (1981) which is based on the principles of hypotonic lysis developed by Dodge et al (1963).

Fresh but exposed human blood samples obtained from Liverpool Blood Transfusion service, Merseyside, England were used for the preparation of erythrocyte ghost membranes (EGM).

The whole blood was centrifuged at 5800 x g for 10 min. The plasma and buffy coat were removed by aspiration to obtain packed erythrocytes. The erythrocytes were washed twice in 5 times volumes of isotonic buffer. Each time the cell suspension was centrifuged at 6000 x g for 10 min. and the supernatant removed by aspiration. The cells were haemolyzed in 5 times volumes of hypotonic buffer and centrifuged at 20000 x g for 20 min. This step was repeated four times. The membranes were then washed five times with the washing buffer by suspending the pellet in the buffer and centrifuging at 20000 x g for 20 min. The haemoglobin-free ghost membranes deficient in calmodulin were finally suspended in the storage buffer and stored at -20°c till required for use.

2.3 Protein determination.

The Lowry procedure of protein determination (Lowry et al., 1951) is based on the use of Folin-Ciocalteu reagent (a solution of phosphomolybdictungstic complex) which reacts with and is reduced by tyrosine residues in protein at alkaline pH to give a blue colour. To buffer the pH around 10 and to neutralize the phosphoric acid produced by the degradation of the phosphomolybdictungstic complex at alkaline pH, a mixture of $Na_2CO_3$-NaOH is added to the assay system. Furthermore, pretreatment of the protein sample with alkaline copper markedly increased the colour developed.

Reagents

(A) Stock Solutions

(i) Reagent A : 2 % $Na_2CO_3$ in 0.1 N NaOH.

10 g of anhydrous $Na_2CO_3$ (BDH Chemicals Ltd., England) was dissolved in about 400 ml of 0.1 N solution of NaOH (4 g NaOH (BDH Chemicals Ltd., England) dissolved in 1 litre volumetric flask and made up to the 1 litre mark with distilled water). The $Na_2CO_3$ solution was made up to the 500 ml mark in a standard volumetric flask with 0.1 N NaOH solution.

(ii) Reagent B : 2 % $Na^{\pm}/K^{\pm}$-Tartrate

2.0 g of Sodium Potassium tartrate (BDH chemicals Ltd., England) was dissolved in 100 ml of distilled water using a volumetric flask.

(iii) Reagent C : 1 % Copper Sulphate

1.0 g of Copper sulphate (BDH Chemicals Ltd., England) was dissolved in 100 ml distilled water using a volumetric flask.

B) Working Solutions

(i) Reagent D : Alkaline copper Solution:

This reagent was prepared by mixing 50 ml of reagent A with 0.5 ml of reagent B and 0.5 ml of reagent C in that order just before use.

(ii) Reagent E : Folin-Ciocalteu reagent

This was obtained in commercial form from Sigma chemicals Co., U.S.A.

(iii) Bovine Serum albumen (BSA) : 1 mg/ml BSA

This was prepared by dissolving 20 mg of BSA (Sigma Chemicals Co., U.S.A) in 20 ml of distilled water using a volumetric flask.

Procedure

Membrane protein determination was carried out as described by Lowry et al. (1951). First, a protein calibrated curve was plotted using the procedure contained in Table 5. The obtained results were used to plot a protein calibrated curve Fig.12. Thereafter, 20 µl of the membrane sample was pipetted into a test-tube and 580 µl of distilled water added to it. Another test-tube containing 600 µl of distilled water served as control. To the two test-tubes were added 3 ml of reagent D and mixed. The mixture were allowed to stand for 10 min after which 0.3 ml of reagent E was added and rapidly mixed. After incubation at room temperature for 30 min, the absorbance at 750 nm was taken and the protein content extrapolated from the protein calibrated curve (Fig.12).

Table 5: Protocol for Protein determination

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 mg/ml BSA (µl) | - | 10 | 20 | 40 | 60 | 80 | 100 |
| $H_2O$ ( µl) | 600 | 590 | 580 | 560 | 540 | 520 | 500 |
| Reagent D (ml) | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Mix rapidly and wait for 10 minutes | | | | | | | |
| Reagent E (ml) | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Mix rapidly and wait for 30 minutes | | | | | | | |
| A 750 nm | | | | | | | |
| Protein Content (µg) | - | 10 | 20 | 40 | 60 | 80 | 100 |

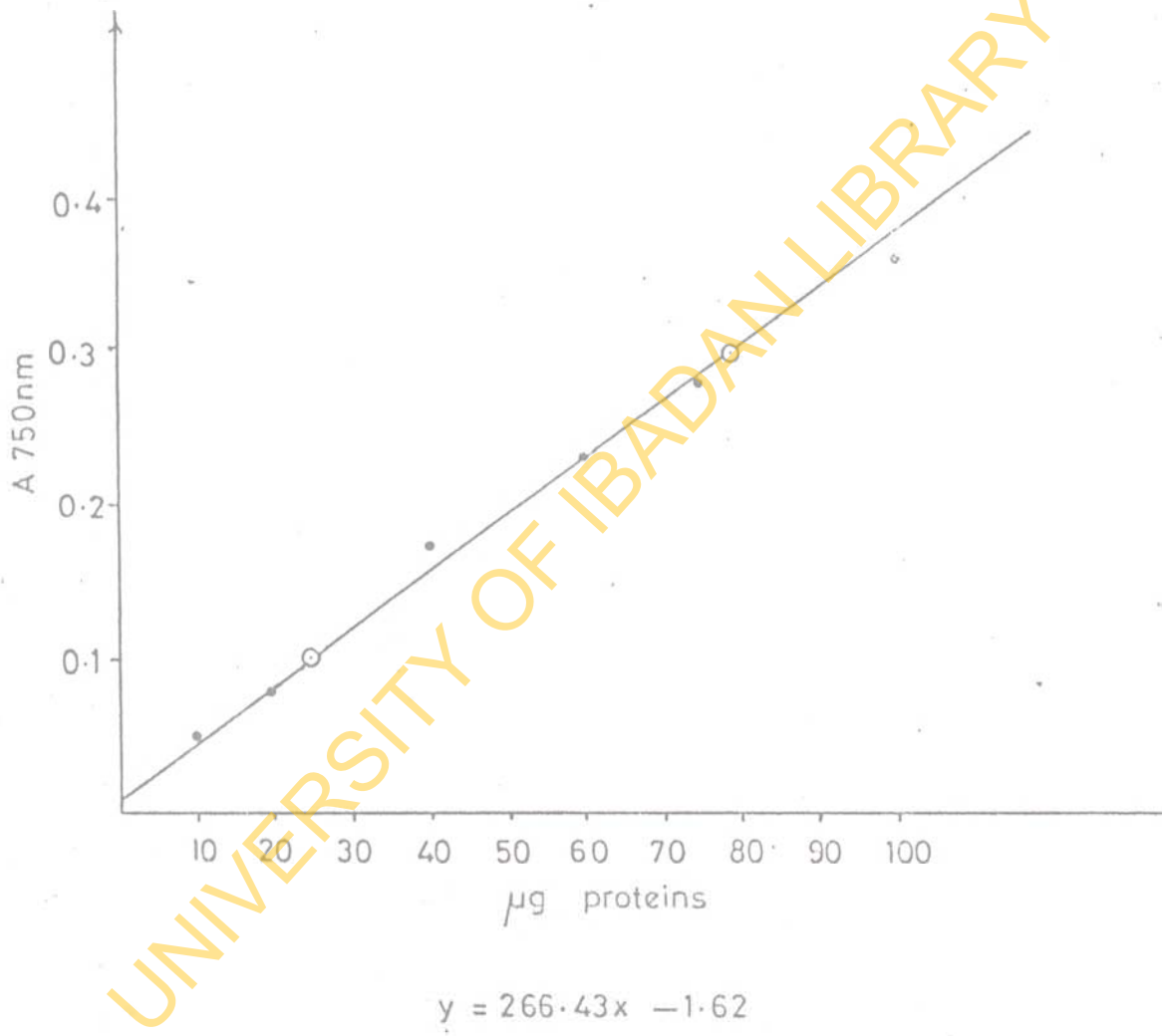





Fig. 12 Protein calibrated plot

## 2.4 Enzyme assays and microsomal haemoprotein content determination.

### 2.4.1 Determination of the activity of membrane-bound rat liver microsomal $Ca^{2+}$-ATPase.

Endoplasmic reticulum, the $Ca^{2+}$ store, is the most important intracellular structure in the fine regulation of intracellular $Ca^{2+}$ concentration. It takes up $Ca^{2+}$ through an $E_1-E_2$ ATPase, and can release $Ca^{2+}$ when acted upon by a number of agonists via the action of inositoltrisphosphate (Carafoli, 1987).

The ability of the ER to accumulate $Ca^{2+}$ requires the hydrolysis of ATP; with the release of its $\gamma$-$P_i$. Determination of the $Ca^{2+}$-ATPase activity is based on the estimation of the released $\gamma$-$P_i$ in the reaction pathway.

REAGENTS

(A) Stock Solutions

(i) 3.25 M KCl: 121.23 g potassium chloride (BDH Chemicals Ltd., England) was dissolved in 1 litre distilled water using a volumetric flask.

(ii) 100 mM Tris: 12.114 g Tris (hydroxymethyl) methylamine (BDH Chemicals Ltd., England) was dissolved in 1 litre volumetric flask and the volume made up to the 1 litre mark with distilled water.

(iii) 100 mM $NaN_3$ : 0.6501 g of Sodium azide (BDH Chemicals Ltd., England) was dissolved in about 90 ml of distilled water and the volume adjusted to 100 ml in a volumetric flask.

(iv) 1 mM digitoxygenin: 0.003745 g of digitoxygenin (Sigma Chemicals Co., USA) was dissolved in 10 ml DMSO (BDH Chemicals Ltd., England).

(B) Working Solutions

(i) 40 mM $CaCl_2$ : 1.4702 g of calcium chloride 2-hydrate (BDH Chemicals Ltd., England) was dissolved in 250 ml distilled water using a Volumetric flask.

(ii) 40 mM ATP: 0.44088 g of disodium Salt of ATP (Sigma Chemicals Co., U.S.A) was dissolved in 20 ml distilled water and kept frozen at -20°c.

(iii) 40 mM EGTA: 1.5216 g of ethyleneglycol-bis- (p-amino ethyl ether) NN'N'N-tetraacetic

acid (Sigma Chemicals Co., USA) was suspended in about 80 ml distilled water and 1 M NaOH added until the suspension became clear. The pH was adjusted to 7.4 and the volume made up to 100 ml using a Volumetric flask.

(iv) 10 % S.D.S: 50 g of Sodium dodecyl sulphate (S.D.S) (BDH Chemicals Ltd.,England) was dissolved in 500 ml distilled water using a volumetric flask.

(v) 9 % Ascorbic acid: 45 g of L-ascorbic acid (BDH Chemicals ltd., England) was dissolved in 500 ml distilled water using a volumetric flask.

(vi) Reagent A: 1.25 % Ammonium molybdate in 6.5 % (v/v) $H_2SO_4$

6.25 g of ammonium molybdate (Sigma Chemicals Co. USA) was dissolved in dilute Sulphuric acid (32.5 ml of conc. $H_2SO_4$ (BDH Chemicals Ltd., England) in 400 ml distilled water) in a beaker. The volume was adjusted to 500 ml with distilled water in a volumetric flask.

(vii) Microsomal $Ca^{2+}$-ATPase assay buffer : 40 mM Tris, 200 mM KCl, 2 mM $NaN_3$ and 40 µM digitoxygenin

The buffer was prepared by mixing 100 ml of 100 mM Tris with 5 ml of 100 mM $NaN_3$, 15.625 ml of 3.25 M KCl, 10 µl of 1 mM digitoxigenin and about 100 ml of distilled water in a beaker. The pH was adjusted to 8.0 and the volume made up to the 250 ml mark with distilled water.

PROCEDURE

Determination of the microsomal $Ca^{2+}$-ATPase activity was carried out as described by Famulski and Carafoli (1982). The assay medium was prepared according to Table 6.

The reaction was initiated with the addition of 100 µl of 40 mM ATP and terminated after 30 min of incubation (at 30°c in a water bath) with the addition of 200 µl of 10 % S.D.S. The difference in the amount of Pi released in the presence and absence of 1.8 mM $CaCl_2$ (final concentration) was used for the calculation of enzyme activity i.e the difference between the $Mg^{2+}$-ATPase and the $Ca^{2+}/Mg^{2+}$-ATPase activity. The ATP blank served as control. The phosphate liberated was determined by the Fiske-Subbarow method (1925). For this, 1ml of ammonium molybdate solution (reagent A) was added to each test tube followed immediately with the addition of 1 ml of 9 % ascorbic acid. The colour developed after 30 min. was read on a spectrophotometer at 660 nm. The amount of phosphate was extrapolated from a phosphate calibrated curve (Fig. 13).

Table 6: Protocol for assay for Microsomal $Ca^{2+}$-ATPase activity

| | ATP blank | Enzyme blank | $Mg^{2+}$-ATPase | $Ca^{2+}/Mg^{2+}$- ATPase |
|---|---|---|---|---|
| Microsomal assay Buffer pH 8.0 (µl) | 400 | 400 | 400 | 400 |
| 40 mM EGTA (µl) | 40 | 40 | 40 | 40 |
| 40 mM $CaCl_2$ (µl) | 36 | 36 | - | 36 |
| $H_2O$ (µl) | 304 | 224 | 240 | 204 |
| 10 µg/µl Membrane (µl) | 20 | - | 20 | 20 |
| 40 mM ATP (µl) | - | 100 | 100 | 100 |

Construction of phosphate calibrated curve

The construction of a standard curve for phosphate (Fig.13) was done using 10 mM $Na_2HPO_4$ as standard; according to Table 7.

Calculation of $Ca^{2+}$-ATPase activity:

The $Ca^{2+}$-ATPase activity was calculated according to the equation below:

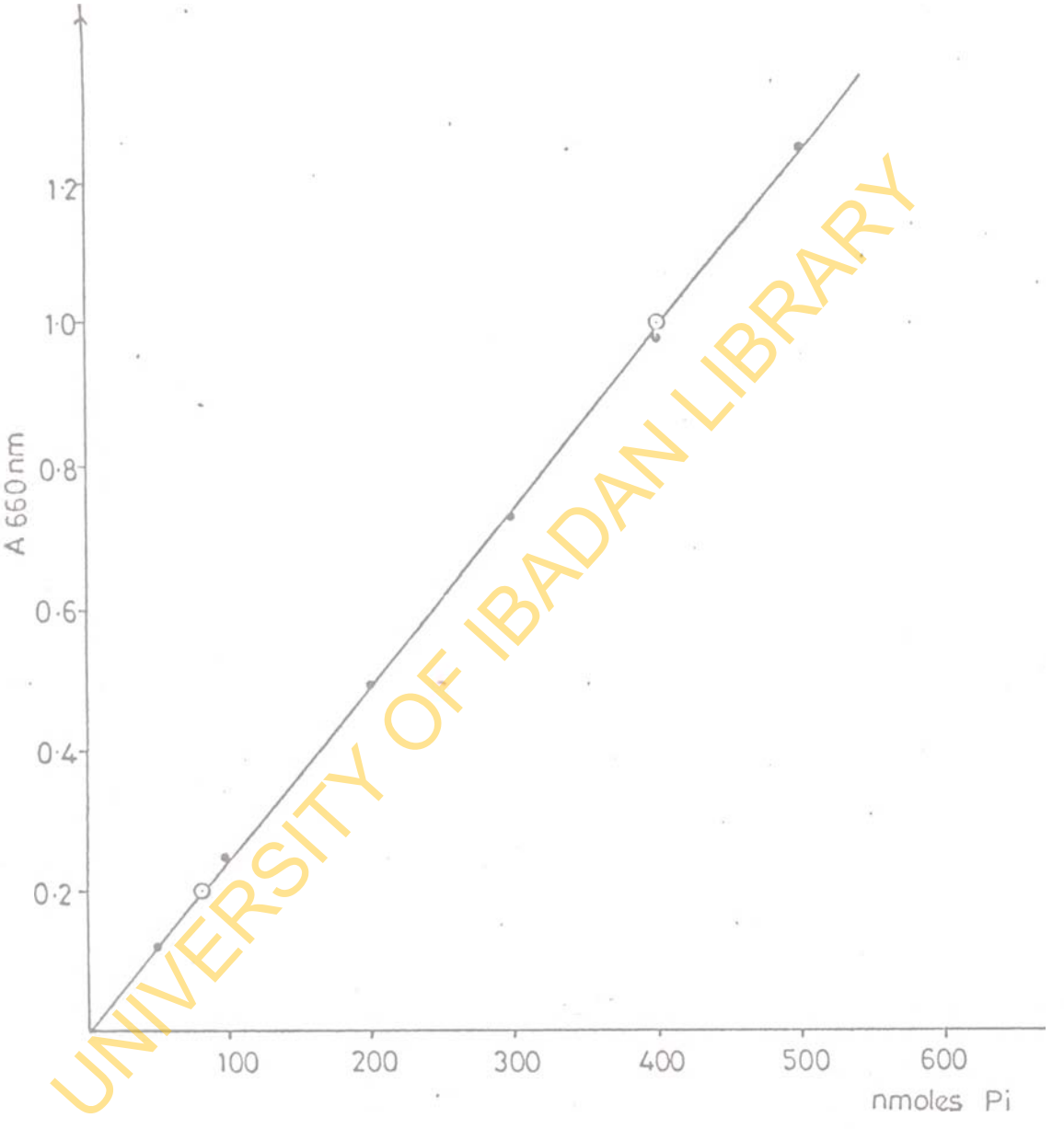
$Ca^{2+}$-ATPase activity = 2x/Y µmoles $P_i$/mg Protein/hr.

where x = $P_i$ liberated in 30 min. (in µmoles)

Y = Protein concentration of membrane (in mg)

Table 7: Protocol for inorganic phosphate calibration

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $H_2O$ | (µl) | 1000 | 995 | 990 | 980 | 970 | 960 | 950 |
| 10 mM $Na_2HPO_4$ | (µl) | - | 5 | 10 | 20 | 30 | 40 | 50 |
| Reagent A | (ml) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ascorbic acid | (ml) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Mix rapidly and wait for 30 min. | | | | | | | | |
| $A_{660}$ nm | | | | | | | | |
| $P_i$ (nanomoles) | | - | 50 | 100 | 200 | 300 | 400 | 500 |




$y = 402 \cdot 31x + 0 \cdot 85$

Fig. 13 Phosphate calibrated plot

2.4.2 Determination of the activity of the membrane-bound erythrocyte ghost membrane $Ca^{2+}$-ATPase.

The plasma membrane $Ca^{2+}$-ATPase functions by pumping out $Ca^{2+}$ from the cell against a concentration gradient. This process is ATP-driven and is accompanied with the release of the $\gamma$-$P_i$ of ATP. Determination of the erythrocyte ghost membrane $Ca^{2+}$-ATPase activity like the microsomal $Ca^{2+}$-ATPase activity is therefore based on the estimation of the released $\gamma$-$P_i$.

Reagents

(A) Stock Solutions

(i) 3.25 M KCl: 121.23 g of potassium chloride (BDH Chemicals Ltd., England) was dissolved in 1 litre distilled water using a volumetric flask.

(ii) 40 mM EGTA: 1.5216 g of ethyleneglycol-bis-(p-aminoethyl ether) N N' N' N' - tetraacetic acid (Sigma Chemicals Co. USA) was suspended in about 80 ml distilled water and 1M NaOH added until the suspension became clear. The pH was adjusted to 7.4 and the volume made up to 100 ml using a volumetric flask.

(iii) 1 M HEPES: 23.830 g of HEPES (N-2-hydroxy ethyl piperazine-N'-2 ethane sulfonic acid) (Sigma Chemicals Co., USA) was dissolved in 100 ml distilled water using a volumetric flask.

(iv) 100 mM $MgCl_2$: 2.3805 g of anhydrous magnesium chloride (BDH Chemicals, Ltd., England) was dissolved in 250 ml distilled water using a volumetric flask.

(v) 40 mM $CaCl_2$: 1.4702 g of Calcium chloride-2-hydrate (BDH Chemicals Ltd., England) was dissolved in 250 ml distilled water using a Volumetric flask.

(vi) 40 mM ATP: 0.44088 g of disodium salt of ATP (Sigma Chemicals Co., USA) was dissolved in 20 ml distilled water and kept frozen at -20°C.

(B) Working Solutions:

(i) EGM assay buffer : 260 mM KCl, 60 mM HEPES pH.74

The buffer was prepared by mixing 20ml of 3.25 M KCl and 15 ml of 1 M HEPES and distilled water to about 200 ml in a beaker. The pH was adjusted to 7.4 and the volume made up to 250 ml in a volumetric flask.

(ii) 8 mM ATP: This was prepared by adding 20 ml of distilled water to 5 ml of 40 mM

ATP. The solution was kept frozen at -20°c.

(iii) 0.8 mM EGTA: The solution was prepared by pipetting 2 ml of 40 mM EGTA into 100 ml volumetric flask and the volume adjusted to 100 ml with distilled water.

(iv) 80 mM $MgCl_2$ : The solution was prepared by pipetting 80 ml of 100 mM $MgCl_2$ into 100 ml volumetric flask and adjusting the volume to 100 ml with distilled water.

(v) 800 µM $CaCl_2$ : The solution was prepared by pipetting 2 ml of 40 mM $CaCl_2$ into 100 ml volumetric flask and adjusting the volume to 100 ml with distilled water.

Procedure

The EGM $Ca^{2+}$-ATPase activity was estimated essentially as reported by Bewaji and Bababunmi (1987). The assay media were prepared as presented on Table 8.

The reaction was initiated with the addition of 100 µl of 8 mM ATP and terminated after 30 min of incubation (at 30°c in a water bath) with the addition of 200 µl of 10% S.D.S. The difference in the amount of Pi released in the presence and absence of 20 µM $CaCl_2$ (final concentration) was used for the calculation of enzyme activity i.e the difference between the $Mg^{2+}$-ATPase and the $Ca^{2+}/Mg^{2+}$-ATPase activity. The ATP blank served as control.

The liberated inorganic phosphate was estimated according to Fiske and Subbarow (1925) using a standard phosphate calibrated curve as contained in section 2.4.2. Calculation of ($Ca^{2+}$ + $Mg^{2+}$) - ATPase activity was also done as contained in section 2.4.2.

Table 8: Protocol for assay for plasma membrane (EGM) $Ca^{2+}$-ATPase activity

| | ATP blank | Enzyme blank | $Mg^{2+}$-ATPase | $Ca^{2+}/Mg^{2+}$- ATPase |
|---|---|---|---|---|
| EGM assay Buffer pH 7.4 (µl) | 400 | 400 | 400 | 400 |
| 80 mM $Mgcl_2$ (µl) | 20 | 20 | 20 | 20 |
| 800 µM $Cacl_2$ (µl) | 20 | 20 | - | 20 |
| 0.8 mM EGTA (µl) | - | - | 100 | - |
| $H_2O$ (µl) | 340 | 260 | 160 | 240 |
| 10 µg/µl Membrane (µl) | 20 | - | 20 | 20 |
| 8 mM ATP (µl) | - | 100 | 100 | 100 |

2.4.3 Determination of the activity of gammaglutamyl transpeptidase (GGT).

Glutamyltranspeptidase (EC.2.3.2.2) catalyzes the transfer of the ɣ-glutamyl moiety of peptides to a variety of amino acid and peptide receptors (Tate and Meister, 1978). The fluorimetric assay procedure for GGT activity is based on the observation that L-glutamic acid-ɣ-(7-amido-4-methyl coumarin) is non-fluorescent while its cleavage product, 7-amino-4-methyl coumarin (AMC) is highly fluorescent - when excited at 370 nm and the fluorescence emission taken at 440 nm (Smith et al., 1979).

Reagents

(A) Stock Solutions

(i) Ammediol buffer : 0.1 M ammediol, 20 mM glycylglycine, and 0.1 % Triton X-100 pH 8.5

10.51 g of 2-amino-2-methyl-1,3, propanediol (ammediol) (Sigma Chemicals Co., USA), 2.642 g of glycylglycine (Sigma Chemicals Co., USA) and 1ml of 100 % Triton X-100 (BDH Chemicals Ltd., England) were dissolved in about 950 ml distilled water and the pH adjusted to 8.5. The solution was transferred into a volumetric flask and the volume made up to 1 litre mark with distilled water.

(ii) 1 mM 7-amino-4-methyl coumarin (AMC):

0.003504 g of 7 amino-4-methyl-coumarin (SigmaChemicals Co., USA) was dissolved in 20 ml of methoxyethanol (BDH Chemicals Ltd., England).

(iii) 10 mM L-glutamic acid-ɣ- (7 amido-4-methylcoumarin (L-ɣ-glutamyl AMC):

0.00761 g of L-glutamic acid-ɣ-(7-amido-4 methyl coumarin) (Sigma Chemicals Co., USA) was dissolved in 2.5 ml of methoxyethanol and sonicated to get a homogenous suspension.

(B) Working Solution:

(i) 0.05 M glycine buffer pH 10.4

1.877 g of glycine (BDH Chemicals Ltd., England) was dissolved in about 450 ml of distilled water (in a beaker) and the pH adjusted to 10.4. The Volume was made up to 500 ml mark in a volumetric flask with distilled water.

(ii) 20 mM Serine/100mM Borate reagent:

0.105 g of L-serine (Sigma Chemicals Co., USA) and 1.907 g of borax

(disodium tetraborate) (BDH Chemicals Ltd., England) were dissolved in about 40 ml of distilled water and made up to the mark in a volumetric flask to 50 ml with distilled water.

(iii) Buffered Substrate for GGT assay

The solution was prepared by adding 0.2 ml of 10 mM L-glutamyl-AMC to 9.8 ml of ammediol buffer pH 8.5.

Procedure

Liver GGT activity was measured according to the procedure of Smith et al., (1979) using L-$\gamma$-glutamy- AMC as substrate. The assay medium contained 142.86 µM substrate, 70mM Ammediol, 14.0 mM glycylglycine, 0.07 % Triton X-100 and liver homogenate. This was prepared by adding 35 µl of liver homogenate (0.25 g/ml) to 250 µl of buffered substrate in a final volume of 350 µl. Sample containing serine/Borate (2 mM/10 mM) as an inhibitor of GGT as suggested by Tate and Meister (1978), was added to check for non-specific protease activity. The mixture was incubated at 37°c for 10 minutes. The reaction was stopped by adding 2 ml of ice-cold glycine buffer pH 10.4. A standard curve was prepared by using 7-amino-4 methyl coumarin (AMC) in ammediol buffer (0.14-3.5 nmoles). The intensity of fluorescence emission was measured at 440 nm after excitation at 370 nm. Slits = 6 and sample sensitivity = 0.3.

Construction of AMC Calibrated Curve

The construction of the 7-amino-4-methyl coumarin (AMC) calibrated curve (Fig.14) was done according to Table 9.

Calculation of GGT Activity

The fluorescence emission of the sample at 440 nm after excitation at 370 nm was used to calculate the amount of AMC released from the substrate (extrapolated from Fig.14) and the value used to calculate the GGT activity as given below. GGT activity was expressed as nmoles AMC/g liver/min.

GGT activity = x/10Y nmoles AMC/g liver/min.

where x = nmoles AMC released after 10 min of incubation.

10 = Period of incubation in min.

Y = Weight of liver (in grams) in pipetted volume of liver homogenate.

Table 9: Protocol for AMC calibration/estimation

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Ammediol buffer pH 8.5 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 |
| 7 µM* or 70 µM+ | | | | | | | | | |
| AMC (µl) | - | 20* | 40* | 50* | 75* | 10+ | 20+ | 40+ | 50+ |
| $H_2O$ (µl) | 100 | 80 | 60 | 50 | 25 | 90 | 80 | 60 | 50 |
| 0.05M Glycine buffer pH 10.4 (ml) (ml) | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| nmoles AMC | - | 0.14 | 0.28 | 0.35 | 0.53 | 0.70 | 1.40 | 2.80 | 3.50 |
| Fluorescence Emission ( ext = 370 ; emis = 440) | | | | | | | | | |

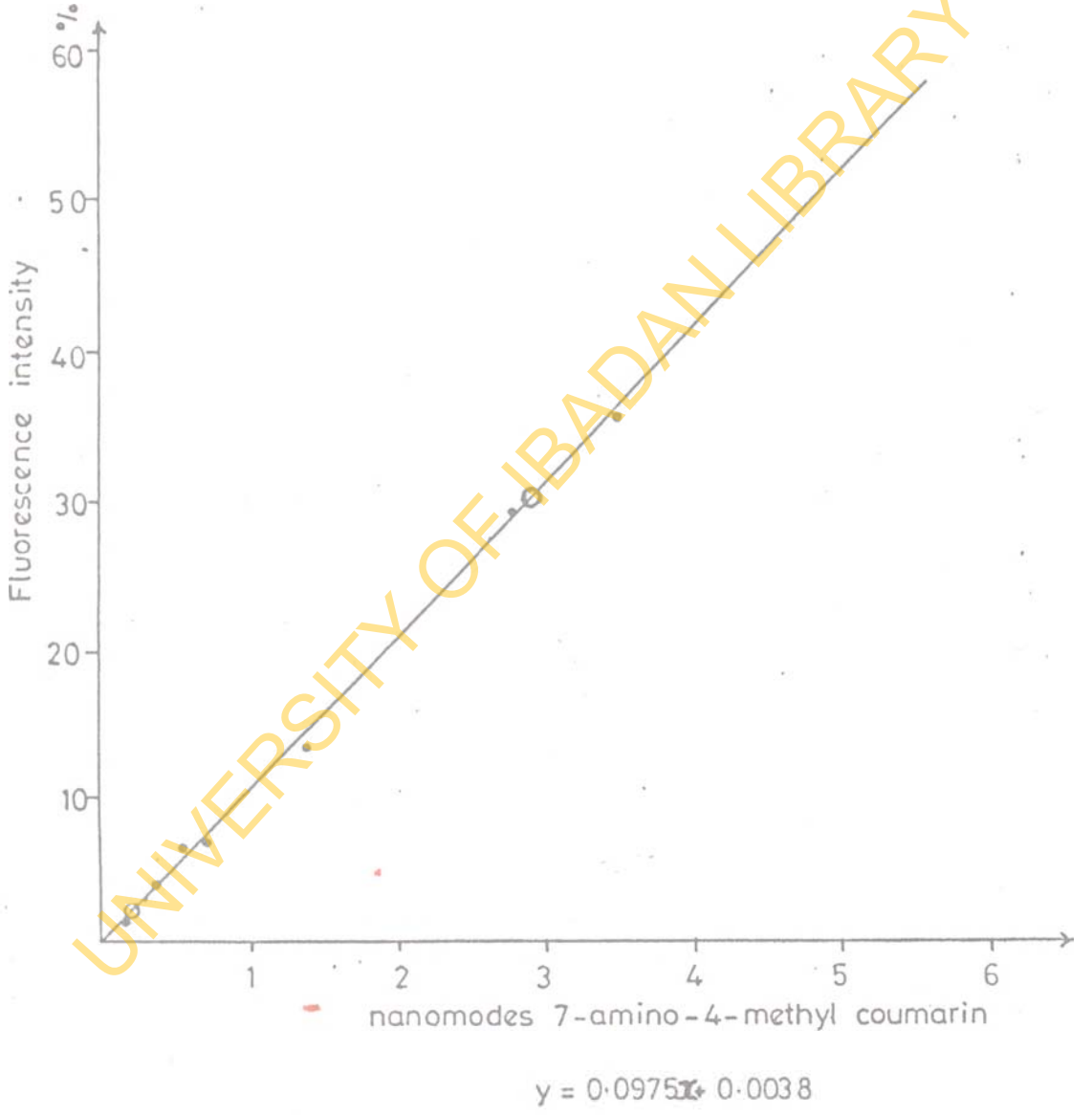





Fig. 14 AMC calibrated plot

2.4.4 Determination of microsomal Cytochrome P450 Content.

Cytochrome $P_{450}$ is a family of haemoproteins involved in the detoxification of a wide range of chemical compounds. Use is made of the fact that when the haem iron is reduced and complexed with carbon monoxide, a characteristic absorption spectrum results. The reduced, carbon monoxide difference spectrum of cytochrome $P_{450}$ absorbs maximally at around 450 nm (hence the name) and the extinction coefficient for the wavelength couple 450-490 nm has been accurately determined to be 91 $nM^{-1}$ $cm^{-1}$ thus allowing quantitative determination of this haemoprotein.

Reagents

(i) 0.1 M Tris pH 7.4 containing 20 % (v/v) glycerol:
6.057 g of Tris (hydroxymethyl) methyl amine (BDH chemicals Ltd., England) was dissolved in about 350 ml of distilled water. 100 ml of glycerol (BDH chemicals Ltd., England) was added and the pH adjusted to 7.4. The solution was made up to the 500 ml mark with distilled water in a volumetric flask.

(ii) Solid Sodium dithionite (BDH chemicals Ltd., England).

(iii) Carbon monoxide (British Gas, U.K.)

Procedure

Cytochrome $P_{450}$ content was determined according to Omura and Sato (1964). 100 µl of microsome sample was added to 1900 µl of Tris/glycerol buffer. The solution was mixed and distributed equally into 2 cuvettes (a sample and a reference cuvette) with a path length of 1 cm. A few grains of solid sodium dithionite were added to each cuvette and stirred gently. The sample cuvette only was then gently bubbled with carbon monoxide for approximately 1 minute. The spectrum was scanned from 400-500 nm on a Cecil series 6000 double beam spectrophotometer.

Calculation

The absorbance difference between 450 and 490 nm was used for the calculation of cytochrome $P_{450}$ content knowing

(1) that the extinction coefficient (450-490) equals 91 $nM^{-1}$ $cm^{-1}$ and

(2) the protein concentration in mg/ml.

$$\text{Cytochrome } P_{450} \text{ content} = \frac{Y \times 1000}{91 \times a} \text{ nmol mg}^{-1}$$

where $Y$ = absorbance difference $(A_{450} - A_{490})$

$a$ = Protein concentration in mg/ml.

2.5 Separation of membrane proteins by SDS-PAGE.

The investigation of the molecular constitution of membranes was revolutionized by the solubilization of membranes in SDS and separation of the polypeptides by elec trophoresis in a poly acrylamide gel matrix in the presence of sodium dodecyl sulphate (SDS).

It has been observed that the electrophoretic migration rates have a fairly predict able relation to the molecular weight of proteins, if the proteins are dissociated and denatured with sodium dodecyl sulphate before and during electro-phoresis (Weber and Osborn, 1969).

SDS binds to proteins and confers on them a net negative charge. As the peptides become saturated with SDS, they fold into helical rods surrounded by an SDS-shell. The mobility of these rods through a polyacrylamide gel is inversely proportional to the logarithm of the molecular weight of the polypeptide. Thus with this technique, it is possible to separate and identify membrane proteins. In order to get complete monomerization and unfolding of proteins, it is often useful to reduce dis-ulfide bonds with mercaptoethanol followed by a brief exposure at 100°c.

Reagents

(i) Stock A : 30 % Acrylamide/0.8 % Bis-acrylamide

30 g of acrylamide (Sigma Chemical Co., USA) and 0.8 g Bis-acrylamide (Sigma Chemical Co., USA) were dissolved in a little quantity of distilled water and then made up to the mark in a 100 ml volumetric flask. The solution was filtered and stored in a reagent bottle at 4°c for several weeks. This solution is used for low cross-links separating gels and for all stacking gels.

(ii) Stock B : 30 % Acrylamide 1.5 % Bis-acrylamide

30 g acrylamide (Sigma Chemical Co., USA) and 1.5 g Bis-acrylamide were dissolved in a little quantity of distilled water and then made up to the mark in a 100 ml volumetric flask. The solution was filtered and stored in a reagent bottle at 4°c for several weeks. This solution is used for high cross-links separating gels.

(iii) Stock C : 1.5 M Tris-HCl, pH 8.8

18.17 g of Tris (hydroxymethyl)-aminomethane (Sigma Chemical Co., USA) was dissolved in about 90 ml of distilled water and the pH adjusted to 8.8 with hydrochloric acid, HCl. The solution was then made up to the mark in a 100 ml volumetric flask, filtered and stored in a reagent bottle at 4°c for several weeks. This solution is used for buffering separating gels.

(iv) Stock D : 10 % SDS (w/v)

10 g of sodium dodecyl sulphate, SDS (Sigma Chemical Co., USA) was dissolved in a little quantity of distilled water and made up to the mark in a 100 ml standard volumetric flask. The solution was stored in a reagent bottle at room temperature. SDS is an anionic detergent. The solution is used in sample buffer, stacking and separating gel buffers. It denatures proteins and imparts a uniform negative charge.

(v) Stock E : 1.25 M Tris-HCl, pH 6.8

15.15 g of Tris(hydroxymethyl)-aminomethane (Sigma Chemical Co., USA) was dissolved in about 90 ml of distilled water and the pH was adjusted to 6.8 with hydrochloric acid, HCl. The solution was made up to the mark in a 100 ml standard volumetric flask with distilled water and stored in a reagent bottle at 4°c for several weeks.

(vi) Glycerol

This was obtained in commercial form from BDH Chemicals Ltd., England; and stored at room temperature.

(vii) TEMED.

N,N,N,N-tetramethylethelenediamine, TEMED (Sigma Chemical Co., USA) was obtained in commercial form and stored at 4°c.

(viii) Ammonium persulphate

(a) Solution for stacking gels.

This was prepared by dissolving 75 mg ammonium persulphate $(NH_4)_2S_2O_8$ (Sigma Chemical Co., USA) in a 5 ml of distilled water. The solution should always be prepared fresh before pouring gel.

(b) Solution for separating gels.

This was prepared by diluting the ammonium sulphate solution for stacking gels in the ration 1:1 with distilled water.

(ix) 10X-Stock Running buffer:250 mM Tris,1.92 M Glycine, 1% SDS, pH 8.3.

30.9 g of Tris(hydroxymethyl)-aminomethane (Sigma Chemical Co., London), 144.1 g glycine (BDH Chemicals Ltd., England), 10 g SDS were dissolved in about 900 ml of distilled water using a magnetic stirrer. The solution was made up to the mark in 1 litre volumetric flask and stored in a reagent bottle at room temperature for several weeks. The pH of this solution should not and was not adjusted.

(x) Working running buffer

This was prepared by diluting the stock running buffer in the ratio 1:9 with distilled water. To do that, 150 ml of 10X stock was added to 1350 ml of distilled water to prepare 1500 ml solution.

(xi) Sample buffer

Sample buffer was prepared by adding 1.0 ml of stock E, 4 ml of stock D, 2 ml of glycerol, 1 ml of β-mercaptoethanol, 0.2 ml of 2 % Bromophenol blue, to 11.8 ml of distilled water. This solution was stored in 1 ml aliquots and kept frozen until required for use.

(xii) Preparation of gel solutions

The running gel and the stacking gel solutions were prepared according to Table 10 and Table 11 respectively.

CAUTION: It should be noted that acrylamide is neurotoxic. Observe extreme caution to minimize skin contact and inhalation.

Apparatus

(i) Power pack: Power supply B605 D/S (OLTRONIX, USA)

(ii) Electrophoretic unit: Vertical slab gel unit SE600 series (Hoefer Scientific Instruments, USA).

Procedure

(i) Setting up the glass sandwich:

Two glass plates (18 x 16 cm each) were thoroughly cleaned and a sandwich of the two made by using the spacers and clamps. The sandwich was then cammed into the casting stand with the rubber gaskets to form a mold for the gel.

(ii) Pouring the gradient gel

The gradient maker was used for this purpose. A long cannula was connected to the free end of the outlet of the gradient maker and serves as a pump for filling the glass sandwich with gel. The mixing chamber of the gradient maker was filled with the heavy (20 %) monomer solution (See Table 10) and the reservoir chamber with the light (5 %) monomer solution (see Table 10 also). A stirrer was placed in the mixing chamber. The valves of the gradient maker were opened and a syringe used to suck the solution to bring about the flow through it of the solution. The free end of the cannula was then placed between the glass plates of the sandwich. When all the gel has been pumped into the glass sandwich, the gel was water-layered. After polymerization, the water was poured off and the surface of the gel rinsed with distilled water. A stacking gel monomer solution was prepared (See Table 11) and poured by using a pasteur pipette. A comb was inserted in the stacking gel to form the sample wells.

(iii) Loading the sample

Using a Hamilton syringe, each well was underlayered with the sample.

(iv) Final assembly of the unit

After the gel has polymerized and samples applied, the top of the gel sandwich was attached to the underside of the upper buffer chamber using the rubber gaskets. This assembly was lifted off the casting stand and lowered into the lower buffer chamber. Buffer was added to each chamber and the electrical circuit was completed by fitting the safety lid onto the apparatus and connecting the leads to power supply.

TABLE 10. PROTOCOL FOR THE PREPARATION OF THE RUNNING GEL

| | 20% | 5% |
|---|---|---|
| STOCK "A" | 11.33 ml | 2.83 ml |
| STOCK "C" | 4.35 ml | 4.25 ml |
| $H_2O$ | - | 9.18 ml |
| STOCK "D" | 0.17 ml | 0.17 ml |
| GLYCEROL | 0.98 ml | 0.23 ml |
| TEMED | 0.01 ml | 0.005 ml |
| AMMONIUM PERSULPHATE | 0.26 ml | 0.335 ml |

TABLE 11. PROTOCOL FOR THE PREPARATION OF THE STACKING GEL

| | |
|---|---|
| STOCK "A" | 2.66 ml |
| STOCK "C" | 5.28 ml |
| STOCK "D" | 0.22 ml |
| $H_2O$ | 11.22 ml |
| TEMED | 28 µl |
| AMMONIUM PERSULPHATE | 0.57 ml |

(v) Staining and detaining. After removing the gel, it was fixed in a solution containing 50 % methanol in 7.5 % Acetic acid for about 30 minutes and then stained with Coomasie brilliant blue solution for 45 minutes. The gel was destained with 20% methanol in 7.5 % acetic acid solution.

2.6 Analysis of data: All data were statistically analyzed using the software package "MINITAB" (MINITAB Inc., U.S.A.).

# CHAPTER THREE

# EXPERIMENTS AND RESULTS

## Experiment 1: Characterization of rat liver microsomal membrane-bound $Ca^{2+}$-ATPase.

### Introduction

The oncogenic transformation of cells from normal to malignant phenotype is associated with a loss of the ability of Calcium to control cell proliferation (Murphy and Fiskum, 1987). In addition, tumour cells generally contain abnormally high levels of endogenous $Ca^{2+}$ (Fiskum, 1985); suggesting that the calcium regulatory system of tumour cells is defective.

The control of calcium is essentially performed by the reversible complexation to specific proteins. Soluble proteins contribute to $Ca^{2+}$ buffering, but membrane-intrinsic proteins play the main role in buffering of cell calcium. They control $Ca^{2+}$ very precisely and with high affinity (ATPases) or with lower affinity (channels, exchangers, the electrophoretic uniporter) (Carafoli, 1987).

The endo(sarco) plasmic reticulum is responsible for the fine regulation of intracellular calcium (Carafoli, 1987). Most of the reports on the sarco(endo) plasmic reticulum were performed with the sarcoplasmic reticulum (SR) mainly because the $Ca^{2+}$-ATPase content of the SR is very abundant, representing as much as 90% of the total protein of SR, whereas the content of $Ca^{2+}$-ATPase in the endoplasmic reticulum (ER) is relatively minor (Carafoli, 1987; Penniston, 1983). More recently, the ER has gained considerable attention because of the discovery that its $Ca^{2+}$ pool is sensitive to inositol 1,4,5 trisphosphate ($IP_3$) the mediator in the release of intracellular stores of $Ca^{2+}$ (Carafoli, 1987).

The paucity of information on the ER $Ca^{2+}$-ATPase and the fact that the ER of the liver cell is the first cellular organelle which is disrupted by chemical poisoning (Moore et al., 1976) has prompted, the partial characterization of the membrane bound ER $Ca^{2+}$-ATPase as a prerequisite to the investigation of the role of the ER $Ca^{2+}$-ATPase in DDT-induced liver tumour promotion.

Procedure

Three male Wistar rats weighing 150-200 g were used for the experiment. Rats were housed together in a plastic cage. The animals were maintained in a 12hour light/12 hour dark regime. Temperature was kept at constant $70^{\circ}F \pm 2^{\circ}F$. The relative humidity was $50 \pm 5\%$. All animals were given MRC 41B diet and water ad.lib.

Animals were $CO_2$ exsanguinated. Livers were quickly removed and placed on ice-cold isolation buffer. Each liver sample was separately weighed and used for the preparation of endoplasmic reticulum (light microsomal fraction) as described in section 2.2.1. The protein content of the membrane preparations was determined as described in section 2.3, and the activity of the membrane-bound microsomal $Ca^{2+}$-ATPase determined as described in section 2.4.1.

Assessment of the pH-dependence of the membrane-bound $Ca^{2+}$-ATPase was carried out by using microsomal assay buffers with pH ranging from 6.0 to 8.5. To establish the $Ca^{2+}$-dependence profile, 40 mM $CaCl_2$ was added to the assay medium to final concentrations between 1 and 4 mM $CaCl_2$. The free calcium concentration was estimated by using a computer programme developed by Fabiato and Fabiato (1979). The ATP-dependence of the membrane-bound enzyme was determined by adding 40 mM ATP to the assay medium to final concentrations ranging from 1 to 7 mM ATP. The S.D.S poly acrylamide gel electrophoresis of microsomal proteins was carried out as described in section 2.5. In order to evaluate the responsiveness of the membrane-bound enzyme to calmodulin and Vanadate, the modulators were added to the assay media to a final concentration of 2.0 µg/ml (calmodulin) and .75 µM (vanadate).

Results

The membrane-bound rat liver microsomal $Ca^{2+}$-ATPase has a specific activity of $4.543 \pm 0.857$ µmole Pi/mg Protein/hr. at pH 8.0 (Fig.15). It has a high affinity for $Ca^{2+}$ (Fig. 16), but the membrane-bound enzyme did not seem to obey the Michaelis-Menten Kinetics (Fig. 17). Furthermore, the enzyme in unfractionated microsomes was not sensitive to calmodulin but slightly sensitive to vanadate (Table 12).

The S.D.S. PAG electrophoresis of microsomal protein and the densitometric scan of the resolved polypeptide revealed the presence of 19 polypeptide (Plate 1,Table 13) with molecular weights ranging between 14,000 and 100,000 dalton.

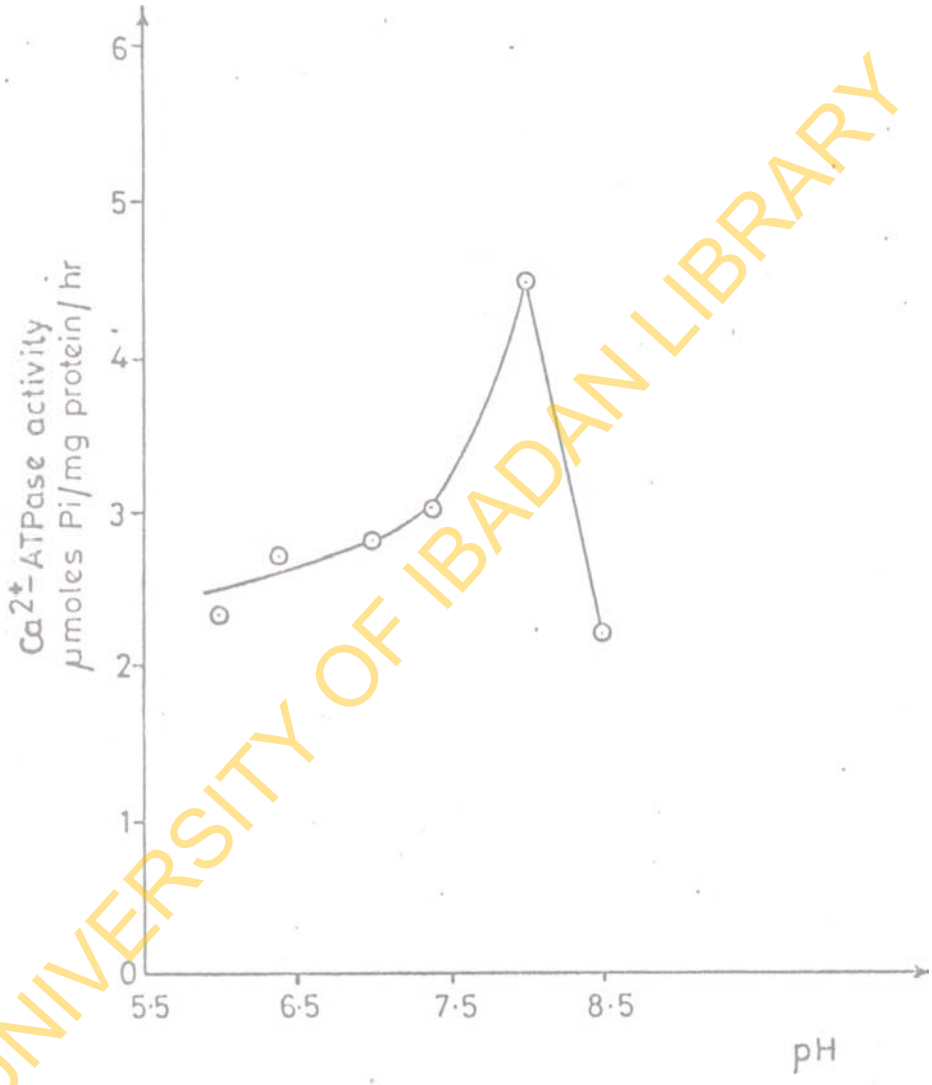





FIG. 15 pH-dependence of the activity of rat liver membrane-bound microsomal $Ca^{2+}$-ATPase

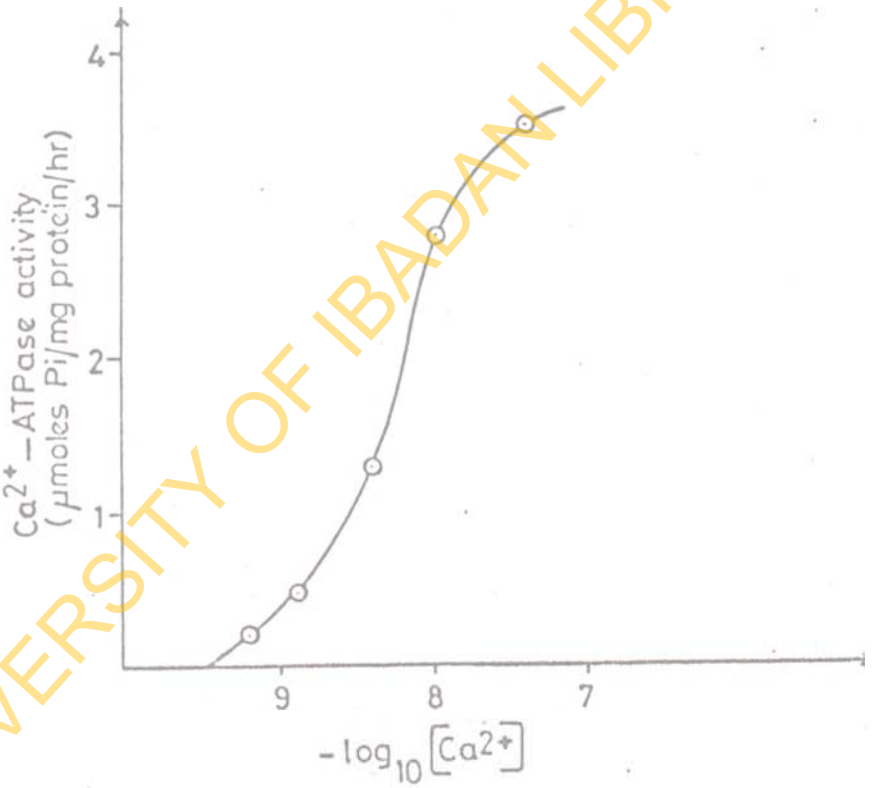





FIG. 16 $Ca^{2+}$-dependence of the activity of rat liver membrane-bound microsomal $Ca^{2+}$-ATPase

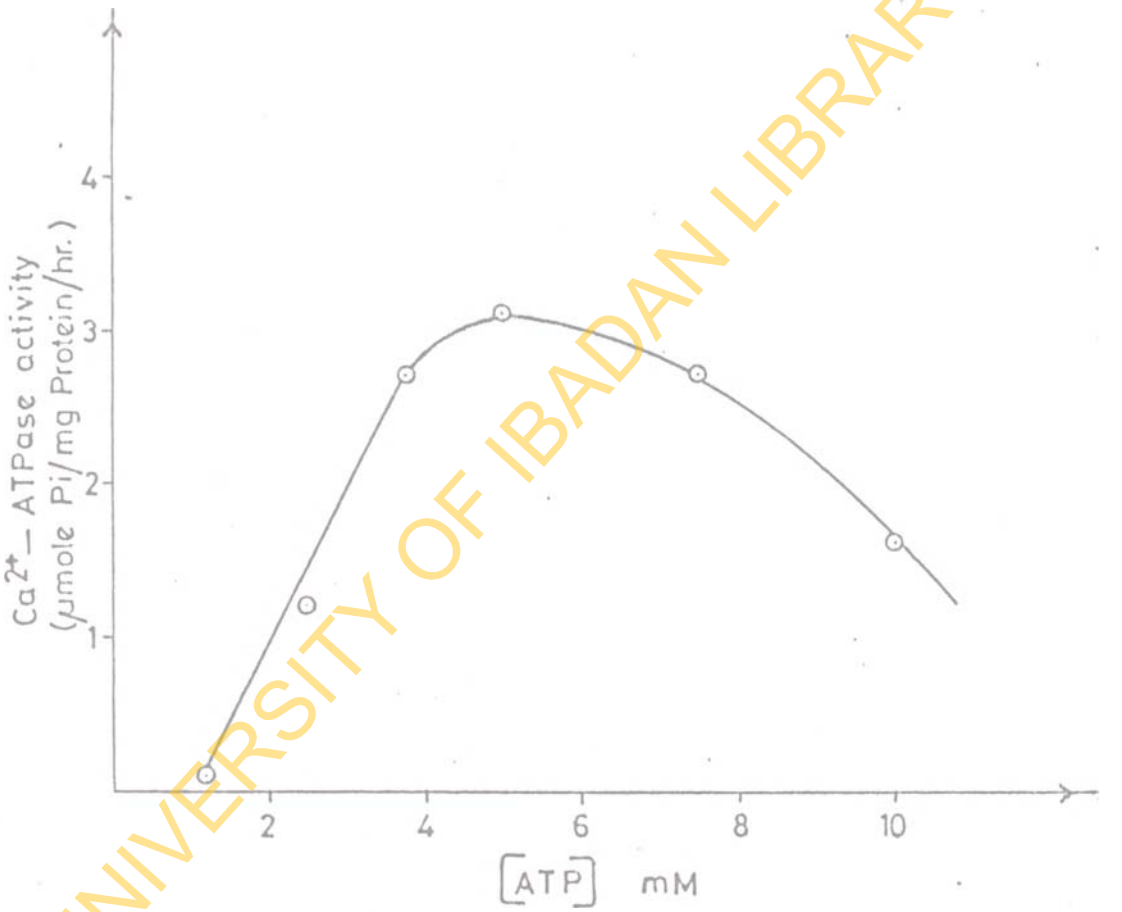





FIG. 17 ATP-dependence of the activity of rat liver membrane-bound microsomal $Ca^{2+}$-ATPase

Table 12: Effects of calmodulin and vanadate on the membrane-bound rat liver microsomal $Ca^{2+}$-ATPase.

| | $Ca^{2+}$-ATPase activity (µmole $P_i$/mg Protein/hr) |
|---|---|
| Control (no additions) | 2.502 ± 0.134 |
| 2 µg/ml calmodulin | 2.415 ± 0.078 |
| 0.75 µM vanadate | 2.030 ± 0.632 |

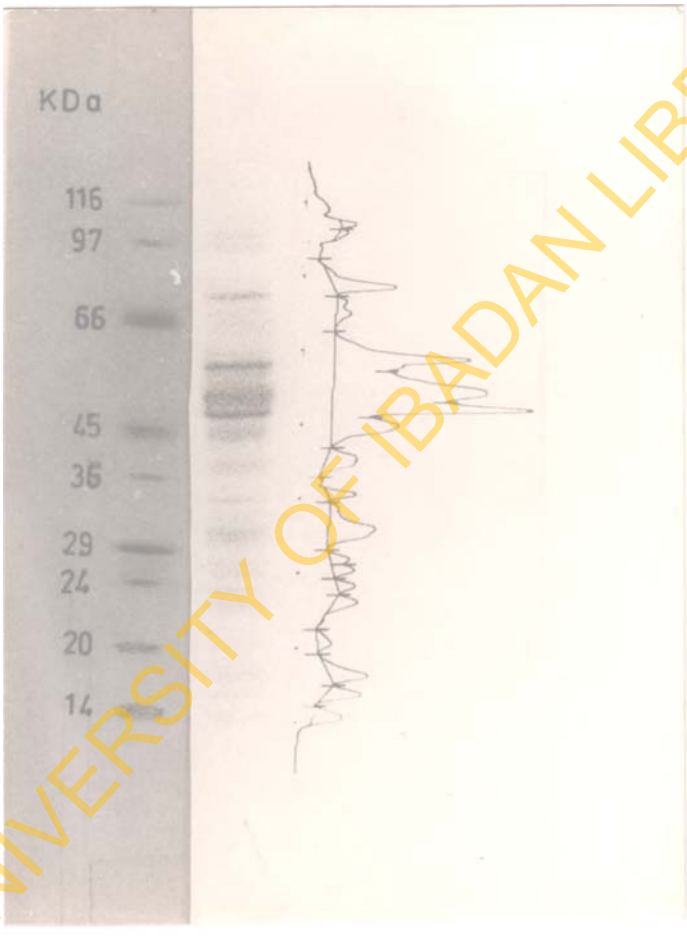





Plate 1 SDS-PAGE and densitometric scan of the microsomal proteins of normal rat.

Table 13: The percentage composition of the microsomal polypeptides of a normal rat as resolved by SDS-Page and the densitometric scanning (Plate1)

| Bands | % Composition |
|---|---|
| 1 | 0.59 |
| 2 | 0.58 |
| 3 | 4.79 |
| 4 | 1.99 |
| 5 | 14.82 |
| 6 | 25.10 |
| 7 | 14.82 |
| 8 | 8.72 |
| 9 | 3.78 |
| 10 | 1.56 |
| 11 | 7.46 |
| 12 | 1.22 |
| 13 | 1.51 |
| 14 | 1.16 |
| 15 | 2.00 |
| 16 | 1.24 |
| 17 | 3.71 |
| 18 | 2.43 |
| 19 | 2.41 |

Experiment 2: Short-term in-vivo studies of the effect of dicophane on the activity of rat liver microsomal membrane-bound $Ca^{2+}$-ATPase.

Introduction

Dicophane (DDT) is a well known liver tumour promoter (Klaunig et al, 1990; Williams, 1983; Williams et al., 1981; Kitchin and Brown, 1987). It has been shown to enhance the effect of 2-acetylaminofluorene, diethylnitrosamine, and 3'-methyl-4-(dimethylamino)-azobenzene in inducing liver tumours in rats and mice when given after these carcinogens (Kitagawa et al., 1984; Nishizumi, 1979; Peraino et al., 1975; Williams and Numoto, 1984). Although the use of this chemical is curtailed or banned in the developed countries, they are still widely used in the developing countries of Africa and Asia (Corbett,1974; WHO/UNEP, 1988; Rojanapo et al., 1988). This is a matter of concern as primary liver cancer (PLC) is one of the most prevalent forms of cancer in Africa and Asia (Parkin et al., 1988).

The mechanism of action of liver tumour promoters is poorly understood. Nevertheless, an inhibition of intercellular communication has been advocated as a mechanism of the action of liver tumour promoters (Klaunig et al., 1990; Williams, 1983; Williams et al., 1981); but it has long been recognized that gap junctional intercellular communication is a function of cytoplasmic calcium concentration. Gap junctional intercellular communication decreases as cytoplasmic calcium concentration increases (Loewenstein, 1979). This observation suggests that the process of intercellular communication is perhaps a secondary event in the action of liver tumour promoters. A rapid and transient increase in cytosolic free $Ca^{2+}$ is part of the first response of cells to mitogens including growth factors and tumour promoters (Berridge, 1987).

The endo(sarco) plasmic reticulum is the most important structure in the fine regulation of intracellular $Ca^{2+}$ concentration (Carafoli, 1987). Endoplasmic reticulum (ER), the intracellular $Ca^{2+}$ store, accumulates $Ca^{2+}$ from the ambient through the ER $Ca^{2+}$-ATPase. Recently, it was reported that thapsigargin-a skin tumour promoter-inhibits the ER-$Ca^{2+}$-ATPase (Thastrup, et al, 1990) and that the inhibition is accompanied with $Ca^{2+}$ mobilization.

Since the tumour promoter-induced inhibition of ER $Ca^{2+}$-ATPase is accompanied with $Ca^{2+}$ mobilization, it appears that the enzyme might play a crucial role in the process of intercellular communication and the mechanism of action of tumour promoters. The aim of the

experiment was to determine the effect of the specific liver tumour promoter,dicophane on the liver ER $Ca^{2+}$-ATPase.

Procedure

Male Wistar rats weighing 150-200 g were used for the experiment. Rats were housed 3 per cage in plastic cages. The animals were maintained in a 12 hour light/12 hour dark regime. Temperature was kept at constant $70^{\circ}F \pm 2^{\circ}F$. The relative humidity was $50 \pm 5\%$. All animals were given MRC 41B diet and water ad.libitum. The experiment was performed in three parts:

Part one was designed to investigate the effect of the liver tumour promoter, dicophane (DDT). To accomplish this, six animals were divided into 2 groups A and B of 3 animals each. Group A (control) were given a single dose of subcutaneous injection of Olive oil and group B (DDT-treated) were given a single dose of subcutaneous injection of 2 µl/g.body wt. of 37.5 mg/ml of DDT in olive oil, corresponding to 75 mg/kg.body wt. of DDT. Animals were fasted overnight on day seven and $CO_2$ exsanguinated on day eight. The dosage is as reported by McLean and McLean (1966).

Part two investigated the effect of another liver tumour promoter, phenobarbital (PB). For this investigation, six animals were also divided into 2 groups A and B of 3 animals each. Group A (control) were injected with 0.1 M NaOH (pH 9.0) intraperitoneally and group B (PB-treated) were injected with 4 µl/g.body wt. of 20 mg/ml of phenobarbital dissolved in 0.1 M NaOH pH 9.0, corresponding to 80 mg/kg.body wt. of phenobarbital once everyday for 4 days. Animals were fasted overnight on day four and $CO_2$ exsanguinated on day five.

Part three was set up to determine the effect of the carcinogenic initiator aflatoxin $B_1$. To do this, six animals were also divided into 2 groups A and B of 3 animals each. Group A (control) were injected with a single dose of 2 µl/g.body wt. of 1 %(v/v) of DMSO intraperitoneally and group B (aflatoxin $B_1$-treated) were injected with a single dose of 2 µl/g.body wt. of 0.36 mg/ml of aflatoxin $B_1$ (dissolved in DMSO) intraperitoneally. Animals were fasted overnight on day six and $CO_2$ exsanguinated on day seven.

Livers of animals were quickly removed after they were sacrificed and placed on ice-cold isolation buffer. Each liver was separately weighed and used for the preparation of endoplas-

mic reticulum (light microsomal fraction) as described in section 2.2.1. The protein content of the microsome preparations was determined as described in section 2.3, and the activity of the membrane-bound microsomal $Ca^{2+}$-ATPase determined as described in section 2.4.1 The S.D.S-PAG electrophoresis and the densitometric scan of the resolved microsomal proteins were carried out as described in section 2.5.

Results

After eight days of the administration of DDT to rats, significant increases ($P < 0.05$) in the relative liver weight (liver weight/body weight) and in the cytochrome $P_{450}$ content were observed for DDT-treated rats compared with control animals (Table 14a). However, the microsomal $Ca^{2+}$-ATPase activity was significantly lower ($P < 0.01$) while an insignificant decrease ($P < 0.05$) in liver GGT activity was observed.

Also, after four days of the administration of phenobarbital to rats, significant increases ($P < 0.05$) were observed in the relative liver weight (liver wt./body wt.) and in the cytochrome $P_{450}$ content (Table 14b). A significant decrease ($P < 0.05$) in the $Ca^{2+}$-ATPase activity was also observed; and the liver GGT activity was significantly increased ($P < 0.05$).

The values obtained for aflatoxin B$_1$-treated rats including the relative liver weight (liver wt./body wt.) cytochrome $P_{450}$ content, and microsomal $Ca^{2+}$-ATPase activity were not significantly different ($P > 0.05$) from the values obtained for control animals (Table 14c).

Although there were changes in the specific activity of the microsomal $Ca^{2+}$-ATPase of promoter-treated rats, the affinity of the enzyme for calcium remained unchanged (Fig. 18). Comparison of the S.D.S. PAG electrophoretic pattern and the densitometric scan of the resolved polypeptide (Plate 2, Table 15) of microsomal proteins of normal, DDT-treated and PB-treated rats showed changes in the relative content of some of the microsomal proteins

Table 14(a-c): Effects of (a) dicophane (DDT), (b) phenobarbital (PB) and (c) aflatoxin-B (AFB ) on the membrane-bound rat liver microsomal $Ca^{2+}$-ATPase activity and other biochemical parameters.

(a)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| control (n =3) | 0.0350 ±0.0013 | 1.256 ±0.162 | 25.160 ±5.550 | 3.290 ±0.754 |
| DDT-treated (n =3) | 0.0451* ±0.0007 | 1.947* ±0.281 | 8.747 ±0.552* | 1.699** ±0.189 |

(b)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| control (n =3) | 0.0388 ±0.0030 | 0.937 ±0.090 | 13.200 ±2.750 | 3.214 ±0.470 |
| PB-treated (n =3) | 0.0489* ±0.0013 | 2.745** ±0.214 | 25.015* ±0.340 | 1.261** ±0.071 |

(c)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| control (n =3) | 0.0387 ±0.0018 | 0.414 ±0.017 | - - | 3.260 ±0.913 |
| AFB -treated (n =3) | 0.0356 ±0.0061 | 0.416 ±0.077 | - | 3.492 ±0.644 |

The value in parenthesis represents the number of animals and microsome preparations.

*p < 0.05 ** P < 0.01

n - no of membrane preparations or animals.

\+ - nmole Cyt. $P_{450}$/mg protein

$ - nmole AMC/g liver/min.

N = μmole Pi/mg protein/hr.

Relative Liver wt. is the ratio of Liver weight to body weight.

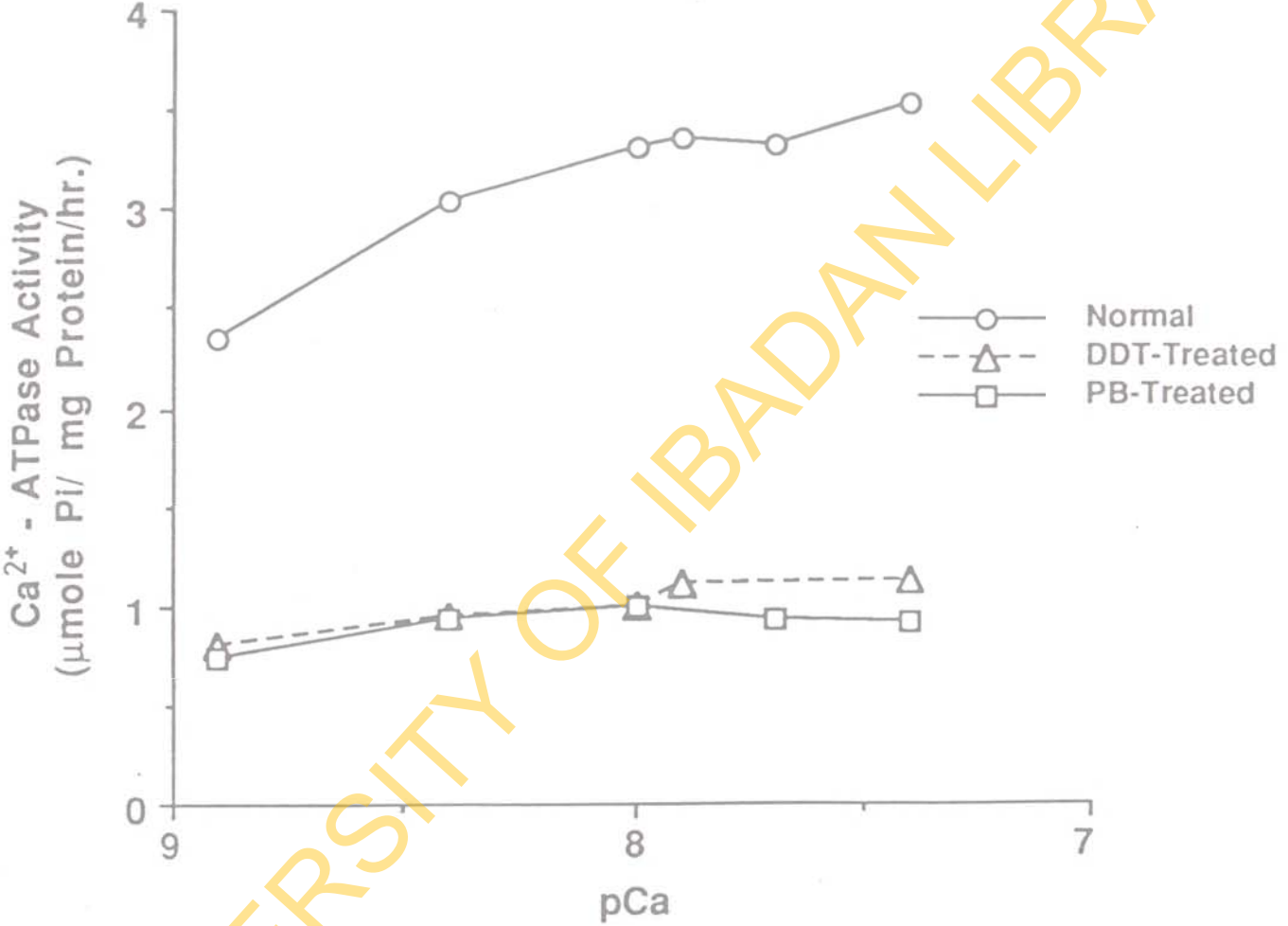



FIG.18 $Ca^{2+}$-dependence of the activity of membrane-bound microsomal $Ca^{2+}$-ATPase. of Normal, DDT- and PB-treated rats

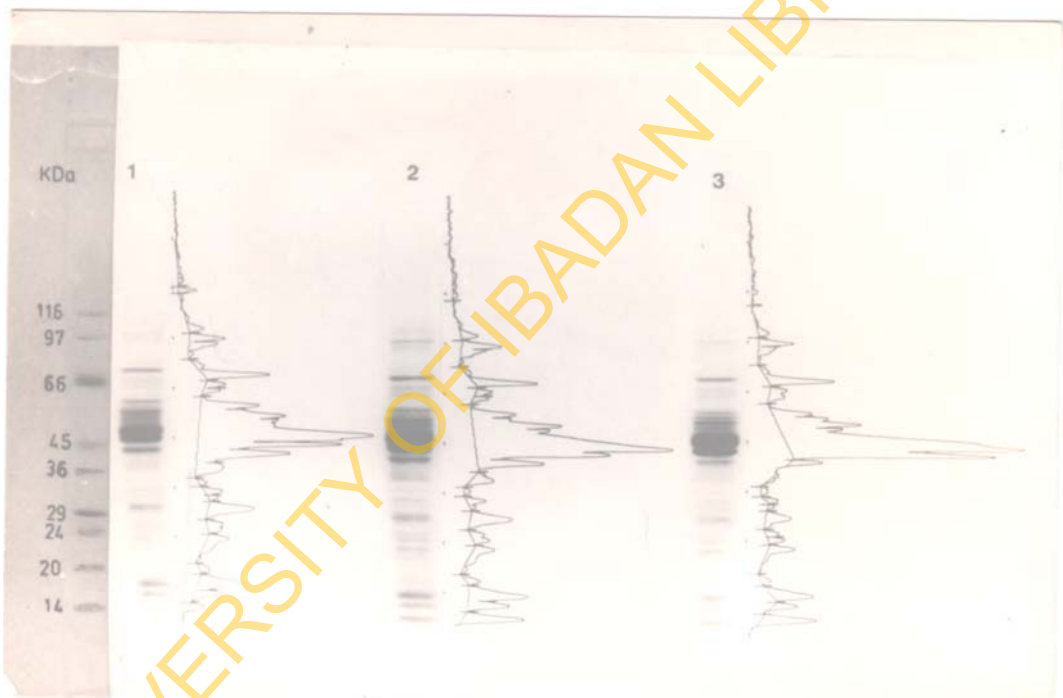





Plate 2. SDS-PAGE and densitometric scan of the microsomal proteins of (1) normal, (2) DDT- and (3) PB-treated rats

Table 15: The percentage composition of the microsomal polypeptides of normal (1), DDT-(2) and PB(3)- treated rats as resolved by SDS-PAGE and the densitometric scanning (Plate 2)

| Bands | % Composition | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 1 | 0.05 | | |
| 2 | 1.41 | | |
| 3 | 0.67 | 1.09 | 1.17 |
| 4 | 1.52 | 1.37 | 0.96 |
| 5 | 1.95 | 2.25 | 1.77 |
| 6 | 2.30 | 2.01 | 1.83 |
| 7 | 4.62 | 3.95 | 4.64 |
| 8 | 1.94 | 0.91 | |
| | | | ) 2.21 |
| 9 | 0.99 | 0.93 | |
| 10 | 4.11 | 3.00 | 2.57 |
| 11 | 7.57 | 7.56 | 8.37 |
| 12 | 32.00 | 33.12 | 41.18 |
| 13 | 8.47 | 10.21 | 13.82 |
| 14 | 8.44 | 4.63 | |
| | | | ) 1.95 |
| 15 | 3.65 | 2.14 | |
| 16 | 0.46 | 0.39 | 0.56 |
| 17 | 1.18 | 0.68 | |
| | ) 1.38 | | |
| 18 | 0.55 | | 0.32 |
| 19 | 4.97 | 4.67 | 2.91 |
| | | | 0.78 |
| 20 | 1.94 | 1.91 ( | |
| | | | 1.01 |
| 21 | 3.06 | 3.00 | 2.34 |
| 22 | 0.82 | 1.07 | 0.73 |
| 23 | 4.78 | 6.05 | 4.17 |
| 24 | 2.30 | 3.47 | 1.47 |
| 25 | 0.96 | 4.79 | 4.48 |

Experiment 3: Investigation of the action of low protein intake on the activity of rat liver microsomal membrane-bound $Ca^{2+}$-ATPase.

Introduction

A number of studies have shown that the development of cancer can be modified either by changing the quality or the quantity of dietary protein. This has been unequivocally demonstrated for tumours of the liver (Tannenbaum and Silverstone, 1953; Rizvi, et al., 1987; Schulsinger, et al., 1989; Mathur and Nayak, 1989). However, the reports of actual experimental studies are conflicting. While some scientists (Rizvi et al., 1987; Mathur and Nayak, 1989) suggest that protein malnutrition could enhance or promote tumour development, some others (Madhavan and Gopalan, 1968; Schulsinger et al., 1989) have reported an inhibitory effect (Rizvi et al., 1987).

Many tumour promoters are almost exclusively membrane active agents (Troll and Weisner, 1985), and low protein intake has been shown to have a pronounced effect on the structural integrity of biomembranes (Coward, 1971). This similarity suggests that low protein diet might act as a tumour promoter.

This experiment was designed to test the hypothesis that low protein intake acts as a liver tumour promoter using the inhibition of liver ER $Ca^{2+}$-ATPase as a mechanistic model.

Procedure

Male weanling Fisher F344 rats were used for the experiment. Animals were divided into two groups A and B of four and seven animals. Group A were given normal (MRC 41B) diet and water ad. libitum while group B were given low protein diet (5 % protein) and water ad. libitum for twelve weeks. All animals were housed in plastic cages and were maintained in a 12 hour light/12 hour dark regime. Temperature was kept at constant $70^{\circ}F \pm 2^{\circ}F$. The relative humidity was $50 \pm 5$ %. Animals were fasted overnight and $CO_2$ exsanguinated after twelve weeks of feeding.

Liver of animals were quickly removed after they were sacrificed and placed on ice-cold isolation buffer. Each liver was separately weighed and used for the preparation of endoplasmic reticulum (light microsomal fraction) as described in Section 2.2.1. The protein content of the microsome preparations was estimated as described in Section 2.3, and the activity of the

microsomal $Ca^{2+}$-ATPase determined as contained in Section 2.4.1. The S.D.S, PAG electrophoresis was carried out as described in Section 2.5.

Results

There was no significant difference ($P > 0.05$) in the relative liver weight (liver weight/ body weight) for protein-malnourished animals compared with well-fed controls (Table 16). The cytochrome $P_{450}$ content and the liver GGT activity also remained normal. A significant depression ($P < 0.05$) in the microsomal $Ca^{2+}$-ATPase activity was, however, observed.

A comparison of the S.D.S. PAG electrophoretic pattern of normal and protein-malnourished animals showed differences in the relative content of some of the polypeptides (Plate 3, Table 17).

Table 16: The $Ca^{2+}$-ATPase activity and other biochemical parameters of normal and protein-malnourished rats.

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| controls (well-fed) (n=4) | 0.0306 ±0.0009 | 0.621 ±0.126 | 22.871 ±17.090 | 3.907 ±1.552 |
| Protein-mal nourished (Low protein diet) (n = 7) | 0.0292 ±0.0012 | 0.729 ±0.093 | 33.015 ±29.201 | 1.226* ±0.268 |

* $P < 0.05$ **$P < 0.01$

\+ nmole cyt. $P_{450}$/mg protein

$ nmole AMC/g liver/min.

N μmole $P_i$/mg protein/hr.

n = no of animals or membrane preparations.

Relative Liver weight is the ratio of Liver weight to Body weight.

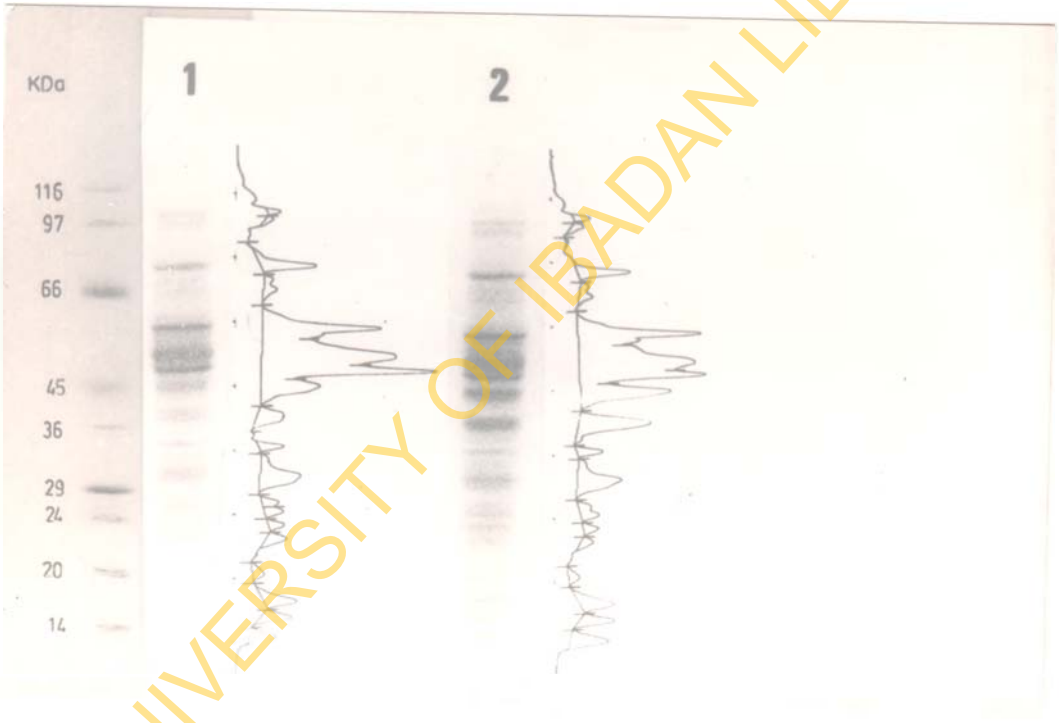





Plate 3. SDS-PAGE and densitometric scan of the microsomal proteins of (1) normal, (2) protein-malnourished animals

Table 17: The percentage composition of the microsomal polypeptides of normal (1) and protein-malnourished (2) rats as resolved by SDS-PAGE and the densitometric scanning (Plate 3).

| Bands | % Composition | |
|---|---|---|
| | 1 | 2 |
| 1 | 0.59 | 0.70 |
| 2 | 0.58 | 0.91 |
| 3 | 4.79 | 3.85 |
| 4 | 1.99 | 1.64 |
| 5 | 14.82 | 14.20 |
| 6 | 25.10 | 22.17 |
| 7 | 14.82 | 10.36 |
| 8 | 8.72 | 11.11 |
| 9 | 3.78 | 9.65 |
| 10 | 1.56 | 1.64 |
| 11 | 7.46 | 7.47 |
| 12 | 1.22 | |
| | | ) 2.29 |
| 13 | 1.51 | |
| 14 | 1.16 | 1.09 |
| 15 | 2.00 | 1.05 |
| 16 | 1.24 | 1.18 |
| 17 | 3.71 | 3.98 |
| 18 | 2.43 | 2.74 |
| 19 | 2.41 | 3.92 |

Experiment 4: Effect of carcinogenic promotion (by dicophane administration) on liver microsomal membrane-bound $Ca^{2+}$-ATPase after carcinogenic initiation (by aflatoxin $B_1$ treatment) in rats.

Introduction

Hepatocellular carcinoma is one of the ten most frequent cancers encountered world-wide, accounting for 4 % of the total. While relatively rare in Europe and the Americas, it is frequent in the People's Republic of China and in Africa (Parkin et al., 1988). The aetiology of this cancer has been associated with two major risk factors, persistent hepatitis B virus (HBV) infection and exposure to dietary aflatoxins, although other aetiological agents, like smoking and some occupational exposures, have also been implicated (Cova et al., 1990).

DDT, the persistent and lipophilic pesticide is widely used in the control of insect vectors of disease in several parts of the world including Africa where the incidence of human primary liver Cancer (PLC) is highest (Bababunmi, 1976; Rojanopo et al.., 1988; Rizvi et al., 1987). Likewise, malnutrition, particularly protein malnutrition is widespread and endemic in developing countries and is universally recognized as the single most important contributor to the high sickness and death rates in childhood in these countries (Hendrickse et al., 1982). It is probable therefore, that dicophane (DDT) and protein malnutrition acting in concert with environmental carcinogens which are also widespread in the developing countries might influence the incidence of hepatocellular carcinoma in Africa and Asia.

Aflatoxin $B_1$ ($AFB_1$), a metabolite of the fungi Aspergillus flavus and Aspergillus paraciticus, is a widespread food contaminant and a potent hepatocarcinogen for several species of animals including primates (Busby and Wogan, 1981). Epidemiological studies from some developing countries suggest a relationship between ingestion of $AFB_1$-contaminated food and increased frequency of human liver cancer (Bababunmi, 1976; Alpert et al; 1971; Shank et al., 1972; Peers and Linsell, 1973).

This experiment examined the effect of carcinogenic promotion on liver microsomal membrane-bound $Ca^{2+}$-ATPase.

Procedure

This experiment was performed in two parts. Part one investigated the long-term effect of the administration of promoter only to rats and also the effect of the treatment after carcinogenic initiation with aflatoxin $B_1$ on the microsomal $Ca^{2+}$-ATPase. Seventeen eight weeks

old Fisher F344 rats divided into four groups A, B, C and D of four, four, five and four animals respectively were used for the investigation. Animals were treated as described in Fig. 19.

Groups A and B were given 2 μl/g.body wt. of 6.25 % (v/v) of DMSO in oil and groups C and D were given 2 μl/g. body wt. of 0.125 mg/ml of aflatoxin $B_1$ (dissolved in DMSO) in oil corresponding to 0.250 mg/kg. body wt. orally for 10 days (5 days per week for 2 weeks). During promotion, group A and D were given a single subcutaneous injection of 2 μl/g. body wt. of oil and groups B and C were given a single subcutaneous injection of 2 μl/g. body wt. of 37.5 mg/ml of DDT in oil corresponding to 75 mg/kg.body wt. of DDT. All animals were given normal (MRC 41B) diet and water ad.libitum for twelve weeks.

Part two investigated the effect of aflatoxin $B_1$ ingestion on the microsomal $Ca^{2+}$-ATPase of animals on low protein diet i.e. protein-malnourished animals. This procedure was adopted because it is more physiological than the pretreatment of animals with aflatoxin $B_1$ followed by placement on low protein diet. Weanling Fisher F344 rats divided into 2 group A and B of seven and nine animals were used for the investigation. All animals were given low protein diet (5 % protein) and water ad. libitum for twelve weeks. After one week of feeding on low protein diet, group A were given 2 μl/g.body wt. of 6.25 % (v/v) of DMSO in oil and group B were given 2 μl/g.body wt. of 0.125 mg/ml of aflatoxin $B_1$ (dissolved in DMSO) in oil orally for 10 days (5 days per week for 2 weeks); corresponding to 0.250 mg/kg.body wt. of aflatoxin $B_1$. The procedure is depicted in Fig.20.

After twelve weeks animals were $CO_2$ exsanguinated. Livers were quickly removed after animals were sacrificed and placed on ice-cold isolation buffer. Each liver was separately weighed and used for the preparation of endoplasmic reticulum (light microsomal fraction) as described in section 2.2.1. The protein content of the microsome preparations was determined as described in Section 2.3, and the activity of the membrane-bound microsomal $Ca^{2+}$-ATPase determined as contained in section 2.4.1. The S.D.S. PAG electrophoresis was carried out as described in Section 2.5.

Results

After four weeks of the administration of DDT to rats, there were no differences in the Cytochrome $P_{450}$ content and the liver GGT activity; but the relative liver weight (liver wt./ body wt.) was significantly different ($P < 0.05$) from the value obtained for control animals. There was also an insignificant decrease in the microsomal $Ca^{2+}$-ATPase activity (Table 18a).


Fig.19 Schematic diagram of the carcinogenic treatment protocol for animals on normal diet

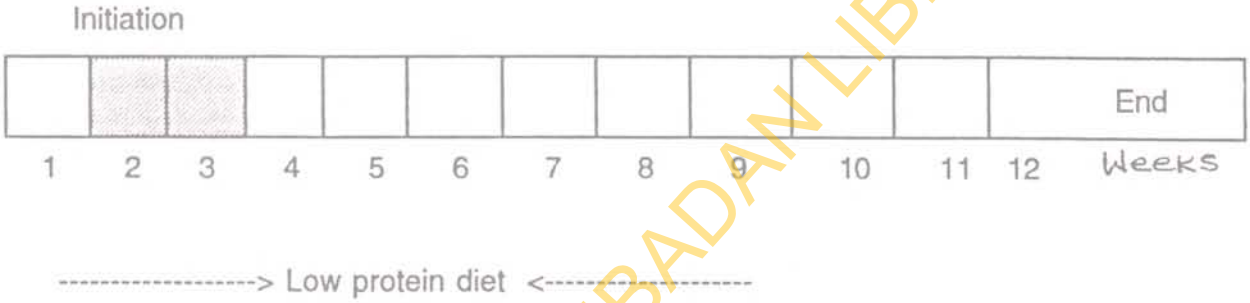



Fig. 20 Schematic diagram of the carcinogenic treatment protocol for animals on low protein diet

The relative liver weight, $Ca^{2+}$-ATPase activity and Cytochrome $P_{450}$ content of DDT-treated animals were not different from those of DDT-treated animals after carcinogenic initiation with aflatoxin $B_1$ (Table 18b). The liver GGT activity was, however, significantly increased ($P < 0.05$).

There were observed increases ($P < 0.05$) in the relative liver weight as well as the $Ca^{2+}$-ATPase activity of protein-malnourished animals treated with aflatoxin $B_1$ compared with protein-malnourished controls; but the cytochrome $P_{450}$ content and the liver GGT activity were not different ($P > 0.05$) from the values obtained for control animals (Table 18c).

Aflatoxin $B_1$ treatment alone (carcinogenic initiation) had no effect on all measured biochemical parameters after nine weeks (Table 18d).

Table 18 (a-d): Long-term study on the effect of dicophane (DDT) administration after carcinogenic initiation with aflatoxin $B_1$ on the rat liver membrane-bound $Ca^{2+}$-ATPase.

(a)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity per g liver/ | $Ca^{2+}$-ATPase activity per mg protein/liver |
|---|---|---|---|---|
| control (n=4) | 0.0302 ±0.0009 | 0.794 ±0.134 | 7.900 ±3.030 | 4.560 ±1.540 |
| DDT-treated (n=4) | 0.0327* ±0.0009 | 1.107 ±0.219 | 7.080 ±2.830 | 2.654 ±0.677 |

(b)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| DDT-treated (n=4) | 0.0327 ±0.0009 | 1.107 ±0.219 | 7.080 ±2.830 | 2.654 ±0.677 |
| $AFB_1$ +DDT (n=5) | 0.0340 ±0.0026 | 1.245 ±0.089 | 13.550* ±4.180 | 2.930 ±1.470 |

(c)

| | Relative Liver wt. | Cytochrome $P_{450}$ content | Liver GGT activity | $Ca^{2+}$-ATPase activity |
|---|---|---|---|---|
| Protein-malnourished (n=7) | 0.0292 ±0.0013 | 0.729 ±0.093 | 33.015 ±29.201 | 1.226 ±0.268 |
| Protein-malnourished and $AFB_1$-treated (n = 9) | 0.0333* ±0.0021 | 0.653 ±0.094 | 19.772 ±13.652 | 1.886* ±0.677 |

(d)

| | Relative Liver wt. | Cytochrome$^{+}$ $P_{450}$ content | Liver GGT$^{\$}$ activity | $Ca^{2+}$-ATPase$^{N}$ = activity |
|---|---|---|---|---|
| Control (n=4) | 0.0305 ±0.0015 | 0.735 ±0.121 | 6.630 ±2.410 | 3.519 ±0.564 |
| AFB$_1$-treated (n=4) | 0.0314 ±0.0009 | 0.798 ±0.076 | 10.590 ± 2.730 | 3.214 ±0.470 |

\+ nmole Cyt. $P_{450}$ /mg protein

$ nmole AMC/g liver/min.

N mole P/mg protein/hr.

* $P < 0.05$

n = no of animals or microsome preparations.

Relative Liver weight is the ratio of Liver weight to body weight.

The S.D.S. PAG electrophoresis of microsomal proteins and the densitometric scan of the resolved polypeptides (Plate 4, Table 19) of protein malnourished animals and their counterparts (protein-malnourished animals after carcinogenic initiation with aflatoxin $B_1$) showed differences in the relative content of some of the proteins.

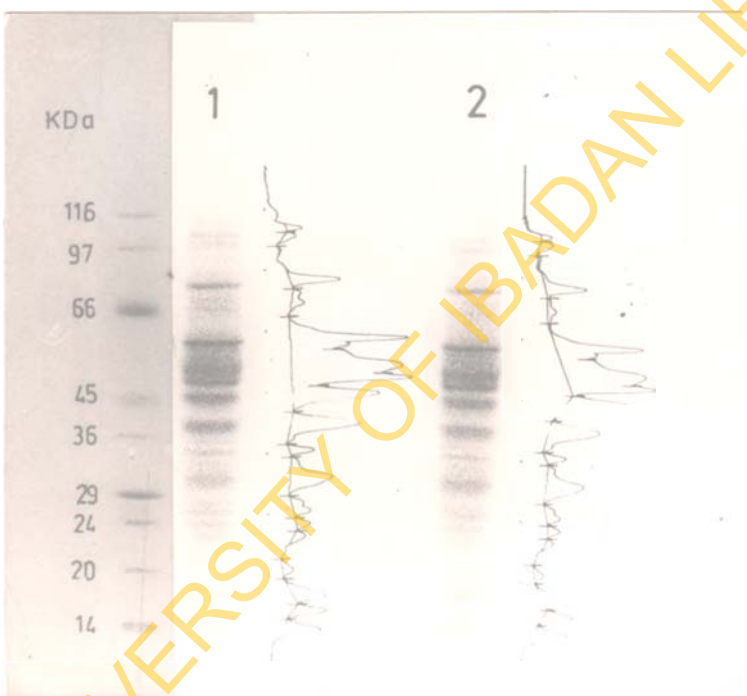





Plate 4 SDS-PAGE and densitometric scan of the microsomal protein of animals on low protein diet (1) without and (2) with aflatoxin $B_1$ treatment

Table 19: The percentage composition of the microsomal polypeptides of protein-malnourished (1) and protein-malnourished rats treated with aflatoxin $B_1$ (2) as resolved by SDS-PAGE and the densitometric scanning (Plate 4)

| Bands | % Composition | |
|---|---|---|
| | 1 | 2 |
| 1 | 0.70 | 0.44 |
| 2 | 0.91 | 1.20 |
| 3 | 3.85 | 4.04 |
| 4 | 1.64 | 0.90 |
| 5 | 14.20 | 12.56 |
| 6 | 22.17 | 21.23 |
| 7 | 10.36 | 8.48 |
| 8 | 11.11 | 9.38 |
| 9 | 9.65 | 10.31 |
| 10 | 1.64 | 1.89 |
| 11 | 7.47 | 9.98 |
| 12 | 2.29 | 2.49 |
| 13 | 1.09 | 1.24 |
| 14 | 1.05 | 0.89 |
| 15 | 1.18 | 1.34 |
| 16 | 3.98 | 5.06 |
| 17 | 2.74 | 3.57 |
| 18 | 3.92 | 4.91 |

Experiment 5: An assessment of the activity of erythrocyte membrane-bound $Ca^{2+}$-ATPase in humans suffering from protein-energy-malnutrition and liver cancer.

Introduction

There is abundant evidence to suggest that an inhibition of the liver endoplasmic reticulum (ER) $Ca^{2+}$-ATPase could be a useful marker of the action of liver tumour promoters (Lowrey et al., 1981; Recknagel et al., 1982; Long and Moore, 1986; Thastrup et al., 1990; Adenuga et al; 1992). However, the limitation of using human liver for diagnostic purposes might hamper the extrapolation of the result to man.

One way to circumvent this problem, is to examine the erythrocyte ghost membrane (EGM) plasma membrane $Ca^{2+}$-ATPase, since blood is more readily available. Furthermore, protein kinase C - the well known receptor of tumour promoters has been shown to interact with and modulate the activity of the EGM $Ca^{2+}$-ATPase (Smallwood et al, 1988). This preliminary investigation was conducted to compare the erythrocyte ghost membrane (EGM) $Ca^{2+}$-ATPase activity of normal humans with those suffering from liver cancer. Furthermore, the $Ca^{2+}$-ATPase activity of kwashiorkor humans was compared with that of their normal counterparts. Kwashiorkor (protein-energy-malnutrition) has been shown to be a positive modulator of hepatocarcinogenesis (Rizvi et al., 1987; Mathur and Nayak, 1989). It has been shown that the action of tumour promoters is accompanied with an inhibition of the plasma membrane $Ca^{2+}$-ATPase (Cerutti et al., 1989; Parola et al., 1990; Lowrey et al., 1981a, 1981b).

Procedure

Blood samples were obtained from patients who were newly identified as having Kwashiorkor (by the paediatric Department) or liver cancer (by the Department of Medicine) of the University College Hospital, Ibadan. The patients were not receiving any dietary therapy or medication at the time of collection of blood. Normal human blood samples were collected from healthy donors in the same age group as the kwashiorkor and liver cancer patients. All samples were collected in acid-citrate-dextrose buffer and stored at $4^{\circ}c$ before they were used for membrane preparation.

Erythrocyte ghost membranes (EGM) were prepared as described in section 2.2.2, and the membrane protein determined as described under Section 2.3. The membrane-bound

$Ca^{2+}$-ATPase activity was measured as described in Section 2.4.2.

Results

The specific activity of the erythrocyte ghost membrane (EGM) $Ca^{2+}$-ATPase of liver cancer patients is not significantly different ($P > 0.05$) from values obtained for adult controls (Table 20). Also, the specific activity of the enzyme in the EGM of kwashiorkor patients although higher, is not significantly different ($P > 0.05$) from values obtained for paediatric controls. However, the enzyme in the EGM of both liver and kwashiorkor patients were less sensitive to the stimulatory effect of calmodulin compared to controls (Fig.21) but these values also did not reach statistical significance ($P > 0.05$).

Table 20: The erythrocyte ghost membrane $Ca^{2+}$-ATPase activity of kwashiorkor and liver cancer patients.

| | $Ca^{2+}$-ATPase activity | |
|---|---|---|
| | - Calmodulin | + Calmodulin |
| Adult Control (5) | 0.189 ± 0.067 | 0.370 ± 0.169 |
| Liver Cancer (5) | 0.202 ± 0.055 | 0.365 ± 0.112 |
| Paediatric Control (6) | 0.092 ± 0.047 | 0.177 ± 0.098 |
| Kwashiorkor (8) | 0.120 ± 0.037 | 0.180 ± 0.064 |

The number in parenthesis represents the number of membrane preparations using different blood samples.

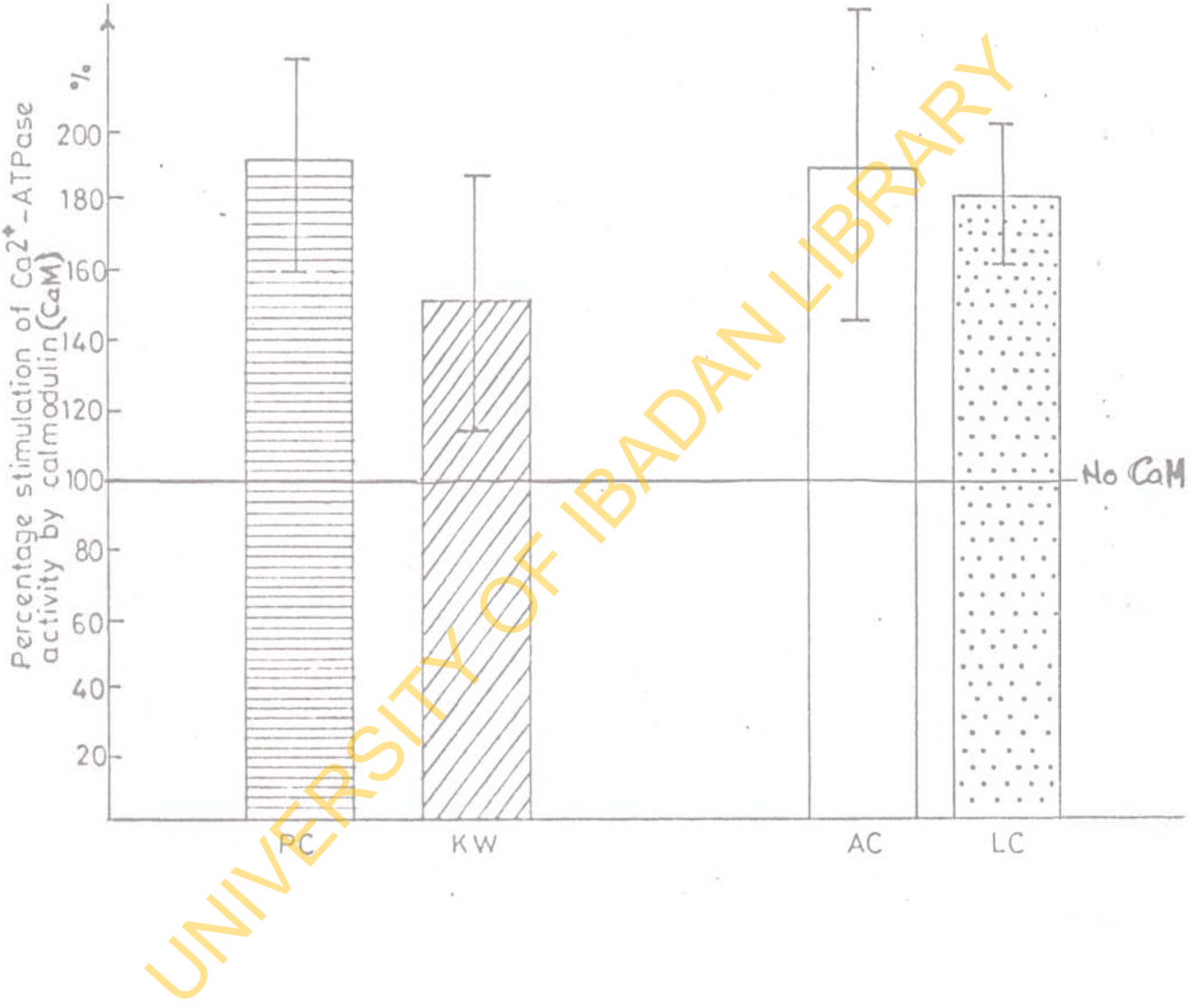



FIG. 21 The responsiveness of the erythrocyte ghost membrane $Ca^{2+}$-ATPase of control, kwashiorkor and liver cancer patients to calmodulin

PC - Paediatric Control
KW - Kwashiorkor
AC - Adult Control
LC - Liver Cancer

# CHAPTER FOUR

## DISCUSSION

The majority (70-90%) of human cancers have been associated with environmental causes. Actually, a number of food additives, pesticides, insecticides, and industrial chemicals introduced commercially during the last 50 years have exhibited carcinogenic properties in animal models. Historically, chemical exposure due to occupation or to drugs has led to human cancers (Weisburger and Horn, 1982).

The liver has received considerable attention as a target for chemical carcinogenesis since the discovery of liver cancer induction with o-amino-azotoluene (Farber, 1980). A variety of chemicals of diverse structure, radiations and two viruses have been implicated in the genesis of experimental liver cancer in several species and various chemicals including hormones and possibly one virus, hepatitis B, in humans (Farber, 1980). Because of its susceptibility to cancer induction under a variety of conditions and because of a large and growing body of knowledge about its cellular and molecular biology andpathology, the liver has been examined many times with many carcinogens for biochemical, physiological and morphological alterations as a function of time during development of cancer. Such studies have consistently found many cellular and tissue changes involving hepatocytes and other types of cells in the liver before the development of unequivocal hepatocellular carcinoma, the commonest form of liver cancer (Farber, 1980). Indeed, primary liver cancer (PLC) is one of the most prevalent forms of cancer in Africa and Asia (Parkin et al., 1988).

Currently, attention is being focused on the mechanisms of action of tumour promoters. Skin is the classical target organ of experimental multi-stage tumourigenesis and the tumour promoter TPA(12-O-tetradecanoyl-phorbol-13-acetate) is the classical skin tumour

promoter. Three biochemical mechanisms have been proposed to explain the action of some skin tumour promoters.

(i) Stimulation of the signal-transduction enzyme protein kinase C ( Castagna et al., 1982).

(ii) Inhibition of several protein phosphatases (Bialojan and Takai, 1988).

(iii) Inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase (Thastrup et al., 1990).

The identification of these biochemical mechanisms for the action of skin tumour promoters calls attention to the importance of understanding the cellular mechanisms of action of other groups of tumour promoters; particularly liver tumour promoters. It has been well documented though, that a rapid and transient increase in cytosolic free calcium is part of the first response of cells to mitogens including growth factors and tumour promoters (Berridge, 1987). This observation suggests that the calcium regulatory system plays a crucial role in the process of tumour promotion.

The endo (sarco) plasmic reticulum is the membrane system responsible for the fine regulation of calcium in the cytosol. It contains an ATPase which has high affinity for calcium. Although there is a wealth of information on this membrane system and its enzyme, most of the work on the membrane system has been traditionally carried out on the microsomal fraction of muscle cells (sarcoplasmic reticulum); and only very little information is available on the ATPase of the microsomal fraction of non-muscle cells (endoplasmic reticulum) (Carafoli, 1987; 1988 a,b; Penniston, 1983). As a prerequisite to this investigation on the role of the ER (microsomal) $Ca^{2+}$-ATPase in carcinogenic promotion therefore, a partial characterization of the membrane-bound (native) enzyme was first conducted.

Most of the results obtained in this study on the characterization of the native microsomal $Ca^{2+}$-pump are in agreement with earlier reports. It was observed that the native microsomal $Ca^{2+}$-ATPase is a high affinity enzyme (Fig.16) as has been reported by other

workers (Moore and Kraus-Friedmann, 1983; Famulski and Carafoli, 1982). This result confirms the assumption that most of the properties of the ER $Ca^{2+}$-ATPase repeat those of the Sarcoplasmic reticulum (SR) ATPase (Carafoli, 1987). Also, the native enzyme is not activated by added calmodulin (Table 12). This result is in agreement with the report of Moore and KrausFriedmann (1983) that the native microsomal $Ca^{2+}$-ATPase contains tightly bound calmodulin; but that the partial removal of the calmodulin by EGTA treatment results in an increased $^{45}Ca^{2+}$ uptake by added exogenous calmodulin and inhibition by trifluoperazine (TFP). The native (membrane-bound) microsomal enzyme was also found to be just slightly sensitive to vanadate (Table 12), the phosphate analog that is now considered as the classic inhibitor of P-type ion-motive ATPases (Carafoli, 1991). This low sensitivity to vanadate observed for the native microsomal enzyme could be due to two factors.

(i) It might be due to the very low level of $mg^{2+}$ in the assay medium. It has been observed that the potency of vanadate as an inhibitor greatly depends on the ionic composition of the medium and on the concentration of ATP (Barrabin et al., 1980). The ions $Mg^{2+}$, $K^{+}$ and $Na^{+}$ enhance the inhibition by increasing the affinity of the pump for $Ca^{2+}$. In fact, vanadate has been shown to be almost ineffective in the absence of $Mg^{2+}$.

(ii) It might also be due to the tightly bound calmodulin, as calmodulin has been shown to protect the pump against vanadate. It is also probable that the two factors act synergistically to bring about the low sensitivity of the pump to vanadate.

However, the observation in this study that the enzyme has a maximum activity (4.543 ± 0.857 µmole $P_i$/mg Protein/hr) at pH 8.0 (Fig.15) contrasts with earlier reports of pH 6.4 - 6.8 usually used for the enzyme assay (Famulski and Carafoli, 1982; Moore and Kraus-Friedmann, 1983). In addition, the enzyme like the liver plasma membrane $Ca^{2+}$-ATPase (Lotersztajn et al., 1981) did not obey the Michaelis-Menten kinetics (Fig.17). This may be a peculiarity of liver $Ca^{2+}$-ATPases (both plasma membrane and microsomal).

Equipped with the kinetic and the physico-chemical properties of the membrane-bound ER $CA^{2+}$-ATPase, the question of the effect of the environmental pollutant and tumour promoter, DDT on the activity of the enzyme wasaddressed. Liver tumour promoters are known to induce cell proliferation and microsomal enzymes and to increase cytochrome $P_{450}$ content (Orrenius, 1965; Orrenius and Ericsson, 1966; McLean and McLean, 1966; Mannering, 1971). In this study, increases in the relative liver weight (liver weight/body weight ratio) and cytochrome $P_{450}$ content were used as criteria of liver tumour promotion. Using these criteria, it was confirmed that DDT is a liver tumour promoter (Table 14a). Surprisingly, the GGT activity of DDT-treated rats were found to be lower than those of normal (control) rats. Elevated GGT levels is widely used as a marker of hepatocellular carcinoma but the role of this enzyme in the carcinogenic process is not yet clear. The cause and the significance of the DDT-induced inhibition of GGT activity is presently not known. However, since GGT is a membrane-bound enzyme and DDT a lipophilic compound, it might be suggested that the inhibition is due to the membrane-active effect of DDT. The drastic depression in the microsomal $Ca^{2+}$-ATPase activity, also, requires comment. The result supports our earlier premise thatmitogen-induced $Ca^{2+}$ release from the microsome could be meaningful only if it is accompanied with an inhibition of the microsomal $Ca^{2+}$-ATPase. The DDT-induced inhibition of the ER $Ca^{2+}$-ATPase could be

(i) the result of a direct effect of DDT on the enzyme as DDT has been shown to activate the SR $Ca^{2+}$-ATPase in vitro (Antunes-Madera and Madera, 1982).

(ii) It could be due to an indirect effect on the enzyme which could be via

  (a) an effect on the membrane or

  (b) the effect of a molecule generated as a result of the metabolism of DDT on the enzyme itself or the membrane.

In addition, tumour promoters are known to induce the formation of free radicals (Cerutti, 1985) and these have been shown to be capable of inhibiting the $Ca^{2+}$-pumping

ATPase (Hebbel et al., 1986).

To confirm whether or not the DDT-induced inhibition of microsomal $Ca^{2+}$-ATPase is peculiar to DDT, the experiment was repeated with another liver tumour promoterphenobarbital (PB). The obtained results (Table 14b) are identical to those obtained with DDT. It was confirmed that PB is also a liver tumour promoter, using the relative liver weight and the stimulation of cytochrome $P_{450}$ as criteria. Here, however, unlike DDT-treated animals, a decrease in body weight was observed; this result could be interpreted to mean that the PB treatment showed some toxicity whereas DDT treatment did not. Also, in contrast with DDT-treated rats, PB-treated rats showed increased GGT activity. It is well documented that phenobarbital treatment leads to increased GGT activity in rats (Ratanasavanh et al., 1979). The differences in the results obtained for DDT and PB-treated animals on changes in body weight and GGT activity suggest that the two promoters might be different in their toxicities to the animals at concentrations used for this investigations.

The differences mentioned above for DDT-treated and PB-treated rats notwithstanding, phenobarbital treatment like DDT treatment also resulted in a significant decrease in the microsomal $Ca^{2+}$-ATPase activity. This result confirms thatthe promoter-induced inhibition of the enzyme is probably a characteristic of tumour promoters. It is worth noting that the promoter-induced depression of the microsomal $Ca^{2+}$-ATPase activity is not due to differences in total microsomal protein content; as there were no significant changes in the total microsomal protein for DDT-or PB-treated rats compared to the control rats. Also, the tumour promoter-induced inhibition of the ER $Ca^{2+}$-ATPase is not accompanied by changes in the affinity of the enzyme for calcium (Fig.18).

Lowrey et al., (1981) had observed that decreased microsomal calcium pumping is one of the earliest signs of chemical hepatotoxicity (carcinogenicity) using haloalkane as their study tools. Recknagel et al. (1982) and more recently Long and Moore (1986) observed that carbon tetrachloride elevates cytosolic calcium in rat hepatocytes and that it is the free

radicals generated from the chemical solvent by the liver ER which inactivate the calcium pump activity of the liver ER. In addition, the consensus of opinion is that the ER of the liver cell is the firstcellular organelle which is disrupted by the chemical poisoning. It has been observed that a naturally occurring tumour promoter, thapsigargin, is a potent inhibitor of the $Ca^{2+}$-pumping ATPase of the ER (Thastrup, et al., 1990). The authors also suggested that the modulation of $Ca^{2+}$-pump activity could provide the means of generating ionic signal based on the observation that thapsigargin induces $Ca^{2+}$-mobilization by ER $Ca^{2+}$-pump inhibition.

Pounds and Rosen (1988) considered the three ways in which a toxicant may alter $Ca^{2+}$ cellular homeostasis:

(a) by substituting for $Ca^{2+}$ at specific sites of $Ca^{2+}$ transport or storage such as a competitive interaction observed with other divalent metals such as lead and cadmium

(b) through non-specific effects on cell function that are biochemically and functionally remote from the processes of $Ca^{2+}$ transport and storage, such as physical-chemical changes in the membrane which are induced by ethanol and other aliphatic alcohols and local anesthetics (includingdibucaine and tetracaine) and

(c) through non-specific effects such as an indirect action of the inactivation of the $Ca^{2+}$-transporter of the smooth ER. It is this third hypothesis that is currently receiving much attention in studies which concern the modulation of $Ca^{2+}$ cellular homeostasis in the process of liver tumour promotion.

To date, the only acceptable biochemical mechanism that has been proposed to explain the action of some liver tumour promoters is the inhibition of gap junctional intercellular communication. However, as earlier suggested and as supported by the results of this investigation, it seems that the inhibition of intercellular communication might be secondary to the inhibition of the microsomal $Ca^{2+}$-pumping ATPase as the former is dependent on

intracellular $Ca^{2+}$ concentration. The present observation on the marked depression of $Ca^{2+}$-ATPase activity in rat liver microsomes may be a useful indicator of the physiological and/or biochemical function of specific liver tumour promoters.

It is well recognized that mitogenesis is an important factor in carcinogenesis particularly at the stage of tumour promotion (Farber, 1980). This notion is supported by the finding that both PB and DDT induced the proliferation of the endoplasmic reticulum as stated earlier on. Three mechanisms have been suggested for the promoter-induced differential rapid growth of initiated cells; these include:

(a) differential inhibition

(b) differential stimulation and

(c) differential recovery (Farber, 1980).

Differential inhibition proposes that the initiator is an inhibitor of cell proliferation except for initiated (resistant) cells. This differential enables the rapid growth of resistant (initiated) cells when a stimulus for proliferation (tumour promoter) is applied.

Differential stimulation proposes that promoter selectively stimulates initiated cell to grow. It also suggests that some promoters might stimulate both initiated and uninitiated cells but that the later loses their abilityto respond while this capability continues in initiated cells. This differential stimulation creates a progressive enlargement or proliferation of initiated cells.

Differential recovery proposes that most initiated cells may undergo reversion to normal - appearing liver - a process called regression. While some cells show rapid reversion, others show no reversion; and that the process of reversion may be similar in principle to the return of the liver to a resting phase ($G_o$) after one or more cycles of cell proliferation. The failure to return to a $G_o$ state could conceivably be part of an altered biochemical programme that prevents normal recovery.

Phenobarbital has been shown to act through the differential stimulation mechanism

(Farber, 1980). It is very probable that DDT acts through same mechanism.

In order to determine whether genotoxic carcinogens such as aflatoxin $B_1$ could also induce an inhibition of the microsomal $Ca^{2+}$-ATPase as observed for the non-genotoxic carcinogens (tumour promoters), the short-term effect of aflatoxin $B_1$ administration on the liver microsomal $Ca^{2+}$-ATPase was investigated. The obtained results (Table 14c) show that genotoxic carcinogens (in this instance aflatoxin $B_1$) do not have any effect on the ER $Ca^{2+}$-ATPase. Aflatoxin $B_1$ is a well known hepatotoxin, hepatocarcinogen and membrane active agent (Cerutti, 1985). Thus, the result shows that the promoter-induced depression of microsomal $Ca^{2+}$-ATPase is probably not due to the membrane-active properties of the tumour promoters.

Having established that liver tumour promoters are inhibitors of membrane-bound microsomal $Ca^{2+}$-ATPase, the effect of another modulator of hepatocarcinogenesis, that is, low protein intake (or protein malnutrition) on the activity of the enzyme was investigated and compared with obtained results for tumour promoters. The experiment was based on the premise that since both liver tumour promoters and low protein intake modify the structural integrity of biomembranes (Troll and Weisner, 1985; Coward, 1971), they might affect the activity of membrane-bound enzymes such as the $Ca^{2+}$-ATPase. The results obtained using the two adopted markers of tumour promotion in this study i.e. cytochrome$P_{450}$ content and the relative liver weight (liver weight/body weight ration) show that low protein diet does not belong to the same class of modulators of hepatocarcinogenesis as DDT and PB. Despite these differences, low protein diet like liver tumour promoters induced a significant decrease in microsomal $Ca^{2+}$-ATPase activity (Table 16). Suggesting that positive modulators of liver cancer are probably inhibitors of microsomal $Ca^{2+}$-ATPase. These results suggest that there is a common mechanism for the inhibition of the microsomal $Ca^{2+}$-ATPase by positive modulators of hepatocarcinogenesis.

Tumour promoters have been shown to be oxidants or to induce cellular prooxidant

state (Cerutti, 1987). Also, certain antioxidant defence systems of protein-energy-malnourished animals and humans have been shown to be depressed (Golden, 1987; 1988; Read, 1990). This condition might result in increased free radical level or an induction of cellular prooxidant state in the affected animal or individual. This is important because the $Ca^{2+}$-ATPase has been shown to be inhibited by free radicals particularlyactivated oxygen (Hebbel et al., 1986). It seems very likely therefore that the inhibition of the microsomal $Ca^{2+}$-ATPase in promoter-treated rats and protein-malnourished animals might be due to the inhibitory effect of free radicals (probably generated during the period) on the enzyme.

As earlier mentioned, the short-term effect of aflatoxin $B_1$ administration does not include an effect on the microsomal $Ca^{2+}$-ATPase. However, it is not known what the effect on the enzyme would be on a long-term basis when preneoplastic foci might (presumably) have been formed. As presented on Table 18(d), aflatoxin $B_1$ administration (cancer initiation) has no effect on the $Ca^{2+}$-ATPase activity after 9 weeks of its administration. It must be noted, however, that the activity of the liver cancer marker enzyme GGT, does not confirm the development of a preneoplatic foci during same period. Although this result cannot be taken as conclusive, it seems that the initiation stage of cancer development and growth is characterized by cells with normal and functional ER $Ca^{2+}$-ATPase activity. On the other hand, depressed ER $Ca^{2+}$-ATPase appears to be a characteristic of the promotion stage.

Since the process of tumour growth and development is a long-term process, some experiments were set up to determine whether the promoter-induced inhibition of $Ca^{2+}$-ATPase is a short-lived or a long-lasting response. As shown on Table 18(a) after 4 weeks of DDT treatment, there were no significant changes in microsomal $Ca^{2+}$-ATPase activity compared to control animals. The results contrast with our earlier observation on the decreased microsomal $Ca^{2+}$-ATPase activity after eight days (DDT) or four days (PB) of the administration of the liver tumour promoters. These results could be interpreted to mean that

the effects of both DDT and PB are short-lived and probably reversible. It is known that the effect of tumour promoters is reversible at least in early stages (Pitot and Sirica, 1980) and that the process of carcinogenic promotion requires the prolonged exposure to the tumour promoter (Faber, 1980; Pitot and Sirica, 1980). It is presently not known what the effect of prolonged exposure to the tumour promoter would be on the microsomal $Ca^{2+}$-ATPase activity. The requirement for a prolonged exposure to tumour promoters is satisfied in the case of chronic dietary protein malnutrition (low protein intake (LPI) for twelve weeks) as earlier reported. The effect of a short-term LPI was not investigated in this study.

Paradoxical enough, aflatoxin ingestion affords some protection against the low-protein intake-induced inhibition of microsomal $Ca^{2+}$-ATPase activity (Table 18c). The mechanism of such protection is not clear. It could be an indirect effect linked to second messenger production or to effects on membrane phospholipids particularly the lipid ambient surrounding the pump. Also, if the free radical theory employed to explain the promoter - and low protein intake-induced inhibition of microsomal $Ca^{2+}$-ATPase is correct, then, it might be possible that aflatoxin $B_1$ ($AFB_1$) - particularly its epoxide-acts as scavenger of free radicals. This last suggestion is interesting; but there are no indications to-date that aflatoxin $B_1$, or its epoxides can act as antioxidants. However, the ingestion of $AFB_1$ does not have a significant effect on the microsomal $Ca^{2+}$-ATPase of DDT-treated rats (Table 18b). This might be due to the fact that the effect of DDT is short-lived as has been discussed earlier.

To determine the status of the plasma membrane $Ca^{2+}$-ATPase in hepatocarcinogenesis, a preliminary study on the activities of the erythrocyte ghost membrane $Ca^{2+}$ pump of liver cancer and kwashiorkor subjects was carried out. The preliminary results show that the activity of the EGM $Ca^{2+}$-ATPase of cancer patients is comparable to that of normal individuals (Table 20). However, a reduced responsiveness of the enzyme to calmodulin was observed even though the value is not significantly different ($P > 0.05$) from control value (Table 20). These results do not support the well-documented observation on

the increased calcium concentration in cancer cells in culture. It however, suggest the tendency of an increased affinity of the enzyme for calmodulin in cancer cells. The results obtained with the EGM $Ca^{2+}$-ATPase of kwashiorkor humans is similar to that obtained for cancer patients (Table 20). This results upports the result on decreased responsiveness to calmodulin reported for the EGM $Ca^{2+}$-ATPase of kwashiorkor patients (Olorunsogo, 1989).

As earlier reviewed (Section 1.8), in all eukaryotic cells so far investigated, and in several transformed cells, the biological message of β -adrenergic agonists, several hormones, tumour promoters,growth factors and oncogene products is intracellularly propagated by receptor-mediated generation of the second messengers diacylglycerol (DG) and inositol 1,4,5 trisphosphate ($IP_3$). $IP_3$ initiates reversible release of $Ca^{2+}$ from the endoplasmic reticulum (ER), or a specialized part of it. The liberated $Ca^{2+}$ now acts as a "third messenger" by activating $Ca^{2+}$-dependent reactions (Heilmann et al., 1989). The $Ca^{2+}$-pool in the ER is refilled by an ATP-energized $Ca^{2+}$-pump. The question on whether the action of $IP_3$ is accompanied by the $Ca^{2+}$-pump activity or inhibition has not been well studied.

One of the major results of this study is that the $Ca^{2+}$-pump is inhibited during the action of the above-mentioned agonists (particularly liver tumour promoters andlow protein intake). This appears reasonable as the existence of a normal pump would have made the action of $IP_3$ a futile one and without effect. Despite the fact that there have been reports on the effects of some hepatotoxins (Lowrey et al., 1981, Recknagel et al., 1982, Long and Moore, 1986) and other non-hepatic tumour promoters (Thastrup et al., 1990) on the activity of the microsomal $Ca^{2+}$-ATPase, it has been difficult to make this generalization since the microsomal fraction is known to contain two distinct classes of $Ca^{2+}$-pumping organelle (Famulski and Carafoli, 1982; Gill et al., 1989).

The enzyme described in this study, obtained as the light microsomal fraction, has been shown to originate from the endoplasmic reticulum (Famulski and Carafoli, 1982). It seems reasonable therefore to conclude that the inhibition of the liver ER $Ca^{2+}$-ATPase is a characteristic of liver tumour promoters.

## 4.2 Summary of results

The specific activity of membrane - bound microsomal $Ca^{2+}$-ATPase of the livers of untreated rats was 4.543 $\pm$ 0.857 µmole $P_i$/mg protein/hr. at pH 8.0 and was insensitive to calmodulin. The specific activity of the enzyme was significantly decreased ($P < 0.01$) following subcutaneous administration of a single dose of 75 mg dicophane/kg body wt.; the affinity of the enzyme for $Ca^{2+}$ was however unaffected. Similarly, liver microsomal $Ca^{2+}$-ATPase activity was significantly diminished following the ingestion of low protein diet by rats for 12 weeks. The mean $Ca^{2+}$-ATPase activity of $AFB_1$-treated animals (in the absence of dicophane) was not statistically different ($P > 0.05$) from that of $AFB_1$-treated rats which subsequently received dicophane. In contrast, liver microsomal $Ca^{2+}$-ATPase activity of animals fed low protein diet prior to and after AFB ingestion was higher ( $P < 0.05$) than that of animals which were fed low protein diet only. Basal activity of erythrocyte $Ca^{2+}$-ATPase of paediatric controls and those having kwashiorkor (protein-energy-malnutrition) were similar ($P > 0.05$); similar observations were made between normal adults and those suffering from PLC. Erythrocyte $Ca^{2+}$-ATPase of either PLC or kwashiorkor patients was however, somewhat, less sensitive (15-40 %) to the stimulatory effect of calmodulin, an endogenous activator of the $Ca^{2+}$-pump.

## 4.3 Contributions to knowledge

The highlights of the contributions of this thesis to knowledge include the observations:

(1) That the inhibition of microsomal $Ca^{2+}$-ATPase appears to be a characteristic of liver tumour promoters.

(2) That low protein diet mimics the effect of liver tumour promoters with respect to the inhibition of the rat liver microsomal $Ca^{2+}$-ATPase.

(3) That the genotoxic carcinogen aflatoxin $B_1$ despite its membrane-active property does not affect the membrane-bound microsomal $Ca^{2+}$-ATPase.

(4) That aflatoxin $B_1$ protected the membrane-bound $Ca^{2}_{+}$-ATPase against LPI-induced inhibition of microsomal $Ca^{2+}$-ATPase in a way that is not yet understood and finally

(5) That the erythrocyte ghost membrane-bound $Ca^{2+}$-ATPase of both kwashiorkor and primary liver cancer patients are less sensitive to the stimulatory effect of calmodulin.

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Appendix 1

# Diet 41B 10mm [441]

## Calculated Analysis

### Nutritional Information

| | | |
|---|---|---|
| Crude Oil | % | 2.82 |
| Crude Protein | % | 16.18 |
| Crude Fibre | % | 6.35 |
| Total Ash | % | 9.06 |
| Neutral Detergent Fibre | % | 22.00 |
| Hemicellulose | % | 14.9 |
| Acid Detergent Fibre | % | 7.1 |
| Starch | % | 36.11 |
| Sugars | % | 3.04 |
| Gross Energy | Mj/kg | 15.5490 |
| Digestible Energy | Mj/kg | 12.2455 |
| Metabolisable Energy | Mj/kg | 10.1399 |
| Essential Fatty Acids | % | 1.3815 |
| D.C.P | % | 13.2952 |
| Lysine | % | 0.6594 |
| Methionine | % | 0.2752 |
| Methionine & Cystine | % | 0.4873 |
| Threonine | % | 0.5127 |
| Tryptophan | % | 0.1793 |
| Arginine | % | 0.9990 |
| Calcium | % | 1.3665 |
| Phosphorus | % | 0.9301 |
| Available Phosphorus | % | 0.5461 |
| Sodium | % | 0.4686 |
| Potassium | % | 0.7240 |
| Salt (NaCl) | % | 1.1386 |
| Magnesium | % | 0.2734 |

### Vitamins & Trace Elements Added by Supplementation

| | | |
|---|---|---|
| Iron | mg/kg | 50.00 |
| Copper | mg/kg | 5.00 |
| Manganese | mg/kg | 50.00 |
| Zinc | mg/kg | 15.00 |
| Iodine | mg/kg | 0.50 |
| Cobalt | mg/kg | - |
| Selenium | mcg/kg | - |
| Vitamin A | IU/kg | 15000.00 |
| Vitamin D | IU/kg | 2000.00 |
| Vitamin E | mg/kg | 70.00 |
| Vitamin K | mg/kg | 10.00 |
| Vitamin B1 | mg/kg | 5.00 |
| Vitamin B2 | mg/kg | 6.00 |
| Vitamin B6 | mg/kg | 7.50 |
| Vitamin B12 | mcg/kg | 7.50 |
| Folic Acid | mg/kg | 2.00 |
| Nicotinic Acid | mg/kg | 12.00 |
| Pantothenic Acid | mg/kg | 15.00 |
| Choline | mg/kg | 1000.00 |
| Biotin | mcg/kg | 200.00 |
| Vitamin C | mg/kg | - |



### Notes

1. The specifications quoted above are those at the time of printing and are only intended as a guide. Changes in growing conditions will alter thus effecting the values given above. When this occurs it will be recorded on the bag label or an ammended extended analysis sheet will be issued.
2. Values in the left hand set of figures are total calculated values.
3. Figures for trace elements and vitamins are the amounts added by supplementation.
4. 1 MJ = 239.005 Kcal
5. 1 U of Vitamin A = 0.344 mcg pure Vitamin A Acetate.
6. 1 U of Vitamin D = 0.025 mcg pure Vitamin D2/D3
7. 1 U of Vitamin E = 1mg DL Alpha Tocopherol Acetate.
8. Further analytical information can be provided for batches as an extended analysis.

# Appendix II

## Composition of SDS's low Protein diet

| Nutritional Information | (%) |
|---|---|
| Volume | 100.00 |
| Mositure | 11.212 |
| Crude Fat | 2.733 |
| Crude Protein | 5.253 |
| Crude Fibre | 1.760 |
| Ash | 5.202 |
| Total Dietry Fibre | 4.574 |
| Starch | 66.593 |
| Sugar | 3.615 |
| Digestibe Crude Fat | 2.574 |
| Digestible Crude Protein | 4.555 |
| Calcium | 0.705 |
| Phosphorus | 0.542 |
| Phytate Phophorus | 0.052 |
| Sodium | 0.238 |
| Chlorine | 0.341 |
| Magnesium | 0.168 |
| Potasium | 0.698 |
| Lysine | 0.208 |
| Methionine & Cystine | 0.130 |

| Vitamins and Trace elements Added by supplementation | | |
|---|---|---|
| Iron | 0.071 | g/kg |
| Copper | 0.015 | " |
| Marganese | 0.062 | " |
| Zinc | 0.037 | " |
| Cobalt | 0.517 | mg/kg |
| Iodine | 0.645 | " |
| Solenium | 0.119 | " |
| Retinol | 1.598 | " |
| Cholecalciferol | 0.025 | " |
| X-Tocopherol | 0.051 | g/kg |
| Vitamin B1 | 7.029 | mg/kg |
| Vitamin B2 | 6.253 | " |
| Vitamin B6 | 5.931 | " |
| Vitamin B12 | 5.499 | mcg/kg |
| Folic Acid | 0.928 | mg/kg |
| Nicotinic Acid | 23.584 | " |
| Pantothenic Acid | 13.658 | " |
| Choline | 0.400 | g/kg |
| Inositol | 0.693 | " |
| Biotin | 0.093 | mg/kg |