SOME CONSEQUENCES OF THE BINDING OF AFLATOXIN B_1 WITH PLASMA MEMBRANE ON THE REGULATION OF INTRACELLULAR Ca^{2+} HOMEOSTASIS.

BY

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ABSTRACT

The possible influence of aflatoxin B_1 a potent hepatocellular carcinogen on the regulation of intracellular Ca^{2+} homeostasis has been studied using the red cell as a model.

Preliminary work on the interaction of the toxin with the red cell membrane using spectrofluometric analysis indicated that the toxin binds spontaneously and irreversibly to the red cell membrane. The binding is highest at pH 4 and least at pH 10. Results obtained from studies using equilibrum dialysis technique show that about 4 nmoles of the toxin bind to one microgram membrane protein. Although the exact membrane component to which aflatoxin B_1 binds is not known, experiments carried out to determine the influence of aflatoxin B_1 on the activity of the calcium pumping protein revealed that the toxin inhibited the calmodulin-stimulated erythrocyte membrane Ca^{2+}-ATPase activity by about 50 percent, while it has little or no effect on its basal activity. Kinetic analysis of the

inhibition shows that, the toxin reduces the V_{max} and K_m of the calmodulin-stimulated enzyme by 50 percent in a non-competitive manner, On the other hand, the carcinogen had no significant influence on the kinetic parameters of the enzyme in the non-activated state.

Similar results were obtained for the triton X-100 solubilized and calmodulin affinity chromatographed enzyme. In this instance aflatoxin B_1 inhibited the calmodulin-stimulated purified enzyme by 50 percent with or without preincubation on ice for half an hour. Again, the toxin had little or no effect on the basal activity of the enzyme in the absence of calmodulin. Analysis of the results obtained using varying concentrations of ATP shows that the K_m and V_{max} of the non-activated enzyme were not altered by the toxin while both the V_{max} and K_m values were reduced by about 50 percent in the presence of calmodulin.

In addition aflatoxin B_1 inhibited Diphosphotidyl glycerol (cardiolipin) by about 28% while it has no effect on the basal activity of the enzyme. Although,

the inhibition of the membrane bound or purified Ca^{2+} ATPase by the toxin is concentration dependent, varying concentrations of phosphatidyl serine and phosphatidyl choline do not affect the inhibition of the purified enzyme by aflatoxin B_1.

Results obtained with triton X-100 solubilized enzyme shows that triton X-100 alone could not activate the enzyme. Thus at triton X-100: protein ratio of 2, the enzyme was stimulated by calmodulin. This activity was sensitive to inhibition by the toxin. In this instance, the calmodulin-stimulated activity was inhibited by about 50%, while at lower ratios of the triton X-100 to protein there was no significant inhibition of enzyme.

Results of experiments carried out on the 124KDa fragment, which was produced as a result of exposure to calpain a Ca^{2+}-dependent cysteine protease, indicated that the toxin has no effect whatsoever on the activity of the fragmented enzyme, Similarly experiments on limited proteolysis of the Ca^{2+} ATPase by trypsin to give the 90KDa fragment which still retains its calmodulin binding

domain and the 76KDa fragment which has lost its calmodulin binding domain revealed that the aflatoxin B_1 inhibited the 90KDa fragment by about 50% while the 76KDa fragment is not affected at all.

Altogether, these findings show that aflatoxin B_1 inhibits the plasma membrane Ca^{2+}-pumping ATPase by interacting with the enzyme at the calmodulin binding domain. The nature of the exact amino acid residue to which the toxin binds is however not known. The implication of these observations is that Ca^{2+} extrusion may be hampered in situations where the cell is poisoned by the aflatoxin.

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CERTIFICATION

We certify that this work was carried out by Miss Aderonke Olanrewaju Adebayo in the Department of Biochemistry, University of Ibadan, Ibadan, Nigeria.

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TABLE OF CONTENTS

LIST OF PLATES

LIST OF FIGURES

LIST OF TABLES

BB

ABBREVIATIONS

ATP	Adenosine 5' triphosphate
ATPase	Adenosine 5' trisphosphatase
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	3'5'-cyclic adenosine-5'-monophosphate
Ca^{2+}-ATPase	Mg^{2+}-dependent Ca^{2+}-pumping Adenosine triphosphatase
DTT	Dithiothreitol
EDTA	ethylene diamine tetracetic acid
IOVs	Inside-out vesicles
Fig	Figure
g	gram
hPMCA	human plasma membrane Ca^{2+}-ATPase
HEPES	4-(2-hydroxyethyl)-piperazine ethanesulphonic acid
hr	hour
Km	Michaelis constant
M	Molar
mg	milligramme
Mr	Molecular weight
nMoles	nanomoles
ND	not determined

pc	phosphatidyl choline
pH	a measure of hydrogen ion concentration
PMSF	phenylmethylsulfonylfluoride
PS	Phosphatidyl serine
rpm	revolution per minute
rPMCA	rat Plasma Membrane Ca^{2+} ATPase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-p-phenylenylene diamine
Tris-HCl	Tris (hydroxymethyl) amino methane hydrochloride
ug	microgramme
umole	micromole
uM	micromolar
V_{max}	maximal velocity of enzymatic reaction.

CHAPTER ONE

INTRODUCTION

1:1 The Control of Intracellular Calcium

The recognition of the importance of calcium in cell functions dates back to the pioneering work of Ringer (1882, 1883) who established that calcium ions are important in the contraction of frog heart muscle. The work was later confirmed and extended by Locke (1894). Research activity in this area remained dormant until 1947 when Heilbrunn and Wiercinski showed that, the injection of a small amount of Ca^{2+} into a muscle fibre causes it to contract.

Calcium ion is now well known to mediate a wide variety of cellular responses and processes such as cell motility, endo-and exocytosis and more complicated processes such as cell proliferation, fertilization and hormone secretion (Table 1).

It is now generally accepted that Ca^{2+} is a very important and possibly the most important second messenger in living cells. It has also been suggested that Ca^{2+} may play the role of a primary messenger since it, directly interferes with

TABLE 1

Calcium-dependent cellular reactions and processes.

Enzyme/process	Reference
Phosphorylase kinase (glycogenolysis)	Cohen, Burchell and Foulkes, 1978.
Phospholipase A_2	Wong and Cheung, 1979.
Myosin light chain Kinase	Walsh, Cavadore, Vallet and Demaille 1980.
Erythrocyte Ca^{2+}-ATPase	Schatzmann 1966
Adenylate cyclase	Bronstom, Bronstom and Wolff 1977.
Cell motility	Tash and Mann 1973
Muscle contraction	Ebashi 1958.
Exo and endocytosis	Linden, Dedman, Chafouleas, Means and Roth, 1981.
Cell division and proliferation	Welsh, Dedman, Brinkley, Means 1978.
Fertilization	Epel, Patton, Wallace and Cheung 1981

the generation of signals at the level of plasma membranes by regulating K^+, Na^+ and even Ca^{2+} currents (Carafoli and Crompton, 1978). An important consequence of the messenger role of Ca^{2+} is, the necessity for its precise regulation. Thus the control of the intracellular level of Ca^{2+} is also an essential step in metabolic regulation. The steady state concentration of Ca^{2+} is about 10^{-8} to 10^{-7}M in mammalian cells, a value that is three or four orders of magnitude lower than the free Ca^{2+} concentration in the extracellular environment estimated to be about 1.5mM (Rasmussen and Goodman, 1977; Carafoli and Crompton, 1978). Maintenance of this concentration difference depends on the extrusion of the cation through the plasma membranes to balance its continous passive influx, sequestration within subcellular organelles such as mitochondria and sarcoplasmic reticulum and binding of Ca^{2+} to non-membranous ligands in cytosol. However, in erythrocytes, the low intracellular Ca^{2+} concentration is regulated mainly by the presence of membrane-bound pump that

drives calcium out of the cell against the concentration gradient. Thus Ca^{2+} is transported across biological membrane by four basic mechanisms as shown in Table 2. In most eukaryotic cells, the same membrane system may transport Ca^{2+} by more than one of these mechanisms (Carafoli, 1984). Na^{+}/Ca^{2+} exchangers have been reported in plasma membrane and the inner mitochondrial membrane.

1.2 Discovery and general properties of Ca^{2+} pump

The existence of a Ca^{2+}-dependent adenosine triphosphatase (ATPase) in the erythrocyte membrane was first reported by Dunham and Glynn (1961) who noted that the simultaneous presence of Mg^{2+} and Ca^{2+} increases the total ADP hydrolysis by isolated membranes from human erythrocytes, and at the same time inhibits the Na^{+}, K^{+} ATPase. This observation was confirmed by Hoffman (1962), who concluded that the site of Ca^{2+} inhibition of the Na^{+}, K^{+} ATPase

TABLE 2

Calcium transporting systems in biological membrane

Transport System	Membrane types	References
ATPase	Plasma membranes	Schatzmann 1966
	Sarcoplasmic Reticulum	Hasselbach and
	Endoplasmic Reticulum	Makinose, 1961.
Exchangers	Plasma membranes Na^+/Ca^{2+} exchanger except mature erythrocytes.	Reuter and Seitz, 1968.
	Inner mitochondria membrane (Na^+/Ca^{2+};H^+/Ca^{2+})	Carafoli, Tiozzo Lugli, Grovetti and Kratzing, 1974.
Channels	Plasma membranes	Fatt and Ginsborg 1958.
Electrophorectic Uniporters	Inner Mitochondria membranes	Vasington and Murphy, 1961.

is on the inner side of the membrane. However, it was five years later that Schatzmann (1966) showed that the ATPase actually transported Ca^{2+} out of erythrocytes. The different kinds of plasma membrane of eukaryotic cells containing the calcium pump are shown in Table 3. It is now widely recognized that the activity of the plasma membrane Ca^{2+} pump is of critical importance to the maintenance of cellular Ca^{2+} homeostasis (Schatzmann 1982; Carafoli, 1981), this pump also catalyses the ATP dependent exchange internal Ca^{2+} for external H^{+} (Niggli, Sigel and Carafoli, 1982; Smallwood, Waisman, Lafreniere and Rasmussen, 1983), and is responsible for the maintenance of a 5,000 - 10,000 fold Ca^{2+} concentration gradient across the plasma membrane. Previous, attempts to study the properties of the enzymes in unfractionated erythrocyte ghosts were difficult because of the presence of a Mg^{2+}-ATPase in the membrane.

Solubilization and purification of the ATPase had been difficult due to the lability of the enzyme (Niggli, Ronner, Carafoli and Penniston 1979) its low concentration (Graf, and Penniston, 1977)

TABLE 3

Calcium pumps in the plasma membrane of eukaryotic cells

Cell type	Approximate molecular mass	Reference
Skeletal muscle		Michalak, Famulski and Carafoli 1984; Mickelson, Beaudry and Louis 1985; Sulakhe, Drummond and Ng 1973.
Sarcolemma	140,000	
T-tubules	ND	Brandt, Caswell, Brunschwig 1980; Hidalgo, Gonzales and Garcia 1986.
Heart	140,000	Caroni and Carafoli 1980; Morcos and Drummond 1979; Caroni and Carafoli 1981; Kuwayama and Kanazawa 1982; Lamers and Stinis 1981; Tuana, Dzurba, Panagia, Dhalla 1981.
Smooth muscle	140,000	Morel, Wibo and Godfraind 1981; Popescu and Ignat, 1983; Wuytack, Deschutter and Casteels 1980; Wuytack et al 1981.
Kidney tubules	140,000	Desmedt, Paris, Borghgraefan Wuyfack 1981; 1983; 1984; Ghijsen, Gmaj and Murer 1984; Gmaj, Murer and Carafoli 1982; Gmaj, Murer and Kinne 1979; Gmaj, Zurini, Murer and Carafoli 1983; Moore, Fitzpatrick, Chen and Landon 1974.
Nervous cells		
Squid axon	ND	Dipolo 1979.

Cell type	Approximate molecular Mass	Reference
Synaptosomes	140,000	Hakim, Itano, Verma and Penniston, 1982; Papazian, Rahamimoff and Goldin 1979; Sorensen and Mahler 1981.
Optic nerve	ND	Condrescu, Asses, Dipolo 1984.
Neurohypophysis	ND	Desmedt et al 1983.
Intestinal epithelium	115000-130,000	DeJonge, Ghijsen and Van Os 1981; Ghijsen and Van Os 1979 Hildmann Schmidt Murer 1982; Nellan and Popovich 1981 Vancorven, Roche and Van Os 1985; Waisman Walters and Weiser, 1988.
Endocrine Pancreas	ND	Kotagal, Patker landt; McDonald and Colca 1982. Pershadsingh, McDaniel, Lamdt, Bry, Lacy and McDonald 1980.
Exocrine Pancreas	100,000	Ansah, Molla and Katz 1984. Bayerdoerfer, Streb, EcKhardt, Hasse and Schulz, 1984 Imamura and Schulz 1985.
Adipocytes	ND	Pershadsingh and McDonald 1979; 1980.
Osteoblasts	ND	Shen, Kohler and Peck 1983.

Cell type	Approximate Molecular Mass	Reference
Leucocytes		
Lymphocytes	150,000	Lichtman, Segel Lichtman 1981; Sarkadi, Enyedi and Gardos 1980.
Monocyctes	ND	Morimoto, Birge, Shen and Avioli, 1985; Scully, Segal and Lichtmann 1982.
Neutrophils	ND	Ochs and Reed 1983; Prentiki Wollhem, and Lew.1984; Volpi, Naccache and Sha'afi 1983
Macrophages	132,500	Lew and Stossel 1980; Schneider, Mottola and Romeo 1979.
Ehrlich ascites cells	ND	Klaven, Pershadsingh Henius, Laris, Long and McDonald 1983; Spitzer, Bohmer and Grosse 1983.
Plant cells	ND	Dieter and Marme 1981; Gross and Marme 1978; Marme and Dieter 1983: Nguyen and Siegenthaler 1985.

Cell type	Approximate Molecular Mass	Reference
Liver	70,000-105,000	Bach, Famulski, Mirabelli and Carafoli 1985; Chan and Junger 1983; Iwasa, Iwasa and Krishnaraj 1983; Iwasa, Iwasa, Higashi Matsui and Miyamoto 1982; Kraus-Friedmann Biber, Murer and Carafoli 1982; Lotersztajn, Hanoune, and Pecker 1981.

1981) and the fact that the ATPase has a molecular weight and solubilization properties similar to those of Band 3, the anion channel, one of the most abundant components of the erythrocyte membrane (Ronner, 1978). Purification of the enzyme was carried out by applying affinity chromatographic techniques which involved the coupling of calmodulin to a sepharose 4B matrix (Niggli, Penniston and Carafoli 1979; Gietzen, Tejeka and Wolf, 1980). The CaM column was loaded with a Triton X-100 solubilizate of erythrocyte ghosts from which endogenous CaM had been removed by EDTA washing. The detergent, column and elution buffers contained phosphatidyl choline (PC) to maintain the Ca^{2+} ATPase in the bilayer state. Elution of the column was done with EDTA buffer, the EDTA -elution peak was shown by SDS-polyacrylamide gel electrophoresis to have a major polypeptide of Mr 138,000 dalton.

The properties of the Ca^{2+} ATPase of erythrocyte plasma membrane (Table 4) resemble, in many respects, the analogous enzymes in the sarcoplasmic reticulum

TABLE 4

Properties of the plasma membrane Ca^{2+}-ATPase

Property	References
Mr (SDS-PAGE) 130,000-140,000	Niggli et al, 1979 Villalobo et al, 1986 Wang et al, 1988 Papp, et al, 1989.
Calculated molecular weight 129,500-139.000	Shull and Greeb 1988; Verma et al, 1988
$K_{0.5}$ (Ca) -low affinity mode: 4-20 µM	Niggli et al, 1981: Wang et al, 1989 Villalobo et al, 1986; Wang et al, 1989.
-high affinity mode: 0.2-0.7 µM	Papp et al, 1989; Enyedi et al, 1987; Kosk-Kosicka and Bzdega, 1988.
$K_{0.5}$ (ATP) -low affinity site: 120-290 µM	Wang et al, 1989
-high affinity site: 0.9-4 µM	Villalobo et al, 1986.
Ca^{2+}: H^+ ratio 1:1 (electrogenic)	Villalobo and Roufogalis 1986; Kuwayama, 1988; Romero and Ortiz 1988
Ca^{2+}: ATP ratio: 1:1 (reconstituted	Carafoli 1991; Wang et al, 1989; Niggli et al, 1981; Romero and Ortiz 1988.
$K_{0.5}$ (Calmodulin) 2-6nM	Niggli et al, 1981; Villalobo et al, 1986; Wang et al, 1988; Kosk-Kosicka and Bzdega 1988.

of muscle cells except that it is regulated by calmodulin. The plasma membrane Ca^{2+}-pump is the largest of all known P-type ion motive ATPases i.e. those that forms an acyl phosphate intermediate during the reaction cycle (Knauf, Proverbio and Hoffmann, 1974). The calculated molecular weight is between 129-139KDa (Strehler Streuler-page, Vogel and Carafoli 1989). Specific activities reported for the purified Ca^{2+} ATPase preparations range from 9.0 - 186 μmoles mg $prot^{-1}$ $hour^{-1}$, compared to the usual values of 0.3-3.0 μmoles mg $prot^{-1}$ $hour^{-1}$ for whole membranes. (Gietzen et. al. 1980, Niggli, Adunyah and Carafoli 1981). The purified enzyme preparation when reconstituted into artificial liposomes is activated by calmodulin about 7 fold, in analogy with the findings on the membrane bound enzyme, also for the reconstituted enzyme the Ca^{2+}: ATP ratio is 1:1 (Carafoli 1991; Wang, Roufogalis and Villalobo 1989). Calmodulin shifts the purified and membrane bound ATPases from a low Ca^{2+}-affinity state (Km for Ca^{2+} about 4-20 μM) to a high Ca^{2+}-affinity state, (Km for Ca^{2+} about 0.2-0.7 μM) (Niggli, Adunyah, Penniston and Carafoli, 1981; Wang, 1989;

Villalobo, Brown and Roufogalis 1986; Wang, Villalobo, Roufogalis, 1988; Enyedi, Flura, Sarkadi, Gardos and Carafoli, 1987). Also the Km for the ATP is shifted from a low ATP-affinity state (Km for ATP 120-290μM) (Wang et al, 1989; Villalobo et al 1986), to a high ATP state (Km for ATP 0.9 - 4 μM) (Villalobo and Roufogalis, 1986, Kuwayama 1988; Romero and Ortiz 1988; Clark and Carafoli, 1983).

1:3 Primary structure of the Ca^{2+}-translocating ATPase

The elucidation of the primary structure of plasma membrane Ca^{2+} pumping ATPase posed a unique problem to researchers despite the fact that the enzyme had been obtained in extremely pure form for many years (Niggli et al, 1981). These initial difficulties arose because of 2 main reasons. Firstly, the enzyme occurs in extremely low concentration in the membrane and secondly, it is larger than all other P-type ATPases known. Attempts to determine the primary structure of the protein

therefore focused on the sequencing of fragments of important domains of the protein. It was indeed in 1987 that, Filoteo, Gorski and Penniston successfully isolated and sequenced a tryptic fragment containing the fluorescein isothiocyanate (FITC) binding domain. This domain is assumed to be part of the binding site for ATP.

To obtain the tryptic fragment, these workers labelled the purified human erythrocyte Ca^{2+}-pump with FITC and then cleaved the enzyme with trypsin. The fragment containing the label was separated by High Performance liquid chromatography. The domain had the sequence F-S-K-G-A-S-E of which K-G-A residues are highly conserved in all the P-type ion-motive ATPases.

Almost simultaneous with the sequencing of ATP binding site was the determination of the phosphorylation site. The sequencing of the phosphorylation site was based on the fact that the phosphorylated intermediate of majority of P-type ATPases is an aspartyl phosphate located in an highly conserved sequence stretch of amino acids. Thus James, Zvaritch,

Hakhparamov and Carafoli (1987) isolated and sequenced a Cyanogen Bromide (CNBr) digest peptide corresponding to the phosphorylation domain. This sequence contains the predicted aspartic acid as the phosphate group acceptor C-S-D-K-T-G-T and it is flanked on both sides by the amino acid sequence corresponding to all other ATPases of the same group.

The sequencing of the calmodulin binding domain has been much more difficult. The use of a bifunctional photoactivable cross linker has made the sequencing possible,(Fig.1) the Denny-Jaffe reagent was linked through an oxysuccinimide moiety at one of its ends to a lysine residue of calmodulin following linking. Calcium stimulated CaM was covalently bound to its binding site in the pump through photoactivation of arylazide located at other end of the reagent. The reagent was then cleaved with dithionite at the azo linkage located between photoactivable and oxysuccinimide. CaM was removed by dialysis, while the pump was still radioactively labeled. The labeled pump

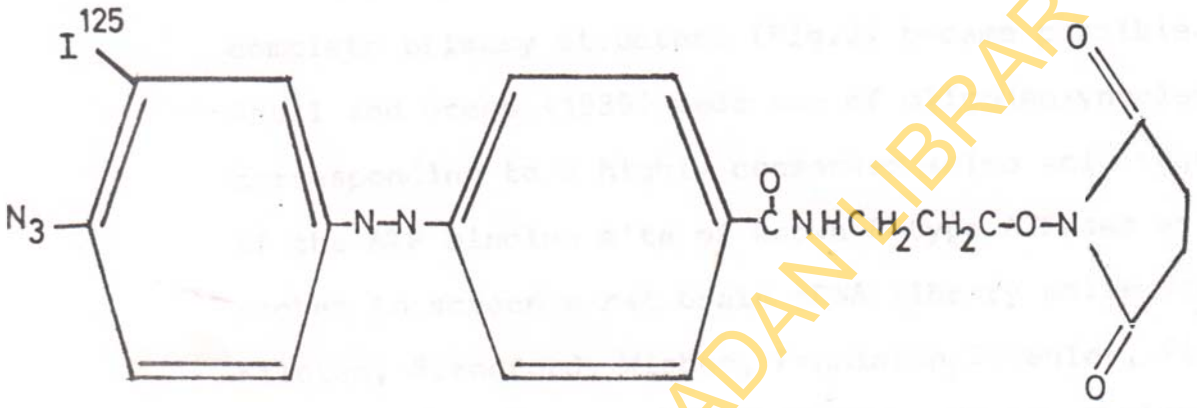


Fig.1 THE DENNY-JAFFE REAGENT
Denny and Blobel 1984

was cleaved by Cyanogen Bromide (CNBr) and the fragment was separated by HPLC. The labeled fragment contained the sequence NH_2 -E-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-L-H-E-F.

Shortly afterwards, the determination of the complete primary structure (Fig.2) became possible. Shull and Greeb (1988) made use of oligodeoxynucleotides corresponding to a highly conserved amino acid sequence of the ATP binding site of known P-type ATPases as probes to screen a rat brain cDNA library while Verma, Filoteo, Standford, Wieben, Penniston, Strehler, Fisher Heim, Vogel , Mathews Strehler-page, James, Vorherr, Krebs and Carafoli (1988) used oligodeoxynucleotides, but this is designed on the basis of the amino acid sequence of 2 short specific peptide from human erythrocyte Plasma Membrane Ca^{2+} ATPase (PMCA) as probes to isolate the corresponding cDNA. The translate sequence contain 1220 amino acids with calculated molecular weight of 134,683 daltons (Verma et al, 1988).

The location of the functional domains of the pump is now established from its complete cDNA

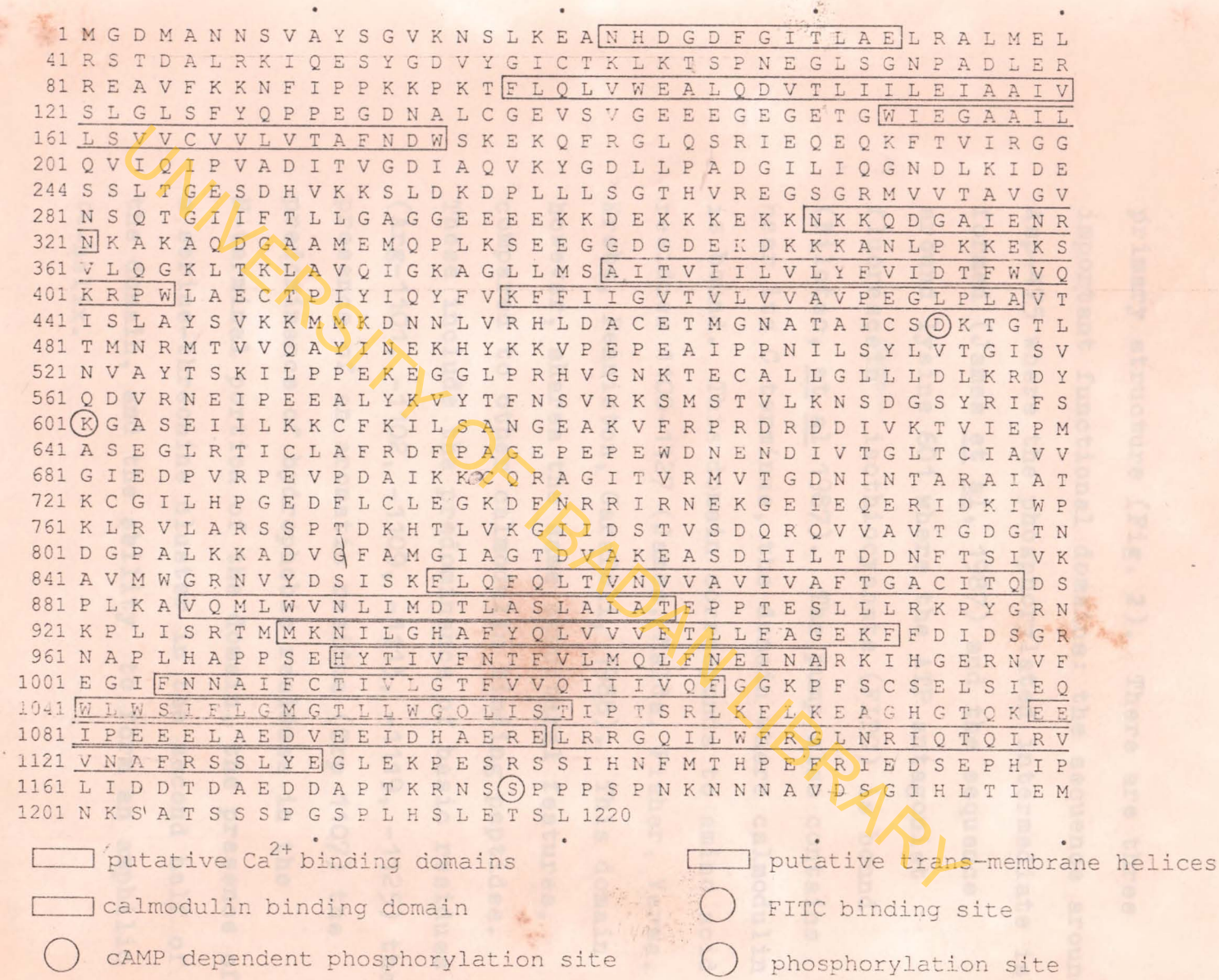
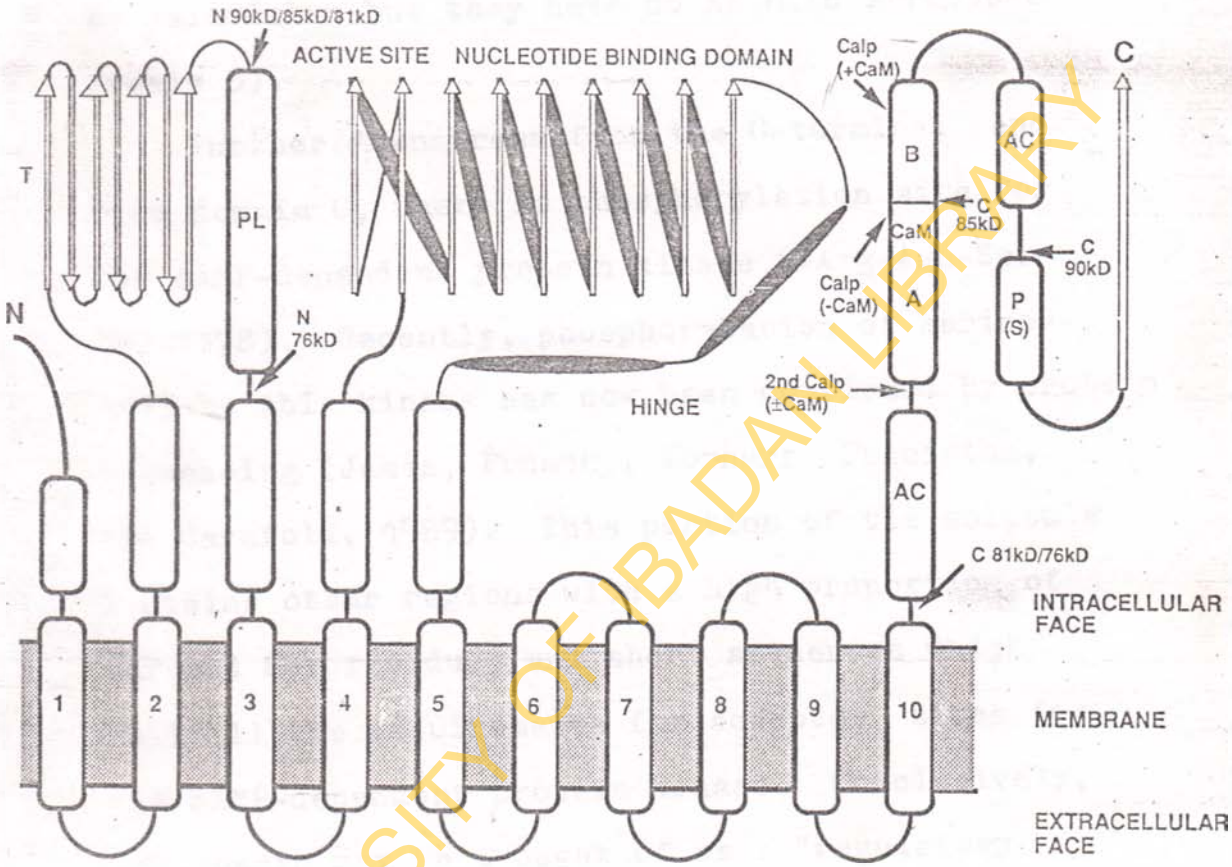


Fig. 2: The complete primary structure of plasma membrane Ca^{2+}-ATPase.

primary structure (Fig. 2). There are three important functional domains: the sequence around Asp-475 where the phosphorylated intermediate is formed (James et al, 1987) and the sequence around lysine 601 where the ATP antagonist fluorescein isothiocyanate (FITC) is bound (Filoteo, et al 1987). The pump also contains near its C terminus, the domain where calmodulin is bound. This domain corresponds to amino acid residues 1100-1127 (James, Malda, Fisher, Verma, Krebs, Penniston, Carafoli, 1988). This domain however, shares the same structural features, compared to other calmodulin binding peptides. These include the predominance of basic residues (Arg-1101, -1102, -1109, -1113, -1119, -1125) the presence of an aromatic residue (Trp 1107) the predominance of hydrophobic residues in the N-terminal portion of the domain, the presence of a serine-threonine cluster in the second half of the domain, and the ability to form an amphilic α-helix.

Further studies have indicated, that the calmodulin binding domain (Fig. 3) is flanked by two regions bearing a strong negative charge (residues 1079-1094 and 1153-1170). In the case of the 1079-1094 residues which was identified as domain A, this is found upstream of the calmodulin domain, it was speculated that because of the position and the charge of this stretch it was believed that it may interact with the calmodulin-binding domain (domain C) and that removal of the domain C by interaction with calmodulin (or by proteolysis) frees this domain to bind Ca^{2+} with the expected high affinity. The other strongly acidic regions which is part of domain B is downstream of domain C containing residues 1153-1170. However, it is not certain, whether domain B contributes to a high affinity Ca^{2+} because any cleavage that removes the calmodulin domain would also remove domain B and the enzyme is known to still retains high Ca^{2+} affinity even after the removal of domain C (Zurini, Krebs, Penniston and Carafoli, 1984; Benaim, Zurini and

Fig.3: Proposed model for the overall topology of PMCAs. A planar representation of the PMCA is shown, including the putative transmembrane topology (TM 1 to 10) and the assignment of important domains. Open rods and black cigar-shaped bars correspond to putative alpha-helices, and arrows denote beta-sheet secondary structural elements. The N-terminal (N 90KD/85KD/81KD,N 76KD) and C-terminal location (C 90KD, C 85KD C 81KD) of the tryptic cleavage sites leading to the production of major proteolytic fragments is also indicated, as are the sites of calpain attack in the presence (Calpl + CaM) and absence (Calpl - CaM) of calmodulin, The site of secondary, calmodulin-independent, calpain attack is labeled 2nd Calp(±CaM). AC, acidic regions flanking the calmodulin binding domain; C,C-terminus;CaM, calmodulin-binding domain consisting of subdomains A and B; N,N-terminus: T.transduction domain;P(S), region containing the serine residue susceptible to phosphorylation by the cAMP-dependent protein kinase; PL. phospholipid-sensitive region. Adapted from Carafoli 1990.



portions of the molecule. The sequence also

Carafoli, 1984). Several studies have shown that demains A and B, have sequence similarities to calmodulin but they have no EF hand structure (Table 5).

Further downstream from the C-terminal end from domain C, there is phosphorylation site for the cAMP-dependent protein kinase (-Arg-Asn-Ser-Ser-1178). Recently, phosphorylation of serine-1175 by this kinase has now been confirmed by protein sequencing (James, Pruschy, Vorherr, Penniston, and Carafoli, 1989). This portion of the molecule contains other regions with a high proportion of Thr and Ser residues and short sequences which fullfill the requirements for acceptor sites for the cAMP-dependent protein kinase. Conclusively, the domain may be thought of as a "regulatory region" with regulation occurring by a number of mechanisms such as calmodulin binding or cAMP directed phosphorylation. The region of Glu 296 and Lys-375 seems to account for greater spacing between the hydrophobic regions of the N-terminal portions of the molecule. The sequence also

TABLE 5

Ca^{2+} pump sequences showing similarities to mammalian calmodulin

1079	EEIPEEELAEDVEEIDHAERE	1099	Domain A
2	DQLTEEQIAEFKEAFSLFDKD	22	Calmodulin
1141	IHNFMTHPEFRIEDSEPHIPLIDDTDAEDD	1170.	Domain B
105	LRHVMTNIGEKITDEEVDEMIREADIDGDG	134	Calmodulin
13	GVKNSLKEANHDGDFGITLAELRALM	38	Domain E
84	EIREAFRVFDKDGNGYISAAELRHVM	109	Calmodulin

Carafoli, 1990

contains two stretches which are very rich in Glu and Lys residues: the first contains a consecutive sequence of 14 residues (residues 296-309) made up entirely by Lys, Glu and an Asp. The second contain, (residues 310-321) from the same region, and this is similar to the loop of Ca^{2+} binding regions of calmodulin, troponin C and parvalbumin i.e. an EF hand type sequence (Kretsniger 1980). Residues 22-23, which is found near the N-terminus of the sequence, is also known to resemble the EF hand sequence, is also known to resemble the EF hand sequence. These regions are called domain D (296-321) and domain E (residues 22-33). Unlike domain A, B, E which have similarities to calmodulin, domain D is not similar to them (Table 5) which shows the sequences of the Ca^{2+} pump that are similar to calmodulin. But, it is shown that domain D and E are similar to the EF hand sequence of calmodulin. Based on the earlier speculation by Verma et al (1988), that domain A binds to domain C, and that domain C, when it binds CaM became free and this can now bind Ca^{2+} with the expected high affinity. Enyedi, Vorherr, James

McCormik, Filoteo, Carafoli, Penniston, 1989) now confirmed that the calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts both with calmodulin and with another part of the pump. These workers also indicated that the C-terminus of the domain C strengthens the domain C-pump interaction perhaps by binding to another part of the molecule. It is speculated that the other part of the molecule might be the domain A, based on the reasons stated earlier, Vorherr, James, Krebs, Enyedi, McCormick, Penniston and Carafoli (1990) further confirmed that indeed, the domain C binds to domain A and thus limiting its accessibility to bind to calcium (Fig. 4a). They based their work on the suggestion made by Klee (1980) that work done on a number of calmodulin regulated enzymes (Benaim et al, 1984; Pearson, Wenttenhall, Means, Hartshore and Kemp, 1988) has shown that the calmodulin binding domain, or a portion of it (Benaim et al, 1984), may function as a natural inhibitor for the enzymes limiting access

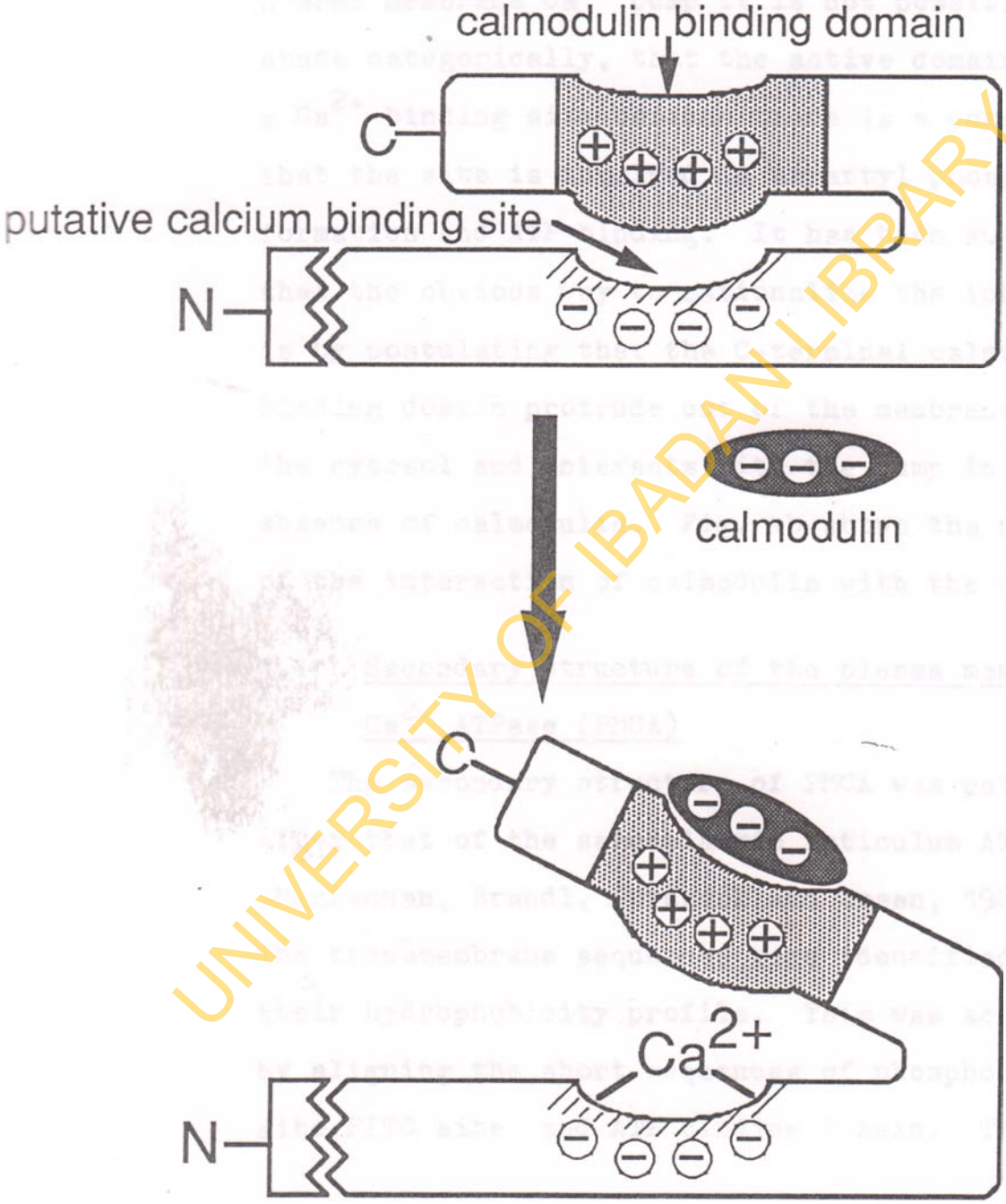


Fig. 4a: Hypothetical scheme of the function of the calmodulin binding domain in the plasma membrane Ca^{2+}-pump. Adapted from Vorherr et al, 1991.

to the active site. However in the case of the plasma membrane Ca^{2+} pump it is not possible to state categorically, that the active domain is a Ca^{2+} binding site because there is a possibility that the site is involved in aspartyl phosphate formation and ATP binding. It has been suggested that the obvious way to rationalize the inhibitions is by postulating that the C-terminal calmodulin binding domain protrude out of the membranes into the cytosol and interacts with the pump in the absence of calmodulin. Fig. 4b shows the model of the interaction of calmodulin with the pump.

1.4 Secondary structure of the plasma membrane Ca^{2+} ATPase (PMCA)

The secondary structure of PMCA was patterned after that of the sarcoplasmic reticulum ATPase (MacLennan, Brandl, Korczark and Green, 1985). The transmembrane sequences were identified by their hydrophobicity profile. This was achieved by aligning the short sequences of phosphorylation site, FITC site and ATP binding domain. The profile

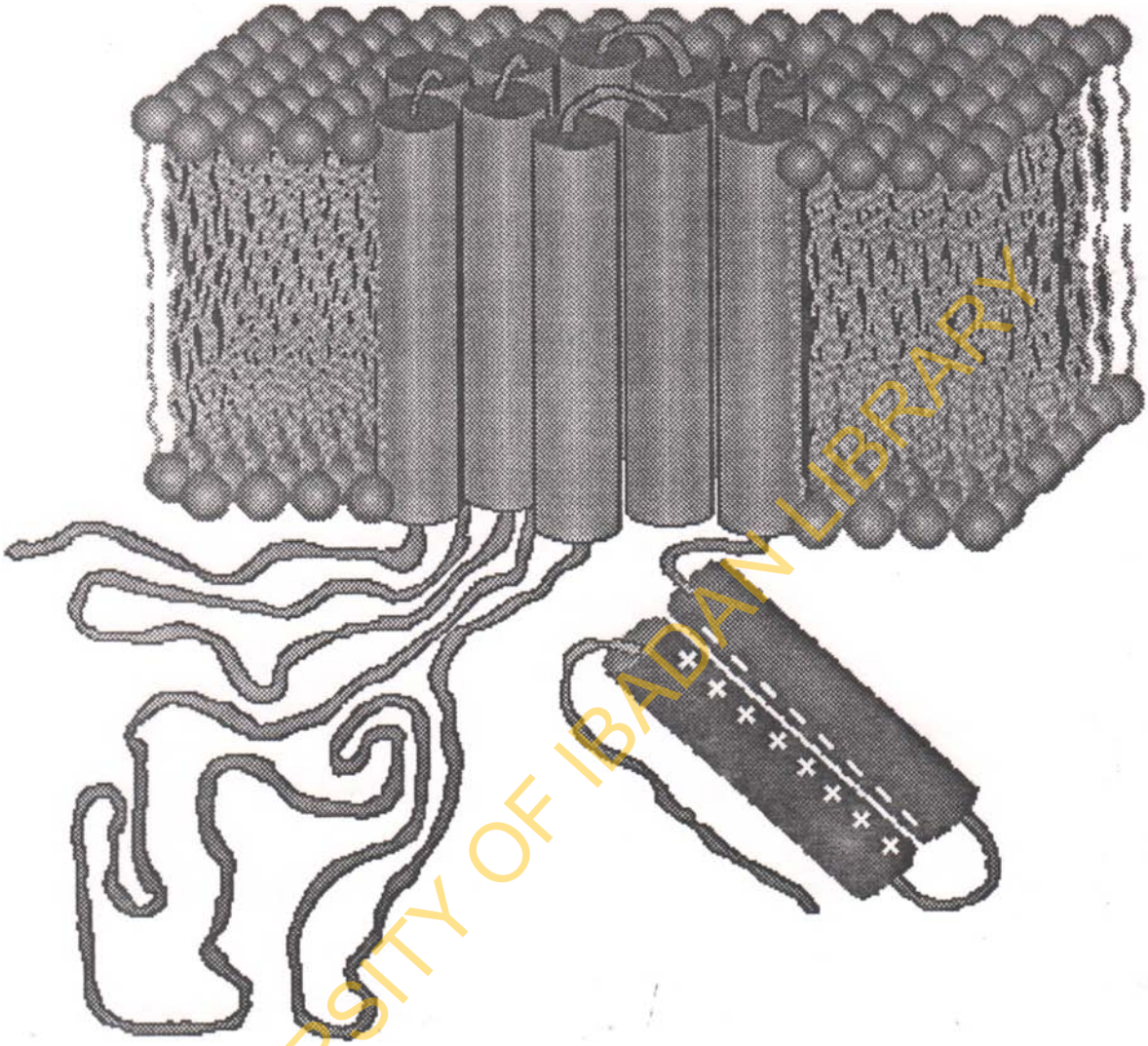
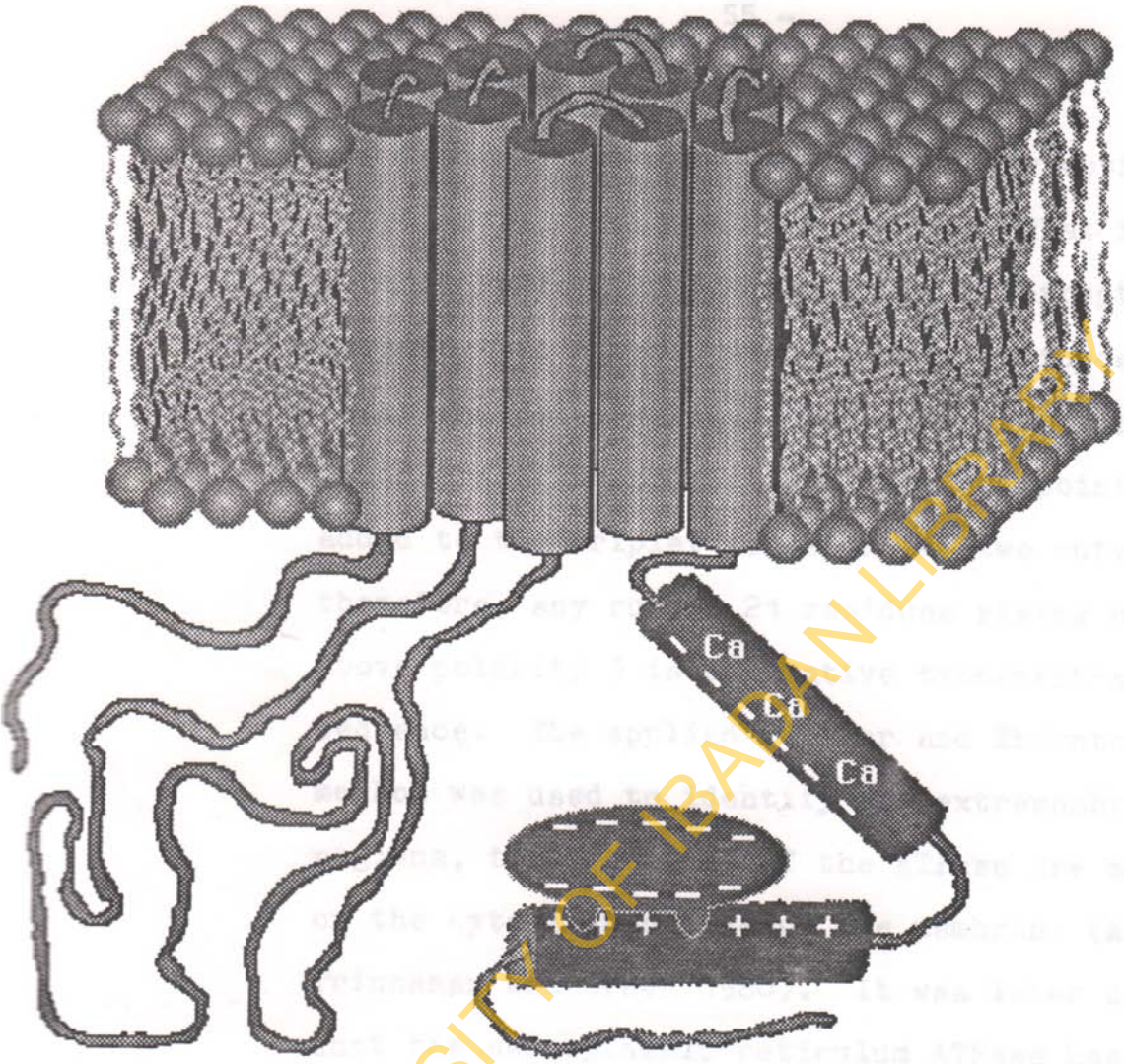


Fig. 4b: A model of the interaction of calmodulin with the plasma membrane Ca^{2+} pump. Adapted from Carafoli, James and Strehler, 1990.



was designed to emphasize polar interruptions of potential transmembrane helices, that is in a small helical bundle, any polar interruption greater than 2 residues is likely to force at least one polar residue out into the hydrophobic lipid phase. For this reason, penalty points are added to the triplet score of the two outer residues therefore, any run of 21 residues rising nowhere above polarity 3 is a putative transmembrane sequence. The applied (Taylor and Thornton 1984) method was used to identify the extramembranous regions, these regions of the ATPase are mainly on the cytoplasmic side of the membrane (Allen, Trinnaman and Green 1980). It was later concluded that the sarcoplasmic reticulum ATPase has the transmembrane domain which consists of the hydrophobic helices M_1-M_{10} (Fig. 5), the stalk of amphipathic connecting helices S_1-S_5; while the phosphorylation and the nucleotide-binding domains are antiparallel β and parallel β sheets respectively, and the remaining sub domain is α-helix. Therefore,

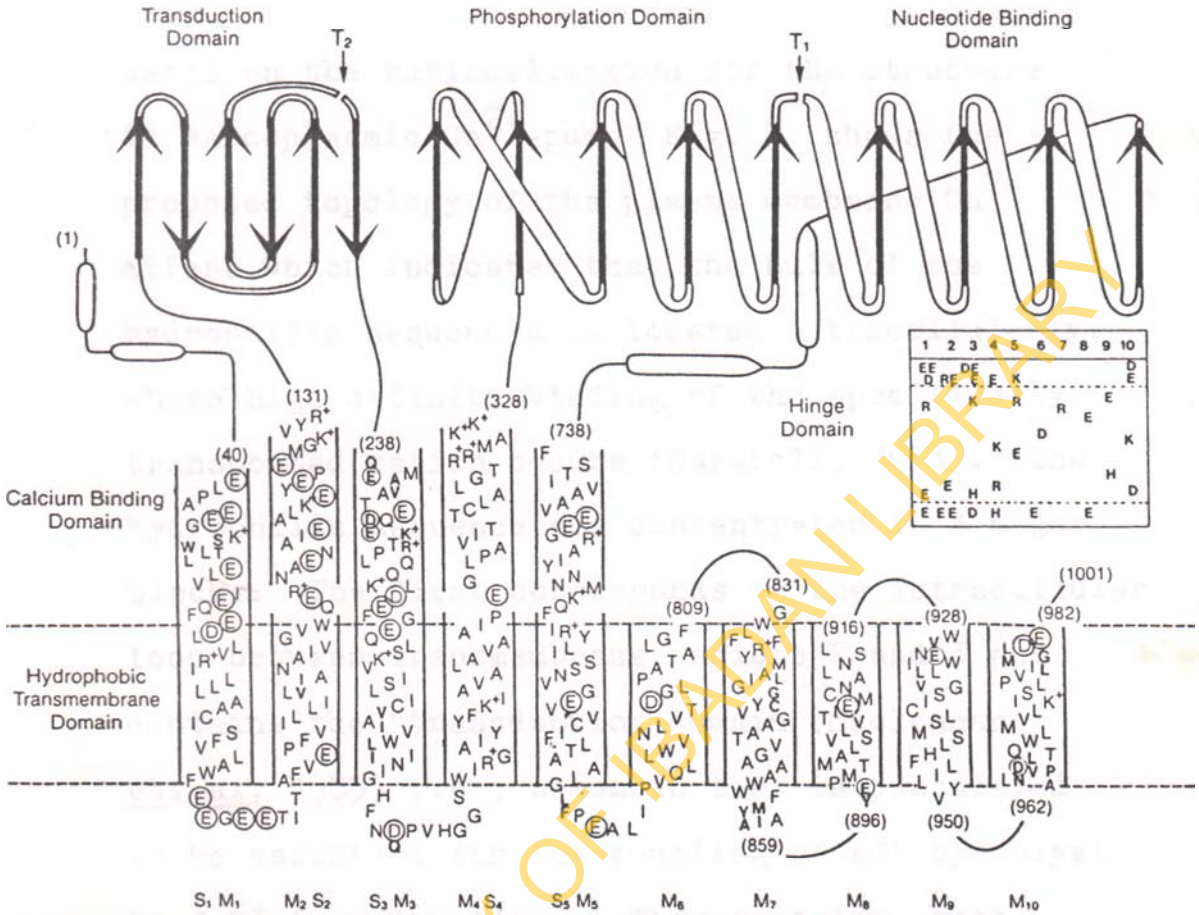


Figure 5. Structural Diagram of the ATPase Molecule Based on Predicted Domain Structure and Hydrophobicity Plots

The detailed arguments for the structure and function assignments shown have been given elsewhere (MacLennan et al., 1985). In the alpha-helical segments of the stalk and the transmembrane region the individual residues are shown on an alpha-helical net. The scale of this section of the diagram is, therefore, larger than that of the globular domains. The folding of the latter is indicated schematically in accordance with the general principles established for antiparallel or parallel beta-sheet domains (Richardson, 1981). The connections between individual beta-strands and alpha-helices are arbitrary since we have not attempted to predict this aspect of the folding pattern. Note the alpha-helical subdomain, which may form a hinge between the phosphorylation and nucleotide binding domain. Both of these domains would interact with the antiparallel beta transduction domain in a trigonal fashion, the planar representation being only a diagrammatic convenience. Similarly, the arrangements of the helices of the stalk and membrane are not planar but would be clustered to provide a channel for translocation of Ca^{2+}. Amino acid differences between the fast twitch and slow twitch Ca^{2+} ATPases are indicated by two residues, the sequence of the slow form being above the fast in boldface type. Acidic residues are circled while basic amino acids are highlighted by plus signs. Inset: location of negative charges (D, E) and positive charges (K, R, H) on the membrane surfaces or within the transmembrane helices. The numbers 1–10 refer to transmembrane helices 1–10.

based on the rationalization for the structure of sarcoplasmic Ca^{2+}-pump Fig. 3 shows the proposed topology of the plasma membrane Ca^{2+} ATPase which indicates that the bulk of the hydrophilic sequences is located intracellularly, where high affinity binding of the specifically transported cation occurs (Carafoli, 1991). The hydrophilic sequence are concentrated in 3 major blocks. The first corresponds to the intracellular loop between transmembrane regions 2 and 3 and contains the "transduction domain (Maclennan et. al, 1985) i.e.; a domain that is postulated to be essential for the coupling of ATP hydrolysis to Ca^{2+} translocation. This contains both -helical and antiparallel B-sheet domains, with one of the -helical domains containing the putative phospholipid responsive sequences (Zvaritch, James, Vorherr, Falchetto, Modynavov and Carafoli 1990). The second block which is the largest contains 430 residues and is located between transmembrane segments 4 and 5 it also contains the "hinge" region, the phosphory-

lation and nucleotide binding domains (MacLennan et al, 1985). This was suggested to have some reasonable degree of flexibility to allow the two catalytic sites to come close in space during the reaction cycle. The third corresponds to the C terminal "regulatory" domain binding site and the cAMP-dependent phosphorylation.

The model also shows that only short stretches of the protein make up the loops between adjoining transmembrane segments and thus facing the extracellular face. It seems probable that this may be responsible for the difficulties encountered in obtaining specific antibodies recognizing extracellular parts of the p-type pumps (Caride, Gorski and Penniston, 1988; Ovchinnikov, Luneva, Arystarkhova, Gevondyan, Arzamazova, Kozhich, Nesmeyanov and Modyanov, 1988). Invariably, there seem to be four transmembrane segments between the N-terminus and the bulk intracellular catalytic/ ATP binding domain (Serano, 1988). The N-terminus is therefore on the cytoplasmic side as the other

major hydrophilic domains (Hennessey and Scarborough (1990). Since, in PMCAs the regulatory domain containing the calmodulin binding and cAMP-dependent phosphorylation site must be located on the cytoplasmic face of the plasma membrane an even number of membrane-spanning regions should be present in the C-terminal half of these molecules (Shull and Greeb, 1988; Verma et al, 1988). It was only speculated that there might be (transmembrane segments in the C-terminal portion of these ion transporters, but workers have shown that, for other P-type pumps, it might be between 2 and 4 transmembrane segments (Serano, 1988; Silver, Nucifora, Chu and Misra, 1989) in fact the C-terminal transmembrane topology of Na^+/K^+ ATPase α-subunits differ tremendously from PMCA pump. Experimental evidence has shown that the C-terminus is on the extra cellular face and it has 3 transmembrane segments in their C-terminal region (Ovchinnikov et al, 1988). Therefore, the precice topology of the C-terminal portion has not yet been solved for any of the P-type ATPases.

1:5 Calcium Pump Isoforms

Elucidation of the primary structure of the Ca^{2+} ATPase of the rat brain plasma membrane (Shull and Greeb, 1988), showed that the pump is structurally similar to that of human erythrocytes (Verma et al, 1988). The two pumps have estimated molecular weights of 138,000 ± 8,000 and stimulated by calmodulin (Hakim, Itano, Verma and Penniston, 1982). However, studies carried out on the cross-reactivity of the rat brain enzyme with antibodies raised against the erythrocyte pump and differences in the degree of calmodulin sensitivity (Hakim et al, 1982), indicated that the brain pump may be a different isoform from that expressed in the erythrocyte. Furthermore, research observations of two distinct vanadate sensitivities in synaptic plasma membrane raises the possibilities of at least 2 isoforms (Michaelis, Kitos, Nunley, Leckiyse and Michaelis 1987).

It has therefore become apparent that the calmodulin-sensitive plasma membrane Ca^{2+} ATPase is

not a single enzyme, but rather consists of a family of enzymes encoded by multiple genes. Table 6 is the summary of all the different isoforms of the Ca^{2+}-pump studied up to date. It seems possible therefore that, tissue-specific differences in the regulation of the Ca^{2+} pump may be due in part to tissue-specific expression of different isoforms of the enzyme. The first isoform of the pump that was discovered in the rat brain is the rat plasma membrane Ca^{2+} ATPase 1a (rPMCA 1a). The pump has 1176 amino acid residues, a molecular weights of 129,500. The second rPMCA 2b consists of 1,198 amino acid residues, it has a molecular weight of 136,605. However, on comparing the two isoforms it shows that each of these enzymes has an even number of transmembrane domains and that their C-termini are located on the cytoplasmic side of the membrane. In addition, an arginine-rich sequence that is highly characteristics of the "A" domain of a calmodulin binding site is located near the C-termini of both isoforms. Therefore this putative A domain is identical in isoforms

TABLE 6

Isoforms of the plasma membrane Ca^{2+}-pump

Isoform[1] designation	Source	Splicing variants[2]	Length in amino acid resedues	Calculated Mr.
PMCA 1	Rat, human	1a [r,h]	1176	129,500
		1b [r,h]	1220	134,700
		1c [h]	1249	137,800
		1d [h]	1258	138,800
		1e [r]	1209	133,500
PMCA 2	Rat, human	2(b) [r]	1198	132,600
		2(f) [h]	1099	121,300
PMCA 3	Rat	3a	1159	127,300
PMCA 4	Human	4a	1170	129,400
		4b	1205	133,900
		4g	1169	130,300
(PMCA 5)	Bovine [8]	5(?)	(71)	(7,700)

(1) Shull and Greeb, 1988; Verma et al 1988).

(r) cDNA from rat source only

(h) cDNA from human source only

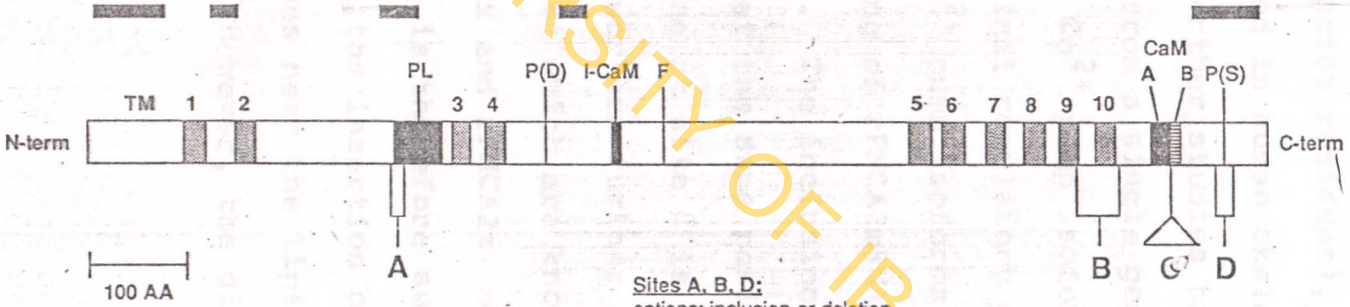
(2) The lettering follows a some what historical pattern of the detection of alternative splicing variants.

1a and 2b but, their B domains and the remaining C-terminal sequences are very different. Furthermore, 3^{1}-untranslated sequences of the isoform 1a transcript have the potential to endode a calmodulin binding B domain and a C-terminus that is very similar to that of isoform 2b. Greeb and Shull (1989) however demonstrated another isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase. This isoform is called rPMCA3a. It has 1159 amino acid residues and has a molecular weight of 127,300. It exhibits 81% and 85% amino acid identity, respectively, to isoforms rPMCA1a and rPMCA 1b. According to the workers the tissue distribution of mRNAs encoding isoforms rPMCA1a, rPMCA1b, rPMCA1c have been determined by Northern blot hybridization analyses. The findings showed that rPMCA2b, rPMCA3a mRNAs exhibit a high degree of tissue specificity. It has also been shown that rPMCA2b mRNAs are expressed predominantly in brain and skeletal tissue.

The isoform from the human teratoma library, however, contains 1220 amino acids and has a molecular

weight of 134,683 as earlier explained by Verma et al (1988). This isoform hPMCA1b appears to correspond to the first rat brain isoform, with which it shares greater than 99% of the first 1,117 residues. The alternative splicing of hPMCA1b (Fig. 6) shows the location of isoform variable regions. It is believed that alternative splicing of hPMCA1b pre-mRNA leads to four different isoforms (Strehler, Strehlerpage, Vogel and Carafoli, 1989). The process of alternative splicing involves an optional 154 base pairs (bp) exon and it results in an mRNA coding for a pump protein with a shorter C-terminal amino acid sequence that lacks a consensus site for phosphorylation by the cAMP-dependent kinase (Strehler et al, 1989), The complete inclusion of this exon leads to PMCA1a (1176 amino acids residue; Mr 129,500), which has already been described in rat brain. Shull and Greeb (1988) whereas exclusion of this exon results in the isoform PMCA1b (1220 amino acids residues; Mr 135,000), which has been isolated from a human teratoma library (Verma et al,

Fig. 6: TM, putative transmembrane domain; PL, putative acidic phospholipid-sensitive region; P(D), site (aspartate residue) of acylphosphate formation. I-CaM "Inhibitory" region that interacts with the calmodulin binding region in the absence of Ca^{2+}/Calmodulin; F, fluorescein isothiocyanate (FITC) binding site (= part of the ATP binding region) CaM, Calmodulin binding region separated into subdomains A and B P(S), Site (serine residue) of phosphorylation by the cAMP-dependent protein kinase.



Sites A, B, D:
options: inclusion or deletion

Site C:
options: full inclusion, partial inclusion, deletion

cDNA examples:

Site A: rPMCA2(b):42 bp deletion
hPMCA2(f):42 bp inclusion

Site B: hPMCA4b: 108 bp inclusion
hPMCA4g: 108 bp deletion

Site C: hPMCA1a: 154 bp inclusion
hPMCA1b: 154 bp deletion
hPMCA1c: 87 bp inclusion
hPMCA1d: 114 bp inclusion

Site D: rPMCA1a: 33 bp inclusion
rPMCA1e: 33 bp deletion

Fig. 6: Domain assignment in the plasma membrane calcium pump and location of isoform variable regions.

1988). Finally, Strehler et al (1989) have suggested that inclusion of 87 nucleotides or 114nt of the 154 bp exon gives rise respectively to PMCA1c (1249 amino acids) and PMCA1d (1258 amino acids residues), these have both been detected in human skeletal muscle.

Further studies have shown that, these mRNAs from a single gene (hPMCA1b) however could encode Ca^{2+} pump isoforms that differ in their C-terminal regulatory domains. More recently, a new Ca^{2+} pump isoforms were generated by alternative splicing of rPMCA2mRNA by Adamo and Penniston (1991). The inclusion or exclusion of 229 base pairs at the same position as the alternative splicing at site C is operative in both genes. These workers further observed that different isoforms which are known as rPMCA2w, rPMCA2y, rPMCA2x and rPMCA2z originate on splicing at site A. It is therefore suggested that the splicing causes the insertion of 0, 14, 31 or 45 amino acid residues near the lipid binding domain of the molecule. However, the distribution of rPMCA2mRNA

varies among the tissues. It has been shown that in brain and heart the rPMCA2z isoform predominates; rPMCA2w is found in kidney and uterus while brain is the only tissue which expresses important amounts or rPMCA2x. These 229 bp inclusions occur in the middle of the calmodulin domain, thus changing the length of this domain and affecting calmodulin and its binding site.

Up to date no other isoform has been found in the case of the Pig smooth muscle, which also has 1220 amino acids. (De-Jaegere, Wuytack, Eggermont Verboomen and Casteels, 1990).

A clear distinction and similarity therefore emerges between rat brain and human teratoma sequences Firstly is the similarity in the catalytic domains (the aspartylphosphate site and secondly is the ATP (FITC) binding site). The so called "hinge" domain (Brandl, Green, Korczak and MacLennan, 1986) that connects the two previous sites permiting them to come close in space during the functional cycle, is also similar and well conserved.

The isoforms also share the same essential topological features i.e. in both species ~ 80% of the total weight of the pump is located outside the membrane domain, protruding into the intracellular space. The major difference appears in C-terminal region, mostly occuring in the second half of the calmodulin-binding domain (Shull and Greeb 1988).

The N-terminal half on the other hand is well conserved and shows absolutely no variation among isoforms and across species (Table 7). Its sequence is LRRGQILWFRGLNRIQTQ (Table 7). It has been shown that calpain can cleave at ...NR... to produce a 125 KDa fragment that is still capable of binding and stimulated by calmodulin (Wang et al 1988); James et al, 1989). This however suggested that the N-terminal half of the calmodulin-binding domain is very important. But at the C-terminal half of the calmodulin-binding sequence, there is a degree of variation only Val and Phe are highly conserved out of the 9 residues.

TABLE 7

Calmodulin-binding domain of various isoforms of the Ca^{2+}-ATPase

Isoforms	Calmodulin Sequences N-terminal half (Subdomain A)	C-terminal half (Subdomain B)	References
rPMCA 1	LRRGQILWFRGLNRIQTQ	IRVVNAFRSS	Schull and Greels 1988.
hPMCA 1	LRRGQILWFRGINRIQTQ	IRVVNAFRSS	Verma et al 1988.
rPMCA 1a	LRRGQILWFRGINRIQTQ	MDVVNAFQST	Shull and Greels, 1988.
hPMCA 1a	LRRGQILWFRGINRIQTQ	MDVVNAFQSG	Verma et al 1988; Strehler et al, 1989.
rPMCA 2	LRRGQILWFRGINRIQTQ	IRVVKAFRSS	Greeb and Shull 1989.
rPMCA 3	LRRGQILWFRGINRIQTQ	IRVVKAFRSS	Greeb and Shull, 1989.
rPMCA 3a	LRRGQILWFRGINRIQTQ	IRVVKAFRSS	Greeb and Shull, 1989.
hPMCA 4	LRRGQILWFRGINRIQTQ	IKVVKAFHSS	Strehler et al 1990.
bPMCA 4a	LRRGQILWFRGINRIQTQ	IDVINTFQTQ	Mann, Brandt, Sisken and Vanaman 1990.
pPMCA 1	LRRGQILWFRGINRIQTQ	IRVVNAFRSS	DeJaegere et al, 1990.

PMCA stands for plasma membrane Ca^{2+}-ATPase. Species origin is denoted by the preceding letter (r-rat, h-human, b-bovine - pig), the number that follows stands for isoforms e.g. rPMCA 1 is human isoform 1; alternative splicing is followed by "a" e.g. rPMCA 1a.

It seems from the foregoing that, highly conserved sequences are likely to represent domains essential for the basic catalytic and transport function and may also reflect specific constraints imposed upon structural elements of the enzyme. In contrast, however, the highly divergent sequences probably specify isoform-specific regulatory and functional specializations of the pump that are adapted to the physiological needs of the tissue/ cell type in which the corresponding enzyme is expressed.

1:6 Mechanism of the Ca^{2+} ATPase pump

The catalytic mechanisms of all P-type ion motive pumps, has been shown to begin with the Ca^{2+} dependent transfer of the terminal phosphate of ATP to the pump, with the formation of a phosphorylated intermediate (Katz and Blostein 1975; Knauf et al 1974). The mechanisms of the Ca^{2+} ATPase is similar to that of all other P-type phosphorylated intermediate formed during its catalytic cycle. (Pederson and Carafoli 1987a, b). It has been shown that the Ca^{2+} dependent phosphorylation of the

membrane bound pump is very rapid (Rega and Garrahan 1975; Garrahan and Rega, 1978; Schatzmann and Burgin, 1978) thus allowing the differentiation of the Ca^{2+}-ATPase from other phosphorylatable proteins of the erythrocyte membrane. Several workers have established the principla characteristics of the subsequent transport cycle which can be summarized in the scheme presented in Fig. 7 (Richards, Rega and Garrahan, 1978; Szasz, Hasitz, Sarkadi and Gardos 1978; Burgin and Schatzmann, 1979; Muallem and Karlish, 1979; 1980; Rega and Garrahan, 1986 and Adamo, Rega and Garrahan, 1988).

Rega and Garrahan (1986) showed that E_1 is the only conformer of the enzyme that catalyzes rapid phosphorylation from ATP. Several group of workers, such as Krebs, Vasak, Scarpa and Carafoli (1987) in the experiment on the mechanisms of this enzyme, showed that the membrane-bound enzyme undergoes E_1- E_2 conformational transitions during the catalytic cycle and that the equilibrum between these conformational forms is influenced by various

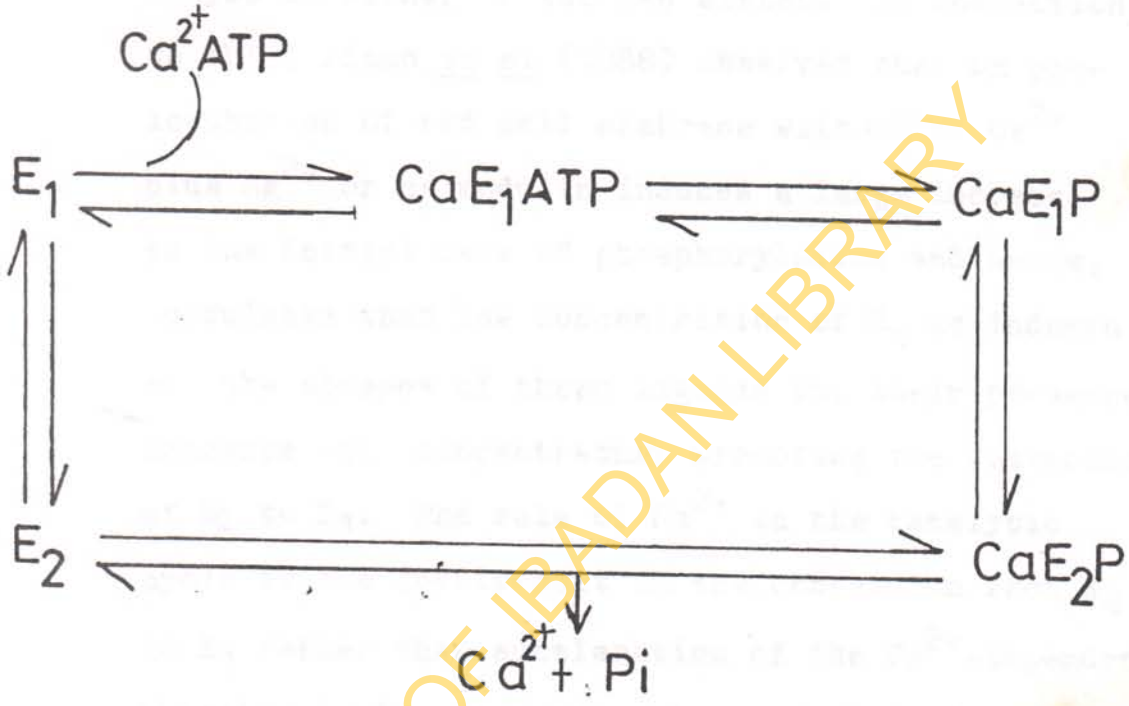


Fig. 7. Scheme for $(Ca^{2+}+Mg^{2+})$ ATPase transport cycle

ligands which preferentially react with the enzyme in either of the two states. In connection to this, Adamo et al (1988) observed that on pre-incubation of red cell membrane with either Ca^{2+} plus Mg^{2+} or calmodulin induces a large increase in the initial rate of phosphorylation and hence, postulated that low concentration of E_1 is induced by the absence of these ligands but their presence increase its concentration promoting the conversion of E_2 to E_1. The role of Mg^{2+} in the catalytic cycle is its involvement in the conversion from E_2 to E_1 rather than acceleration of the Ca^{2+}-dependent phosphorylation as previously suggested (Garrahan and Rega, 1978; Adamo et al, 1988). Furthermore the E_1 form of the membrane-bound and purified enzyme is shown to be stabilized by Ca^{2+} ions (Krebs et al, 1987; Wrzosek, Famulski, Lehosky and Pikila, 1989) while in the absence of Ca^{2+}, orthovanadate (Krebs et al 1987) or EGTA (Wrzosek et al, 1989) shifts the conformational equilibrum to and stabilizes the E_2 form. In addition, Wrzosek and his group in

the same year, were able to used circular dichroism and steady-state fluorescence methods to demonstrate that the E_1-E_2 transition of the purified enzyme involves a relatively major refolding of the polypeptide chain. These workers, concluded from their data that the change in conformation of the enzyme differ because of the interaction of this enzyme with calmodulin or phosphatidyl serine (PS)

Based on the hypothesis that only E_1 catalyses phosphorylation and that in the absence of ligands, near 90% of the Ca^{2+}-ATPase is in the E_2 conformation, Adamo et al (1990) estimated the relative abundance of the two conformers of the enzyme and the rates of their interconversion by measuring the initial velocity of phosphorylation of the enzyme at 37°C. These workers, suggested that in intact membranes, Ca^{2+}-stabilized E_1 possibly through Ca^{2+}-transport sites and that the Ca^{2+}-induced E_2-E_1 transition was strongly accelerated by Mg^{2+}. The reaction cycle starts all over again.

1:7 Modes of activation of plasma membrane Ca^{2+}-pump

Work during the last several years has shown that the plasma membrane Ca^{2+}-pump, which is one of the targets of calmodulin activation (Gopinath and Vinzenci, 1977) can also be activated by a number of alternative treatments (Table 8). These include the effect of acidic phospholipids and polyunsaturated fatty acids (Niggli et al, 1981), controlled proteolytic treatment with a number of proteases Zurgni et al, 1984; Benaim et al, 1984), intracellular Ca^{2+} dependent protease calpain (Wang et al, 1988; James et al 1989) and phosphorylation by the cAMP-dependent protein kinase (Caroni and Carafoli, 1981; Neyses, Reinlib, and Carafoli, 1985). Protein kinase C has also been shown to activate the pump (Smallwood, Gugi and Rasmussen, 1988). More recently it has been shown that the pump can be activated by oligomerization (Kosk-Kosicka, Bzdega and Johnson, 1990).

TABLE 8

Effects of various modulators on the plasma membrane Ca^{2+}-ATPase

Activator	Effects	References
Calmodulin	Increase Vmax decrease K(Ca) 0.5 to 0.4-0.5 µM	Gopinath and Vincenzi (1977); Jarrett and Penniston; (1977) Niggli et al, 1981; Villalobo et al 1986
Acidic phospholipid	Increase Vmax decrease $K_{0.5}$(Ca) to 0.2µM become insentive to CaM.	Niggli et al, 1981; Papp et al 1989 Enyedi et al 1987
Protein kinase A	Increase Vmax decrease $K_{0.5}$(Ca) to 2µM remain sensitive to CaM.	Caroni and Carafoli 1981; Dixon and Haynes; 1989; James et al 1989.
Calpain	Increase Vmax decrease $K_{0.5}$(Ca) to 0.4-0.7µM become insensitive to CaM.	Wang et al 1989; 1988 Au 1987; James et al 1989.

Activator	Effects	References
Protein kinase C	Increase Vmax $K_{0.5}$(Ca) unchanged remain sensitive CaM.	Smallwood et al 1988.
Oligomerization	Increase Vmax decrease $K_{0.5}$ (Ca) become insensitive to CaM.	Kosk-Kosicka, Bzdega and Johnson 1990; Vorherr, Kessler, Hofmann and Carafoli 1991.

1:8 Modulation of Ca^{2+}-ATPase by calmodulin

The activation of the PMCA dates back to 1973 when Scharff and Schatzmann (1973) demonstrated that the specific activity was depressed in erythrocyte membrane isolated from EDTA concentration buffers in compared to membranes isolated in the presence of calcium. Based on these findings (Scharff and Foder 1977) proposed that the Ca^{2+}-pump exists in 2 conformations A and B. The former is believed to be induced by the presence of chelators in the medium. The state of conformation A is characterized by low Ca^{2+} affinity and transport rate. State B is thought to be induced by the presence of micromolar (μM) concentration with a high affinity and transport rate. It became obvious that the transition from A to B is as a result of the removal of an activator (Farrance and Vincenzi, 1977; Hanahan, Taverna, Flynn and Ekolm, 1978; Scharff and Foder, 1978) when it was discovered that the erythrocyte cytosol contained a protein activator of the pump (Bond and Clough, 1972). Jarret and Penniston (1977) and

Gopinath and Vincenzi (1977) demonstrated that it is calmodulin. The protein is a single polypeptide with a molecular weight of 16,700, has 148 amino acids, thermal stable and acidic in nature. Some 30 percent of its amino acids consist of aspartate and glutamate, this accounts for the ionic strength (PI) of 4.3. The protein also contains no cysteine, hydroxyproline or tryptophan, but it does contain a trimethylated lysine at position 115. A tertiary structure highly flexible to interact with its receptor proteins has been attributed to lack of cystine and hydroxy proline/calmodulin displays a distinctive ultra violet absorption pattern with five peaks at 253, 259, 365, 269 and 277 nm and a shoulder at 282 nm probably because of the high ratio of phenylalaline (eight residues) to thyrosine (two residues). The protein has now been isolated and purified from other varieties of sources including invertebrate and vertebrate and animal species as well as from both lower and higher plants. (Table 9). In addition, the chain of calmodulin has 4 domains (No. 1-1V) each binding

TABLE 9

Representative organisms from which calmodulin has been isolated.

Organisms	Classification	References
A) Plants spinach	Angiospermae	Watterson, Sharief and Vanaman 1980
Peanuts	Angiospermae	Anderson Charbonneau, Jones, McCann and Cormier 1980.
Chlamydomonas	Chlorophyta	Gitelman and Witman 1980.
B) Lower Animals		
Amoeba	Protozoa	Kuznicki, Kuznicki and Drabikowski, 1979.
Englena	Protozoa	Kuznicki et al 1979.
Tetrahymena	Protozoa	Jamienson et al 1979.
Sea anemone	Coelenterata	Yazawa Sakuma and Yagl 1980.
Renilla reniformis (Sea pansy)	Coelenterata	Jones, Besch, Fleming, McConnaughey and Watanabe 1979.
Sea Urchin	Echinodermata	Garbers, Hansbrough, Radany, Hyne and Kopf 1980.

Organisms	Classification	References
Higher Animals		
Cow	Mammalia	Wang and Desai 1976; Watterson et al, 1980.
Human	Mammalia	Jarrett and Penniston 1978.
Pig	Mammalia	Yazawa et al, 1980.
Rabbit	Mammalia	Nairin and Perry, 1979.
Rat	Mammalia	Dedman, Jakson Schreiber and Means 1978.
Sheep	Mammalia	Autric, Ferraz, Kilhoffer, and Demaltte 1980.

one Ca^{2+} ion and the four Ca^{2+}-binding sites have a dissociation constant ranging from 4 to 18 µM. (Lin, Liu and Cheung, 1974). Each binding site is a loop containing aspartate and glutamate side chains that form ionic bonds with Ca^{2+}. Also, the oxygen atoms on the side chains of threonine, serine, thyrosine and asparagine residues also participate in Ca^{2+} binding. The structure of calmodulin is called α-helix E Ca^{2+} binding-loop-α-helix F where the Ca^{2+} binding loop is the EF hand. Fig. 8 shows the sequence of bovine brain calmodulin.

Calmodulin itself is not active, its active form is the calmodulin Ca^{2+} complex (Lin et al 1974). Consequently, once it is bound to Ca^{2+}, calmodulin assumes a more helical conformation to become the active species, which binds reversibly to the apoenzyme resulting in the formation of an active holoenzyme.

Calmodulin + Ca^{2+} ⟺ (Calmodulin*. Ca^{2+})

$(E)_{less\ active}$ + (Calmodulin*. Ca^{2+})active

⟺ (E) (Calmodulin* Ca^{2+})active

(Lin et al, 1974).

Over the years, calmodulin has been found to be involved in many cellular functions including enzyme regulation, regulation of cell cycle and calcium transport across plasma membranes (Table 10.) However, the general interpretation therefore is that the effect of calmodulin on the rate of the pump is the increase of its turnover; Calmodulin has also been found to stimulate both the rate of phosphorylation of the pump and that of its dephosphorylation (Jeffery, Roufogalis and Katz 1981, Luthra, Watts, Scherer and Kim 1980; Muallem and Karlish, 1980).

TABLE 10

Calmodulin mediated enzymes and processes

Enzyme/Process	References
Cyclic nucleotide phosphodiesterase	Cheung, 1970.
Adenylate cyclase	Brostrom et al, 1977.
Myosin light-chain kinase	Wolff, Cook, Goldhammer, and Berkowctz 1980; Walsh, Cavadore, Vallet and Denaille 1980; Nairn and Perry, 1979.
$(Ca^{2+} + Mg^{2+})$-ATPase	Jarrett and Penniston, 1978.
Ca^{2+}-transport	Larsen and Vincenzi 1979.
Phosphorylase kinase	Cohen, Cohen, Shenolikai Nairn and Victor, 1979.
NAD^{+} Kinase	Anderson et al, 1980.
Phospholipase A_2	Wong and Cheung, 1979.
Microtubule assembly and disassembly	Welsh Dedman, Brinkley and Means, 1978, 1979.
Neurotransmitter release	Grab, Berzins, Cohen, and Siekevitz, 1979
Cell cycle regulation	Chafouleas, Wade, Hiroyos, Aubrey and Antheny 1982.

Further studies have shown, that the tryptic cleavage of calmodulin in the presence of Ca^{2+} results in two main fragments which have been identified by analysis of the amino acid composition as 1-77 and 78-148 (Guerini, Krebs, and Carafoli, 1984) while, only fragments 78-148 and 1-106 are still able to stimulate the purified Ca^{2+}-ATPase of erythrocytes. This fragment cannot stimulate the calmodulin-dependent cyclic nucleotide phosphodiesterase (Newton, Oldewurtel, Krinks, Shiloacl and , Klee, 1984). This study shows however the importance of the carboxyl-terminal half of calmodulin and especially of Ca^{2+}-binding region III (Fig.8) in the interaction of calmodulin with the Ca^{2+} ATPase and provides clear evidence that calmodulin interacts differently with different targets (Guerini et al 1984). In the presence of Ca^{2+}, calmodulin stimulate the Ca^{2+} ATPase activity of human red cell membranes, as well as active Ca^{2+} transport by membrane vesicles (Bond and Clough 1973; Gopinath and Vincenzi 1977; Larsen and Vincenzi 1979). Similarly, it appears that, one calmodulin

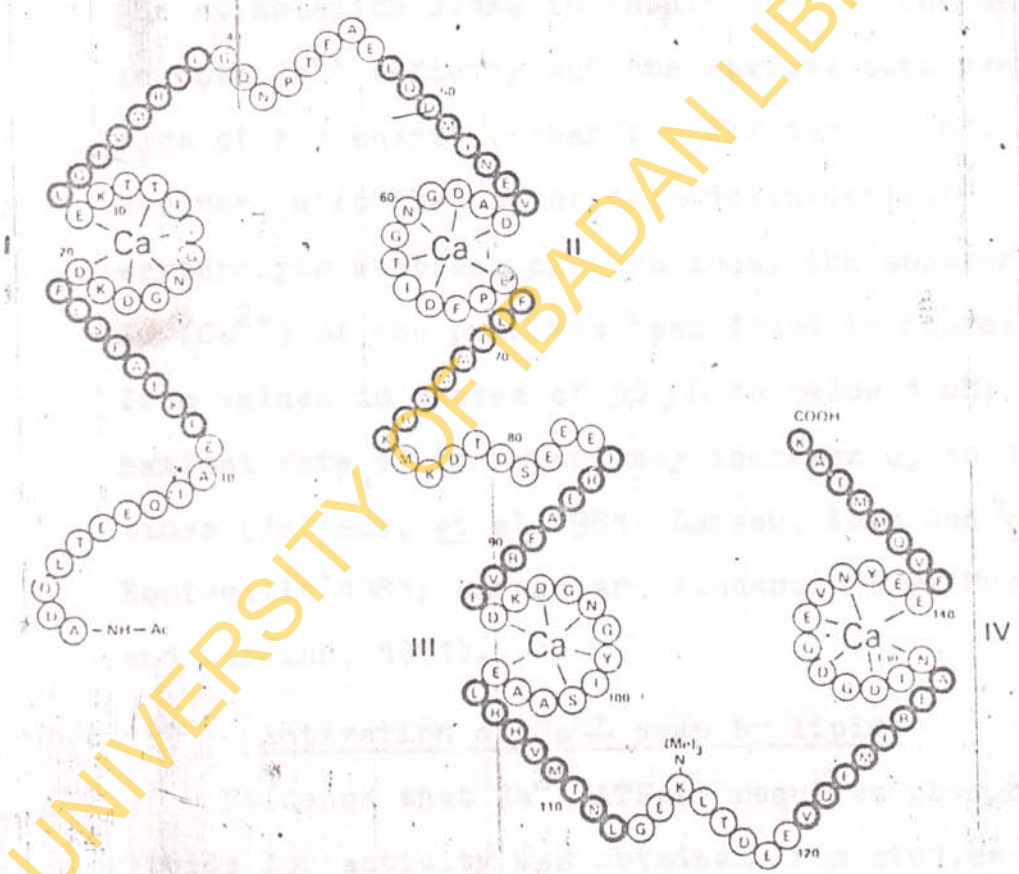


Fig.8: Sequence of calmodulin. The sequence of bovine brain calmodulin (Watterson *et al.*, 1980) is shown utilizing the one-letter code for amino acid residues. A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr. The four proposed Ca^{2+}-binding domains (see text) with the stretches of α-helix (darker circles) are indicated in Roman figures I-IV.

binds per ATPase polypeptide (Graf and Penniston 1981), although this appears to be the case in most studies (Hinds and Andreasen 1981), a 2:1 calmodulin-to-pump binding ratio has also been found in studies using the purified erythrocyte enzyme (Zurini, et al, 1984). The stimulation seems to result from an increase in both Ca^{2+} affinity and the maximal turn over rate of the enzyme (Scharff and Foder, 1978). However, with the use of calmodulin-depleted erythrocyte membrane preparations, the apparent Km (Ca^{2+}) of the pump has been found to decrease from values in excess of 30 µM to below 1 µM; the maximal rate of transport may increase up to 10 times (Jeffery, et al 1981; Larsen, Katz and Roufogalis 1981; Larsen and Vincenzi 1979; Muallem and Karlish, 1981).

1:9 Activation of Ca^{2+} pump by lipids

Evidence that Ca^{2+}-ATPase requires phospholipids for activity was obtained from studies on delipidated membrane Ca^{2+} pump (Roelofsen and

Schatzmann, 1977; Ronner, Gazzotti and Carafoli, 1977, Niggli et al 1981). It was shown that calmodulin is not unique as an activator of the enzyme. Like in calmodulin stimulation the transition from a low to the high Ca^{2+}-affinity state can be induced, by a variety of acidic phospholipids (Phosphatidyl serine, phosphatidyl inositol and phosphatidic acid). These phospholipids increase the V_{max} and decrease the Km for Ca^{2+} of the isolated enzyme to the same extent as calmodulin. Furthermore it was shown that, the activation by acidic phospholipids was due mainly to an increase of the Ca^{2+} sensitivity of the pump with smaller effects on the turn over of the enzyme Niggli et al 1981; Aljobore and Roufogalis, 1981) since a similar increase in Ca^{2+} sensitivity is seen in the activation by calmodulin. It was suggested therefore that the activators are amphiphilic in nature, since both contain acidic groups and a hydrophobic component (Gietzen, Sadorf and Bader, 1982). Also, the activation by acidic phospholipid is kinetically indistinguishable from the activation by calmodulin

(Niggli et al 1981; AlJobore and Roufogalis 1981) and the molar ratio of phospholipid to Ca^{2+} transport ATPase is considerably higher than that for an equivalent activation by calmodulin (Niggli et al 1981). Based on these observations it was suggested that the mode of binding of these two activators are not identical.

Further experiments showed that, the stimulation of the Ca^{2+} ATPase activity by negatively charged phospholipids is based on a binding of these lipids to the Ca^{2+} ATPase and that the negative charges are a major modulatory factor for this interaction (Verbist, Theodurus, Gadella, Racymaekers, Wuytack, Wirtz and Casteels, 1991). However, it has been shown that the actual position of the lipid regulatory domain may be tentatively placed at the NH_2-terminal 5KDa part of the 81 KDa tryptic fragments (Papp, et al 1989) as suggested by the change in the transport kinetics, and also by the large number of positively charged residues in this area in the amino acid sequence of the plasma membrane calcium pump (Verma et al, 1988). More

recent work has shown that, the site of phospholipids interaction is at the C-terminal portion of the highly charged stretch of 44 amino acid residues separating the N-terminus of the fragment of 76 KDa from those of the 81, 85 and 90 KDas (Zvaritch et al 1990). Further studies have shown that, there is another domain for phospholipid interaction apart from the one stated by Zvaritch et al 1990); the other domain, is at the calmodulin binding domain, (Brodin et al, 1991).

On the other hand, there are some lipids that inhibit the enzyme, such as oleic acid which was shown to competitively inhibit calmodulin activation of red cell ghost enzyme and compete with calmodulin binding (Wetzker, Klinger and Frunder, 1983) although this does not preclude competition at an allosteric site or at overlapping site.

1:10 Activation of the purified Ca^{2+}-ATPase of the erythrocyte membrane by controlled proteolysis

The stimulation of the membrane bound calcium pump by proteolysis was first shown by Taverna and Hanahan (1980) in which they used chymotrypsin and trypsin on isolated erythrocyte membranes. However, a detailed study of the phenomenon was performed shortly thereafter on inverted erythrocyte vesicles by Sarkadi, Enyedi and Gardos (1980). They were also able to show that trypsin mimicked the effects of calmodulin. It was observed that the activated pump was no longer stimulated by calmodulin. Further studies have indicated that the treatment reduced the steady state level of the phosphorylation in the absence of Mg^{2+}.

Stieger and Schatzmann (1981) have shown that the activation by trypsin corresponds to a decrease of the $K_m(Ca^{2+})$ of the pump to a level that is much lower than that of calmodulin. Moreover, when it

is maximally activated, the pump became fragmented into a number of products, the most prominent having molecular weights of ~ 100, KDa and 40 KDa. Fragments in 1981 Stieger and Schatzmann suggested that the calmodulin receptor was the fragment of ~ 40 KDa that was removed from the main body of the pump, because there was a loss of calmodulin sensitivity and this confirmed tha work done by Enyedi, Sarkadi, Szasz,Bot and Gardos (1980). Zurini,Krebs, Penniston and Carafoli (1984), did a comprehensive study on the controlled tryptic fragmentation of the purified erythrocyte enzyme and this has led to a rapid fragmentation of approximate molecular weights of 90 KDa, 81 KDa and 76 KDa. fragments it was shown that I^{125} lodoazido calmodulin crosslinked to the 90 KDa fragment product, thus showing that calmodulin interacting domain was contained in a fragment of 9 KDa fragment removed from the 90 KDa product as proteolysis reduced it to 81 KDa fragment. This is in contrary to the study of (Stieger and Schatzmann 1981) which indicated that about 40 KDa fragment is removed. It also showed that

the treatment almost immediately removed from the enzyme a fragment of molecular weight ~ 33 KDa which interacted better than any other of the cleavage products with the hydrophobic probe M- ^{125} Iodophenyl diazirine. This fragment was suggested to contain hydrophobic intramembrane regions of the pump. The acylphosphate intermediate was not formed by products having molecular weights lower than 76 KDa fragment. A product of 48 KDa fragment which most likely was derived from the 76 KDa fragment bound a radioactive ATP analogue and was thus suggested to contain the ATP binding site.

Benaim, Zurini and Carafoli (1984) were able to add significant information to the organization of the pump, this is as a result of the milder conditions they used. They observed that an additional 85 KDa fragment and activitymeasurements revealed it to be less responsive to calmodulin than the product of molecular weight 90 KDa fragment (Benaim, Clark and Carafoli, 1986; Benaim et al, 1984). However, these two different proteolysis conditions seemed to promote the E_1 and E_2 conformations. The

prteolysis scheme is shown in Fig. 9. Furthermore, and based on the work of Benaim et al (1986), it was possible to deduce that the products 90 KDa and 85 KDa fragments bound calmodulin,wwhereas the product of 81 KDa fragment did not bind calmodulin. It was also shown that the product of 76 KDa had higher activity in the absence of calmodulin. The 90 KDa fragment responded normally to calmodulin the 85 KDa fragment was less able to do so, despite the fact that it could still bind it. It seems therefore probable that the calmodulin binding domain acts as an inhibitory sequence i.e. it would bind to a domain in the pump that is essential for full action as earlier explained. Zvaritch, et al (1990) were able to give a conclusive support for the trypsin proteolysis scheme in (Fig. 9). Trypsin proteolysis has also been used to map the functional domains in the plasma membrane Ca^{2+}-pump (Fig. 10). This showed that the 90 KDa fragment produced the C-terminal sequence ASK, and it corresponds to residue 1161 in the structure of human plasma membrane calcium ATPase 4 (hPMCA 4) isoform and

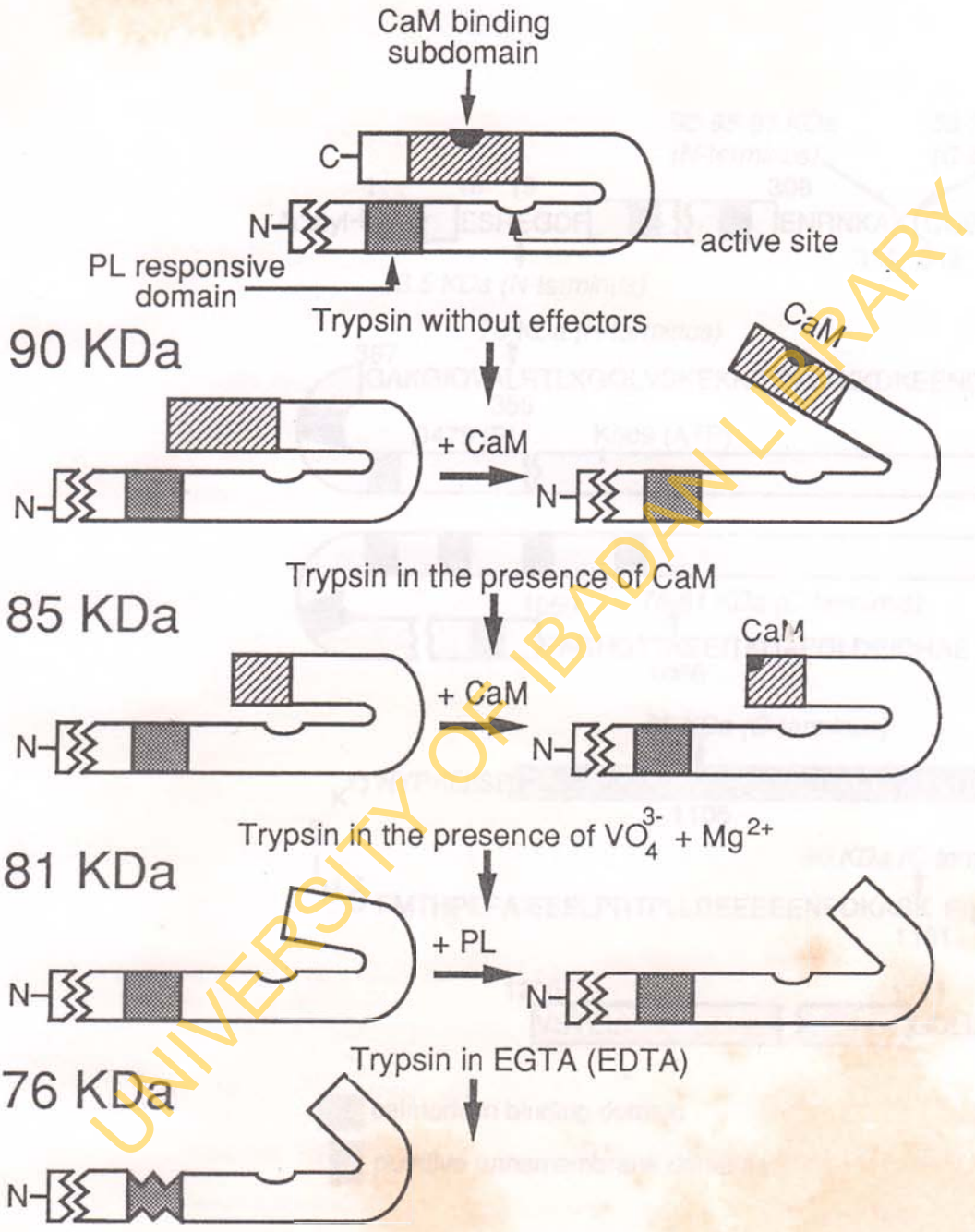


Fig. 9: Scheme for trypsin proteolysis of purified Ca^{2+}-pump in the presence of different effectors. Adapted from Carafoli, 1991.

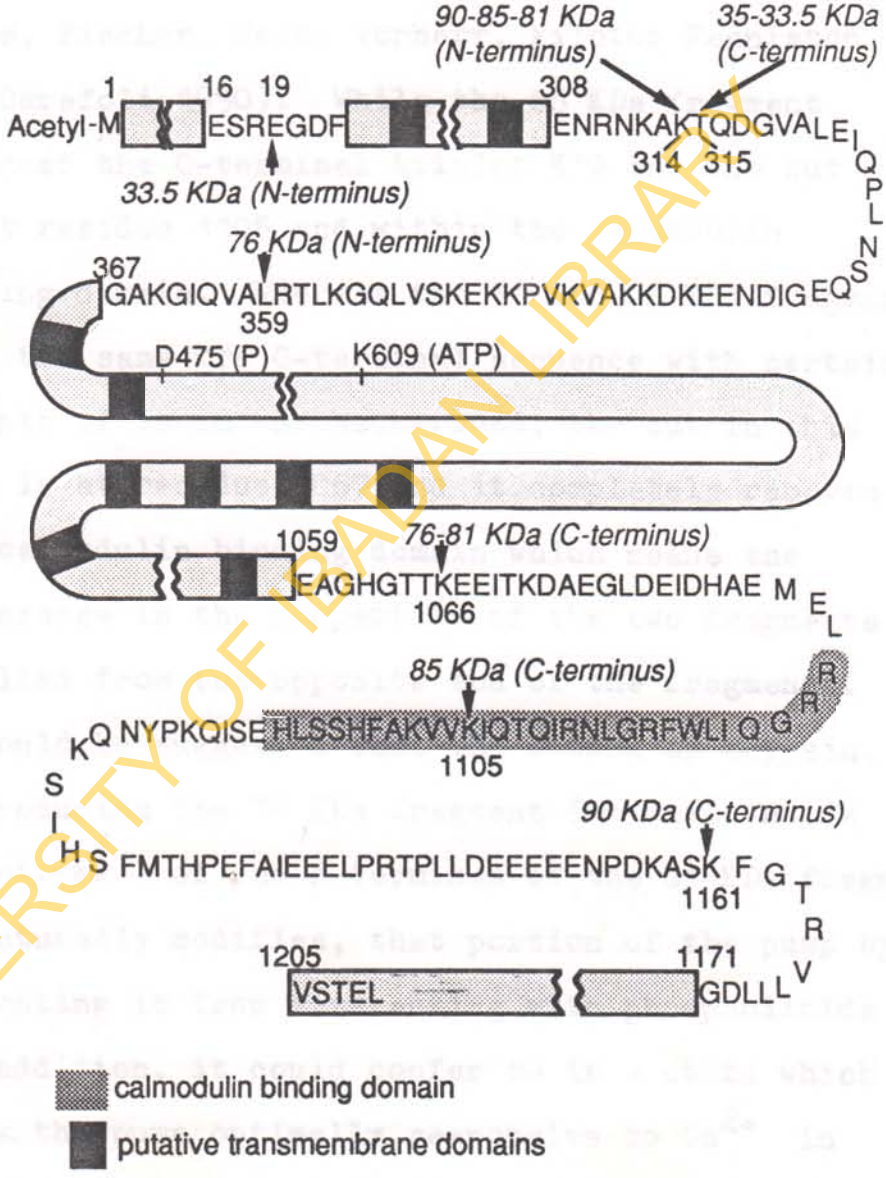


Fig. 10: Location of NH_2 and COOH termini of tryptic fragments of molecular masses 90, 81 and 76 KDa. Adapted from Carafoli, 1991.

the cut occurs just outside the calmodulin binding domain (Verma et al, 1988; Strehler James, Fischer, Heim, Vorherr, Filoteo Penniston and Carafoli 1990). While the 85 KDa fragment produced the C-terminal triplet K1Q and the cut is at residue 1105 and within the calmodulin binding domain. Whereas the 81 and 76 KDa fragments gave the same TTK C-terminal sequence with certain amounts of it in the background, the cut in this case is at residue 1067 and it completely removes the calmodulin binding domain which means the difference in the properties of the two fragments resulted from the opposite end of the fragments. It could be suggested that the attack by trypsin, in producing the 76 KDa fragment (44 amino acids downstream of the N-terminus of the 81-KDa fragment), structurally modifies, that portion of the pump by preventing it from interacting with phospholipids "In addition, it could confer to it a state which makes the pump optimally responsive to Ca^{2+} in the absence of phospholipids. Subsequently, the N-terminal sequence of the functionally inactive,

tryptic fragments of 35 and 33.5 KDa were also determined (Zvaritch et al, 1990).

However, in the case of the 90 KDa fragment the cut was at position 315 while the 85 and 81 KDa fragments coincided with that of the 90 KDa fragment and the region is rich in basic residues, their cuts wereeat positions 338 and 348. But in the case of the 76 KDa fragment this occurred further downstream in the human isoform of the pump (Zvaritch et al, 1990) and the cut is at leucine 359 while the cut 33 KDa and 35.5 KDa fragments were at residue 19. It is not known, whether trypsin attack between putative transmembrane helices 2 and 3 i.e., the fragments of 33.5 - 35 KDa are removed from the main body of the pump in the absence of SDS. However, it was suggested that the properties of the products resulting from cleavages between transmembrane helices 2 and 3 reflect those of the entire sequence of the enzyme from the N-terminus to the cleavage point(s) in the hydrophilic domain protruding from the 10th putative transmembrane helix.

1.11 Modulation of Ca^{2+}-ATPase by calpain

It is well established that, calcium dependent neutral cysteine proteases, collectively called calpain, regulate cellular functions by limited proteolysis of specific proteins. For instance the protease is involved in the degradation of myofibrillar protein (Dayton, Goll, Zeece, Robson and Reville 1973), activation of protein kinase C (Melloni, Pontremoli, Michetti, Sacco, Sparatore and Horeckes 1986), limited proteolysis of erythrocyte membrane cytoskeleton proteins (Lang, Wickenden, Wunne and Lucy), degradation of B-hemoglobin in erythrocytes (Pontremoli, Melloni, Sparatore, Michetti and Horecker 1984) and the modification of calmodulin-binding proteins Wang, Villalobo and Roufogalis (1989).

This protease is present as an inactive proenzyme (procalpain) which can be converted through Ca^{2+}-dependent autoproteolysis to a mature form (Calpain fully active at micromolar Ca^{2+}-concentration.

(Pontremoli and Melloni, 1986). The procalpain is composed of a catalytic 80 KDa subunit and a 30KDa subunit (Pontremoli and Melloni, 1986). It was shown that, the primary structures of both human subunits have been deduced from the nucleotide sequences of cDNA (Suzuki, 1987) and found to contain, distinct domains. (Fig. 11) shows the domain structure of calpain. In the case of the 80KDa it is composed of four domains (I, II, III, IV) whereas the 30KDa subunit has two (V and IV). Domains IV and IV' are homologue and have four consecutive EF hands (Kretsinger, 1975).

Two different forms of calpain have been demonstrated. The characterization of these forms was based on their Ca^{2+} sensitivities (Suzuki 1987; Pontremoli, Melloni, Murachi 1983). Calpain 1 and Calpain 2 are active only at micromolar and milliomolar. Independently Au (1987) and Wang et al (1988), found that treatment of the plasma membrane-bound Ca^{2+}-ATPase with calpain 1 activated the Ca^{2+}-ATPase activity in an irreversible

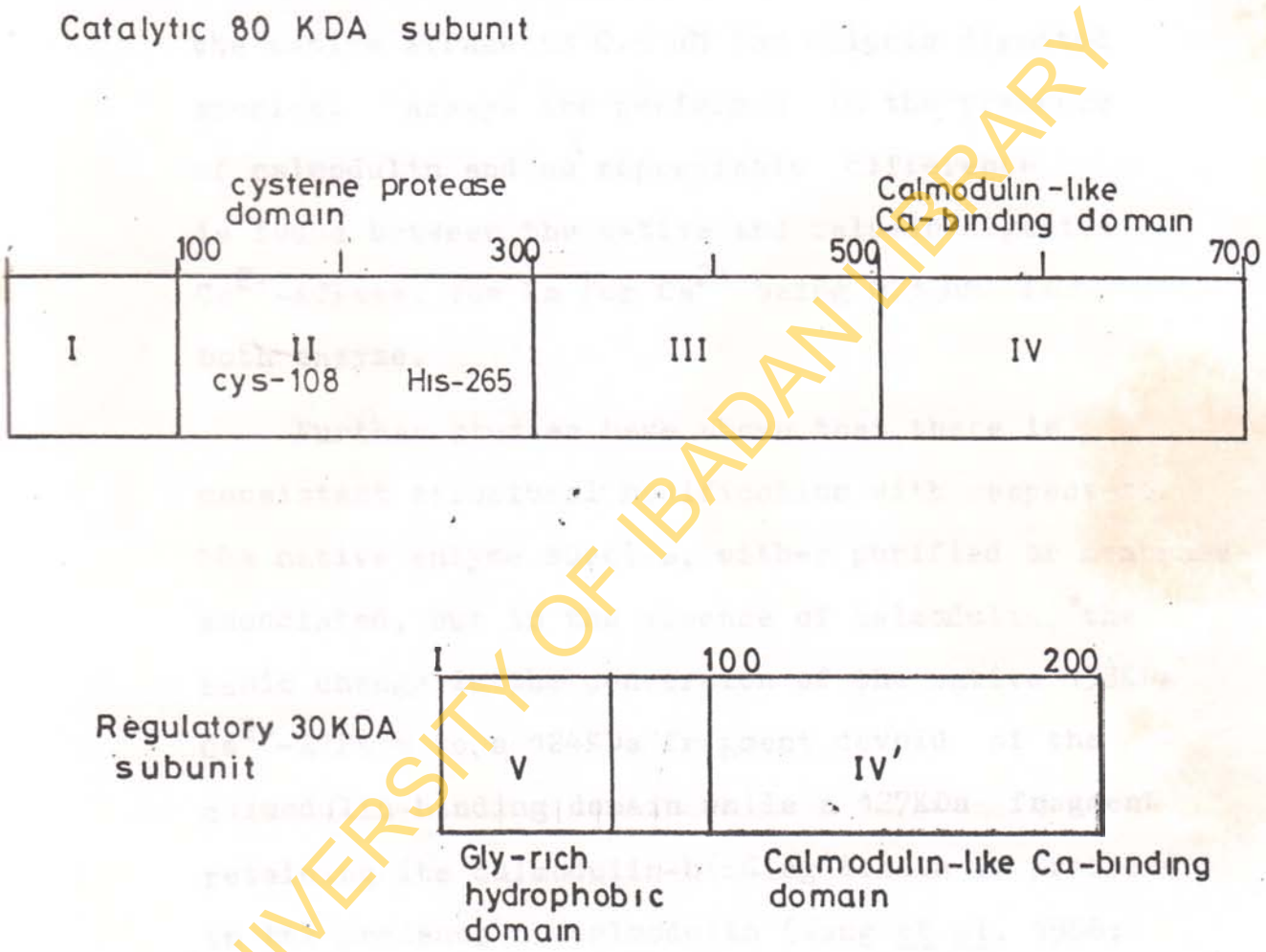


Fig .11. Domain structure of Calpain

Suzuki, 1987

manner and rendered it calmodulin insensitive. However, in the case of the calpain activation, the Km for Ca^{2+} is shifted from a value of 6 μM for the native ATPase to 0.5 μM for calpain digested species. Assays are performed in the presence of calmodulin and no appreciable difference is found between the native and calpain digested Ca^{2+}-ATPase, the Km for Ca^{2+} being 0.3 μM for both enzyme.

Further studies have shown that there is consistent structural modification with respect to the native enzyme species, either purified or membrane-associated, but in the absence of calmodulin, the basic change is the conversion of the native 138KDa Ca^{2+}-ATPase to a 124KDa fragment devoid of the calmodulin-binding domain while a 127KDa fragment retaining its calmodulin-binding domain is produced in the presence of calmodulin (Wang et al, 1988; James et al, 1989). Although, calpain cleaves and leaves a fragment of 124KDa in the absence of calmodulin, studies, have also shown that, calpain actually

cuts at 2 different portions of the Ca^{2+} ATPase molecule. The first cut occurs in the middle of the calmodulin binding domain producing a fragment of about 14KDa and a (calmodulin binding) fragment of about 124 KDa while a second cut occured closer to the N terminus of the calmodulin binding domain producing a fragment of about 124 KDa and accounted for the loss of calmodulin binding at prolonged times of incubation of the ATPase with calpain. The 124 KDa fragment has been demonstrated to have an increased rate of Ca^{2+} transport in a liposome reconstituted system (Wang et al, 1989).

1:12 Activation of Ca^{2+} pump by kinase mediated phosphorylation

Smallwood and Rasmussen (1988) have shown that, the activated form of purified protein kinase C was found to stimulate Ca^{2+} transport in alkaline phosphatase-pretreated inside out vesicles (IOVS) suggesting that the Ca^{2+} ATPase may already be partly phosphorylated in the cells in Situ. This

activation is however in the presence of specific activators such as phorbol ester and diacylglycerol and the physiological concentrations of free Ca^{2+} was between 0.08 - 5μM. Some other studies have indicated that both, protein kinase C and phorbol ester increased the maximum velocity of erythrocyte IOV Ca^{2+} transport but had no significant effect upon the apparent Km for Ca^{2+}. In contrast, calmodulin increased both the maximum velocity and the Ca^{2+} sensitivity of the pump while a combination of the two activators (protein kinase C and 12-O-tetradecanoyl phorbol-13-acetate (TPA) or protein kinase C and diolein a phospholipid plus calmodulin showed additive effects at maximal doses of each and suggesting therefore that calmodulin and protein kinase C may exert their effects through different mechanisms (Smallwood and Rasmussen 1988). In addition, these workers, have shown that protein kinase C and TPA or protein kinase C and diolein have been found to stimulate the activity of the erythrocyte purified calcium pump ATPase. (Fig. 12) shows a proposed model of the activation

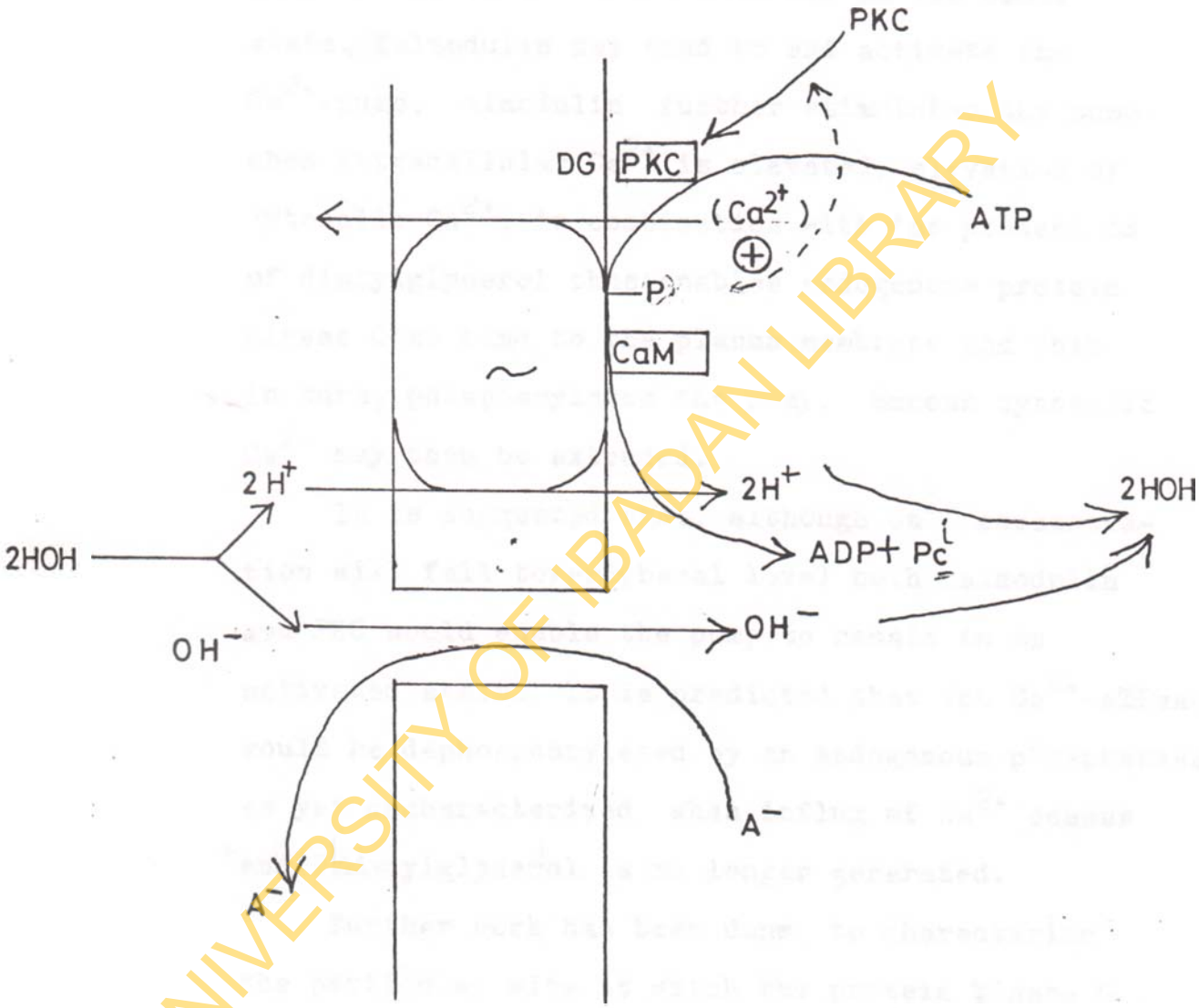


Fig. 12: Model of the erythrocyte membrane calcium pump. Smallwood et al, 1988.

of Ca^{2+}-pump by protein kinase C. According to this model, in an intact cell and in its basal state, Calmodulin may bind to and activate the Ca^{2+}-pump, almodulin further stimulates the pump when intracellular Ca^{2+} is elevated, elevation of cytosolic Ca^{2+}, in combination with the production of diacylglycerol thus enables endogenous protein kinase C to bind to the plasma membrane and this in turn, phosphorylates the pump. Excess cytosolic Ca^{2+} may then be extruded.

It is suggested that, although Ca^{2+} concentration will fall towards basal level both calmodulin and PKC would enable the pump to remain in an activated state. It is predicted that the Ca^{2+}-ATPase would be dephosphorylated by an endogenous phosphatase as yet uncharacterized when influx of Ca^{2+} ceases and diacylglycerol is no longer generated.

Further work has been done, to characterize the particular site at which the protein kinase C phosphorylates. In this regard Wang, Wright, Machan, Allen, Conigrave and Roufogalis (1991) demonstrated that the phosphorylation site is located within a

12KDa a region at the carbonyl terminal end of the molecule and that the single threonine within the calmodulin-binding domain is a likely site of phosphorylation. Another serine residue located at the carboxyl-terminal to the calmodulin binding domain has been suggested to be a possible additional phosphorylation site. In the same work, a mixture of isozymes (α, β, γ) of PKC was shown to activate the purified Ca^{2+}-ATPase by 5-15%, this activation was increased from 40 to 60% when A23187 was included in the assay on the other hand the α-isozyme of PKC alone appears not to activate but to reduce activation by calmodulin (Wang et al, 1991). It is suggested that the differences in activation might have various explanation firstly, PKC could phosphorylate the purified Ca^{2+}-ATPase only substoichiometrically thereby producing only a moderate activation. Secondly, PKC could also phosphorylate directly or indirectly other membrane components, possibly including some phospholipids, which could as well indirectly stimulate the Ca^{2+}-ATPase and, thirdly it seems likely that, the solubilized and purified Ca^{2+}-ATPase

may not allow the full expression of the effect of PK-C phosphorylation.

Furthermore, the plasma membrane Ca^{2+}-ATPase from both heart sarcolemma and erythrocyte cells are also activated as a result of the phosphorylation by cAMP-dependent protein kinase A *in vitro* (Caroni and Carafoli 1981; Neyes *et al*, 1985). Recent work has demonstrated that PK-A phosphorylation leads to increased affinity for Ca^{2+} as well as a 2 fold increase in Vmax (Dixon and Haynes 1989; James, Pruschy, Vorherr, Penniston and Carafoli, 1989) (Table 8). It is worth noting therefore, that in the erythrocyte the increase in Vmax produced by PK-A is non-additive to that of calmodulin (James *et al*, 1989). In contrast to protein kinase C, cAMP-dependent protein kinase was shown to phosphorylates the plasma membrane Ca^{2+}-ATPase at a single serine residue located between the calmodulin-binding domain and the carboxyl terminus of the enzyme about 5 KDa from the carboxyl terminal end (James *et al*, 1989). It was therefore speculated that activation could be achieved in like manner by

modulation of calmodulin-binding domain.

1:13 Activation by Oligomerisation

Another way by which the Ca^{2+}-ATPase is stimulated is through dimerization or oligomerization (Kosk-Kosicka, et al 1989). It has been shown thatea dimerized pump could form the transmembrane ion channel between two subunits, whereas a monomeric pump would have to possess an intramolecular pathway for the translocations of the enzyme. Using the sarcoplasmic reticulum Ca^{2+}-ATPase as a model, Hymel, Maurer, Berenski, Jung and Fleisher (1984) suggested that Ca^{2+} pump could exist as a dimer. Thiswas later confirmed by Anderson (1989). Its seems plausible, that sarcoplasmic Ca^{2+} ATPase could exists as a dimer because of its low molecular weight and high concentration in the membrane. However, in the case of the plasma membrane Ca^{2+}-ATPase this is not likely, because of its high molecular weight and low concentration of the enzyme (0.1%) in the membrane (Knauf, et al 1974). Therefore it is possible that, the occurence of the dimer would be something more specific rather

than a randomized collision-mediated association process.

Further studies by Kosk-Kosicka et al (1989) using fluorescence resonance energy transfer showed that energy transfer occurred between enzyme molecules, the effect being half maximal at 10-20μM. It was suggested that concentration dependent stimulation is due to oligomerisation. These workers demonstrated further that 1 mole of calmodulin binds to 1 mole of monomeric Ca^{2+}-ATPase whereas only 0.5 mole of calmodulin was bound to 1 mole of the oligomeric form of the enzyme. These findings suggest that the notion that at high concentration, the enzyme most likely exist as dimers.

Further studies, by Vorherr, Kessler, Hofmann and Carafoli (1991) reveal that plasma membrane Ca^{2+}-ATPase truncated at the COOH terminus by calpain to a fragment of 124 KDa does not contain the calmodulin binding domain could not oligomerize with that intact ATPase, suggesting

that calmodulin binding domain mediate the process of oligomerisation of the Ca^{2+}-pump.

1.14 Historical Background

Intensive research on the aflatoxins started in 1960 when the mysterious Turkey 'X' disease killed young turkeys, ducklings and young phesants in England and certain parts of Africa (Blount, 1961). About the same time an outbreak of trout hepatoma was reported in the U.S.A. (Wolf and Jackson, 1963).

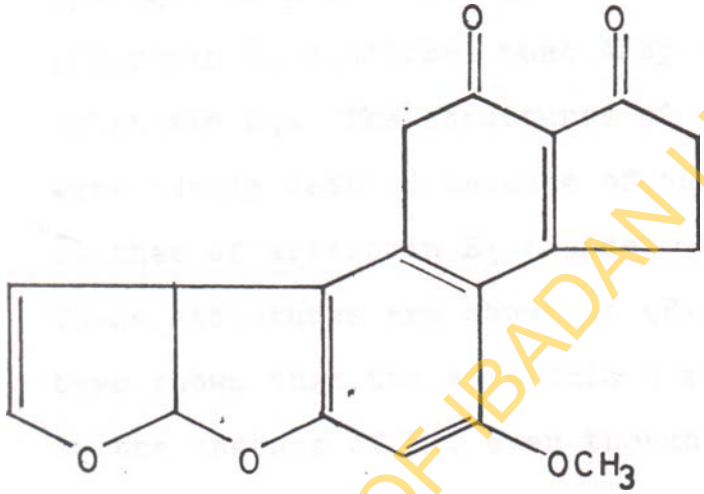
Earlier on, Burnside, Sippel, Forgacs, Caril, Atwood and Doll (1957) had reported a fatal disease characterized by liver lesions in swine fed on mouldy corn. Sargeant, Sheridan and O'Kelly (1961) and Spensley (1963) eventually showed that the new disease was a mycotoxicosis caused by the ingestion of groundnut meal infested with the mould Aspergillus flavus. Almost simultaneously Hartley, Nesbitt and O'Kelley (1963) showed that this mould when cultured in appropriate ~~media~~ produced toxic metabolites collectively called "aflatoxins or toxins of Aspergillus flavus.

TABLE 11

Local (Nigerian) Foodstuffs known to support aflatoxin production.

Medium	Aflatoxin metabolites produced by Aspergillus flavus strains			
Soybeans	B_1	B_2	G_1	G_2
Paw Paw	B_1	B_2	G_1	G_2
Beans (Black eye)	B_1	B_2	G_1	G_2
Banana	B_1	B_2	-	-
Groundnuts	B_1	B_2	G_1	G_2
Red pepper	B_1	B_2	-	-
Millet	B_1	B_2	G_1	G_2
Rice	B_1	B_2	G_1	G_2
Corn	B_1	B_2	G_1	G_2
Yams (Yam flour)	B_1	B_2	G_1	G_2
Garri (Manihot flour)	B_1	B_2	G_1	G_2

chromatoplates were designated aflatoxins B_1, B_2, G_1, and G_2 in order of decreasing R_f value; because only relatively small amounts of the toxins were available, the structural elucidation made use of modern organic chemistry relying almost solely upon interpretation of UV, IR, NMR and mass spectra. Using these tools it was found that the major toxin, aflatoxin B_1 exhibited a blue fluorescence, has a melting point of 268-269°C, and UV spectrum $\lambda_{max}^{E^+OH}$ 223, 265nm and 362nm. It's molecular weight was found to be 312 by mass spectrometry and this correlates well with the empirical formula $C_{17}H_{12}O_6$. A comparison of the infrared spectra of aflatoxin B_1, coumarin and tetrahydro-deoxoaflatoxin indicated that the two absorption bands 1760cm^{-1} and 1084cm^{-1} in the spectrum of the natural product are due to the presence of a coumarin and ketone carboxyl groups in the molecule. Further studies using NMR technique aided the complete structural elucidation of aflatoxin B_1 (Fig. 13) (Asao, Bucchi, Abdet-



AFLATOXIN B_1

Fig. 13: Structure of aflatoxin B_1

Kader, Chang, Wick and Wogan, 1965). Reports by Vander Merwe, Fourie and Scott (1963) on the interuption of the catalytic hydrogenation of aflatoxin B_1 after the uptake of one mole of hydrogen to give a product which was identical with aflatoxin B_1 confirmed that AFB_2 is dihydro aflatoxin B_1. The structures of other aflatoxins were easily deduced because of their similarity to that of aflatoxin B_1 (Vander Merwe, 1963). These structures are shown in (Fig. 14). It has been shown that the aflatoxin G series seldom occur in the absence of B_1, even though cases of the G series contaminated products have been reported (Wogan, 1977). Aflatoxins M_1 and M_2 which are hydroxylation products of B_1 are as toxic as the parent aflatoxin (Purchase 1967b). Dutton and Heathcote (1963) described two other hydroxy derivatives of B_2 and G_2 designated aflatoxin B_{2a} and G_{2a}. Another compound assigned the name aflatoxin R_o, was found to be less toxic than B_1 (Detroy and Hesseltine, 1970).

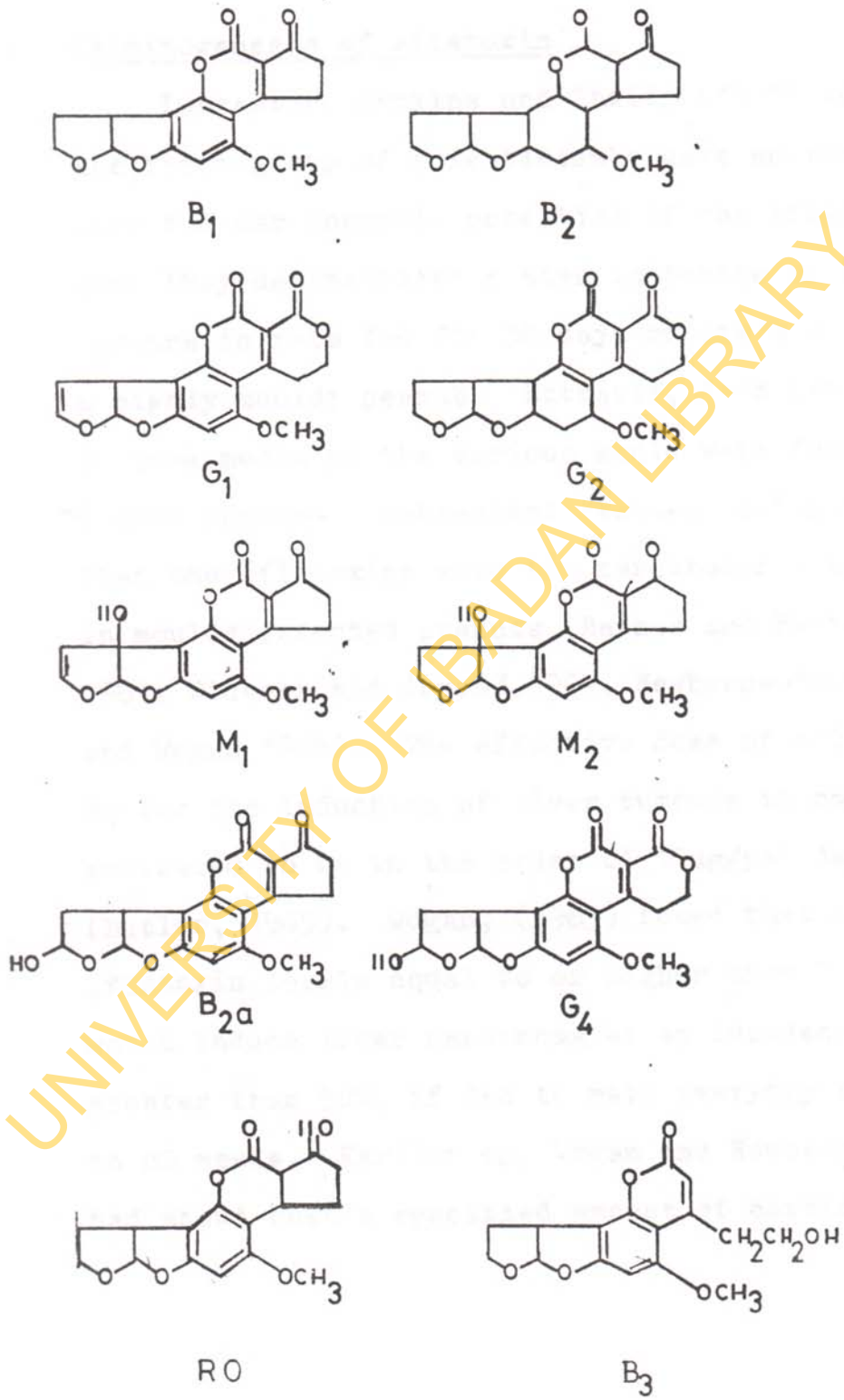


Fig. 14: Structures of the different types of aflatoxin. Van der Merwe (1963)

1:16 Carcinogenesis of aflatoxin

Lancaster, Jenkins and Philip (1961) were the first group of scientists who gave an insight into the carcinogenic potential of the aflatoxins when they demonstrated a high incidence of liver tumours in rats fed for 30 days on diets containing a highly mouldy peanut. Extracts, from their culture media of the various mould were found to induce tumours. Subsequent studies, established that the aflatoxins were the carcinogenic agents in mouldy infected peanuts (Barnes and Butter 1964, Dickens and Jones, 1964, Newberne, 1965; and Wogan 1966). The effective dose of aflatoxin B_1 for the induction of liver tumours in rats was estimated to be in the order of 10µg/per day (Butler, 1965). Wogan, (1963) found that dietary aflatoxin levels equal to or higher than 0.1ppm would induce liver carcinoma at an incidence greater than 50%, if fed to rats everyday for up to 80 weeks. Earlier on, Wogan and Newberne (1967) had shown that a specified amount of carcinogen

given at a low dosage over a long period of time has a greater carcinogenic effect than a higher dose given over a shorter period of time. A linear correlation between liver tumour incidence and dietary aflatoxin intake over the 0.07ppm - 1.0ppm have been established for the rat (Newberne, 1965).

Several factors have been found to come into play in the carcinogenesis of aflatoxin. For instance, Wogan (1973) showed that female animals develop tumours more slowly than males, although the incidence of tumour is similar in both sexes.

Apart from hepatomas, carcinomas in many other tissues of the rat have been reported, these lesions affect for example, the glandular portion of the stomach (Butler and Barnes 1966), and the renal tubules (Butler, Greenblatt, Lijinsky 1969). Lesions such as focal lipid accumulation, parenchyma cell hyperplasia, bile duct hyperplasia have also been reported.

Furthermore, certain species of animals have been shown to be more susceptible to aflatoxin than

others. In particular, trouts developed a 10% liver tumour incidence when fed 0.1 ug dietary aflatoxin while ducks are one of the most susceptible to the carcinogen. In contrast mice and sheep are resistant to aflatoxins carcinogenesis, despite their comparable ability to metabolize these compounds to their predominant metabolite (Bassir and Emafo, 1970; Bassir and Emerole, 1975). Histological assessment of aflatoxin-induced tumours showed that there are two main types of tumours developed in rats fed aflatoxin, those derived from hepatic parenchymal cells which later formed nodules, and those derived from the bile duct epithelia (Lancaster, 1968). Injections of aflatoxin B_1 (38%) and G_1 (56%), B_2 and G_2 (traces) in arachus oil into rats show that the animals developed sarcomas and fibrosarcomas at the injection sites (Dicken and Jones, 1964). Tumours in other animal species have suggested that the sunbird trout is more sensitive to the hepatocarcinogenic effect than the rat (Sinnhusber and Walee 1965). In addition, Gopalan (1972 described a liver carcinoma in a rhesus monkey

after feeding aflatoxin for a period of 6 months to 3 years. In man, however, the actual carcinogenic potency of the aflatoxin B_1 has remained rather speculative although there is considerable evidence to show that several food consumed by man are contaminated by Aspergillus flavus.

1:17 Occurrence of aflatoxin in Some Nigerian foods

So far only two species of moulds namely Aspergillus and Penicillum have been found to be very versatile in producing the aflatoxins. Mould strains obtained from the Nigerian enviroment have been shown to produce aflatoxins (Bababunmi and Bassir, 1976). Previous work in this laboratory have been shown that several of the Nigerian food stuffs can serve as suitable media for the growth of these moulds (Emerole and Uwaifo, 1980), of all the food stuffs studied pawpaw gave the lowest yield of crude aflatoxin (Table 11).

This finding gave the impetus for screening some Nigerian food stuffs for possible contamination by aflatoxin. Aflatoxin screening of the chloroform

extracts of some Nigerian foods show that both the B and G series of the aflatoxins are actually present in certain food stuffs. Different levels of aflatoxin ranging from 0.04mg in rice and 1.70mg/kg in groundnuts were found (Emerole, Uwaifo, Thabrew and Bababunmi,1982).

1:18 Inhibition of DNA synthesis by aflatoxin B_1

Inhibition of DNA synthesis in cells exposed to aflatoxin preparation has been demonstrated under several experimental systems, thus, under in vivo conditions, this response is particularly evident in rat liver undergoing regeneration after subtotal hepatectomy. However, Frayssinet, Lafarge, Recondo and Le Breton (1964) reported that aflatoxin B_1 inhibited the net synthesis of liver DNA when immediately after hepatectomy at dose levels of 30 or 60ug/animal; this showed that the enzymes responsible for DNA synthesis remained fully active under these conditions, hence the toxin exterted its inhibitory effects by interacting with DNA in such a way as to impact its ability to act as a primer for DNA synthesis. This was confirmed by Rogers

and Newberne (1967), who found that a single large dose (3mg/kg) of aflatoxin B_1 given to rats caused marked reduction of tymidine - 3H labeling of liver cell nuclei,' But, the evidence regarding the mechanism of inhibition is not yet conclusive, it tends to support the hypothesis that this action is a consequence of interaction of the compound with DNA.

1:19 Inhibition of protein synthesis by aflatoxin B_1

Earlier studies have shown that the mechanisms of gene transcription and translation, alterations in DNA-dependent RNA synthesis would be expected to result in changes in protein metabolism. Therefore, it has been shown that exposure of liver tissue to aflatoxin B_1 results in marked alterations in gene transcription as evidenced by impaired synthesis of nuclear RNA. Associated with this effect are significant and persistent losses of cytoplasmic RNA and polyribosoma disaggregation. Impaired synthesis of total liver proteins has been observed upon exposure of liver slices to aflatoxins in vitro. However, under in vivo conditions synthesis

of specific proteins (inducible enzymes) is inhibited by the toxin, whereas total liver protein synthesis is not markedly affected (Wogan, 1968). Clifford and Rees (1963) demonstrated that the in vivo synthesis of certain liver enzymes is inhibited in animals treated with aflatoxin B_1, characteristics of inhibition in these instances suggest that the observed effects on protein synthesis are secondary to inhibition of RNA synthesis. This was however, based on studies of toxin effects on the inducibility of tryptophan pyrrolase, an enzyme present in mammalian liver at low levels (Feigelson and Greengard, 1962). Wogan (1968) however concluded that the action of aflatoxin B_1 *in vivo* involves suppression of the synthesis of specific liver proteins through its alteration of RNA metabolism.

Aflatoxin has also been shown to cause an irreversible displacement of polysomes from rough endoplasmic microsomal membrane (Rabin, Blyth Doherty, Freedman and Williams, 1974), by acting directly on polysomes and thus inhibiting mRNA

synthesis. The monosomes produced contained amino acyl transfer RNA.

1:20 Alterations of RNA metabolism by aflatoxin B_1

Altered RNA metabolism as a result of aflatoxin B_1 treatment has been demonstrated under a variety of experimental conditions. It has been shown for example that *in vivo* administration of the compound to rats or direct exposure of liver slice preparations *in vitro* results in rapid and dramatic inhibition of precursor incorporation into RNA, particularly in the nucleus (Wogan, 1968). Subsequently, the impaired RNA synthesis was attributed to inhibition of RNA polymerase activity (Gelboin, Wortham, Friedman and Wogan 1966). Further studies by Friedman and Wogan (1967) confirmed also that the toxin produced inhibition of RNA polymerase activity that persisted for several days after dosing.

In view of the marked effects of the toxins on nuclear RNA synthesis, it might be anticipated that cytoplasmic RNA metabolism would be affected as well (Fr edman and Wogan 1966; Svododa and Soga 1966)

In certain studies, it was observed that the primary effect of aflatoxin is to block RNA synthesis, on binding to the DNA molecule (Hayes, Platt, Tilzer, and Chiza 1975). Also, the intercalation of aflatoxin with the DNA template results in the release of an inhibitor which selectively inhibits the transcriptional activity of RNA polymerase II (Yu, 1977).

1:21 Effect of aflatoxin B_1 on nucleolar morphology

Alterations of nuclear RNA metabolism resulting from treatment with aflatoxin B_1 are associated with changes in the morphology of nucleoli as observed by electron microscopy of affected cells. However, work done by Bernhard, Frayssinet, Lafarge and Le Breton (1965) on liver cell nucleoli of rats, showed that there were some lesions. These lesions consisted of segregation of the granular and fibrillar components of the organelle, with the formation of so-called nucleolar "Cap". The structural change was developing within 30 minutes after administration of the toxin (0.5mg/kg), but proved to be reversible, since the nucleolar

morphology was essentially normal 24 hours after injection. Subsequently Lafarge, Frayssinet and Simard (1966) also confirmed the result and they concluded that inhibition of nucleolar RNA synthesis preceded development of the morphologic lesion, as the former process was essentially maximal 20 minutes after treatment with the toxin, whereas the latter took longer to develop. Wogan (1968) was able to conclude that the marked alterations in nucleolar morphology induced by single doses of aflatoxin B_1 are associated only with acute effects of the toxin but do not occur after chronic treatment.

1:22 Interaction with Proteins

Bassir and Bababunmi (1973) demonstrated that aflatoxin B_1 is firmly bound at least at one site unto either bovine or human serum albumin *in vitro*. It was shown that aflatoxin B_1 interacts unspecifically with different proteins showing the same behaviours as described for BSA (Evrain, Cittanova and Jayle 1978). Subsequently, binding of ^3H aflatoxin B_1 to rat plasma was investigated *in vivo* and *in vitro*.

It was observed that, aflatoxin B_1 binds an apolar site with an association constant of $30mM^{-1}$ at pH 7.4 and 20°C. The association constant was pH-dependent, increasing about 1.7 fold as the pH increased from 6.1 to 8.4. This was ascribed to a pH-induced conformational change in the albumin molecule. Thermodynamic studies, indicated that the aflatoxin-albumin interaction was exothermic (H - -29.3 KJi mol^{-1}), with a S value of -13.8 J. mol^{-1} K^{-1} (Heini and Schabort, 1986).

Furthermore, earlier studies have shown the effect of functional groups on the interaction of aflatoxin B_1 and G_1 with starch and cellulose; seven other cellulose derivatives were later studied by Uwaifo and Bassir (1977) using equilibrium dialysis. Their finding further emphasized the functional roles of amino groups in the interaction of aflatoxin with macromolecules.

1:23 Interactions of aflatoxins with other cellular compounds

It has been suggested that aflatoxin binds to the membrane to produce degranulation of the rough

endoplasmic reticulum Garvican and Rees (1974) Gurtoo and Dahms, (1974) showed that the absence of toxicity of aflatoxin B_{2a} might be due to its binding to extra cellular proteins of the membrane, thus preventing its entry into the cell. The hydroxyl of C_2 atom appears to prevent it's re-entry into the cell. It has been shown that, the OH of C_2 atom does not have this same effect since the labelled forms of aflatoxin, M_1 and GM_2 are incorporated into other aflatoxins. The interaction of cellular organelles has been demonstrated. For instance, the toxins interacts with some cellular organelles such as lysosomes which contain dhydrolytic enzymes such as ribonuclease (Pokrovsky, Krowchenko, and Tutlyan, 1972). Lysosomes are known to release degradative enzymes glycosidase, hyaluronidase, proteases and collagenase during tumour maliginant formation (Cameron, 1966; Balaz and Von Euler, 1952; Fiszerszarfar and Szafard 1973; Harris, Stephens, Ghosh and Taylor, 1977). The persistence of hyaluronidase release in neoplastic cell proliferation is absent in normal cell proliferation.

To establish the relationships of mitochondria chemical carcinogen interactions to the normal to neoplastic transition. Graffi showed as early as (1940), that carcinogenic hydrocarbon are accumulated by the mitochondria of animal cells. In more recent years, the interactions of chemical carcinogens with mitochondria have been studied in vitro, and the effect of carcinogen feeding to rats on the functional properties of freshly isolated mitochondria has been studied as well (Belt and Campbell, 1973).

Aflatoxin B_1 exerts several types of effects on freshly isolated rat liver mitochondria. These include a partial inhibition of respiration, a partial inhibition of RNA synthesis, an induction of mitochondria swelling and an enhancement of uncoupler-stimulated ATPase activity (Doherty and Campbell, 1973, Belt and Campbell, 1973, 1975; Bababunmi and Bassir, 1976).

1:24 Activation and Toxicity of aflatoxin

Many carcinogenic agents are electrophilic in nature and are able to react with nucleophilic

cellular constituents (Miller, 1970). It is well established that aflatoxin B_1 does not react towards nucleophiles (Sporn, Dingman, Phelps and Wogan 1966; Clifford and Rees 1967). Aflatoxin B_1 2,3-oxide was first suggested by Schoental (1970) as an intimate carcinogenic metabolite of aflatoxin B_1. In general, the 2,3-epoxide acts as an alkylating agent for macromolecules (Dickens and Jone 1964). The existence of aflatoxin 2,3-oxide was demonstrated by Swenson, Miller, Miller (1974) on isolating the 2,3 dihydro 2-3 dihydroxy (dihydrodiol) (Fig.15). Aflatoxin B_1 form an acid hydrolysate of the r-RNA of aflatoxin adduct form by hepatic microsomal oxidation of aflatoxin B_1 in the presence of r-RNA. The instability and high reactivity of aflatoxin 2-3 oxide made it impractical to isolate it from microsomal reaction mixtures or to synthesize it chemically.

Results of several studies have shown that the toxicity of aflatoxin may be classified into two; namely subacute toxicity and acute toxicity. It has been shown that adult animal would not die for several days after an intake of sublethal

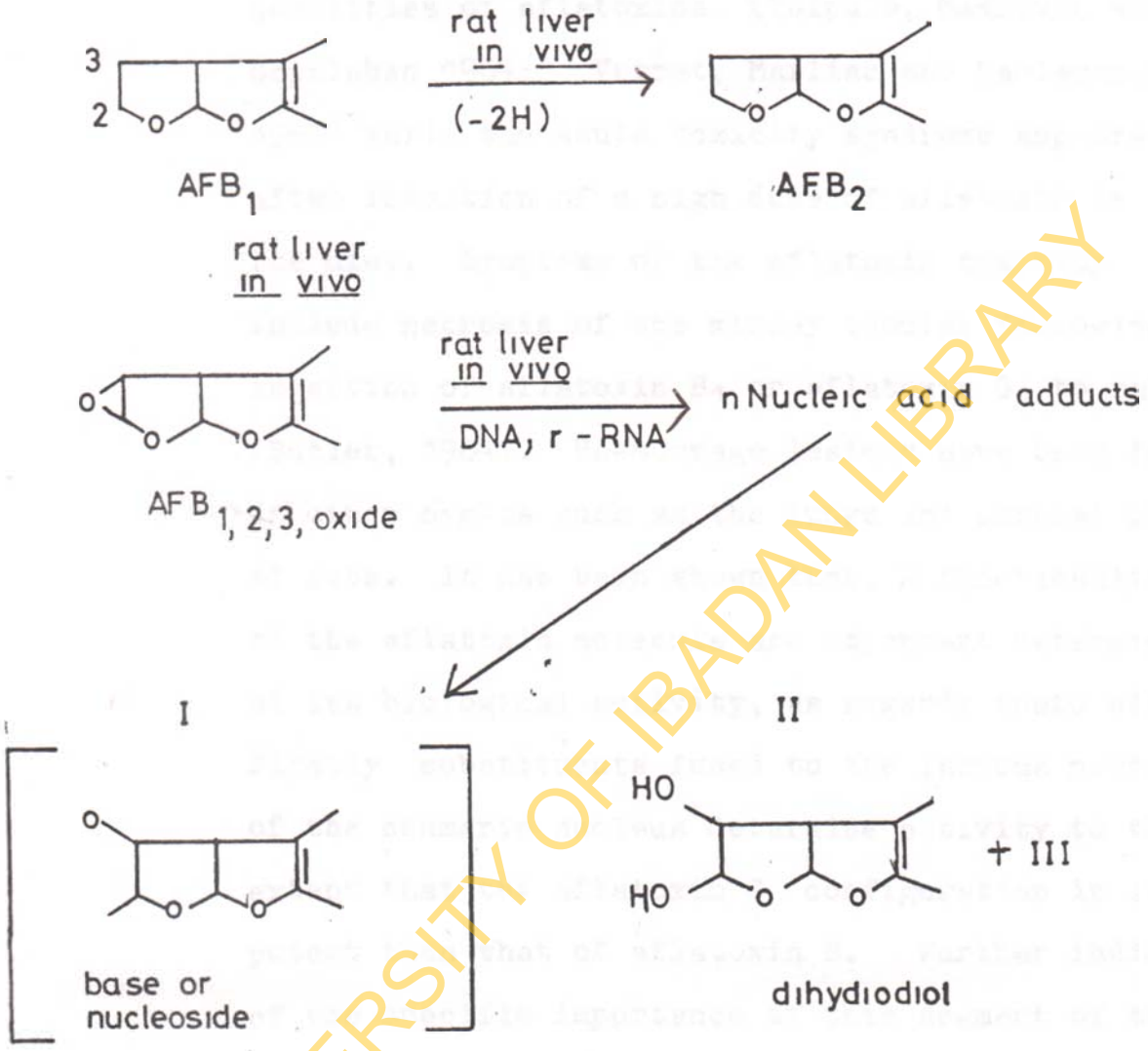


Fig. 15: The metabolic activation of aflatoxin B_1 by rat liver. Swenson et al, 1975.

quantities of aflatoxins (Tulpule, Madhavan and Gopalahan 1964; Verret, Marliac and Maclaughlin 1964) while the acute toxicity syndrome appears after ingestion of a high dose of aflatoxin in the diet. Symptoms of the aflatoxin toxicity include necrosis of the kidney tubules following ingestion of aflatoxin B_1 or aflatoxin G_1 to rat (Butler, 1964). Haemorrage lesions have been found in other organs such as the lungs and adrenal glands of rats. It has been shown that, 2 functionalities of the aflatoxin molecule are important determinants of its biological activity, as regards acute effects. Firstly substituents fused to the lactone portion of the coumarin nucleus determine activity to the extent that the aflatoxin G configuration is less potent than that of aflatoxin B. Further indication of the specific importance of this segment of the molecule is provided by the recent finding that "aflatoxicol" the hydroxylated derivative produced by reduction of the carbonyl group of aflatoxin B_1, is less toxic than B_1 to ducklings (Detroy and Hesseltine, 1970). Consequently, more evidence

points to the importance of the dihydrofurofuran portion of the aflatoxin B_1 molecule. Secondly, reduction of the vinyl ether double bond in the terminal furan ring brings about significant reduction in potency in most systems and nearly total loss of activity with respect to acute toxicity to rats.

1:25 Properties of aflatoxins

Ultra violet light and infra-red spectra have been used for the characterisation of the physical properties of aflatoxins. The physico-chemical properties of the aflatoxins are shown in (Table 12). The chemical properties of aflatoxins deal with the oxidation or the addition reaction they undergo. Aflatoxins are unstable in alkaline medium (Vander, Zijden and Barret, 1962), in the presence of methyl lamine or ammonia (Dollear and Mann, 1968) in methanol, air and ultra-violet light. Aflatoxins B_1 and B_2 possess a reactive carbonyl group in their cyclopentenone ring. This takes part in ketonic reactions reacting with 2,4 dinitrophenyl-hydrazine and hydroxylamine to form phenyl hydrazones and oximes (Crisan and Grefig, 1967). Aflatoxins B_1

TABLE 12

Physico-chemical properties of the aflatoxins.

Aflatoxin	Chemical formulae	Molecular weight	Fluorescence under u.v 363mu
Aflatoxin B or Difurocoumarocyclopentenone series.			
B_1	$C_{17}H_{12}O_6$	312	Blue
B_2	$C_{17}H_{14}O_6$	413	Blue
B_{2a}	$C_{17}H_{14}O_7$	330	Blue
M_1	$C_{17}H_{12}O_7$	328	Blue
M_2	$C_{17}H_{14}O_7$	330	Blue
Ro	$C_{17}H_{14}O_6$	314	Blue
Q_1	$C_{17}H_{12}O_6$	328	Blue
P_1	$C_{17}H_{12}O_6$	298	Blue
M_{2a}	$C_{17}H_{14}O_8$	346	Turquoise
Aflatoxin G or Difurocoumarolactone series.			
G_1	$C_{17}H_{12}O_7$	328	Turquoise
G_2	$C_{17}H_{14}O_7$	330	Turquoise
G_{2a}	$C_{17}H_{14}O_8$	346	Turquoise
G_{M1}	$C_{17}H_{14}O_8$	344	Turquoise
G_{M2}	$C_{17}H_{12}O_8$	346	Turquoise
G_{M2a}	$C_{17}H_{14}O_9$	362	Turquoise
B_3	$C_{17}H_{14}O_6$	302	Blue

and G_1 react with ozone (Dwarakanath, Rayner, Mannaand Dollear, 1968), benzoyl peroxide, osmium tetroxide and potassium iodine complex (Trager and Stoloff, 1966). Aflatoxins B_1, B_2, G_1 and G_2 also react with acetic acid and trifluoroacetic acid employing thionylchloride as a catalyst to form derivatives with R_f values different from that of the parent compound (Androlles and Reid, 1964; Stoloff, 1967; Crisan and Grefig, 1967).

1:26 OBJECTIVE

The aflatoxins are highly hepatocellular carcinogenic chemicals; aflatoxin B_1 being the most potent of these toxins. Binding studies using equilibrium dialysis technique have shown that aflatoxin B_1 binds firmly to serum albumin (Bassir and Bababunmi, 1973) and polysaccarides (Uwaifo and Bassir, 1977). Further binding studies using Bacillus brevis revealed that 66% of cell-bound toxin was associated with the cell membrane fraction of the bacterium (Uwaifo and Bassir, 1978). The finding that a significant amount of the toxin occurs in blood following a single intraperitoneal injection of the toxin suggests that the toxin must have contact with the membrane (Wogan and Shank, 1977). It is not known, whether the binding of aflatoxin B_1 to proteins and polysaccarides in vitro could be extrapolated to its behaviour in the blood. It appears that if aflatoxin B_1 binds firmly to red cell membrane proteins, as it does to serum albumin, the properties and functions of the membrane may be modified. Such modifications could involve transport of cations especially Ca^{2+}, across the red cell

membrane, since the proteins responsible for carrying these cations require membrane phospholipids in their immediate environment for maximum activity. Moreover, binding of the toxin directly to transport proteins could significantly affect the catalytic activity of the pumps.

Presently, there are 2 main types of ion motive ATPases that are present in the membrane of the red cell. The Na^+/K^+-ATPase pumps $3Na^+$ against $2K^+$ at the expense of ATP while Ca^{2+}-ATPase regulates the intracellular Ca^{2+} concentration by pumping Ca^{2+} out of the cell (Schatzmann, 1966). Although, Ca^{2+} has to fulfill several messenger functions within the cell, elevated intracellular Ca^{2+} concentration has been shown to cause an increase of K^+ leak transport, an inhibition of Na^+, K^+ ATPase, and a destruction of the plasticity and normal shape of red blood cells (Sarkadi and Tostenson, 1979). It is now well established that Ca^{2+}-pumping ATPase is responsible for the active extrusion of Ca^{2+} from erythrocytes, thus maintaining the erythrocyte intracellular concentration below 1μM while the Ca^{2+} level in the surrounding plasma is about 1-2mM

(Schatzmann and Vincenzi 1969; Roufogalis, 1979). Recent studies have shown that different types of genes code for different isoforms of the pump (Shull and Greeb 1988, Verma et al, 1988), hence they differ in their tissue specificity, localization and functions. In human, however, the most abundant is the hPMCA4 and it is the major isoform in erythrocytes.

In spite of the fact that a number of substances have been shown to inhibit the calcium pump, to date no known specific inhibitor to the pump has been identified pharmacologically. From pharmacology standpoint, a specific inhibitor of the pump will be useful in controlling the intracellular concentration of Ca^{2+}. Such a drug will certainly have important value in experimental studies on the role of calcium in control of muscle contraction. Potentially, the drug may find particular use in overcoming heart failure and in increasing blood pressure in hypotension. Furthermore, availability of a specific inhibitor will alleviate the current difficulties encountered in the design of experiments aimed at evaluating the physiological and pathological roles of the pump.

The overall objective therefore was to study the influence of the toxin on the functional integrity of the pump and thus its effects on the regulation of intracellular Ca^{2+} homeostasis in cells using the erythrocytes as model. The effect of the toxin on the catalytic activity of the pump was studied after establishing that the toxin binds to the red cell membrane.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection of Samples

Blood samples (500ml) were obtained from healthy individuals who visited the Blood bank of the Ibadan University College Hospital. Fresh human blood was also obtained from the Blood bank of University of Zurich Teaching Hospital, Zurich.

2.2 Preparation of erythrocyte ghost membrane

Reagents

(i) Isotonic Buffer: 130mM KCl + 10mM Tris pH 7.4 9.69g of potassium chloride, KCl (Hopkins and Williams, England) and 1.21g of Tris (hydroxymethyl) amino methane (Sigma Chemical Co., London) were dissolved in about 900ml of distilled water and the pH was adjusted to 7.4 with a E-520 model pH meter. The solution was made up to 1 litre in a standard volumetric flask and stored at $4^{o}C$.

(ii) Hypotonic Buffer: 10mM Tris + 1mM EDTA, pH 7.4 1.21g of Tris (hydroxymethyl)-amino methane (Sigma

Chemical Co., London) and 0.380g of the tetra-sodium salt of ethylene diamine tetra acetic acid, EDTA (Sigma Chemical Co., USA) were dissolved in about 900ml of distilled water and the pH was adjusted to 7.4. The solution was made up to 1 litre and stored at $4^{o}C$.

(iii) Washing Buffer: 10mM HEPES, pH 7.4

2.38g of N-2 hydroxyethyl piperaizine-N^{1} ethanesulphonic acid, HEPES (Sigma Chemical Co., London) was dissolved in about 900ml of distilled water and the pH was adjusted to 7.4. The solution was made up to 1 litre.

(iv) Storage Buffer: 10mM HEPES, 50µM $CaCl_2$, 500µM $MgCl_2$, 130mM KCl

2.38g of N-2-hydroxyethyl piperazine-N-ethane-sulphonic acid, HEPES (Sigma Chemical Co., London). 7.4mg of hydrated calcium chloride, $CaCl_2 . 2H_2O$ or 5.5mg of anhydrous calcium chloride, $CaCl_2$ (BDH Chemicals Ltd., England), 9.69g of potassium chloride, KCl, (BDH Chemicals Ltd., England 0.102g of Magnesium chloride hexahydrate, $MgCl_2 . 6H_2O$

(BDH Chemicals Ltd., England) were dissolved in about 900ml of distilled water and the pH was adjusted to 7.4. The solution was made up to 1 litre with distilled water and stored at 4°C.

(v) Addition of phenylmethylsulfonylfluoride (PMSF)

Phenylmethylsulphonyfluoride (PMSF) solution in DMF was added to Buffers (ii) and (iii) at a final concentration of 0.2mM just before use. 500mM PMSF solution was normally prepared by dissolving 0.4355g of PMSF (Sigma Chemical Co., London) in 5ml of Dimethyl formamide, DMF.

Procedure

Haemoglobin-free ghost membranes deficient in calmodulin were prepared by the procedure of Niggli et al, (1981) which is based on the principle of hypotonic lysis developed by Dodge, Mitchell and Hanahan (1963). All steps of the membrane preparation were carried out at 4°C.

Whole blood samples were centrifuged at 6,000 rpm for 10 mins in an MSE refrigerated centrifuge and Damon/IEC PR-600 refrigerated centrifuge. The plasma and "buffy" layers (containing white blood cells) were removed by aspiration to obtain packed erythrocytes. The erythrocytes were washed thrice in 10 volumes of isotonic buffer pH 7.4 to remove plasma constituents. Each time, the cell suspension was centrifuged at 6,000 rpm the supernatant was always removed by aspiration.

Washed erythrocytes were lysed in 10 volumes of hypotonic buffer pH 7.4, and centrifuged at 18,000 rpm for 20 minutes. Also, the washed erythrocytes were lysed in 10 volumes of hypotonic buffer 7.4 and RC 5C Sorvall Zonal centrifugation was used. Membranes were washed four times in the hemolysis solution and eight times in 10mM HEPES containing 0.2mM PMSF, pH 7.4 in order to remove calmodulin, haemoglobin and EDTA. The haemoglobin-free ghosts were finally resuspended in a storage buffer $CaCl_2$ and stored at $-80^{o}C$ and used within 2 weeks.

2.3 Determination of Protein

Erythrocyte ghost membrane protein was estimated by the procedure of Lowry et al, 1951 using bovine serum albumin as standard.

Principle

The colour reagent used in this method is a phospho-18-molybdotungstic complex (a mixture of several molecular forms such as $3H_2O.P_2O_5.9M_oO_3$ and $3H_2O.P_2O_5$ 10 $WO_3.8M_oO_3$) which can be reduced by 4 phenol groups to give a blue colour at alkaline pH. Tyrosine (and tryptophan) residues present in the protein are responsible for the reduction of the phosphomolybdo tungstic complex. This complex also called phenol reagent, is very unstable and decomposes in alkaline solutions. Since it interacts with tyrosine, only at alkaline pH, an excess of the reagent must be added in order to get complex reaction. However, these high concentrations of

the phosphomolybdo tungstic acid can cause turbidity due to the formation of insoluble salts. Folin and Ciocalteu (1927) have found that the turbidity can be prevented by adding to the reagents lithium salts (e.g. Li_2 So_4). In their mixture, called Folin-Ciocalteu's reagent, they also added some bromine water to maintain the phosphomolybdo tungstic reagent in the oxidized state during storage. Lowry, Rosebrough, Fan and Randall (1951) found that pretreatment of the protein sample with alkaline copper solution markedly increased the colour developed in the reduction of the phosphomolybdo tungstic reagent. Their assay system include a mixture of Na_2CO_3-NaOH to buffer the pH around 10 to neutralize the phosphoric acid produced by the degradation of the phosphomolybdo tungstic complex at alkaline pH.

Reagents

(i) Reagent A: 2% Na_2CO_3, 0.4% NaOH, 0.16% Na-tartrate, 0.8% SDS.

20g of sodium carbonate (Na_2CO_3) (BDH Chemicals Ltd, England), 4g of sodium hydroxide pellets, NaOH, (BDH Chemicals Ltd., England), 1.6g sodium-potassium tartrate ($Na\text{-}KC_4O_6.4H_2O$) (BDH Chemicals Ltd., England), 18g of sodium dodoecyl sulphate SDS (Sigma Chemical Co. London) were dissolved in a little quantity of distilled water in a 1 little quantity of distilled water in a 1 litre standard volumetric flask and made up to the mark with distilled water.

(ii) Reagent B: 4% copper sulphate

1g of copper sulphate ($CuSO_4.5H_2O$), (BDH Chemicals England) was dissolved in a small volume of distilled water and made up to the 100ml mark in a volumetric flask.

(iii) Reagent C: Alkaline copper solution

This was prepared fresh just before use by mixing 100ml of reagent A with 1ml of reagent B.

(iv) Reagent E: Folin-ciocalteu Reagent

100g of sodium tungstate ($Na_2WO_4.2H_2O$) (BDH Chemicals Ltd., England) and 25g of sodium

molybdate, ($Na_2Mo_0O_4.2H_2O$) (BDH Chemicals Ltd., England) were dissolved in 700ml of distilled water in a round-bottomed quick-fit flask. 50ml of 85% ortho phosphoric acid (BDH Chemicals Ltd., England) and 100ml of conc. HCl BDH Chemicals Ltd., England) were added to the solution and the mixture was gently refluxed for 10 hours. To the resulting mixture were added 150g of lithium sulphate and 2 or 3 drops of Bromine water. The mixture was boiled for 15 minutes in a fume hood without condenser to remove excess bromine. After cooling, the solution was made up to 1 litre, filtered and stored at 4°C. The 2N solution of folin ciocalteu was usually diluted to 1N with distilled water just before use.

Standard protein solution

10ml of 4ml/ml Bovine serum albumin (BSA) (Sigma Chemical Co., London) was prepared by dissolving 40mg of BSA in 10 mins of water. 1ml of the prepared solution was diluted with distilled water (about 16-19ml) to obtain a solution with an absorbance of 0.140 at 279nm.

Based on the fact that the molecular extinction coefficient (E) of BSA is 45,000 and its molecular weight is 65,000, this absorbance will give a concentration of 200 ug/ml albumin for the solution.

Procedure

Assay was carried out in duplicate. The reagents were dispensed into the various test tubes as outlined in the following protocol. The contents of each tube were rapidly mixed on addition of reagent C and left to stand at room temperature for 10 minutes and reagent D was later added and the contents of the tube was vigorously mixed. Mixing is very important since reagent D is unstable at alkaline pH and can be destroyed before it has reacted with the protein-copper complex. The absorbance of the solution in each tube was measured at 660nm after 45 minutes in WPA spectrophotometer. A standard curve was generated from the absorbance values obtained.

TABLE 13

Protocol for protein Estimation According to the Modification Procedure of Lowry et al 1951

Test tubes in duplicates	1	2	3	4	5	6	7	8
Standard BSA solution (200µg/ml)	-	100µl	200µl	300µl	400µl	500µl	-	-
Samples	-	-	-	-	-	-	100µl	200µl
Distilled water	800µl	700µl	600µl	500µl	400µl	300µl	700ul	600µl
Reagent C	2.4ml	2.4ml	2.4ml	2.4ml	2.4ml	2.4ml	2.4ml	2.4ml
Reagent D	240µl	240µl	240µl	240µl	240µl	240µl	240µl	240µl

2.4 Measurement of protein concentration of purified Ca^{2+} ATPase enzyme samples

Total protein concentration was determined by the procedure of Lowry et al (1951) as modified by Markwel, Hassi, Tolbert and Bierber (1981), using bovine serum albumin as standard of the interfering materials such as HEPES, dithiothreitol, Triton X-100 etc, the protein was first precipitated with deoxycholate and trichloroacetic acid as described by Bensadoun and Winstein (1976).

Reagents

2% Sodium Deoxycholate

2g of the sodium salt of deoxycholic acid (Fluka AG, Switzerland) was dissolved in 100ml of distilled water andstored at room temperature.

24% Trichloroacetic acid

24g of trichloroacetic acid, CCl_3 COOH, (BDH Chemicals Ltd., England) was dissolved in 100ml of distilled water and stored at room temperature.

Procedures

The protein was first precipitated using a mixture of 6% trichloroacetic acid (TCA) and 125ug/ml

sodium deoxycholate, since TCA alone does not produce quantitative protein precipitation at low protein concentrations (1-25 ug). An aliquot of the protein sample (10-50ul) was made up to a final volume of 3ml with distilled water. Twenty five micro litre of 2% sodium deoxycholate was then added and the solution was mixed vigorously on a vortex mixer and allowed to stand for 15 mins. One mililitre of 24% trichloroacetic acid was then added and the solution was again mixed vigorously and centrifuged at 3,000Xg for 30 mins. The supernatant was gently removed by used of a pasteur pipette connected to a vacuum line; the last 0.5ml was removed gently by tilting the tube and maintaining the tip of the suction pipette in contact with the side of the test tube without disturbing the pellet.

The pellet was then dissolved in a 3ml of Lowry reagent C, by mixing vigorously on vortex, and allowed to stand for 10 min. Folin-Ciocalteu reagent (D) (0.3ml) was then added, and the solution mixed rapidly and allowed to stand for 30 minutes

after which the absorbance was read at 660nm. Bovine serum albumin was used as standard. A blank containing bovine serum albumin was similarly treated.

2.5 Assay of membrane-bound (Ca^{2+} + Mg^{2+})-ATPase activity

Reagents

(i) 9% Ascorbic Acid (Reagent A).

22.5g of L-ascorbic acid, (Sigma Chemical was dissolved in a quantity of distilled water in a 250ml standard volumetric flask and made up to the mark. The solution was then stored in a brown reagent bottle and kept in a refridgerator at $4^{o}C$.

1.25% Ammonium Molybdate in 6.5% Sulphuric acid

Reagent B

6.25g Ammonium molybdate $(NH_4)_6\ Mo_7O_{24}\ 4H_2O$; (Hopkins and Williams Ltd., England) was dissolved in 500ml of 6.5% sulphuric acid (BDH Chemicals Ltd., England). The latter was prepared by mixing 32.5ml of concentrated

sulphuric acid in water and making up the solution to the mark in a 500ml standard volumetric flask. The reagent was stored at room temperature in a plastic bottle.

(iii) Assay Solution: 2M Potassium Chloride

14.912g potassium chloride, KCl (BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water and then made up to the mark in an 100ml standard volumetric flask. The solution was stored in a reagent bottle at 4°C.

Assay Buffer: 500mM HEPES or 500mM Tris buffer

29.7875g of N-2-hydroxethyl piperazine-N-2-ethanesulphonic acid HEPES (Sigma Chemical Co., London) was dissolved in about 200ml of distilled water and the pH adjusted to 7.4. The solution was then made up to 250ml in a standard volumetric flask and stored at 4°C. In the cases where Tris buffer was used, 15.125g of the Trizma base was dissolved in about 100ml of distilled water and 105ml of 1M HCl was added. The solution was adjusted to pH 7.4 and then made up to the 250ml mark in a standard volumetric flask. The solution was stored at 4°C.

0.1M $MgCl_2$

2.033g of magnesium chloride hexahydrate ($MgCl_2.6H_2O$) (Hopkins and Williams, England) was dissolved in a little quantity of distilled water in 100ml standard volumetric flask. After this, the solution was made up to the mark with distilled water. This was quantitatively transferred into a reagent bottle and stored at 4^oC.

100mM $CaCl_2$

1.4702g of calcium chloride dihydrate ($CaCl_2.2H_2O$) (BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water and the solution made up to the mark in a 100ml standard volumetric flask. This was quantitatively transferred into a reagent bottle and stored at 4^oC. 1mM $CaCl_2$ was prepared from this stock solution by dispensing 1ml of it into water and making up the solution to the 100ml mark with water in a 100ml standard volumetric flask. The solution was also stored at 4^oC in a plastic reagent bottle.

50mM EGTA

0.9509g of Ethylene glycol-bis-(2-aminoethyl) tetra-acetic acid (Sigma Chemical Co., London) was accurately weighed and suspended in a little quantity of distilled water in a 50ml beaker. With constant stirring, potassium hydroxide solution was carefully added to it until the pH is 7.4. The solution was quantitatively transferred into a 50ml volumetric flask and made up to the mark with water. After transfer into a small reagent bottle, the solution was stored at 4°C.

10% SDS

10g of sodium dodecyl sulphate, SDS (BIO-RAD Labs. Richmond, California, USA) was dissolved in 100ml of distilled water. The solution was left to stand on the bench at room temperature.

40mM ATP

0.4842g of disodium salt of Adenosine 5^{1}-triphosphate, ATP (Sigma Chemical Co., London)was carefully weighed and dissolved in 18ml of 10mM HEPES, pH 7.4. The pH of the solution was adjusted

to 7.4 and the solution made up to the mark in a 20ml volumetric flask. The solution was normally dispensed into eppendorf tubes (2ml aliquots) and stored at $0^{o}C$.

10mM Na_2HPO_4

0.142g of disodium hydrogen phosphate (Na_2HPO_4)(BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water and then made to the mark in a 100ml standard volumetric flask.

Calmodulin Solution

1mg calmodulin (Calbiochemicals Ltd., London) was dissolved in 5ml of 10mM HEPES pH 7.4 to produce a 200µg/ml solution of calmodulin.

Procedure

Determination of the Ca^{2+}-ATPase activity in the Membrane

The Ca^{2+}-ATPase activity was assayed by measuring the rate of release of inorganic phosphate from the Y-position of ATP. The phosphate liberated to the medium was determined colorimetrically using

the method of Raess and Vincenzi (1980) based on the procedure of Fiske and Subbarow (1925).

Calmodulin-depleted membranes (100-200µg) were incubated at 37°C for 5 minutes in a final total volume of 1ml. Final concentrations of the constituents in the assay medium was 100mM KCl, 50mM potassium-HEPES pH7.4, 2mM $MgCl_2$, 20mM $CaCl_2$, 2mM EGTA (when added), and 2µg/ml calmodulin (when added) The reaction was started by adding 2mM ATP (Final concentration). After 30 minutes of incubation at 37°C, with constant shaking, the reaction was stopped with 1ml of 10% solution of sodium dodecyl sulphate (SDS) in distilled water, 1ml of reagent B was then added to the reaction mixture and a blue colour was developed with the addition of 9% ascorbic acid solution. After 30 mins the absorbance of the solutions was read at 660nM. Exact timing between the addition of reagent A and reading at 660mm is critical.

The activity of the Mg^{2+}-dependent ATPase (assayed in the absence of added $CaCl_2$ i.e. in the presence of 2mM EGTA) was subtracted from the total assay in the presence of Ca^{2+}.

2.6 Purification of erythrocyte plasma membrane Ca^{2+}-ATPase on a calmodulin affinity column

2M KCl

37.29g of potassium chloride, KCl (BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water in a 250ml standard volumetric flask. The solution was made up to the mark, transferred quantitatively into a reagent bottle and stored at 4°C. 2M NaCl was prepared the same way except that 29.22g of sodium chloride (BDH Chemicals Ltd., England) was used instead of KCl.

20% Triton X-100

20g of Triton X-100 (Sigma Chemical Co., London) was dissolved in 100ml of distilled water and the solution was stored at 4°C.

100mM $MgCl_2$

2.034g of Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) (BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water in a 100ml standard volumetric flask. After

dissolution, the solution was made up to the mark, transferred quantitatively into a reagent bottle and stored at 4°C.

100mM HEPES, pH 7.4

5.95g of N-2-hydroxyethyl piperazine-N^1-ethanesulphonic acid, HEPES (Sigma Chemical Co., London) was dissolved in 200ml of distilled water and the pH was adjusted to 7.4. The solution was made up to 250ml in a standard volumetric flask and stored at 4°C.

100mM $CaCl_2$

1.471g of calcium chloride dihydrate, $CaCl_2.2H_2O$ (BDH Chemicals Ltd., England) was dissolved in a little quantity of distilled water in a 100ml standard volumetric flask. The solution was made up to the mark, transfered quantitatively into a reagent bottle and stored at 4°C.

100mM EDTA pH 7.4

18.6g of the sodium salt of ethylene diamine tetracetic acid, EDTA (Sigma Chemical Co., London) was dissolved in about 40ml of distiled water and

the pH was adjusted to 7.4. The solution was made up to 50ml in a standard volumetric flask and stored at 4°C.

10mg/ml Phosphatidyl Choline

10mg of phosphatidyl choline was dispersed in a tube and was sonicated to clarity. 1ml of storage buffer (130mM KCl, 20mM HEPES, 500µM $MgCl_2$, 50µl $CaCl_2$, pH 7.4) was later added to dissolve the sonicated phospholipid and stored at 4°C.

500mM Dithiothreitol

7.6g of dithiothreitol (DTT) (Aldrich Chemical Co., U.S.A.) was dissolved in a about 70mls of distilled water. The solution was made up with distilled water to 100ml in a standard volumetric flask and stored at 4°C.

87% glycerol

This reagent was purchased from (Fluka Chemical Co., Switzerland) and was stored at room temperature.

0.5% Sodium Azide

500mg of sodium azide (Fluka Chemical Co., Switzerland) was dissolved in about 80mls of distilled water in a volumetric flask. The solution was made up to the mark and transfered in to a reagent bottle and stored at 4°C.

A. Solubilization Buffer pH 7.4

This was prepared as follows:

Stock Solution	Volume used in Buffer preparation	Final Concentration
1M NaCl	13ml	130mM
100mM HEPES pH 7.4	10ml	10mM
100mM $MgCl_2$	0.5ml	0.5mM
100mM $CaCl_2$	0.05ml	0.05µM

Distilled water was added to make up to the final volume of 100ml.

B. Equilibration Buffer pH 7.4

This was also prepared as follows;

Stock Solution	Volume used in Buffer preparation	Final Concentration
20% Triton X-100	2.0ml	0.4%
100mM Hepes pH 7.4	20ml	20mM
NaCl	13ml	130mM
100mM $MgCl_2$	1ml	1mM
100mM $CaCl_2$	0.1ml	100µM
85%	11.49ml	10%
500mM DTT	0.4ml	2mM DTT
5mg/ml phosphatidyl choline	50mg	500µg/ml

Distilled water was added to make up to the final volume of 100ml.

C. Washing Buffer

Stock Solutions	Volume used in Buffer preparation	Final Concentration
20% Triton X-100	0.25ml	0.05%
100mM HEPES pH 7.4	20ml	20mM
1M NaCl	13ml	130mM
100mM $MgCl_2$	1ml	1mM
100mM $CaCl_2$	0.1ml	100µM
85% glycerol	11.49ml	10%
500mM DTT	0.4ml	2mM
5mg/ml phosphatidyl choline	50mg	0.5mg/ml

Distilled water was added to make up to the final volume of 100ml.

D. Elution Buffer pH 7.4

Stock Solutions	Volume used in Buffer preparation	Final Concentration
20% Triton X-100	0.25ml	0.05%
100mM HEPES pH 7.4	20ml	20mM
1M NaCl	13ml	130mM
100mM $MgCl_2$	1ml	1mM
85% glycerol	11.49ml	10%
500mM DTT	0.4 ml	2mM
5mg/ml phosphatidyl choline.	50mg	0.5mg
500mM EDTA	0.4ml	2mM

Distilled water was added to make up to the final volume of 100ml.

Solutions for washing calmodulin Affinity Column After Elution

E. 1st washing medium

Stock solution	Volume use in Buffer preparation	Final Concentration
20% Triton X-100	2.5ml	0.5%
500mM EDTA-Na	0.2ml	1mM

Distilled water was added to make up to the final volume of 100ml.

F. 2nd washing medium

Stock Solution	Volume used in Buffer preparation	Final Concentration
100mM HEPES pH 7.4	10ml	10mM
5% Sodium Azide	4ml	0.02%
100mM $CaCl_2$	0.1ml	0.1mM
1M NaCl	13ml	130mM

Distilled water was added to make up to the final volume of 100ml.

Procedure

The solubilization and purification of Ca^{2+}-ATPasewwas performed at 4°C, except where indicated and was carried out using the procedure of Niggli et al (1981). 20-100ml of white calmodulin-depleted erythrocyte membrane suspension (150-200mg protein) were concentrated by centrifugation at 36,000 rpm for 15 mins in a Beckman Centrifuge. The pellet was diluted with Buffer A and slowly the required amount of Triton X-100 to a final concentration of 0.45% was added (0.9mg Triton/mg protein). The solubilized membranes were stirred on ice for 10 mins to ensure complete solubilization. The solubilized membranes were centrifuged at 38,000 rpm for 35 mins in order to remove unsolubilized material. The supernatant containing the solubilized Ca^{2+}-ATPase was carefully removed and pasteur pipette that has been rinsed several times with cold solubilization buffer and the volume was noted. Phosphatidyl choline was added to a final concentration of 0.5 mg/ml and $CaCl_2$ was added up to 0.1mM and glycerol was also added to make a final volume

of 10%. The calmodulin-sepharose 4B column (4ml bed volume) was equlibrated with 2 bed volumes of buffer B (flow rate 10ml/hr; SG 3/250). The protein solution was passed at the same velocity. The calmodulin affinity column was washed overnight after the column has been loaded with the solubilized enzyme with buffer B and 100ml of buffer C (16ml/hr; SG $^{a}/_{500}$). The Ca^{2+}-ATPase was eluted from the column by using about 100ml of the elution buffer (buffer D) and Ca^{2+}-ATPase assay was performed on 0.6ml fraction (5 min/fraction 7ml/hr; SG $^{1}/_{125}$). Fractions containing Ca^{2+}-ATPase activity were pooled and $MgCl_2$ was added up to 2mM and $CaCl_2$ up to 0.05mM. The Ca^{2+}-ATPase was frozen at -70°C until used specifically for SDS-PAGE.

The calmodulin-affinity column was treated after each use as follows: 100ml of buffer E containing 0.5% Triton X-100 and 1mM EDTA was passed through the column to remove phospholipids. 100ml of Buffer F was also passed several times to remove non-specifically bound proteins and was stored in this buffer at 4°C until used again.

2.7 Purification of the enzyme in the absence of added phosphatidyl choline

The solubilization and purification of Ca^{2+}-ATPase performed at $4^{o}C$ except where indicated was carried out using the procedure of Niggili et al (1981). 20-100ml of white calmodulin-depleted erythrocyte membrane suspension (150-200mg protein) were concentrated by centrifugation at 36,000 rpm for 15 mins in a Beckman centrifuge. The pellet was diluted with Buffer A and the required amount of Triton X-100 to a final concentrations of 0.4%, 0.8%, and 1% was slowly added (0.8mg Triton/mg protein, 1.1mg Triton/mg protein, 2mg Triton/mg protein) and glycerol was added to a final volume of 20%. The solubilized membranes was stirred on ice for 10 mins to ensure complete solubilization. The solubilized membranes were centrifuged at 38,000 rpm for 35 mins in order to remove unsolubilized material.

The supernatant containing the solubilized Ca^{2+}-ATPase was carefully removed with pasteur pipette that has been rinsed several time with cold solubilization buffer.

The calmodulin - sepharose 4B column (4ml bed volume) was equlibrated with 2 bed volumes of Buffer B (flow rate 10ml/hr; Sg $^{3}/_{250}$). Buffer B always contained the amount of Triton X-100 that was used for a particular solubilization. The protein solution was passed at the same velocity. The calmodulin affinity column was washed overnight with buffer B after the column had been loaded with the solubilized enzyme. The Ca^{2+}-ATPase was eluted from the column by using about 100ml of the elution buffer (Buffer C) and Ca^{2+}-ATPase assay was performed on 0.6ml fraction (5 min/fraction, 7ml/hr; SG $^{1}/_{125}$). Fractions containing Ca^{2+}-ATPase activity were pooled and frozen at $-70^{o}C$ until used.

2.8 Assay of purified Ca^{2+}-ATPase

Principle

This method is based on the reaction of phosphomolybdate with the basic dye malachite green to form a coloured complex.

Reagents

0.5% Malachite green hydrochloride

0.5g of malachite green hydrochloride (Fluka Chemicals, Switzerland) was dissolved in about 80ml of distilled water. The solution was stirred very well and the volume was made up to 100ml with distilled water.

10% Ammonium molybdate in 4N HCl

50g of Ammonium molybdate, $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$); (Hopkins and Williams Ltd., England) was dissolved in 500ml of 4N hydrochloric acid (BDH Chemicals Ltd., England).

34% Sodium citrate

34g of Sodium citrate (BDH Chemicals, London) was dissolved in about 80ml of distilled water and stirred for some minutes until all have dissolved. The solution was made up to 100ml with distilled water.

Lanzetta reagent

0.045% of malachite green hydrochloride was mixed with 4.2% ammonium molybdate in 4N HCl in the ratio 3:1 and this solution was stirred for 20 minutes and filtered through Whatman 5 and 0.8ml of 20% Triton X-100 was later added per 100ml of the solution.

Procedure

Phosphate assay technique

The release of inorganic phosphate from ATP was measured according to the method of Lanzetta et al, 1979. 0.2ml of the assay medium was suspended in an eppendorf tube containing the the storage buffer and this was transferred to

Rocomat heater at 37°C and protein sample was added. The reaction was started with 1mM ATP five minutes after incubation at 37°C. 0.1ml of the medium was transferred into a test tube containing 1.6ml of Lanzetta reagent and mixed vigorously. The reaction was stopped 1 minute after, with 0.2ml of 34% sodium citrate $2H_2O$ (BDH Chemicals, London) and 30 minutes after the reaction was measured at 660nm in a Perkin Elmer Spectrophotometer. The blank contained water, color reagent and citrate. Standard inorganic phosphate (2-10n moles) was prepared using a 1mM K-phosphate solution.

2.9 Binding studies on the interaction of aflatoxin B_1 to erythrocyte membrane

Principle of emission intensities

The model 204 fluorescence spectrophotometer is a grating instrument designed to automatically record fluorescence and phosphorescence emission intensity versus wavelength in the ultraviolet and visible regions for a variety of organic and inorganic materials.

Fluorescence is the phenomenon whereby a molecule, after absorbing radiation, emits radiation of a longer wavelength. Thus a compound may absorb radiation in the ultraviolet region and emit visible light. This increase in wavelength is known as stoke's shift. At room temperature most organic molecules are in the ground state. Absorption of photons elevates electrons in these molecules to a higher energy state in less than 10 sec^{-15}. After absorption, energy is lost very rapidly by collision degradation (as heat), resulting in the energy of the excited molecules falling rapidly to that of minimal vibrational energy in the lowest excited state. The energy emitted from these molecules in regaining ground state within a period of less than 10^{-8} sec. gives rise to a fluorescent peak, showing the stokes shift. Aromatic molecules containing delocalized π electrons sometimes fluoresce.

Aflatoxin B_1 molecule is a bifurranocoumarin derivative and it has an α, B unsaturated lactone ring, and if its irradiated with U.V light it will fluoresce

Reagents:

Quinine sulphate 10mg/ml

10mg of Quinine sulphate (Hopkins and Williams, England) was dissolved in 1ml of 50mM conc. H_2SO_4, pH 2. The stock solution was later diluted to the required 0.1µg/ml concentration. The solution was kept in a brown bottle.

100mM sodium carbonate buffer pH 10.0

The buffer was prepared by mixing 50ml of 0.1M sodium carbonate (8.4gNaH CO_3/L) and 10.7ml of 0.1N NaOH. The solution was made up to 100ml with distilled water and the pH checked using a pH meter.

10mM sodium acetate buffer pH 4.0

The buffer was prepared by mixing 41.0ml of 0.1M acetic solution (5.7ml /L) and 9.0ml of 0.1M sodium acetate (8.2g $C_2H_3O_2Na$/L). The solution was made up to 100ml with distilled water after checking the pH to be 4.0 with a pH meter.

Dialysis bag or Visking tubing

Visking tubings were purchased from (Scientific Centre, London, U.K).

Washing of dialysis bag

The visking tubing was rinsed in distilled water followed by boiling in 0.2M EDTA for about three hours. The solution was changed every 30 minutes. The visking tubing was immersed in about 100ml of 0.02M EDTA and stored at 4°C. The dialysis bag was rinsed in distilled water before it was used.

Procedure

Thoroughly washed tubing was securely tied at one end and checked for leakage. 20µg of ghost membrane was suspended in 3.5ml of buffer containing 130mM KCl, 20mM Tris, 50µM $MgCl_2$) in the tubing and then securely tied at the other end. The dialysis bag was immersed in a beaker containing various concentrations of aflatoxin B_1. The beaker was kept on ice with constant shaking for 6 hours. The free aflatoxin B_1 and membrane bound aflatoxin B_1 were determined using Perkin Elmer Spectrofluorimeter 204.

2.10 Proteolysis of Ca^{2+}-ATPase

Reagents

1mg Trypsin

1mg Trypsin (Boehringer Diagonistics Manheim, FRG) was dissolved in about 800µl of distilled water on ice and stirred until dissolved. The solution was made up to 1ml with distilled water and dispensed in 50µl aliquots in an eppendorf tube and kept at $4^{o}C$.

1mg Trypsin Inhibitor

1mg soybean trypsin inhibitor (Boehringer Diagonistics, Manheim, FRG) was dissolved in about 800µl of distilled water and stirred until dissolved. The solution was made up to 1ml, dispensed in 50µl aliquots and stored at 4°C.

10mg Leupeptin

50mg of Leupeptin/Sigma Chem. London) was dissolved in about 8ml of distilled water and mixed vigourously for it to dissolved. Distilled water was added to make 10ml. The solution was stored in a reagent bottle and stored at 4°C.

2mg Aflatoxin B_1

5mg of aflatoxin B_1 (Sigma Chem. London) was dissolved in 10ml of dimethylsulfoxide (DMSO). The solution was stored in a brown bottle at 4°C.

Procedure

The ATPase samples (0.05mg/ml) were digested on ice with 2ug of trypsin 1mg/ml. The reaction was stopped by the addition of a 2 fold weight excess of soybean trypsin inhibitor. In order to

obtain appreciable amounts of 90 KDa and 76 KDa fragments, the proteolysis was performed in an EDTA storage buffer (130mM KCl, 20mM HEPES, pH 7.2 0.05% Triton X-100, 0.05% phosphatidyl choline (PC), 2mM EDTA and 1mM $MgCl_2$) for 5-7 and 90 min respectively. SDS-polyacrylamide Gel electrophoresis was carried out as on page 189. The assay of the ATPase was carried out in the presence of 1µg aflatoxin B_1. The activity was determine according to the procedure of Lanzetta et al, 1979 on page 173.

Calpain digestion was performed in the presence of calpain for 2 hours (1 Iu Calpain per 10µg ATPase) in the presence of 0.5mM total Ca^{2+} with 20nM calmodulin. Proteolysis was stopped by adding 10 fold molar excess of leupeptin. Assay of the ATPase was done in the presence of 1µg aflatoxin and the activity was determined as on page 173.

2.11 Electrophorectic separation of membrane-bound and purified proteins on continuous gradients of sodium dodecyl sulphate-polyacrylamide gels

A very sensitive method for characterizing and

ascertaining the purity of a purified protein is by electrophoresis on a polyacrylamide gel. Electrophorectic procedures are rapid and detection of the polypeptide bands in the gel by staining or autoradiography is sensitive. Electrophorectic migration rates have a fairly predictable relation to the molecular weight of proteins, if the proteins are dissociated and denatured with sodium dodecyl sulphate (SDS) before and during electrophoresis (Weber and Osborne, 1969).

Principle

Membrane proteins are solubilized by treatment with SDS, which binds to proteins, conferring on them a net negative charge. Most proteins bind a fixed amount of SDS per amino acid, and as a result, proteins acquire a fixed charge to mass ratio and differ only in their size. As the polypeptides become saturated with SDS, they change into helical rods surrounded by an SDS-shell . Electrophoresis of such proteins separates them almost strictly according to size.

The mobility of the proteins through a polyacrylamide gel is inversely proportional to the logarithm of the molecular weight of the polypeptide.

In order to ensure that the proteins are unfolded and separated into monomers, the disulphide bonds in them are reduced with dithiothreitol or mercaptoethanol followed by a brief exposure at 100°C.

The polyacrylamide is formed by the co-polymerization of acrylamide and N,N-methylene bisacrylamide in the presence of a catalyst (e.g. ammonium persulphate) and an initiator (e.g. (dimethyl aminopropionitrite or N,N,N' N'-tetra methylethylenediamine). Concentional means of visualizing proteins on electrophoresis gels is by staining using agents like Nigrosin in acetic acid or trichloroacetic blue - zinc sulphate-acetic acid, periodic acid oxidation, followed by treatment with Schiff's reagents (forglycoproteins) etc.

Reagents

(i) Stock A: 30% $^{w}/_{v}$ Acrylamide, 0.8% $^{w}/_{v}$ Bisacrylamide

30g of acrylamide (Sigma Chemical Co., London) and 0.8g of N,N'-methylene-bisacrylamide (BIO-RAD Labs. California, U.S.A.) were dissolved in 100ml of distilled water in a 100ml standard volumetric flask. The solution was filtered and stored at 4°C.

(ii) Stock C: (Separating Gel Buffer) 1.5M Tris HCl pH 8.8

18.17g of Tris (hydroxymethyl) aminoethane, base (Sigma Chemical Co., London) was dissolved in about 90ml of distilled water in a beaker and the pH was adjusted to 8.8 with 1M hydrochloric acid. The solution was quantitatively transferred into a 100ml standard volumetric flask and made up to the mark with distilled water. The solution was stored in a reagent bottle at 4°C.

(iii) Stock E: (Stacking Gel Buffer): 1.25M Tris pH 6.8

15.15g of tris (hydroxymethyl) amino methane base (Sigma Chemical Co., London) was dissolved in

about 90ml of distilled water in a beaker and the pH was adjusted to 6.8 with hydrochloric acid (1M). The solution was quantitatively transferred into a 100ml standard volumetric flask and made up to the mark with distilled water . The solution was stored in a reagent bottle at 4°C.

Stacking Gel Buffer B: 0.5M Tris-Cl, 8mM EDTA, 0.4% SDS, pH 6.8

3.03g of Tris (hydroxymethyl) amino methane base (Sigma Chemical Co., London), 149mg of sodium salt of (ethylenediaminetetraaceticacid) EDTA (Sigma Chemical Co., London) and 0.2g of sodium dodecyl sulphate, SDS (BIO-RAD Labs. California USA) were all dissolved in about 30ml of double distilled water and the pH was adjusted to 6.8 with 2M HCl. The solution was quantitatively transferred into a 50ml standard volumetric flask and made up to the final mark with double distilled water. The solution was filter through 0.4µM filter and was later stored in a reagent bottle at 4°C.

Ammonium Persulphate: 0.75% and 10%

0.075g and 10g of ammonium persulphate, $(NH_4)_2 S_2O_8$ (BIO-RAD Labs. California U.S.A.) was dissolved in 10ml of distilled water respectively. The solution was usually prepared fresh.

N,N, N', N'-tetramethyl ethylene diamine (TEMED)

This reagent was purchased from Sigma Chemical, Co., Ltd., London and was stored in a refridgerator.

Running gel buffer: 1.5M Tris-Cl , 8mM EDTA 0.4% SDS, pH 8.8

45.5g Tris (hydroxymethyl) aminomethane base (Sigma Chemical Co., London), 745mg sodium salt of ethylenediaminetetraaceticacid EDTA (Sigma Chemical Co., London) and 1g of sodium dodecyl sulphate SDS (BIO-RAD Labs. California U.S.A.) were all dissolved in about 150ml of double distilled water and the pH was adjusted to 8.8 with 2M HCl. The solution was quantitatively transferred into a 250ml volumetric flask and was later filtered through, 0.45µM filter and was later stored in the refridgerator.

10X-Stock Running Buffer

30g of Tris (hydroxymethyl)aamino methane (Sigma Chemical Co., London), 144.1g of glycine (Sigma Chemical Co.,London), and 10g of sodium dodecyl sulphate (SDS), were dissolved in water and made up to the mark in a 1 litre volumetric flask, The solution was stored at room temperature in a reagent bottle.

5X-Stock Electrode buffer pH 8.3

150g of Tris (hydroxymethyl) aminomethane (Sigma Chemical Co., London), 720g of glycine (Sigma Chemical Co., London), 25g of sodium dodecyl sulphate (SDS) (BIO RAD Lab. California USA) and 12g of sodium salt of ethylenediaminetetraaceticacid (Sigma Chemical Co., London) were dissolved in a double distilled water and made up to the mark in a 4 litre volumetric flask (the pH of the concentrated solution is slightly higher but it will change upon dilution to the right value. The pH should not be changed with HCl, since chloride anions in the electrode buffer do not allow the formation of a thin band during electrophoresis.

Working Running Buffer

The 10X stock running buffer was diluted ten times (10X) just before use by adding 150ml of the 10X stock to 1350ml of distilled water to produce a 1.5 litre solution. Also the 5X electrode buffer was diluted 5 times (5X) by adding 1 litre of the 5X stock to 4 litres of double distilled water to produce 5 litre solution. This is stored at room temperature until it is finished.

Stock D (Anionic Detergent Solution) 10% SDS

10g of sodium dodecyl sulphate, SDS (BIO-RAD Labs. California, USA) was dissolved in 100ml of distilled water and the solution stored at room temperature.

Sample buffer

This was prepared by mixing 1ml of Stock E, 4.0ml of Stock D, 2.0ml of glycerol (BDH Chemicals Ltd., England), 1.0ml of B-mercaptoethanol (Sigma Chemical Co., London), 0.2ml of 2% Bromophenol blue (BDH Chemicals Ltd., England) and 11.80ml of double distilled water to make a total volume of 20.0ml. The solution was stored at $0^{o}C$ in 1ml aliquots.

Fixing Solution: 10% TCA-50% Methanol

10g of Trichloroacetic acid BDH Chemicals Ltd., England), was dissolved in a little double distilled water. 500ml of methanol (BD Chemicals Ltd., England) was added, and the solution made up to 1 litre with double distilled water.

Stain Solution: 0.25% Coomasie Brilliant Blue, 50% Methanol, 7.5% Acetic Acid

0.5g of Coomasie Brilliant Blue (Serva Feinbiochemica, Heilderberg), was dissolved in 100ml methanol (BDH Chemicals Ltd., England). 15ml of glacial acetic acid (BDH Chemicals Ltd., England) was then added and the solution made up to 200ml with distilled water and stored in a brown bottle.

Destaining Solution: 20% Methanol, 7.5% Acetic Acid

400ml of methanol (BDH Chemicals Ltd., England) and 150ml of glacial acetic acid (BDH Chemicals Ltd., England) were measured into a 2 litre standard flask. Distilled water was then added up to the 2 litre mark.

Procedure A

Making the Gel Sandwich

Two glass plates (18 x 16 x 0.3cm each), spacers and combs were properly washed and cleaned with ethanol to ensure removal of grease which might interfere with gel polymerisation. Grease was sparingly used in the assembly of these materials. The grease was first applied to the spacers and the rubber gasket for the casting stand. A sandwitch of the two glass plates was made by using the greased spacers and clamps. The sandwitch was then clamped into the casting stand with the rubber gasket to form a mould for the gel. Adequate care was taken to ensure that minimum grease enters the space where the gel will be formed since grease retards gel polymerization. Deionized water was then carefully poured into the gel space to test for any leakage.

The running and stacking gels were prepared as shown in Table 14.

TABLE 14

Preparation of running and stacking gels (A)

	Running gel		Stacking gel
	20%	5%	
Stock A	11.33ml	2.83ml	2.66ml
Stock C	4.25ml	4.25ml	-
Stock E	-	-	5.28ml
Water	-	9.18ml	11.22ml
Stock D	0.17ml	0.17ml	0.22ml
Glycerol	0.98ml	0.23ml	-
TEMED	0.01ml	0.005ml	0.028ml
0.75% Ammonium persulfate	0.26ml	0.335ml	-
1.5% Ammonium persulfate	-	-	0.57ml

After stirring, 1ml of solution was removed from each of the 20% and 5% Running gels.

The gradient maker was used for the purpose of mixing the running gel. A long rubber tubing was connected to the free end of the outlet of the gradient maker and served to fill the glass sandwitch with the gel mixture.

The gradient maker was placed on a magnetic stirrer, its mixing chamber containing a small magnetic stirrer was filled with 16ml of the 20% solution while the reservoir chamber was also filled with 16ml of the 5% solution. While stirring, the value of the gradient maker was opened to allow the 5% solution to flow steadily into the mixing chamber. Simultaneously the free end of the rubber tubing was placed between the glass plates of the sandwitch. When all the gel has run into the glass sandwitch, the top of the gel was layered with water. After the gel has polymerized, the water was poured off and the surface of the gel rinsed with distilled water. A comb was inserted and the stacking

gel solution was carefully layered on the running gel using a pasteur pipette. After the stacking gel has polymerized, the comb was removed and the gel sandwitch was filled to the underside of the upper buffer chamber using the rubber gaskets. The upper buffer chamber was filled half-way with the working running buffer; the lower buffer chamber of the electrophoresis unit was also filled to about one quarter full with the same buffer. The gel sandwitch sealed to the upper chamber containing the buffer was lifted off the casting stand and lowered into the lower buffer chamber.

Procedure B

Making the Mini gel Sandwitch

Two glass plates (5cm x 4cm x 1.5mm each) spacers and combs were properly washed and cleaned with ethanol to ensure removal of grease which might interfere with gel polymerisation. A sandwitch of the two glass plates was made by using the spacers and clamps. The sandwitch was then clamped into the casting stand with the gel. Deionized water

was then carefully poured into the gel space to test for any leakage.

The running and stacking gels were prepared as shown in Table 15. The running gel was poured into the gel assembly to a level of about 3cm below the maximal filling level. The gel was immediately overlay with ¼ diluted running gel buffer. The gel was allowed to polymerise, water was poured on the gel to rinse the gel, the water poured off and stacking gel was poured and the comb was immediately inserted. After about one hour the comb was removed and the gel was clamped to the electrophoresis chamber. Both chambers were filled with electrode buffer, with a syringe; bubbles that were found below the gel were removed.

Sample Application and Electrophoresis

The protein or membrane samples for electrophoresis were prepared by mixing 20µl to 50µl of the sample with 150µl - 180µl of the sample buffer in eppendorf tubes. These were incubated in a

TABLE 15

Preparation of running and stacking minigels spacers

	Stacking gel 4%	Running gel 7%
Stock A (ml)	0.250	1.200
Buffer (ml	0.500 pH 6.8	1.250 pH 8.8
TEMED	5µl	5µl
10% Ammonium persulfate	25µl	25µl
Total Volume	2ml	5ml

boiling water for 3 minutes to ensure breakage of the disulphide bonds in the proteins. Equal amounts of the samples (40µg - 50µg) were then carefully applied to the bottom of the sample wells with a Hamilton syringe. The electrophorectic unit was then connected to the power pack unit. Electrophoresis was run at a constant voltage of 60V and a constant current of 25mA in an air-conditioned room until the tracking dye was about 1cm from the bottom of the gel.

After electrophoresis, the gel was carefully removed from the slabs, fixed in a solution containing 50% methanol, and 7.5% acetic acid for about 30 minutes and then stained for about 3 hours with Coomasie Brilliant blue (R250) solution (0.25% Coomasie Brilliant blue, 50% CH_3OH and 7.5% acetic acid). The gel was destained by rinsing it several times in a solution of methanol, acetic acid: water (10:7:83 v/v). The gel was later drained and mounted on a glass plate for photography (Plate 1 and 2)

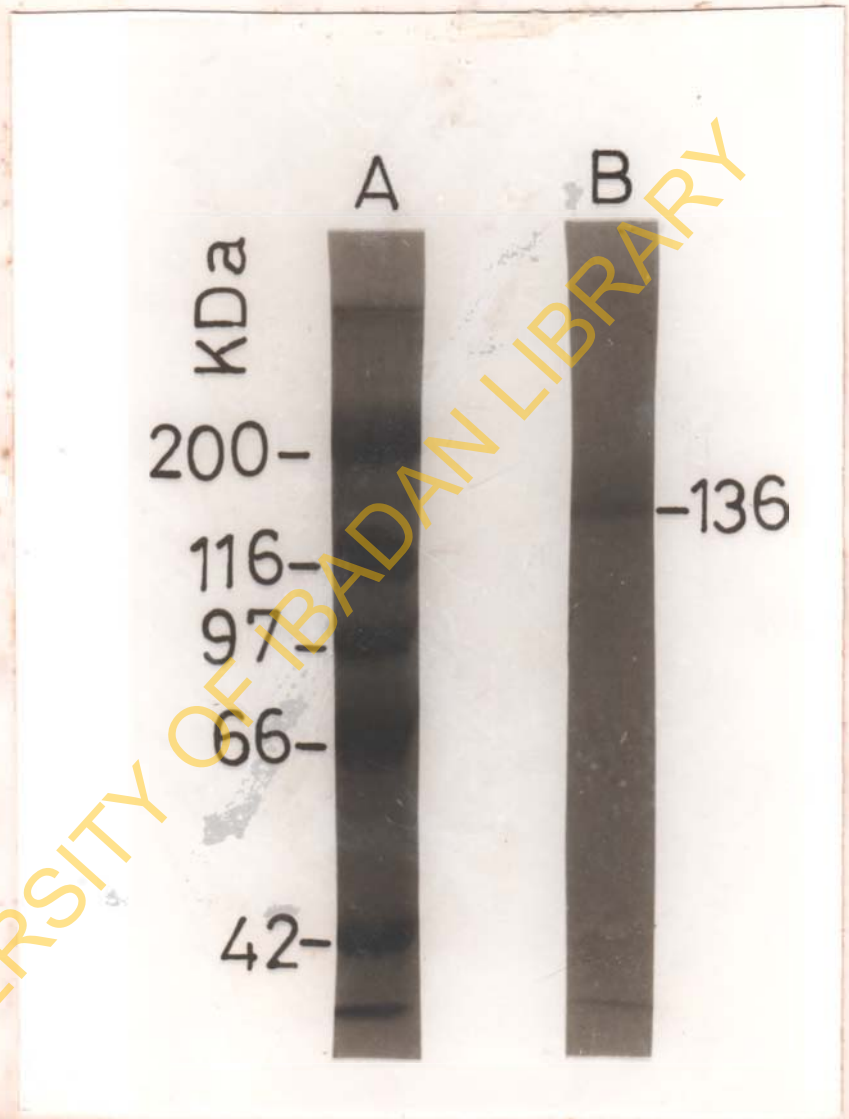


Plate 1: SDS-polyacrylamide gel electrophoresis of erythrocyte purified Ca^{2+}-pumping ATPase from erythrocyte (B). (A) are values (in KDa) indicative of the molecular weights of the protein. The purified enzyme was applied to a 4-7% continuous gradient gel as described in page 194.

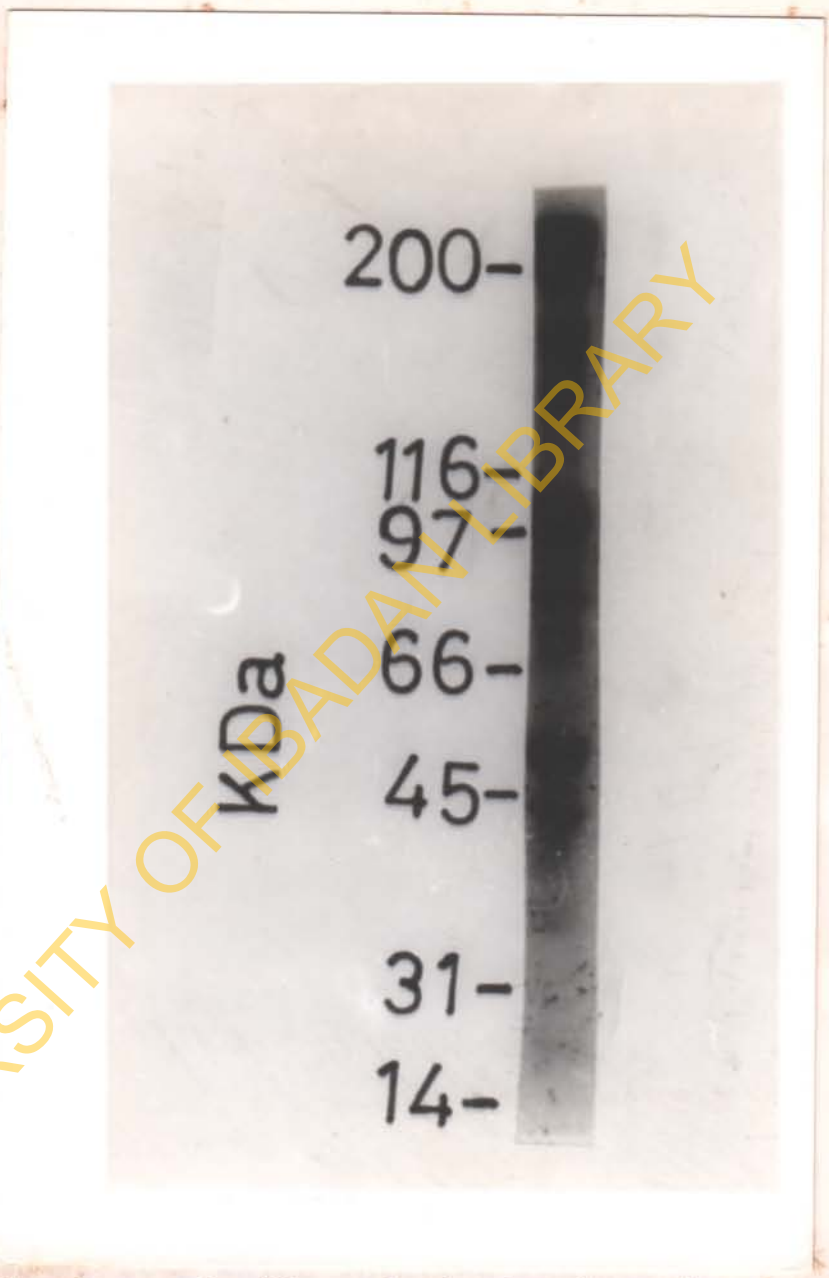


Plate 2: SDS-polyacrylamide gel electrophoresis of erythrocyte ghost membrane. The ghost membrane was applied to a 5-20% continuous gradient gel as described on pp. 190 values above are indicative of the molecular weights of the protein markers.

2:12 Silver staining technique

Principle

The sensitivity of silver staining method is about 50 times greater than with Coomassie blue. It is possible to dectect with this method as low as 5ng of protein. Silver staining technique involves about 5 different steps. The first step involves fixing of the protein in the gel with methanol and acetic acid. This step is needed in order to prevent the elution of proteins from gel during the staining process. Methanol removes some of the gel impurity which might interfere with the staining. However, fixing with methanol causes shrinking of gels while the washing of the gel in ethanol and acetic acid allows the gel to swell to normal size, thus helping to remove contaminating buffer and ions (SDS, glycerol and glycine) which might reduce the sensitivity of the method. The third step which involves the use of an oxidizer favours the interaction of silver ions with the protein and also encourages the reduction

of the bound silver ions to metallic silver during the development step. Albeit, if this step is omitted a negative image of the protein band is obtained and the gel matrix will then be preferentially stained. In addition, the oxidizer enhances the formation of a positive image by converting the protein hydroxyl and sulfhydryl groups to aldehydes and thiosulfate.

The development of the colour of the protein bands which is the last step makes use of formaldehyde, a strong reducing agent in alkaline medium. Formic acid so formed in the reaction is buffered by carbonate solution. The alkaline formaldehyde reagent reduces silver ions that is bound to the protein to metallic silver. Although, silver ions are also complexed with the polyacrylamide gel, however, their reduction occurs at a much slower rate.

Reagents

Fixing solution: 50% methanol + 12% acetic acid

This was made up by mixing 50ml of methanol (Analytical grade), 12ml of acetic acid and 38ml of

distilled water in a beaker. The solution was stored at room temperature.

Washing solution: 10% ethanol + 5% acetic acid

This was made up by mixing 10ml of ethanol (Analytical grade) and 5ml of acetic acid and 75ml of distilled water. The solution was stored room temperature until use.

Oxidizing solution: 2.5g potassium dichromate $K_2Cr_2O_7$ + 380µl 85% H_3PO_4

The oxidizer was made up by dissolving 2.5g of $K_2Cr_2O_7$ (Fluka, Chemicals Switzerland) in distilled water and mixed with 380µl of 85% H_3PO_4 in a 250ml volumetric flask. The solution was made up to 250ml with distilled water, and stored at 4°C in a brown reagent bottle until use. The solution was diluted 1:10 before use.

Staining solution: 5.1g silver nitrate ($AgNO_3$)

5.1g of $AgNO_3$ (Fluka, Chemicals, Switzerland) was dissolved in about 200ml of distilled water in a 250ml volumetric flask. The solution was mixed after making it up to 250ml. The solution was stored at 4°C.

The staining solution was diluted 1:10 before use.

Developing solution: 19.7g Na_2CO_3 + 0.5ml 37% formaldehyde

19.7g of Na_2CO_3 (General purpose Reagent (G.R.P.) Hopkin and Williams, Essex, England) was dissolved in about 800ml of distilled water. The solution was kept at room temperature. Before use, 0.5ml of 37% formaldehyde (37ml + 63ml distilled water) was added and stirred for about 10 minutes. The solution was used up immediately or discarded. Never store the solution.

Procedure

The protein in the gel was fixed for 30 minutes in 150ml solution containing 50% methanol + 12% acetic acid and later the protein in the gel was washed twice for 20 minutes in 150ml of 10% ethanol and 5% acetic acid. It was later allowed to stand in the oxidizer for 10 minutes and then was washed 3 times for 2 minutes with distilled water and immersed in the silver staining solution for 20 minutes.

This staining solution was later rinsed off with distilled water. The colour was developed by rinsing the gel with the colour developer until the protein band becomes visible. The developing solution was always prepared fresh.

CHAPTER THREE

EXPERIMENTS AND RESULTS

Experiment 1

Investigation of the binding of aflatoxin B_1 to erythrocyte membrane

It has been shown that aflatoxin B_1 binds firmly to at least one site on either bovine or human serum albumin *in vitro* (Bassir and Bababunmi, 1973). The effect of functional groups on the interaction of aflatoxin B_1 and G_1 with starch, cellulose and seven other derivatives were later studied by Uwaifo and Bassir (1977) using equilibrium dialysis.

The observation that the amount of aflatoxin B_1 in blood was third highest to that in the liver, 24 hours after injection suggests strongly that aflatoxin B_1 could interact with the red cell membrane. The fact that the liver harbours the highest amount of aflatoxin (Wogan, 1968) suggests also that the toxin penetrates the lipid bilayer with considerable ease probably because of its high lipid solubility.

In this study, the red cell membrane has been used as a model biological membrane mainly because it is readily available. There is no information in the literature on the consequence of the interaction of the toxin with plasma membrane. This is because the carcinogenic effect of the toxin is believed to be due to its effect on DNA synthesis. Nevertheless, the toxic effect of the toxin on the membrane can not be under-estimated since this may be the primary event that takes place in aflatoxin B_1 hepatocellular carcinogens.

The aim of the study was therefore, to establish whether or not aflatoxin B_1 binds to the red cell membrane.

Procedure (A)

80 µg of aflatoxin B_1 was preincubated with 24mg of erythrocyte membrane on ice. The aflatoxin B_1-membrane complex was washed several times with 100 volumes of a buffer containing 130mM KCl, 10mM Tris, 50 µg $CaCl_2$, and 500 µM $MgCl_2$ and centrifuged at 16,000rpm for 30 minutes.

The fluorescence of the resulting solution was measured in a fluorescence spectrophotometer against a blank containing 100µl of untreated membrane. To obtain pH 4 and 10, 100mM acetate buffer and carbonate buffer were used, respectively. The incubation was also performed at 37°C.

The exciter and analyzer wavelength used were those previously established for aflatoxin B_1 (370nm i.e. exciter, 460nm analyzer wavelength). Quinine sulphate dihydrate in 50mM H_2SO_4 (0.1 µg/ml) was used as a fluorescence standard and was irradiated at 365mm and analyzed at 460nm.

(B) Various concentrations of aflatoxin B_1 prepared in Dimethylsulfoxide (DMSO) were made in a beaker containing about 150ml of buffer (130mM KCl and 10mM Tris). 20 ug of erythrocyte membrane protein prepared as described on page 143 was carefully transferred into washed dialysis tubing containing 3.5ml of the buffer and tied at the other end. The tubing was suspended in the buffer containing aflatoxin B_1 and

dialysed with constant shaking as described on page 178. The bag was untied after dialysis emptied into an eppendorf tube. 3ml aliquot was taken out and its fluorescence measured in a Perkin-Elmer fluorimeter 204.

Results

The results obtained by shinning UV light on the supernatants of the first three washing steps, after centrifugation at 16,000 rpm, did not indicate the presence of aflatoxin, as no blue fluorescence was seen in the supernatants; whereas the membrane pellet showed an intense blue fluorescence characteristics of aflatoxin. In addition, supernatants obtained from the washing steps of untreated membranes did not give any blue fluorescence indicating the absence of aflatoxin.

Table 16 shows the various amounts of aflatoxin B_1 bound to the erythrocyte membrane. It seems clear from the data that aflatoxin B_1 still binds firmly to the membrane despite washing the membrane thoroughly. It may be inferredfrom

TABLE 16

Effect of pH and temperature on the binding of aflatoxin B_1 to the erythrocyte ghost membrane.

AFB_1-treated membrane	ng AFB_1 bound per µg membrane protein		
	zero min	10 min	30 min
Control	0.0	0.0	0.0
pH 4.0	3.12 ± 0.10	3.91 ± 0.10	4.63 ± 0.01
	*3.31 ± 0.14	*4.21 ± 0.13	*4.68 ± 0.02
pH 7.4	2.38 ± 0.05	3.11 ± 0.10	3.76 ± 0.03
	*2.43 ± 0.05	*3.18 ± 0.02	*3.83 ± 0.01
pH 10.0	1.24 ± 0.12	1.72 ± 0.03	2.31 ± 0.03
	*1.20 ± 0.17	*1.68 ± 0.01	*2.30 ± 0.02

Pellet obtained after incubating the membrane with aflatoxin solution at 4°C was irradiated at 360nm and analyzed at 460nm.

* Data were obtained from experiment carried out at 37°C

Each value is a mean of five different determinations ± standard error.

the results that aflatoxin B_1 binds spontaneously to the membrane even though more binding seemed to take place on longer period of incubation. Similar results were obtained when binding was studied at 37°C provided that incubation was not allowed to proceed beyond 30 minutes. Studies on the effect of pH on the binding of the toxin to the membrane indicate that more of the toxin molecules are bound at acidic pH while the degree of binding is drastically reduced at alkaline pH.

Results obtained from binding studies using equilibrium dialysis technique confirm that aflatoxin B_1 binds firmly with the cell membrane. Scatchard plot of the data obtained as shown in Fig. 16 indicate that the binding of aflatoxin B_1 to the membrane is a non-cooperative process and that 4.5 nmoles of the toxin was bound per ug membrane protein.

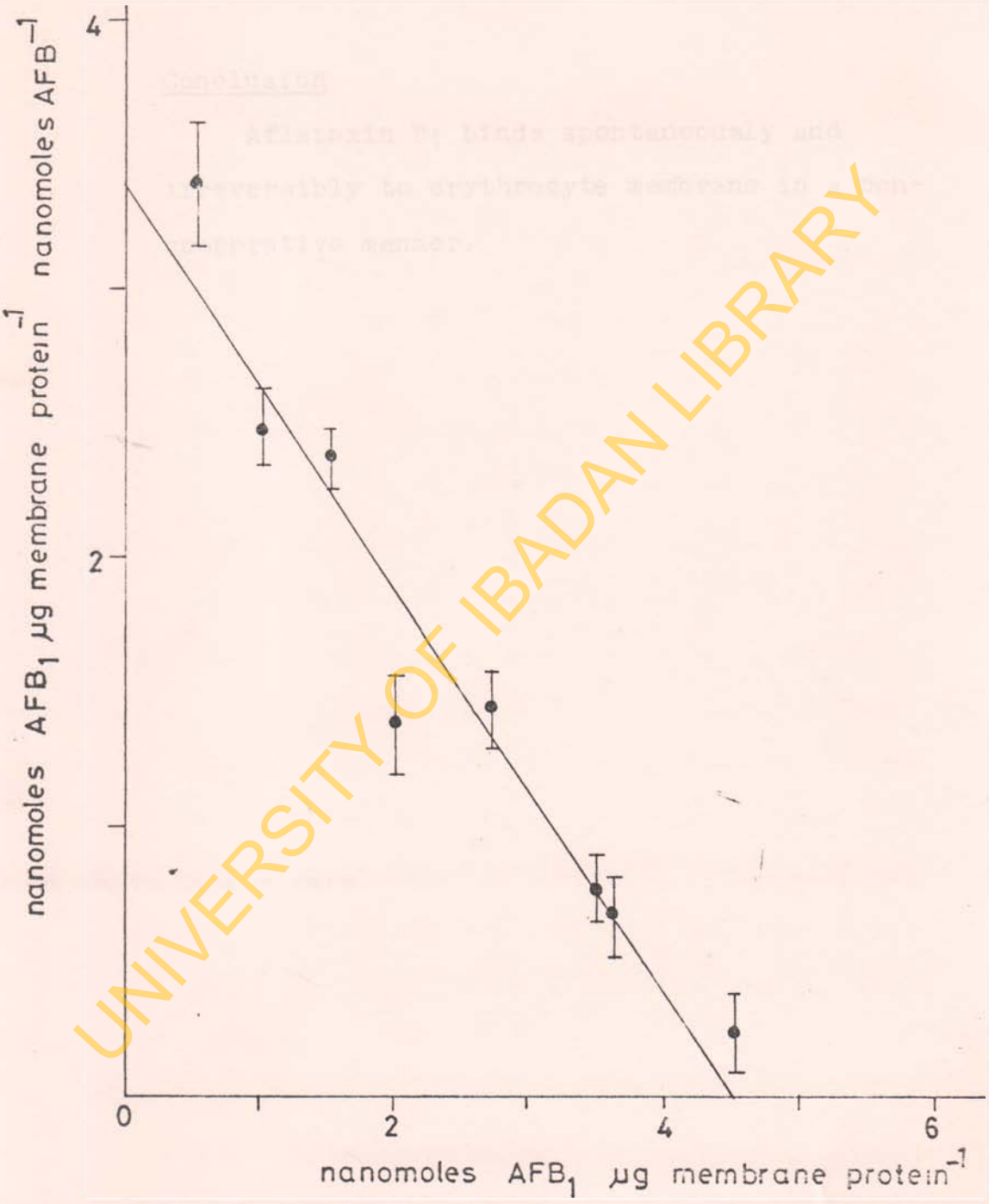


Fig. 16: Scatchard plot of the binding of aflatoxin B_1 to erythrocyte membranes.

Conclusion

Aflatoxin B_1 binds spontaneously and irreversibly to erythrocyte membrane in a non-cooperative manner.

Experiment 2

Influence of aflatoxin B_1 on erythrocyte membrane Ca^{2+} ATPase activity

The Ca^{2+} -pumping ATPase of the plasma membrane is an integral protein with a Mr of about 136KDa (Niggli et al, 1981) and representing less than 0.1% of the erythrocyte total membrane protein (Knauf et al, 1974). It has been suggested that about 700 copies of this protein are present in the erythrocyte membrane (Rega and Garrahan, 1975).

The most important endogenous regulator of the protein is calmodulin which becomes active after binding calcium ions (Lin et al, 1974). The catalytic and the calmodulin functional domains of the protein have been shown to possessimportant amino acids residues such as lysine (Kretsinger, 1980). Concidentally these amino acids have also been shown to be important for the binding of aflatoxin to BSA. It thus seems probable that aflatoxin B_1 could inhibit the Ca^{2+}-pumping ATPase by binding to such amino acid residues, most especially because data reported in experiment I in this thesis

have shown that, although the toxin binds spontaneously and irreversible to the erythrocyte membrane, the binding is favoured by increasing the acidity of the medium.

The aim of this study therefore was to investigate the effect of aflatoxin B_1 on the activity of the pump with or without calmodulin.

Procedure

Haemoglobin-free erythrocyte ghost membranes were prepared from normal fresh erythrocytes according to the procedure of Dodge et al (1963) as described on page 143. Membrane protein was determined according to the procedure of Lowry et al (1951) and as described on page 149. Ca^{2+} ATPase was assayed in a medium containing final concentration (100mM HEPES, 2mM KCl, 100mM $MgCl_2$, 1mM $CaCl_2$, 50mM EGTA) with various concentrations of Aflatoxin B_1. The activity was assayed in the presence and absence of calmodulin described on page 157 under 'materials and methods'. To study the ATP dependence, various concentrations of ATP were used to start the reaction and aflatoxin B_1 concentration with maximum inhibitory

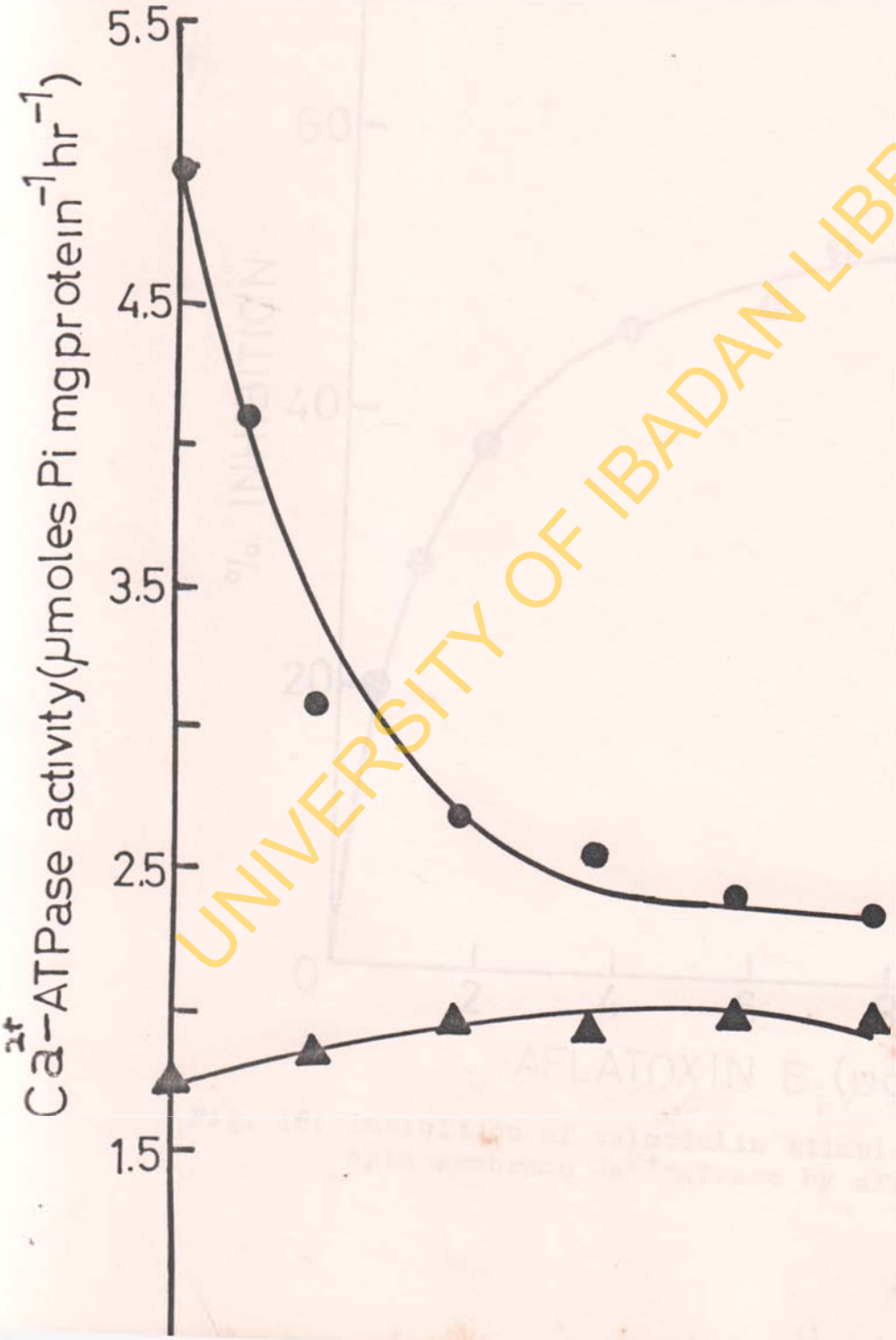
effect was used (10µg aflatoxin). The activity was also assayed as described above. The amount of inorganic phosphate liberated during the ATPase reaction was determined by the method of Fiske and Subbarow (1925) and as described on page 158 under 'materials and methods'.

Results

Fig. 17 shows the effect of aflatoxin B_1 on the basal and calmodulin stimulated enzyme. It can be seen from the figure that aflatoxin B_1 has no significant effect on the basal activity of the enzyme. The profile for the calmodulin stimulated enzyme. shows that aflatoxin B_1 inhibits the calmodulin stimulated enzyme in a concentration dependent fashion; maximum inhibitory effect of about 50% was obtained with almost 10µg aflatoxin B_1 (Fig. 18).

Fig. 19 and 20 show the kinetic analysis of the data obtained from studies on the ATP dependence of the inhibition by aflatoxin B_1 and they shows that the toxin has no effect whatsoever on the

Fig.17: Effect of varying concentrations of aflatoxin B_1 on the basal and calmodulin stimulated activity of erythrocyte plasma Ca^{2+} ATPase. In the presence (circles) and abscence (triangles) of calmodulin.



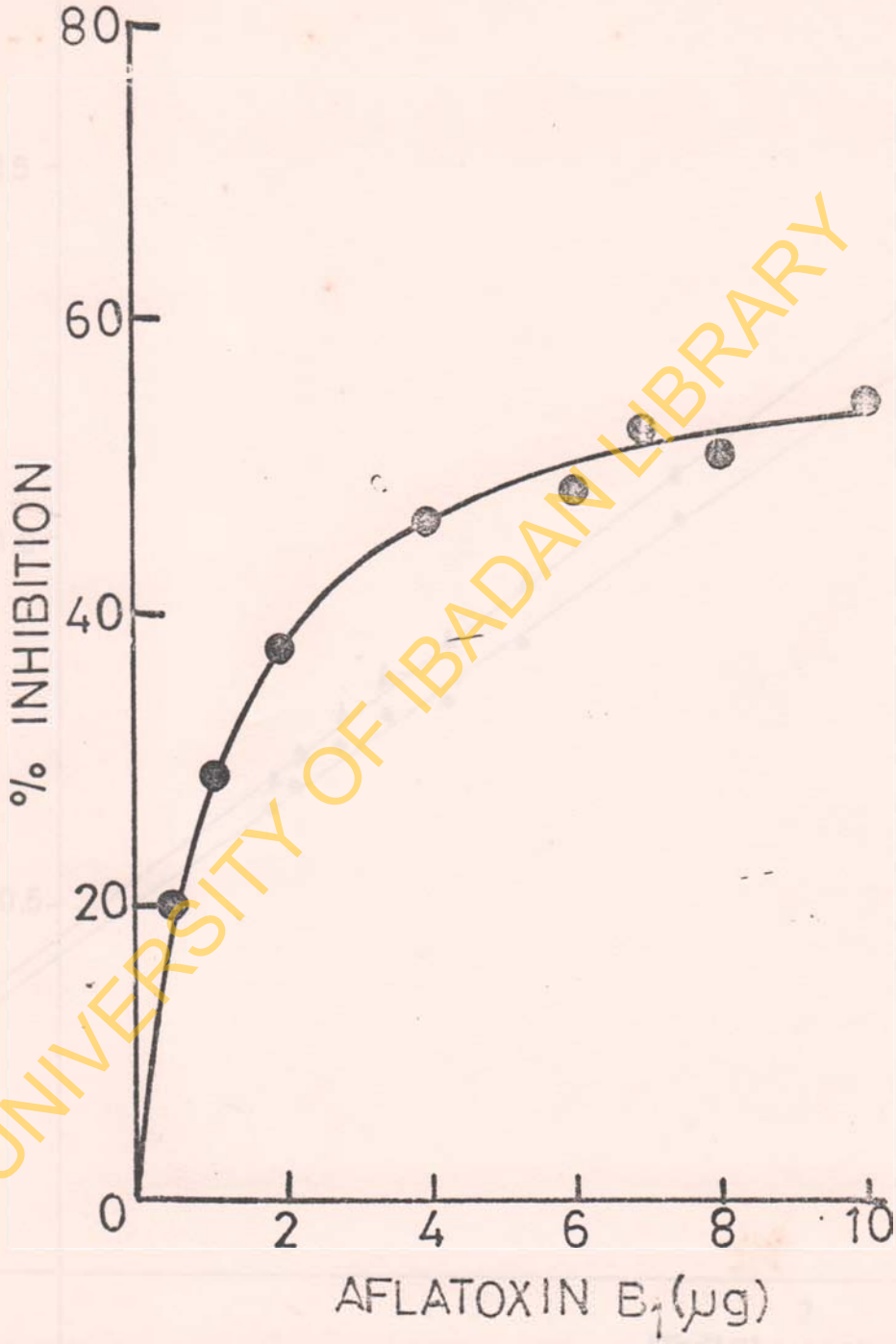


Fig. 18: Inhibition of calmodulin stimulated erythrocyte membrane Ca^{2+}-ATPase by aflatoxin B_1.

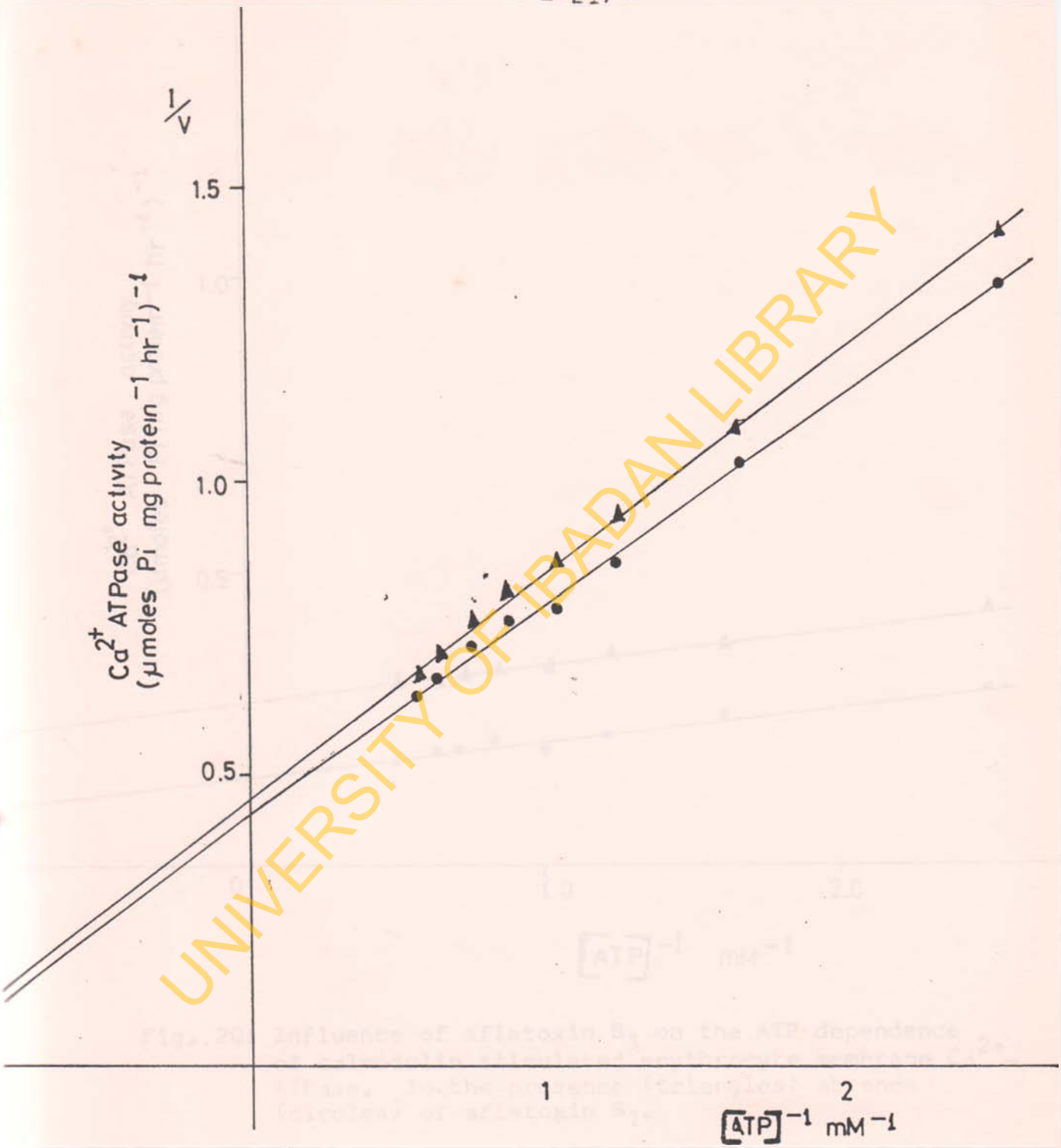


Fig.19: Influence of aflatoxin B_1 on the ATP dependence of erythrocyte membrane Ca^{2+}-ATPase. In the presence (triangles), absence (circles) of aflatoxin B_1.

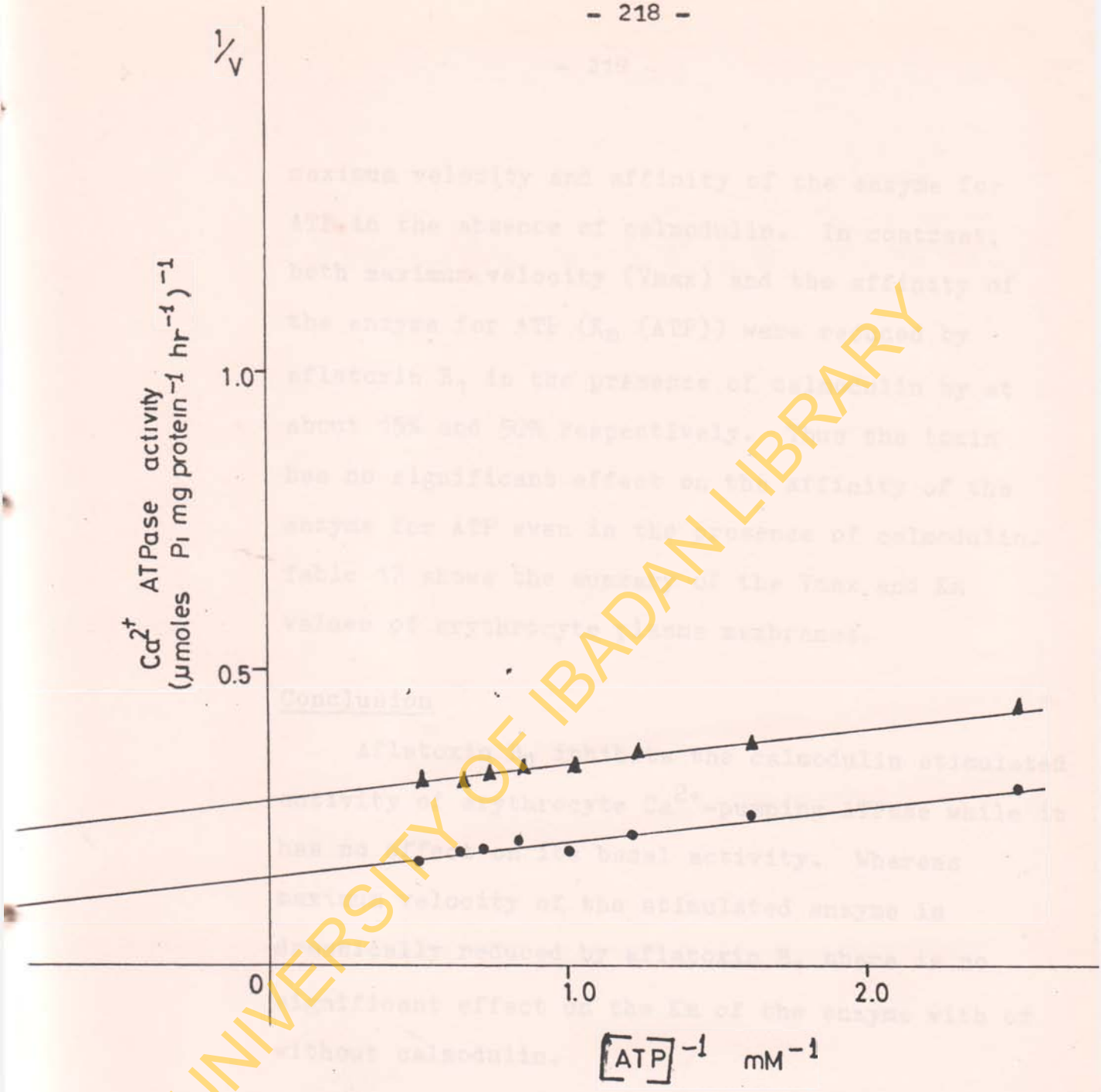


Fig. 20: Influence of aflatoxin B_1 on the ATP dependence of calmodulin stimulated erythrocyte membrane Ca^{2+}-ATPase. In the presence (triangles) absence (circles) of aflatoxin B_1.

maximum velocity and affinity of the enzyme for ATP in the absence of calmodulin. In contrast, both maximum velocity (Vmax) and the affinity of the enzyme for ATP (K_m (ATP)) were reduced by aflatoxin B_1 in the presence of calmodulin by at about 15% and 50% respectively. Thus the toxin has no significant effect on the affinity of the enzyme for ATP even in the presence of calmodulin. Table 17 shows the summary of the Vmax and Km values of erythrocyte plasma membranes.

Conclusion

Aflatoxin B_1 inhibits the calmodulin stimulated activity of erythrocyte Ca^{2+}-pumping ATPase while it has no effect on its basal activity. Whereas maximum velocity of the stimulated enzyme is drastically reduced by aflatoxin B_1 there is no significant effect on the Km of the enzyme with or without calmodulin.

TABLE 17

Effect of aflatoxin B_1 on the Vmax and Km value of the erythrocyte plasma membrane Ca^{2+}-ATPase.

	Vmax (umol Pi $mgprot^{-1}h^{-1}$)	Km (uM ATP)
Ca^{2+} ATPase Erythrocyte membrane (basal)	2.2 ± 0.10 (2.4 ± 0.20)	6600 ± 500 (6800 ± 600)
Ca^{2+} ATPase Erythrocyte membrane (calmodulin)	6.25 ± 0.30 (3.40 ± 0.15)	400 ± 50 (340 ± 80)

( ) were obtained in the presence of aflatoxin B_1.

Experiment 3

Inhibition of erythrocyte purified Ca^{2+} ATPase by aflatoxin B_1

Experiments on the interaction of aflatoxin B_1 with membrane bound Ca^{2+} ATPase have shown that the toxin reduced the maximum velocity (Vmax) of the calmodulin-stimulated enzyme only, without affecting its basal activity and its affinity for ATP whether or not calmodulin is present. Knowledge on the kinetic properties of the pump has shown that calmodulin increases the Vmax of the pump by increasing its affinity for Ca^{2+} and thus increasing its turnover (Jeffery et al, 1981; Larsen et al, 1981). Accordingly, the activation has been shown to stimulate both the rate of phosphorylation of the pump and that of its dephosphorylation (Jeffery et al, 1981; Luthra et al, 1980). Since the inhibition of calmodulin stimulated membrane-bound enzyme is not ATP dependent, it seems logical to suggest that the toxin could be interfering with the pump by binding to some amino acid residues in the calmodulin binding domain, which has a sequence with an obvious predominance of basic amino acid residues.

In order to gain insight into the mechanism of inhibition of the pump by aflatoxin B_1, the enzyme has been detergent-solubilized and purified on calmodulin affinity chromatography. It is thus possible to study the interaction of the aflatoxin B_1 directly with the pump. The aim of this experiment was therefore to establish the mode of inhibition of the calmodulin-stimulated pump by aflatoxin B_1.

Procedure

To study the effect of aflatoxin B_1 with the purified enzyme, various concentrations of aflatoxin B_1 preincubated with 3µg purified ATPase in eppendorf tubes containing the enzyme and aflatoxin B_1 were then incubated at 37°C in a water bath with constant shaking. The assay medium was added to obtain a final concentration (120mM KCl, 1mM $MgCl_2$, 30mM HEPES, 10uM $CaCl_2$, pH 7.4). Calmodulin (2µg) was also added and the reaction was started by addition of 2mM ATP. The reaction was allowed to proceed for 3 minutes. The amount

of inorganic phosphate liberated was measured by the methof of Lanzetta et al 1979 as described on page 173. The experiment was carried out in duplicate.

In addition, series of experiments on the ATP dependence of the effect of aflatoxin were carried out using the concentration of aflatoxin that gave maximum inhibition. ATPase activity was determined as described above and various concentrations of ATP ranging from 0.5-2.5mM were used to initiate the reaction. The experiments were similarly carried out in duplicate.

Results

Fig. 21 shows the influence of aflatoxin B_1 on the basal and calmodulin-stimulated activity of the purified enzyme. It can be seen from the figure that the effects of aflatoxin B_1 on the purified erythrocytes are similar to that seen in case of the membrane-bound enzyme. The data show clearly that aflatoxin B_1 has no significant effect on the basal activity of the enzyme whereas the stimulated enzyme was inhibited in a concentration

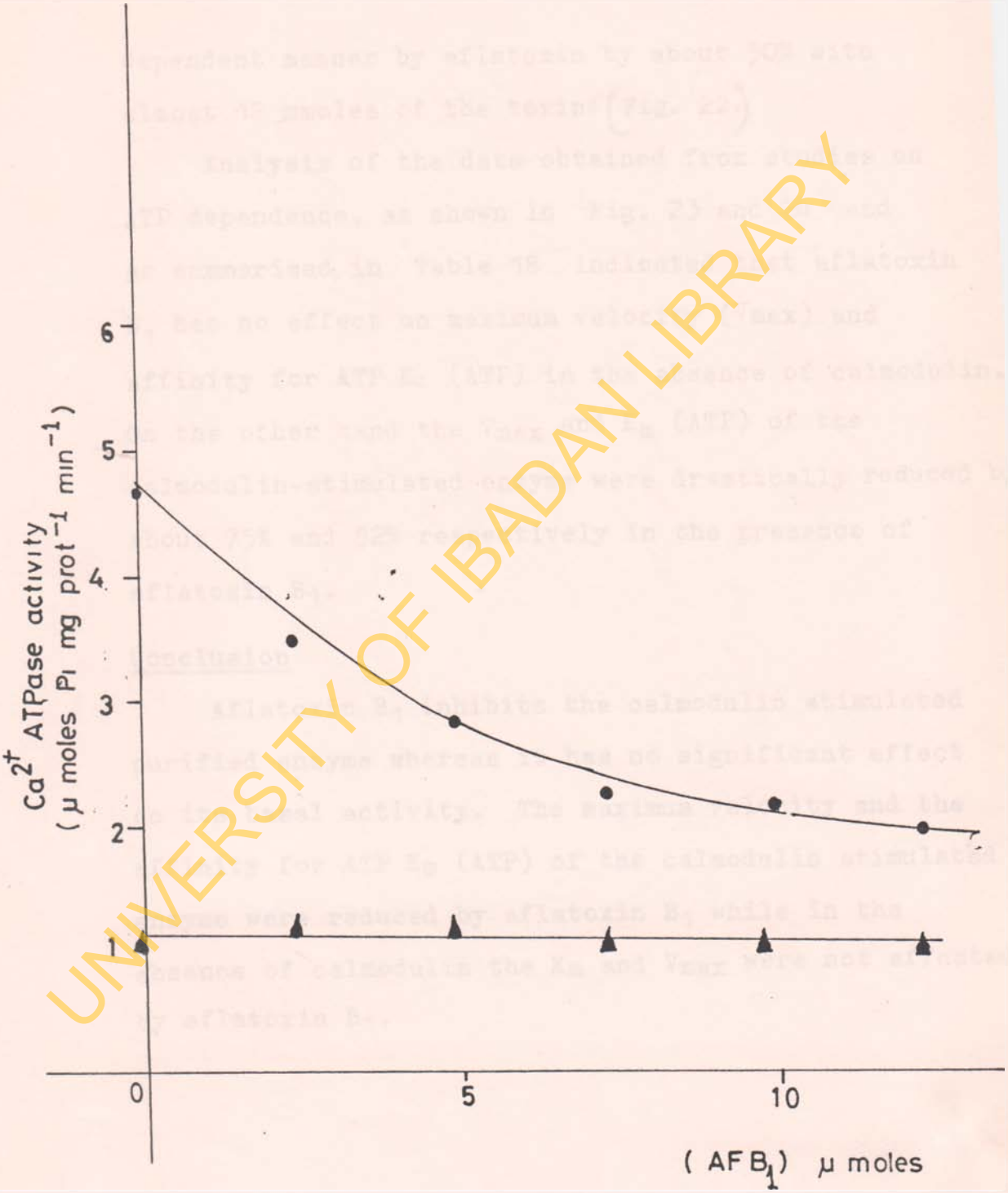


Fig. 21: Effect of varying concentrations of aflatoxin B_1 on the basal and calmodulin stimulated activity of the purified ATPase. In the presence (circles), and absence (triangles) of calmodulin.

dependent manner by aflatoxin by about 50% with almost 12 µmoles of the toxin. (Fig. 22.)

Analysis of the data obtained from studies on ATP dependence, as shown in (Fig. 23 and 24) and as summarised in (Table 18) indicated that aflatoxin B_1 has no effect on maximum velocity (V_{max}) and affinity for ATP K_m (ATP) in the absence of calmodulin. On the other hand the V_{max} and K_m (ATP) of the calmodulin-stimulated enzyme were drastically reduced by about 75% and 52% respectively in the presence of aflatoxin B_1.

Conclusion

Aflatoxin B_1 inhibits the calmodulin stimulated purified enzyme whereas it has no significant effect on its basal activity. The maximum velocity and the affinity for ATP K_m (ATP) of the calmodulin stimulated enzyme were reduced by aflatoxin B_1 while in the absence of calmodulin the K_m and V_{max} were not affected by aflatoxin B_1.

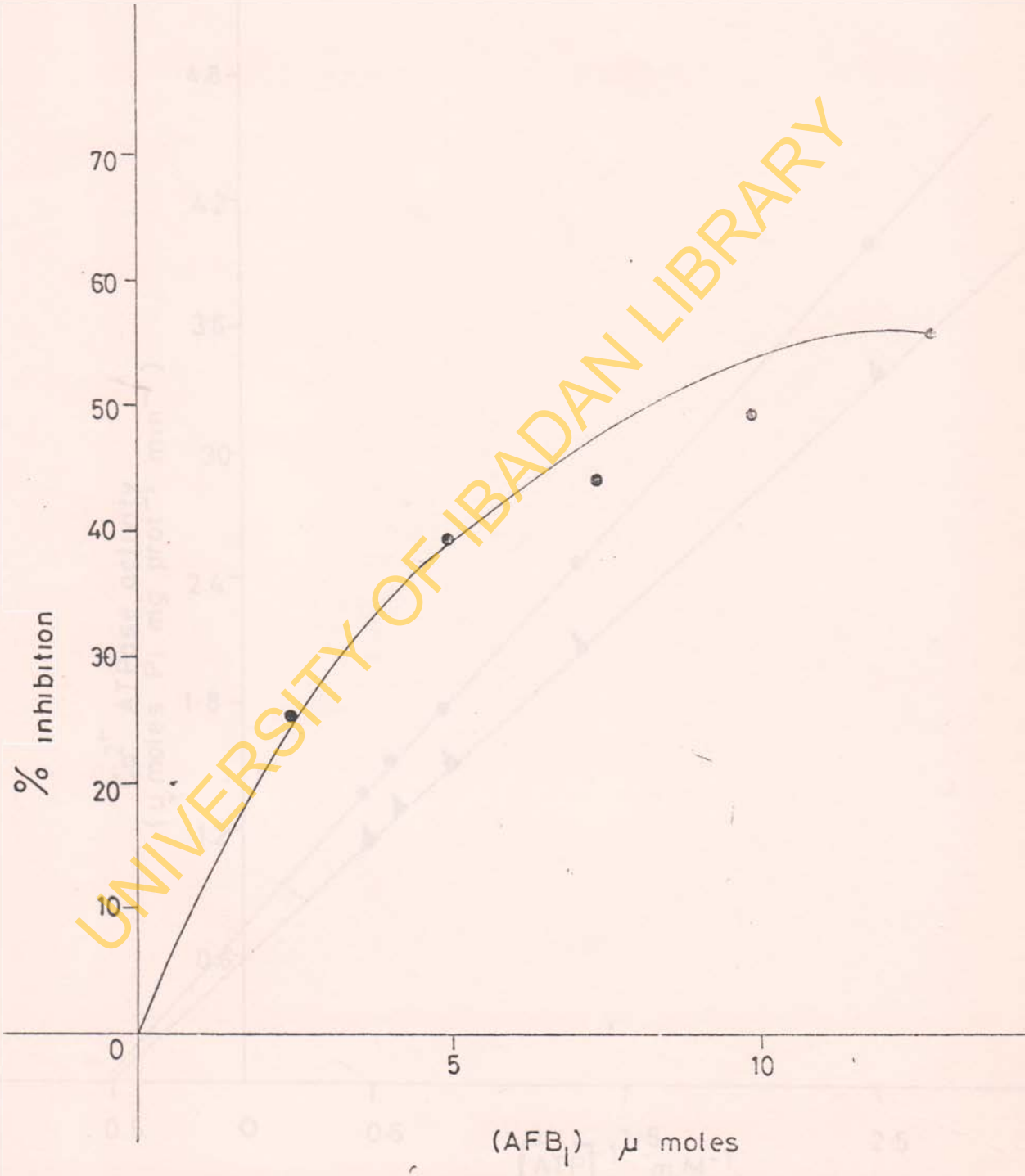


Fig. 22: Inhibition of the calmodulin stimulated purified enzyme by aflatoxin B_1.

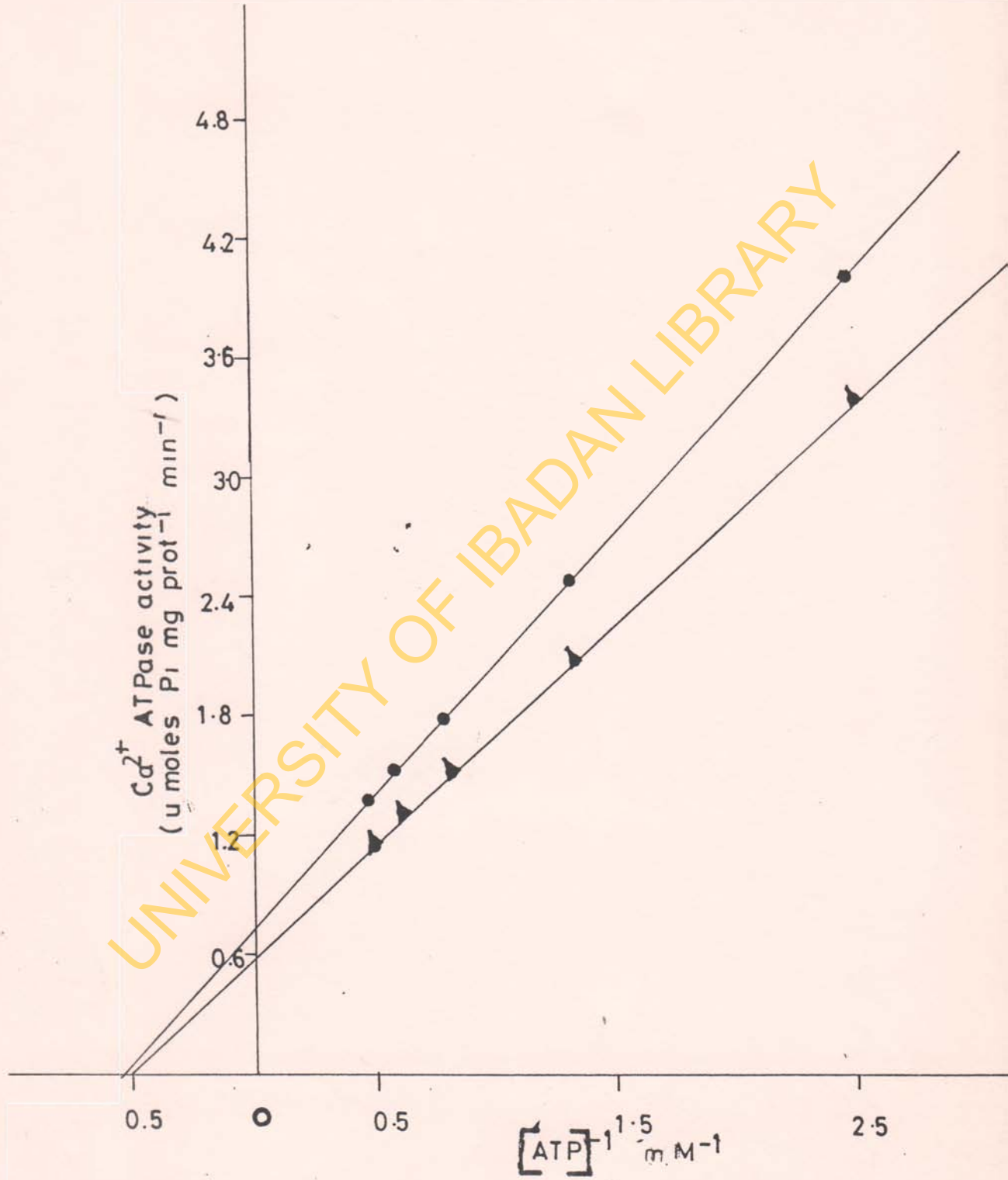


Fig. 23: Influence of aflatoxin B_1 on the ATP dependence of the unactivated ATPase. In the presence (circles) and absence (triangles) of aflatoxin B_1.

Fig. 24: Influence of aflatoxin B_1 on the ATP dependence of the calmodulin stimulated purified ATPase. (in the presence (open circles) and absence (closed circles) of aflatoxin B_1).

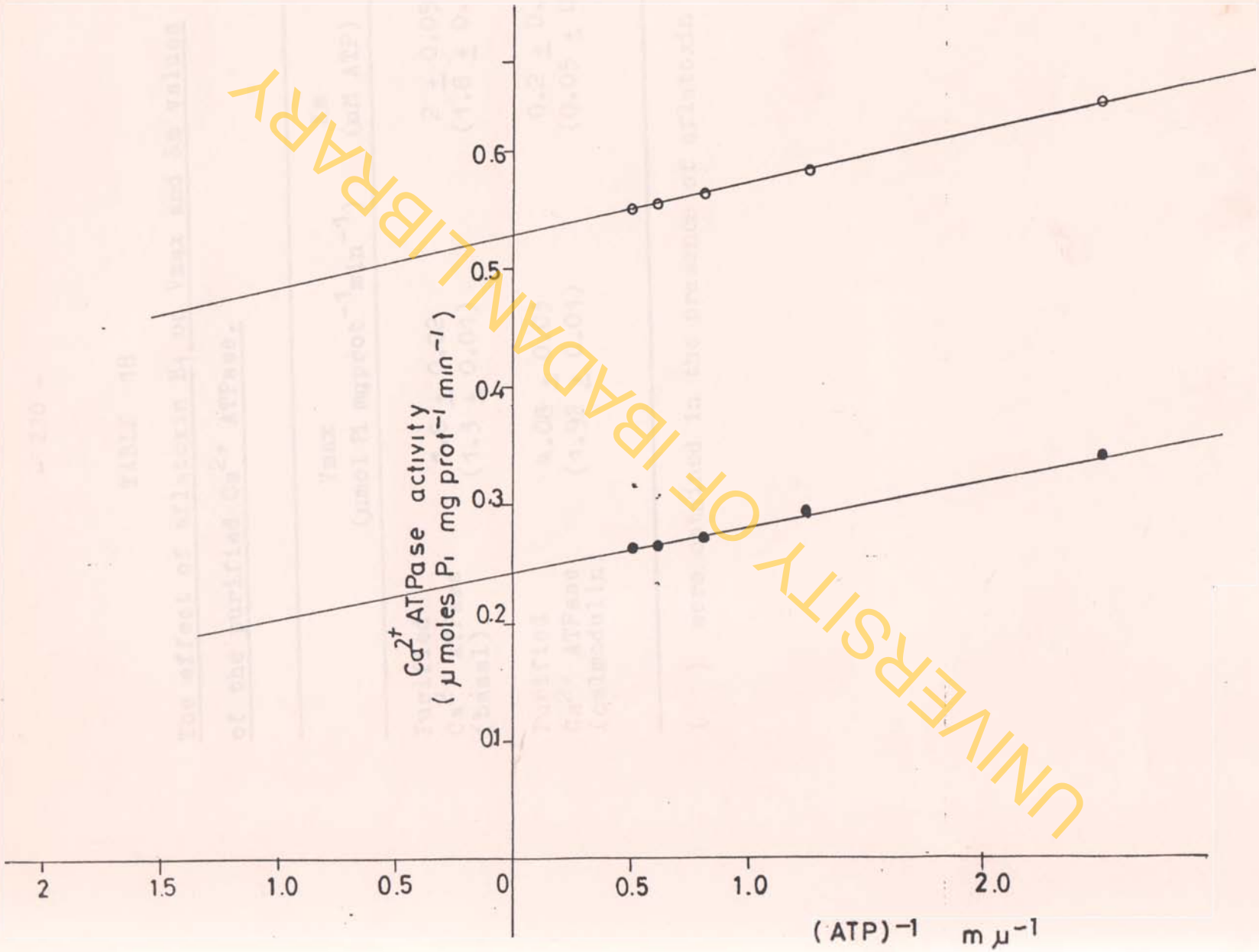


TABLE 18

The effect of aflatoxin B_1 on Vmax and Km values of the purified Ca^{2+} ATPase.

	Vmax (μmol Pi $mgprot^{-1}min^{-1}$)	Km (μM ATP)
Purified Ca^{2+} ATPase (basal)	1.6 $\pm$ 0.02 (1.3 $\pm$ 0.01)	2 $\pm$ 0.05 (1.8 $\pm$ 0.01)
Purified Ca^{2+} ATPase (calmodulin)	4.08 $\pm$ 0.03 (1.92 $\pm$ 0.01)	0.2 $\pm$ 0.02 (0.05 $\pm$ 0.001)

( ) were obtained in the presence of aflatoxin B_1.

Experiment 4

Effect of triton X-100 and acidic phospholipid on the inhibition of Ca^{2+}-ATPase by aflatoxin B_1

It is well established that the Ca^{2+}-ATPase of the erythrocyte ghost is remarkably stable in the membrane-bound form, apparently because the lipid surrounding the protein molecules ensures perfect conditions for preservation of the enzyme functional conformation. Solubilization of this enzyme is frequently accompanied by the loss of its hydrolytic activity (Niggli et al, 1979; Niggli et al, 1981); Scharff 1981. The nature of the detergent used for solubilization nothwithstanding, its concentration is an important factor in achieving maximal ATPaseaactivity. Several workers have shown that in the absence of detergent, sonicated lipid dispersions were unable to support ATPase activity whereas activity was observed when detergent and phospholipid were present (Nelson and Hanahan 1985).

Results of several studies have shown that acidic phospholipids are five to eight times more effective than phosphatidyl choline in promoting activity of the pump by increasing both its maximum velocity and its apparent calcium affinity (Taverna and Hanahan, 1980; Niggli et al 1981; Sarkadi et al, 1982) while causing a significantly lower $K_{\frac{1}{2}}$ (Ca^{2+}) than calmodulin with no co-operativity in the calcium activation (Enyedi et al, 1987; Sharff 1978; Villalobo et al 1986; Enyedi et al 1987).

It has also been shown that, although phosphatidyl serine stimulates the ATPase activity, it was achieved as a result of the low detergent concentration used, where mixed micelle formation was complete (Taverna and Hanahan, 1980). On the other hand, higher detergent concentration resulted in a decrease in ATPase activity and this might be due to a dilution of the lipid by excess detergent.

On subjecting the purified enzyme to tryps inisation, Enyedi et al (1987) were able to deduce that the 81KDa tryptic fragment is still regulated

by acidic phospholipids while the 76KDa fragment is not, Further studies indicated the actual position of phospholipid binding and was tentatively placed at the NH_2-terminal 5KDa of the 81-KDa fragment (Papp et al, 1989). Zvaritch et al (1990) demonstrated that the phospholipid domain has about 44 amino acids residues and it is lysine rich, while Brodin et al (1991) showed that there are possibly two functional domains of phospholipid interaction.

In this study, the effect of triton X-100 and acidic phospholipids on the inhibition of Ca^{2+}-ATPase by aflatoxin B_1 have been assesed, in an attempt to distinguish between the possible site of attack by aflatoxin B_1 since acidic phospholipids and proteolysis mimick the effect of calmodulin and act at different sites on the enzyme.

Procedure

Purified Ca^{2+} ATPase was prepared from normal fresh erythrocytes according to the procedure

described earlier. Protein concentration was determined according to a modified procedure of Markewell et al (1981).

To study the effect of aflatoxin B_1 on cardiolipin-stimulated enzyme, various concentrations of aflatoxin B_1 were prepared in dimethylsulfoxide (DMSO) and these were added as described earlier. ATPase activity was measured by the procedure of Lanzetta et al, (1979) as also described earlier.

In studies on the effect of triton X-100 on the inhibition of the enzyme by aflatoxin B_1 various concentrations of triton X-100 were used for solubilization prior to affinity chromatography on calmodulin column as earlier described. ATPase activity was measured by the procedure of Lanzetta et al (1979) as earlier described after initiating the reaction by addition of ATP solution.

The effects of varying concentrations of phosphatidyl serine (PS) and phosphatidyl choline (PC) on the inhibition of the pump by aflatoxin B_1 were also studied. Similar concentrations of aflatoxin B_1 (1 ug) were added to the enzyme (3 ug). Calmodulin (2 ug) was added to further stimulate the enzyme. ATPase activity was determined as described earlier.

Results

Figs. 25 and 26 shows the effect of aflatoxin B_1 on basal and cardiolipin-stimulated activity of the purified enzyme. The figures show that, aflatoxin B_1 inhibited the activity of the enzyme in a concentration dependent manner by about 28% while it has no effect on the basal activity of the enzyme. Effect of aflatoxin B_1 on the solubilized and purified enzyme (Fig. 27) indicated that the activity of the

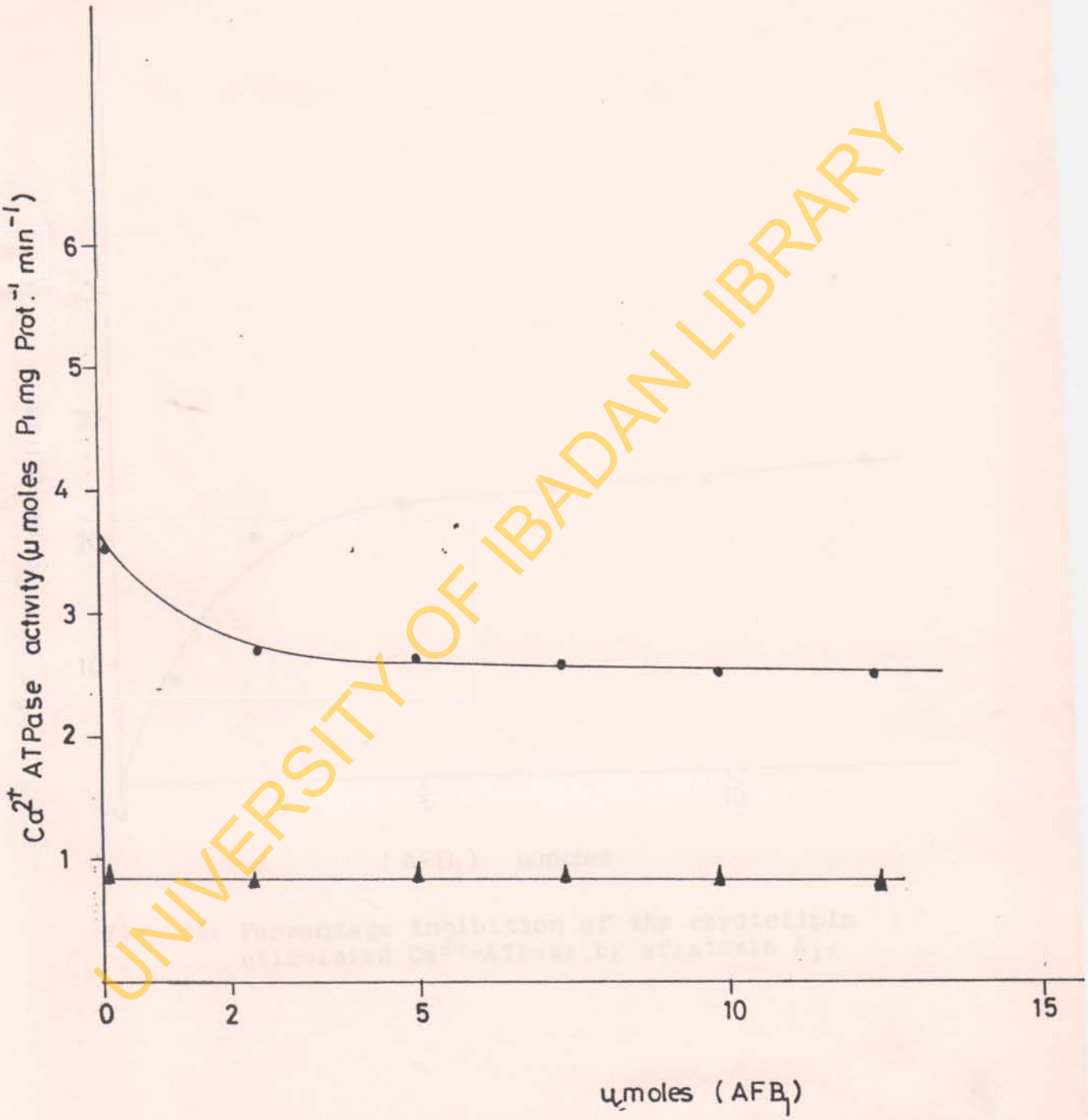


Fig. 25: Effect of varying concentrations of aflatoxin B_1 on the cardiolipin (DPG) stimulated Ca^{2+}-ATPase. In the presence (circles), absence (triangles) of cardiolipin.

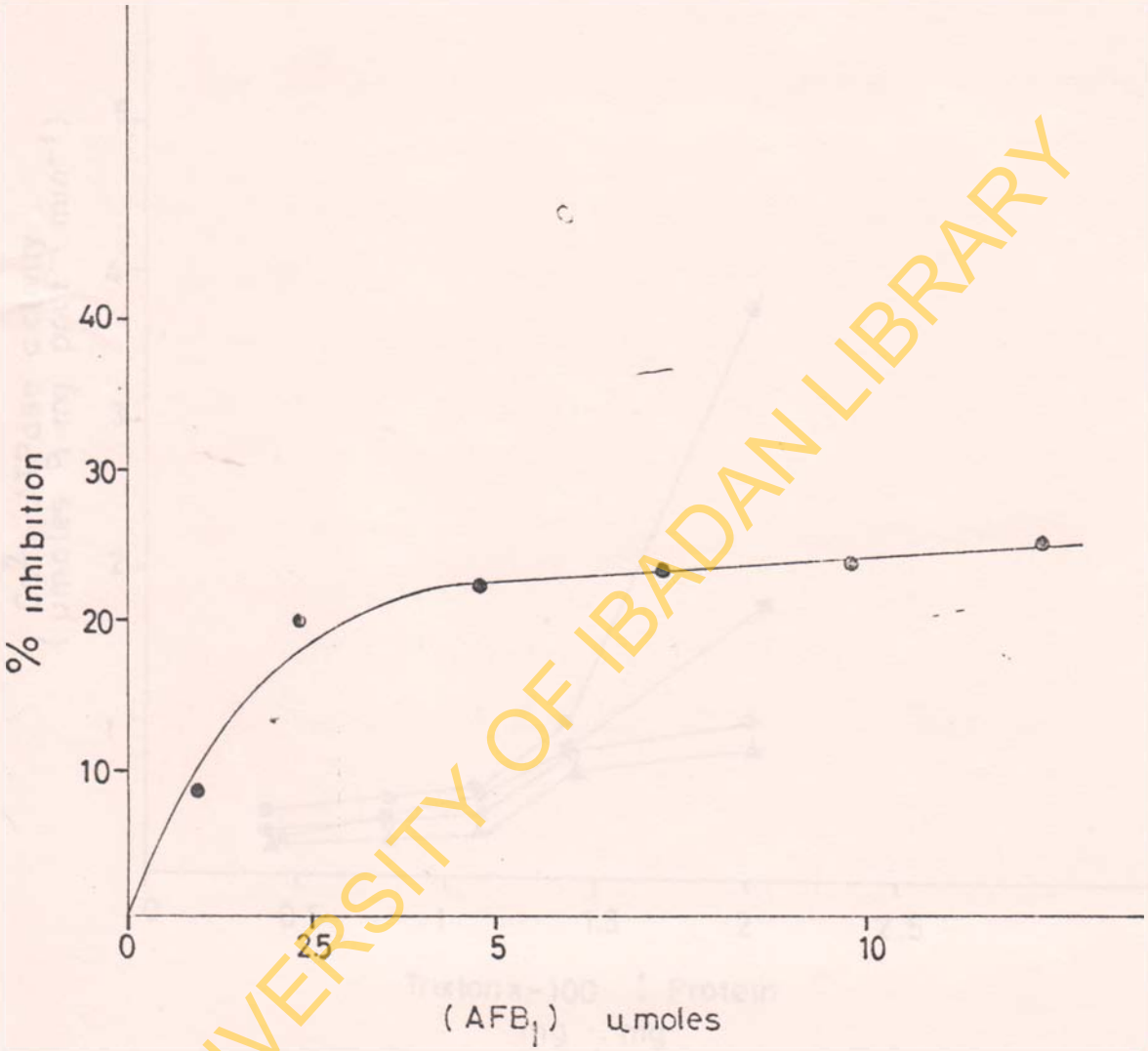


Fig. 26: Percentage inhibition of the cardiolipin stimulated Ca^{2+}-ATPase by aflatoxin B_1.

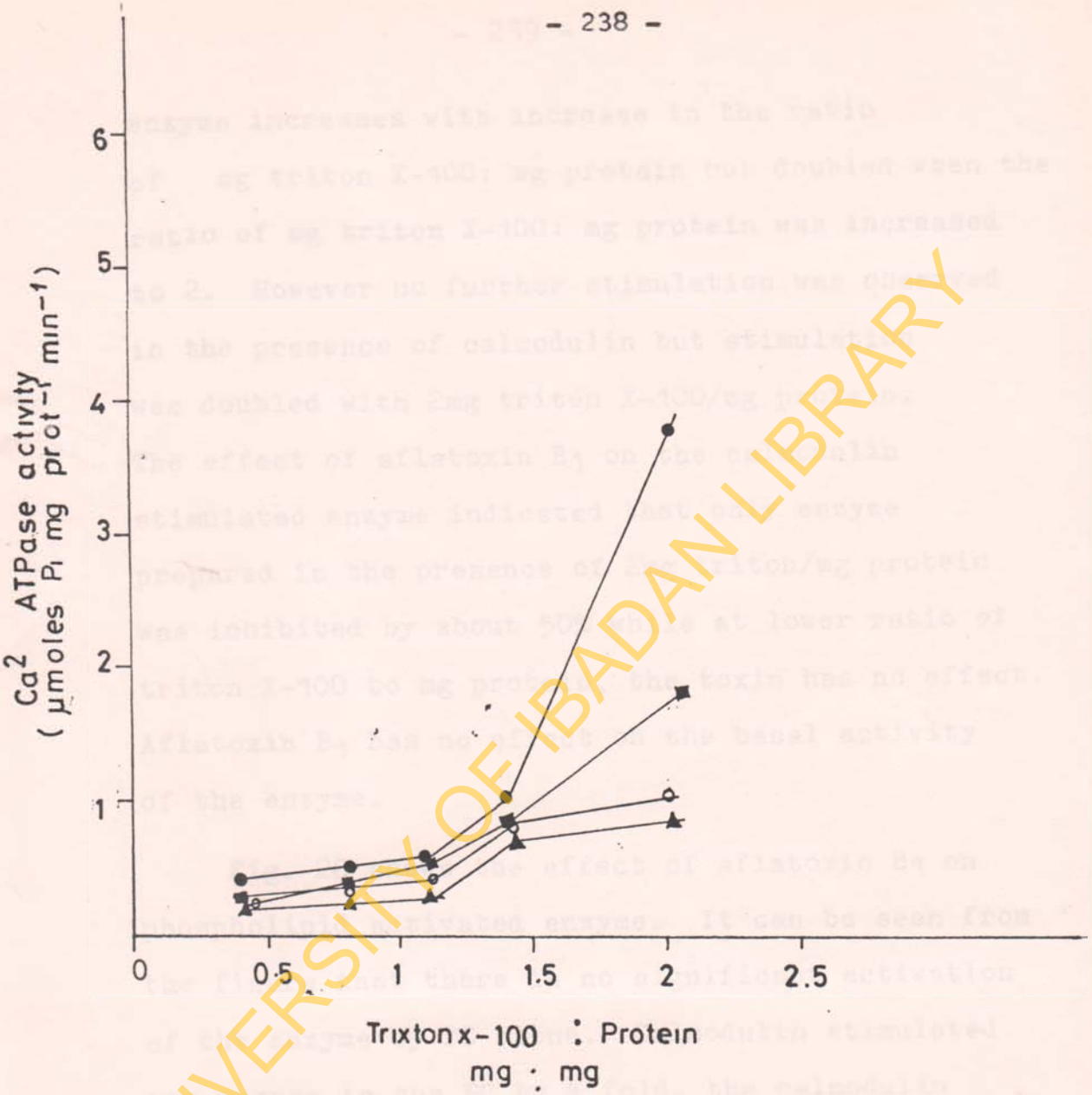


Fig. 27: Influence of varying the ratios of triton X-100 to protein on the inhibition of the Ca^{2+}-ATPase by aflatoxin B_1. In the presence of calmodulin (closed circles) presence of (squares) calmodulin and aflatoxin B_1, presence of (open circles) no calmodulin and aflatoxin B_1, presence of aflatoxin B_1 (triangles).

enzyme increases with increase in the ratio of mg triton X-100: mg protein but doubled when the ratio of mg triton X-100: mg protein was increased to 2. However no further stimulation was observed in the presence of calmodulin but stimulation was doubled with 2mg triton X-100/mg protein. The effect of aflatoxin B_1 on the calmodulin stimulated enzyme indicated that only enzyme prepared in the presence of 2mg triton/mg protein was inhibited by about 50% while at lower ratio of triton X-100 to mg protein, the toxin has no effect. Aflatoxin B_1 has no effect on the basal activity of the enzyme.

Fig. 28 shows the effect of aflatoxin B_1 on phospholipid activated enzyme. It can be seen from the figure that there is no significant activation of the enzyme by PC alone. Calmodulin stimulated the enzyme in the PC by 4 fold, the calmodulin stimulated enzyme in the presence of PC was inhibited by about 50% by aflatoxin B_1. Aflatoxin B_1 has no effect on the basal activity of the enzyme in the presence of PC.

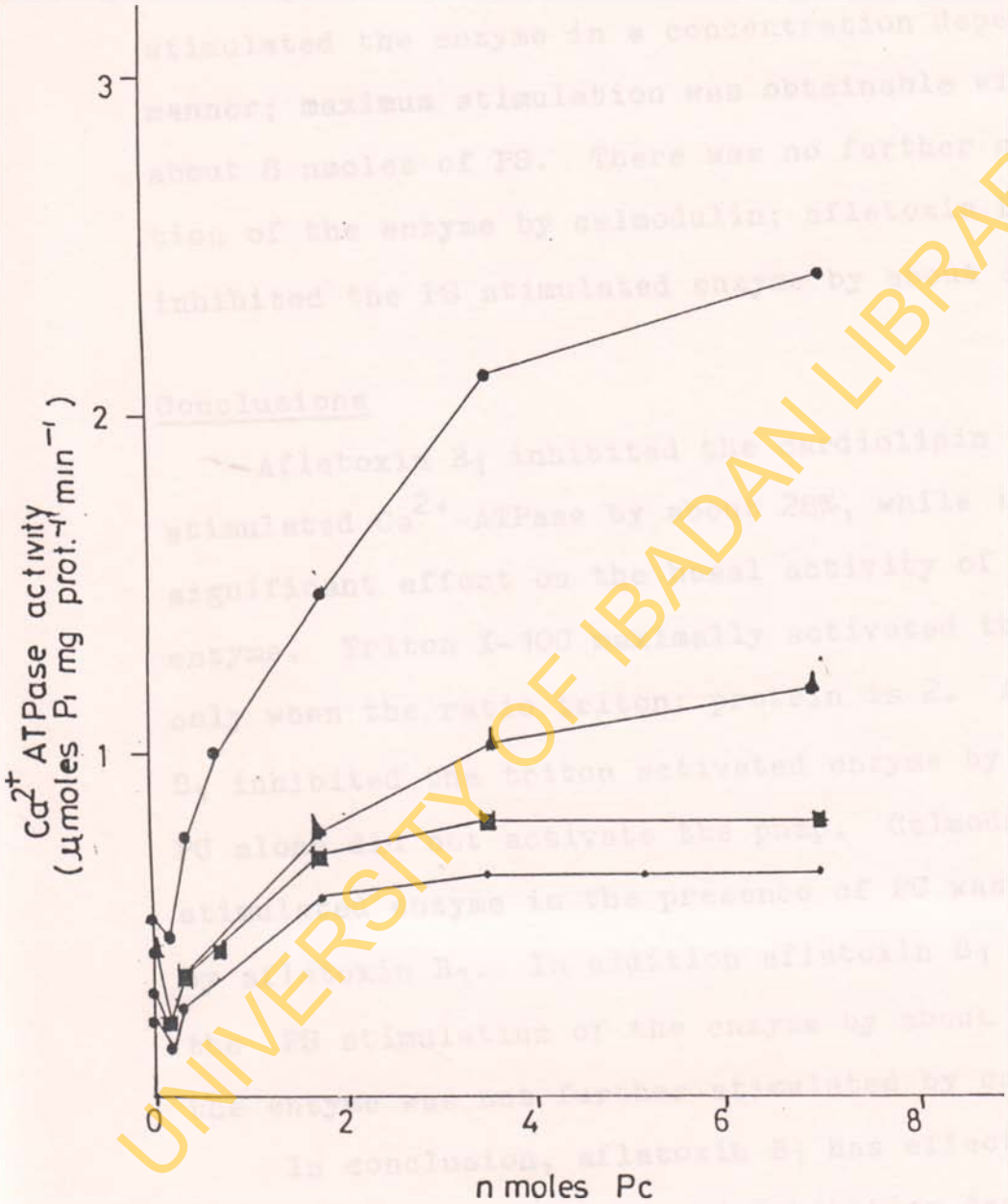


Fig. 28: Effect of varying concentrations of phosphatidyl choline on the inhibition of the Ca^{2+}-ATPase by aflatoxin B_1 (in the presence of: (a) calmodulin (big closed circles); (b) calmodulin and aflatoxin (triangles); (c) absence of calmodulin and aflatoxin B_1 (squares) and (d) presence of aflatoxin B_1 (small circles)).

Fig. 29 shows that phosphatidyl serine (PS) stimulated the enzyme in a concentration dependent manner; maximum stimulation was obtainable with about 8 nmoles of PS. There was no further stimulation of the enzyme by calmodulin; aflatoxin B_1 inhibited the PS stimulated enzyme by about 28%.

Conclusions

Aflatoxin B_1 inhibited the cardiolipin stimulated Ca^{2+}-ATPase by about 28%, while it has no significant effect on the basal activity of the enzyme. Triton X-100 maximally activated the enzyme only when the ratio triton: protein is 2. Aflatoxin B_1 inhibited the triton activated enzyme by about 50%. PC alone did not activate the pump. Calmodulin-stimulated enzyme in the presence of PC was inhibited by aflatoxin B_1. In addition aflatoxin B_1 inhibited the PS stimulation of the enzyme by about 28% while the enzyme was not further stimulated by calmodulin.

In conclusion, aflatoxin B_1 has effect only on the stimulated enzyme and inhibition depends on the type of activator used.

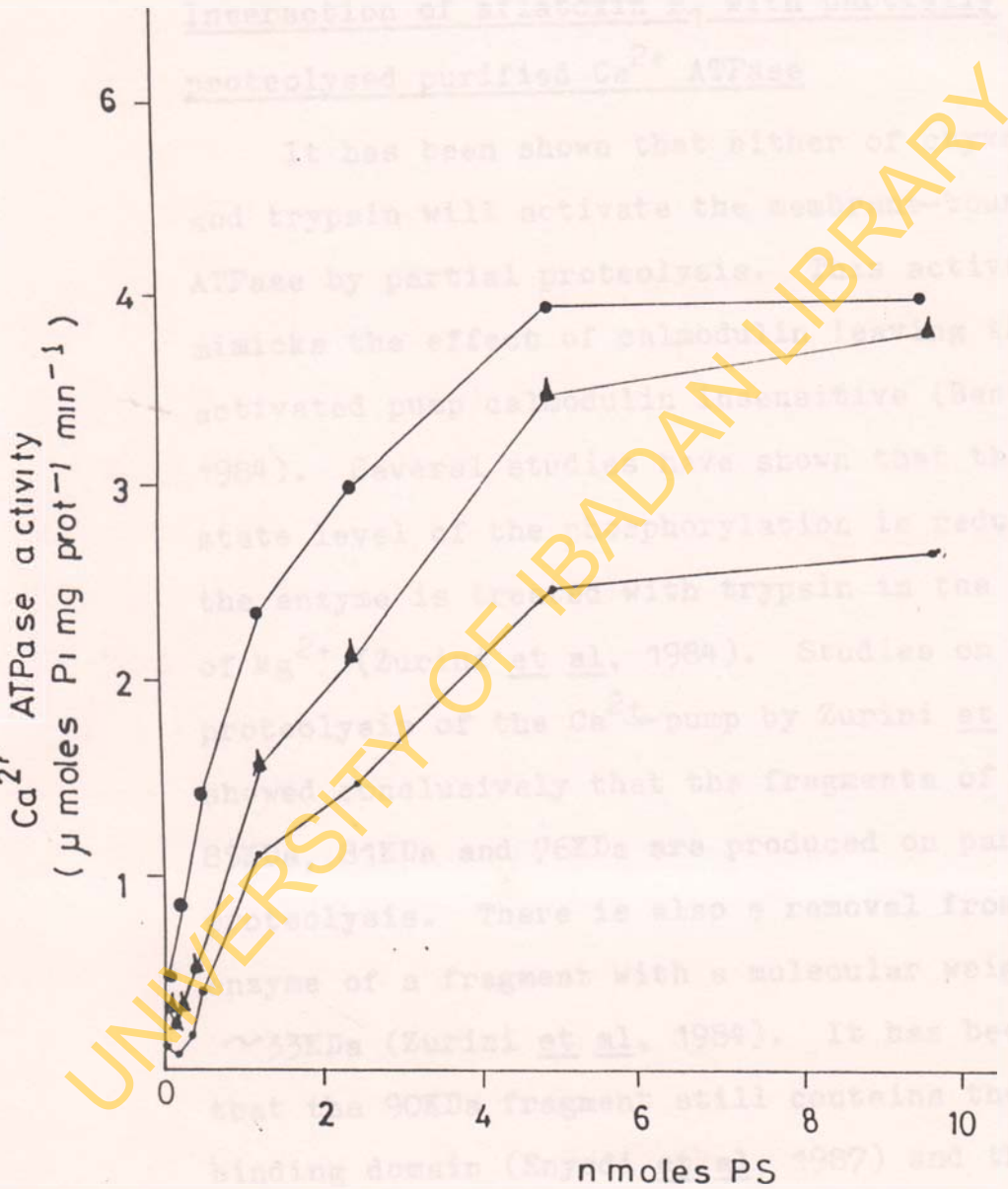


Fig. 29: Effect of varying concentrations of phosphatidyl serine on the inhibition of the Ca^{2+}-ATPase by aflatoxin B_1 (in the presence of; (a) calmodulin (big circles); (b) phosphatidyl serine (triangles; (c) aflatoxin B_1 (small circles)).

Experiment 5

Interaction of aflatoxin B_1 with partially proteolysed purified Ca^{2+} ATPase

It has been shown that either of chymotrypsin and trypsin will activate the membrane-bound Ca^{2+} ATPase by partial proteolysis. This activation mimicks the effect of calmodulin leaving the activated pump calmodulin insensitive (Benaim et al, 1984). Several studies have shown that the steady state level of the phosphorylation is reduced when the enzyme is treated with trypsin in the absence of Mg^{2+} (Zurini et al, 1984). Studies on the proteolysis of the Ca^{2+}-pump by Zurini et al, (1984) showed conclusively that the fragments of 90KDa, 85KDa, 81KDa and 76KDa are produced on partial proteolysis. There is also a removal from the enzyme of a fragment with a molecular weight of ~33KDa (Zurini et al, 1984). It has been shown that the 90KDa fragment still contains the calmodulin binding domain (Enyedi et al, 1987) and thus responds normally to calmodulin, while the 85KDa fragment is

is less responsive to calmodulin, indicating that the calmodulin binding domain is not completely removed by proteolysis in this fragment (Zvaritch et al, 1990). Studies on the 76KDa and 81KDa fragments show that they are no longer responsive to acidic phospholipids and calmodulin. Altogether these findings indicate clearly, that acidic phospholipids and calmodulin interact with the ATPase at two different sites. It is clear that the effects of trypsin and chymotrypsin have no physiological meaning whereas the effects of the intracellular Ca^{2+} dependent cysteine protease, otherwise called calpain could be physiologically relevant (Bond and Clough, 1972; James et al, 1989; Wang et al, 1988).

This protease has been shown to increase the basal activity of the pump in the erythrocyte membrane but does so much slowly than the extracellular proteases, reaching the maximal levels attainable with calmodulin in one to two hrs. It has also been shown that the treatment with calpain reduces

the apparent molecular weight of the enzyme by 12KDa, leaving in the membrane, a component of apparent molecular weight of 124KDa fragment, which is insensitive to calmodulin (Zurini et al, 1984; Zvaritch et al, 1990).

Its seemsppertinent therefore to study the interaction of aflatoxin B_1 on the partially proteolysed Ca^{2+}-pump, so as to assess the possible site of interaction of the toxin on the Ca^{2+}-pump.

Procedure

Calpanized ATPase was prepared from normal fresh erythrocytes according to the procedure described earlier. Protein concentration was determined according to the modified procedure of Markwell et al (1981) as described earlier. Various concentrations of aflatoxin B_1 were preincubated with 3ug of the enzyme in eppendorf tubes for 30 minutes on ice and transferred to a water bath at 37°C. ATPase activity was estimated in the absence and presence of calmodulin according to the procedure of Lancetta as described earlier.

In experiment on the influence of aflatoxin B_1 on trypsinised ATPase, the enzyme was subjected to trypsinisation for 5-7 min and 90 mins respectively in order to obtain the 90KDa and 76KDa fragments. The trypsinised enzyme (50ug) was preincubated in an eppendorf tubes on ice with aflatoxin B_1 (10 ug) for about 30 mins and transferred to a water bath at 37°C. The assay buffer were added to a final concentration of (120mM KCl, 1mM $MgCl_2$, 10uM $CaCl_2$, 30mM HEPES, pH 7.4), the ATPase activity was determined in the presence and absence of calmodulin as previously described under 'materials and methods' page 173 .

Results

Table 19 shows the effect of aflatoxin B_1 on calpanised ATPase. The Table shows that varying concentrations of aflatoxin B_1 have no effect on the activity of the partially proteolysed enzyme in the presence and absence of calmodulin. There was no stimulation of this enzyme by calmodulin.

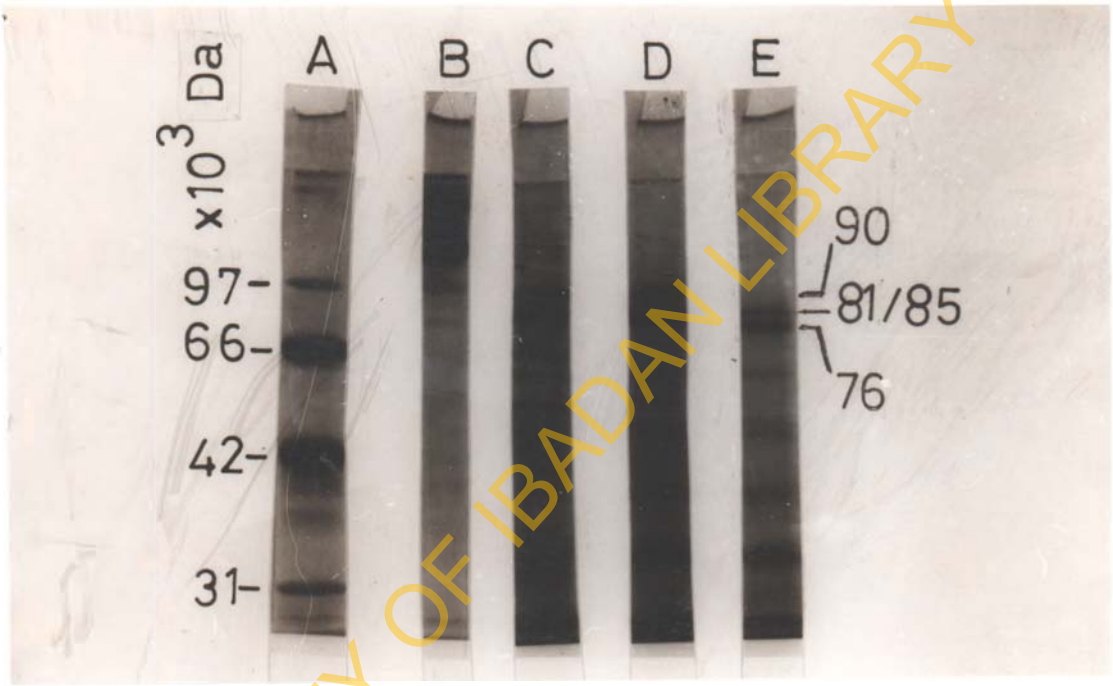


Plate 3: SDS-polyacrylamide gel electrophoresis of partially purified Ca^{2+}-pumping ATPase from erythrocyte. (A) are values ±(in X10^3 Da) indicative of the molecular weights of the protein markers (B) is the purified enzyme. (C) is fragment for 5 mins. (D) is fragment for 15 mins. (D) is fragment for 30 mins. (E) fragment for 1 hr. The partially purified enzyme was applied to a 4-7% continuous gradient gel as described on page 194.

TABLE 19

Effect of aflatoxin B_1 on calpanised ATPase

(Aflatoxin) (μmoles)	Specific Activity μmoles Pi mg prot $^{-1}$ min $^{-1}$ -Calmodulin	+ Calmodulin
0	2.0 ± 0.01	2.2 ± 0.01
2.5	2.1 ± 0.02	2.3 ± 0.02
4.9	2.3 ± 0.01	2.5 ± 0.01
7.4	2.6 ± 0.01	2.7 ± 0.01
12.3	2.8 ± 0.02	2.8 ± 0.03

Each value is a mean of five different determinations ± standrd error.

Table 20 shows the effect of aflatoxin B_1 on the trypsinised enzyme. According to the Table the enzyme fragment produced after 5 minutes which is 90KDa was calmodulin sensitive and was inhibited by aflatoxin B_1 by about 50%. The 76KDa fragment produced after 90 minutes of incubation with trypsin was calmodulin insensitive. The ATPase activity of this fragments could not be inhibited by aflatoxin B_1 even at the fragment that give maximum inhibition.

Conclusion

Aflatoxin B_1 has no effect whatsoever on the calpanised enzyme, whereas the toxin inhibited the ATPase activity of the enzyme partially trypsinised for 5^1 (which correspond to the 90KDa fragments) the toxin has no significant effect on the enzyme trypsinised for 90 which corresponds to the 76KDa fragments of the ATPase.

TABLE 20

Effect of aflatoxin B_1 on partial proteolysis of the Ca^{2+} ATPase by Trypsin

Period of incubation (min)	µmoles Pi $mg\ prot^{-1}\ min^{-1}$	
	-Calmodulin	+ Calmodulin
0	1.00 ± 0.01	4.50 ± 0.05
(Aflatoxin B_1)	(0.86 ± 0.01)	(2.30 ± 0.01)
5[1]	1.19 ± 0.02	4.10 ± 0.03
(Aflatoxin B_1)	(1.05 ± 0.01)	(2.19 ± 0.07)
90[1]	4.65 ± 0.06	4.54 ± 0.01
(Aflatoxin B_1)	(4.40 ± 0.01)	-

( ) in the presence of aflatoxin B_1

Each value is a mean of five different determinations ± stand error.

CHAPTER FOUR

DISCUSSION

A negligible amount of Ca^{2+} is contained in the extracellular and intracellular fluids; most of the calcium of higher organisms is immobilized in the bones and teeth as hydroxy-apatite $Ca_{10}(PO_4)_6 (OH)_2$. Calcium concentration in the extra cellular compartment including blood plasma is regulated by the mobilization of Ca^{2+} from and to bone deposits, and is fixed at about 3mM; approximately half of this amount of Ca^{2+} is in the ionized form. Extracellular or plasma Ca^{2+} is important from the stand point of its relation-ship to the intracellular Ca^{2+}, because the cytosolic calcium performs the fundamental role of transducing signals to a large number of biochemical processes in the various subcellular compartments the link between extracellular Ca^{2+} and the signalling Ca^{2+} inside the cell is not well under-stood, but it seems clear that the extracellular pool provides a relatively large reservoir from which Ca^{2+} is drawn and made to flow into the cell.

The maintenance of the extracellular Ca^{2+} within a narrow concentration range ensures that a constant source of Ca^{2+} will always be available to cells.

Despite the very large inwardly directed Ca^{2+} gradient across the plasma membrane, the background concentration of a free Ca^{2+} in most cytosols oscillates indeed between 0.1 and 0.2µM (Carafoli, 1987). The essential concept of cellular Ca^{2+} homeostasis (Carafoli, 1987) centers therefore on the ability of certain components of the membrane to perform the task of decoding the information of Ca^{2+} and of controlling its cytosolic concentration. These membrane components consist of a large number of specific Ca^{2+} binding protein soluble or insoluble to the membrane. It follows therefore that in the presence of a large Ca^{2+} pressure, even very minor changes of this permeability would result in significant swings in the intracellular concentration of free Ca^{2+} and would thus efficiently influence the

modulation of Ca^{2+} targets. In case of a breakdown of the permeability barrier of the plasma membrane, cellular calcium overload would unavoidably occur and this could result in cell death. It is well established that flooding of the cytosol with Ca^{2+} is indeed a frequent and early event in cell pathology (Niggli, Adunyah, Cameron, Bababunmi and Carafoli, 1982; Olorunsogo, Okudolo, Lawal, Falase, 1985). It seems clear therefore that eucaryotic cells have chosen to survive in a permanent condition of controlled danger, a dynamically convenient but nevertheless perilous choice where the line separating cells from Ca^{2+} poisoning may at times be very tenuous. Clearly the need for systems extruding Ca^{2+} from cells to cancel its downhill influx is glaring. Although eucaryotic cells generally satisfy most of their Ca^{2+} demands by extracting Ca^{2+} from internal Ca^{2+} stores, it is very clear, that long-term maintenance of the Ca^{2+} gradient across the plasma membrane is the result of th

concerted operation of the importing system (the Ca^{2+} channel) and of two exporting systems (the Ca^{2+}-pump and the Na^{+}/Ca^{2+}-exchanger) of the plasma membrane.

These transport systems have different kinetic properties, poised to satisfy the different requirements of cells during their functional cycle (Carafoli, 1987). Indeed, there will be situations where Ca^{2+} must be regulated in the cytosol, or in other cell compartments, very rapidly and with utmost precision e.g. the contraction-relaxation cycle of muscle especially fast muscles. Conversely other situations may require slower movements of bulk amounts of Ca^{2+}. The systems mentioned above are diversified to do just that, since they have different affinities of interaction with Ca^{2+}, and different total Ca^{2+} handling capacity. In general, whenever the need arises to transport Ca^{2+} with high interaction affinity, ATPases are chosen, since this appears to be the only transport mode that confers to the system high Ca^{2+} affinity. As a result, cells rely

solely on ATPases for the fine tuning of their Ca^{2+}. On the other hand, more options are open to situations that demand the movement of bulk amounts of Ca^{2+} with intermediate affinity. Exchangers, channels and electrophoretic uniporters are all low Ca^{2+} affinity systems. However, in red cells the major extrusion mechanisms is the Ca^{2+}-ATPase (Schatzmann, 1966).

The availability of a specific inhibitor for the pump has several implications and these include the ease with which experiments on the evaluation of the physiological and pathological role of the pump will be designed and the application of the chemistry of such substance to the development of a drug which may find particular use in overcoming heart failure. Although, aflatoxin B_1 effects its carcinogenic action by intercalating with DNA through N^7-guanyl residue to form an adduct, the effect of its direct interaction with membrane components is still unknown. It seems however, reasonable to surmise that since aflatoxin B_1 binds

amino acid residues of serum albumin (Bassir and Bababunmi, 1973) and certain functional groups of polysaccarides (Uwaifo and Bassir, 1977) it seems likely the toxin could bind to the red cell membrane components with or without modifying the properties of the membrane. It is based on this reasoning therefore, that the toxin has been used as a tool to inhibit the pump. It is convenient to use red cell membrane as a model for two main reasons. Firstly, red blood cells are readily available in sufficient quantities and secondly, they have no nucleus and no organelles, hence uncontaminated haemoglobin-free erythrocyte ghost membranes are readily obtained from these cells. In order to determine whether or not aflatoxin binds to the red cell membrane, advantage has been taken of the fluorescent properties of the toxin.

The finding that aflatoxin B_1 binds spontaneously and irreversibly to red cell membrane (Table 16) suggests that the toxin interacts with certain components of the membrane. Although, certain functional groups have been found to be

necessary for the binding of the toxin to proteins and to carbohydrate (Uwaifo and Bassir, 1977), it is not known if the same groups are involved in the binding of the toxin to the red cell membrane. It seems likely, however, that groups such as the amino and carboxyl groups of either amino acid residues of proteins or other groups on the lipid and carbohydrate components of the membrane could possibly be involved in the binding. Depending on the number of aflatoxin B_1 molecules that bind to the membrane, there is the possibility that spontaneous and irreversible binding of the toxin to the membrane could cause pertubation of the membrane and also disrupt the assymetry of the phospholipids and fluidity of the membrane. It is also possible that since the toxin is lipid soluble it could trasverse the membrane and subsequentl bind to a lipid soluble component on the cytoplasmic face of the membrane.

Data obtained from studies on the temperature and pH dependence of the binding of the toxin to

the membrane reveal that more binding occurred at acidic pH 4.0, indicating that the binding of the toxin is enhanced by the degree of protonation of the groups or amino acid residues responsible for binding the toxin to the membrane. It seems likely that protonation of the membrane could affect the conformation of the various components of the membrane in such a way that more toxin molecules are bound. The results also show that there was a reduction in the binding of the toxin to the membran at alkaline pH (pH 10). Since coumarins are labile at alkaline pH, due to hydrolytic cleavage of the unsaturated lactone ring of the coumarin nucleus (Fujimoto and Ohba, 1975) there is thus the possibility that there will be a reduction in the fluoresence of aflatoxin B_1 at pH 10.

Using equilibrum dialysis technique the amount of aflatoxin B_1 bound to the membrane was evaluated spectrofluorimetrically. Scatchard plot of the data obtained (Fig. 16) revealed that 4.5 nmoles of aflatoxin B_1 bind per µg membrane

protein. The implication of this finding is that the binding of the toxin to the membrane is likely to be to specific groups on the membrane. Furthermore, it seems that if such a small amount of the toxin binds to the membrane it could be binding to a component of the membrane that has a very low concentration in the membrane.

The fact that Ca^{2+} is the most preponderant cation in the human body and the observation that its concentration is carefully regulated in the cell interior, despite the passive influx of this cation into cells and its function in the regulation of metabolism, suggest that aflatoxin B_1 binding to the membrane could cause a change in the permeability of the cell membrane to the cation in addition to modifying its efflux mechanism. Since the cell membrane is generally permeable to calcium, it appears that aflatoxin B_1 effect would be more drastic or pronounced on the efflux mechanism. The studies reported in this thesis cente mainly on the effect of the toxin on membrane-bound and purified erythrocyte Ca^{2+}-ATPase.

Experiments on the membrane-bound enzyme should yield information on the effect of the toxin on the pump protein in its natural environment, while studies conducted on the purified enzyme are relevant from the perspective of the exact mechanisms of interaction of the toxin with the enzyme protein.

Architectural models of the secondary structure of the plasma membrane calcium pump have been predicted and developed by simple analogy with the structure of the sarcoplasmic reticulum Ca^{2+}-ATPase and on the basis of the homology in the amino acid sequences of the two pumps (Shull and Greeb, 1988; Verma et al, 1988). According to Carafoli et al (1991), the three functional domains of the pump, namely the FITC binding domain (Filoteo et al, 1987) that is normally assumed to be part of the binding site for ATP, the domain surrounding the aspartyl phosphate (James et al, 1987) and the calmodulin binding domain (James et al, 1988) are located on the cytoplasmic face of the membrane. The calmodulin

binding domains are made up of positively charged and hydrophobic residues in the N-terminal portion of the domain.

In order to assess therefore, the effect of aflatoxin B_1 binding to the membrane on the Ca^{2+}-ATPase activity of erythrocyte membrane, the toxin was preincubated with the membrane so as to allow for the mobility of the toxin from the carrier solution dimethylsulfoxide, into the membrane. Ca^{2+}-ATPase activity was assayed thereafter. The data (Fig. 17) obtained indicated that the ATPase activity is drastically reduced in the presence of calmodulin.

It seems likely that the toxin could interact with calmodulin and thus prevent it from activating the enzyme. The finding that the basal activity of the enzyme was not significantly affected may be explained by the reasoning that aflatoxin B_1 could bind to the enzyme only when calmodulin/Ca^{2+} complex is bound.

This fact is supported by the observation that calmodulin stimulated enzyme was inhibited by at least 50% by the toxin. Although, the possibility

that aflatoxin B_1 could bind to the same site as calmodulin or even to calmodulin itself could not be ruled out, it seems possible also that the toxin could bind to the calmodulin-induced conformational state of the enzyme. In this regard, increasing calmodulin concentration has no effect whatsoever on the inhibition of aflatoxin B . This finding demonstrates clearly that the toxin does not bind directly to calmodulin and thus its inhibitory effect is due to its interaction with the pump.

The effect of ATP on the extent of inhibition of the enzyme by aflatoxin B_1 was studied in order to determine whether the toxin could bind to any of the two ATP binding sites. Several workers have shown that the enzyme has two ATP binding sites, one with low affinity (145-400 uM) the other with high affinity (1-2.5 uM) (Muallem and Karlish, 1979; Richard et al, 1977). The data obtained in this study (Fig. 19 and 20) demonstrated that the toxin does not significantly affect the affinity of the enzyme for ATP at the low-affinity site with or without calmodulin. Consequently, increasing ATP

concentration does not reverse the inhibition by aflatoxin. This observation suggests that the toxin does not interfere with the regulation of the enzyme by ATP at the ATP regulatory site. This observation also indicates that the toxin does not bind to the lysine residue (601) responsible for binding ATP at this site. Kinetic analysis of the results (Fig. 20) indicated that only the V_{max} of the calmodulin-stimulated enzyme was reduced in the presence of aflatoxin B_1. This finding suggests that the toxin probably affects the turnover rate of the enzyme.

The membrane bound enzyme was solubilized in the presence of triton X-100 and eluted from calmodulin-agarose affinity column with a buffer containing, EDTA, phosphatidyl choline and 10% glycerol. The purified enzyme, has a molecular weight ranging between 136-140KDa. It is well established that calmodulin has two major effects on the purified Ca^{2+}-pumping ATPase; an increase in its maximum velocity and an enhanced Ca^{2+}-

sensitivity, mainly as a result of the conversion of the low Ca^{2+}-affinity component to a high Ca^{2+}-affinity component (Knauf et al, 1974; Gopinath and Vincenzi, 1977). The effect of the toxin on the purified enzyme (Fig. 21 and 22) further confirmed that, the toxin inhibited the enzyme by about 50%, while it has no effect on the basal activity of the enzyme. It could thus be postulated that irrespective of the state of the enzyme; whether membrane bound or not, the toxin binds to the enzyme protein either 'in situ' or purified. This indicated that the toxin has a specific site to which it binds.

Analysis of the influence of the toxin (Fig. 23 and 24) on the kinetic properties of the enzyme showed that the Km and Vmax values of the purif: enzyme are reduced by about 75% and 50%, respectively when compared to the results obtained in the absence of aflatoxin B_1. This observation further showed that the toxin could either bind to the calmodulin—binding domain thus preventing the effector from binding, or to another site on the enzyme

in such a way that the affinity of the enzyme for camodulin is affected. In either case, the stimulation of the enzyme by calmodulin will be reduced in the presence of aflatoxin B_1.

Calmodulin is a well characterized endogenous calcium-dependent modulator of the calcium pump. (Gopinath and Vincenzi, 1977). Results of studies by Knauf *et al* (1974); Niggli *et al* (1981) have shown that the catalytic activity of the membrane bound and purified enzyme are enhanced several folds by calmodulin. These workers deduced that calmodulin alone is not active, except when it binds Ca^{2+}. The conformation of the calmodulin Ca^{2+} complex becomes more helical and it binds to the inactive Ca^{2+}-ATPase reversibly, the inactive enzyme thus becomes an active calcium pump. This Ca^{2+} modulator is not the only activator of the enzyme. Ronner *et al* (1979) have shown that acidic phospholipids activate the enzyme by increasing its Ca^{2+}-sensitivity and thus increases its rate of turnover (Niggli *et al*, 1981; Aljobore and Roufogalis, 1981). Although Ca^{2+} are needed during

the activation of calmodulin it is not required in the case of phospholipids (Carafoli and Zurini 1982), also the molar ratio of phospholipid to Ca^{2+} transport ATPase is considerably higher than that for an equivalent activation by calmodulin (Niggli et al, 1981), therefore the modes of binding of the two are not the same. Experiments have shown that the number of phosphatidyl serine molecules required for maximal ATPase activity was near 50 per micelle, implying that a single complete layer of phospholipid molecules surround each of the transmembrane helixes of the ATPase for maximal activity to occur. (Nelson and Hanahan, 1985). Furthermore, Zvaricth et al, (1990) were able to show that the amino acid residues between 81 and 76KDa are the phospholipid binding domain. This domain is lysine rich and it consists ofaabout 44 amino acid residues. Several workers have shown that the amount of calmodulin and phospholipids required for stimulation are dramatically different; while calmodulin activates the enzyme at a 1:1 molar

ratio, optimal activation by phospholipids requires hundred of molecules per molecule of ATPase (Enyedi et al, 1987; Zvaricth et al, 1990; Brodin et al, 1991). Enyedi et al, (1987), have shown that acidic phospholipids are apparently more effective than calmodulin i.e. that they decrease the K_m (Ca^{2+}) to values lower than calmodulin;this has led to the suggestion that acidic phospholipids and calmodulin activate the Ca^{2+}-ATPase by separate mechanisms involving different binding sites.

In order to understand the mechanisms of inhibition of the calmodulin activated enzyme by aflatoxin B_1 the purified enzyme was subjected to phospholipid activation in the presence of aflatoxin B_1. Results (Fig. 25) reported in this thesis showed that the toxin inhibited cardiolipin stimulated enzyme by about 28%. This result indicated that because, phospholipid and calmodulin bind at different sites (Zvaritch et al, 1990), the activation by calmodulin is more significantly

inhibited than that of phospholipid. The result also indicated that the toxin has preference for calmodulin stimulated enzyme. The only reasonable interpretation for the selective inhibition of calmodulin stimulation is that the toxin binds to the enzyme only at a site where further binding to calmodulin is hindered while the toxin does not bind as such to the phospholipid binding site. This interpretation finds support from knowledge on the sequences of the amino acid residues of both the phospholipid and the calmodulin binding domains. Although, aflatoxin B_1 has been shown to bind to lysine rich histones, it appears that the lysine-rich phospholipid-domain of the Ca^{2+}-ATPase does not have a significant affinity for the toxin while the calmodulin-binding domain has groups or residues that could bind aflatoxin B_1. One possible candidate is tryptophan 1107, The possibility that the toxin could bind to this residues is supported by the finding that tryptophan is the target for aflatoxin B_1 binding to

Bovine serum albumin (BSA) (Heini and Schabot, 1986).

Several studies have shown that detergent alone could not support ATPase activity (Tarverna and Hanahan, 1980) although this depends on the ratio of the detergent to protein used, and on the type of detergent used choice of detergent is also very important since the effect is based on the critical micelle concentration (CMC) of the detergent used. (Taverna and Hanahan, 1985). For instance triton N-100 has a CMC which is about one third that of triton X-100. Triton X-100 is also a very effective detergent for solubilizing the Ca^{2+}-ATPase and less is required. The effect of the toxin on the triton X-100 solubilized enzyme Fig. 27 shows that the toxin inhibited by about 50%, the calmodulin triton X-100 activated enzyme when the ratio of triton X-100 to mg protein was about 2, while no effect of toxin was observed at lower triton X-100 to protein ratio.

This result indicated that, triton X-100 alone could not support activation, although it could also

mean that triton X-100 could possibly be mimicking the effect of phosphatidyl choline when the ratio is 2. The finding that there is no effect at low ratios of triton X-100 to protein shows that the enzyme did not have the right phospholipid or triton X-100 environment required for activity.

Apart from calmodulin and phospholipids, limited proteolysis by trypsin and calpain could also activate the enzyme (Wang et al, 1988). However, studies of limited proteolysis of the purified enzyme have yielded information of great interest on the organization of the functional domains in the molecule. Stieger and Schatmann (1981) have shown that the activation by trypsin corresponds to a decrease of K_m (Ca^{2+}) of the pump to levels that under experimental conditions, were even lower than those obtained with calmodulin. However, when it was maximally activated the pump became fragmented into a number of products. Zurini et al (1984) have shown that proteolysis for 5-7 mins

yielded 90KDa fragment that is responsive to both calmodulin and acidic phospholipids. Results reported in this thesis (Table 20) showed that calmodulin stimulated the ATPase activity of the 90KDa fragment. This activity is inhibited by the toxin. This means that, the site at which the toxin inhibits is somewhere within 90KDa fragment and confirms also that the site of inhibition is the calmodulin binding domain as previously discussed. Further proteolysis of EDTA-treated enzyme showed that the 76KDa product obtained after 90 mins of preincubation was not stimulated by calmodulin and was also not inhibited by the toxin (Table 20). The results indicated that because the calmodulin binding domain has been cleaved in the 76KDa fragment (Wang et al, 1989) the toxin could no longer inhibit the truncated and activated enzyme since the inhibition of the enzyme is at the calmodulin binding domain. This domain has abundance of basic amino acid residues such as tryptophan and lysine residues. (Blumenthal, Takio, Edelman, Charbonneau, Titatni, Walsh, Krebs,

1983). It seems possible therefore that aflatoxin B_1 could be binding to lysine residue, since findings reported by Swenson et al (1974) shown that the toxin binds to lysine histone preparations. The toxin may also bind to tryptophan as postulated by Heini and Schabort (1986). The mechanism of the binding of the toxin to the calmodulin binding domain may be explained from the standpoint of the structure of the calmodulin binding domain (Fig. 4A). Enyedi et al (1989); Vorherr et al (1990) have shown that in the absence of calmodulin, the calmodulin binding domain (domain C) binds to domain A and thereby limits assesibility to Ca^{2+}, while in the presence of calmodulin, calmodulin binds to calmodulin-binding domain, and domain A is free to bind Ca^{2+}. However, in the presence of aflatoxin B_1, the toxin may bind to domain C thereby preventing the stimulation of the enzyme by calmodulin.

To further confirm the binding that the toxin could be binding to the calmodulin binding

domain another proteolytic agent was used. It has been shown that the Ca^{2+} dependent cystein-protease also known as calpain, will cleave about 14KDa fragment from the whole molecule of Ca^{2+}-ATPase, thereby, making the enzyme insensitive to calmodulin. The cleavage removes a part of the domain A required in calmodulin binding. Results of experiments reported in this thesis (Table 19) showed that increasing concentration of aflatoxin B_1 has no effect on the calpanized ATPase and thus indicated that the possible target of the toxin has been cleaved from the enzyme, since the 124KDa is devoid of a part of the calmodulin binding domain.

It is difficult to know which amino acid residue is really involved in aflatoxin binding. However, it could be speculated that both lysine and tryptophan may be possible targets. In order to determine exactly the amino acid residue to which the toxin binds, radio-active labelled aflatoxin B_1 will be required. Furthermore, such experiments will involve the

use of High performance liquid chromatography (HPLC) to purify and separate tryptophan fragments to which the toxin is bound. Thus aflatoxin B_1-labelled fragment will then be subjected to sequencing. Alternatively, the fragments may be run on an SDS-polyacrylamide gel electrophoresis and the band transferred by electroblotting to PVDF/Imobilon P followed by amino acid sequencing of the band corresponding to the aflatoxin-labelled fragment in a sequenator or automatic amino acid sequencer.

Several workers have shown that some drugs such as phenothiazine neuroleptics do inhibit calmodulin-stimulated enzyme at low concentrations while at high concentrations of the compounds they also inhibit the basal ATPase activity stimulated by acidic phospholipids and limited proteolysis. Based on these rationalization, it seems possible therefore that the aflatoxin could be mimicking the action of an anticalmodulin drug. In general, it seems possible, therefore that enzymes that have the identical calmodulin binding domain such as Ca^{2+} transporting ATPase could be inhibited by

aflatoxin B_1. One major example is cyclic AMP phosphodiesterase which functions in the formation of cAMP. Also, Hidaka, Sasaki, Tanaka, Endo, Ohno, Fujii and Nagata (1981) haveesshown that when anticalmodulin drugs such as phenothiazine, thioxanthene and naphtalene sulfornamide were used to treat CHO cells, these workers deduced that, the growth of these cells were arrested at both the G/S boundary and in S phase. It seems possible therefore that the toxin could arrest these cells at bothG/S and S phase. This could however be the primary event in the toxicity of this toxin.

SUMMARY OF RESULTS

1. Incubation of the ghost membrane with aflatoxin B_1 shows that the toxin binds spontaneously and irreversibly to erythrocyte membrane in a non-co-operative manner.

2. Aflatoxin B_1 inhibits the calmodulin stimulated activity of erythrocyte Ca^{2+}-pumping ATPase while it has no effect on its basal activity, whereas maximum velocity of the stimulated enzyme is drastically reduced by aflatoxin B_1. Also there is no significant effect on the K_m of the enzyme with or without calmodulin.

3. Aflatoxin B_1 inhibits the calmodulin stimulated purified enzyme whereas it has no significant effect on its basal activity. The maximum velocity and the affinity for ATP K_m (ATP) of the calmodulin stimulated enzyme were reduced by aflatoxin B_1 while in the absence of calmodulin the K_m and V_{max} were not affected by aflatoxin B_1.

4. Aflatoxin B_1 inhibited the cardiolipin stimulated by about 28%, while it has no significant effect on the basal activity of the enzyme. Experiments on the effect of aflatoxin B_1 on the stimulation of the enzyme by Triton X-100 shows that Triton X-100 maximally activated the enzyme only when the ratio triton: protein is 2 and aflatoxin B_1 inhibited the triton activated enzyme by about 50%.

5. Results from this thesis shows that PC alone did not activate the pump. Calmodulin-stimulated enzyme in the presence of PC was inhibited by aflatoxin B_1. In addition, aflatoxin B_1 inhibited the PS stimulation of the enzyme by about 28% while the enzyme was not further stimulated by calmodulin.

6. Results of the effect of aflatoxin B_1 on proteolysed enzyme shows that aflatoxin B_1 has no effect whatsoever on the calpanised enzyme, whereas the toxin inhibited the ATPase activity of the

enzyme partially trypsinised for 5' which correspond to the 90KDa fragment. The toxin has no effect on the enzyme trypsinised for 90' which corresponds to the 76KDa fragment of the ATPase.

CONTRIBUTION TO KNOWLEDGE

In this thesis, evidence has been presented to show that:

1. Aflatoxin B_1 binds to the membrane and can alter the function of the membrane.
2. Aflatoxin B_1 has no effect on the basal activity of the membrane bound and purified ATPase.
3. Aflatoxin B_1 inhibits the activity of the calmodulin stimulated membrane bound and purified ATPase.
4. Aflatoxin B_1 inhibits the stimulation of the membrane bound and purified enzyme activity by acidic phospholipids.
5. The K_m and V_{max} of the stimulated enzyme are reduced by aflatoxin B_1.
6. Aflatoxin B_1 seems to bind at the calmodulin binding domain of the enzyme.

REFERENCES

Adamo, H.P., Rega, A.F. and Garrahan, P.J. (1988). Pre-steady-state phosphorylation of the human red cell Ca^{2+}-ATPase. J. Biol. Chem. 263: 17548-17554.

_____ (1990). The $E_2 \rightleftharpoons E_1$ transition of the Ca^{2+} ATPase from plasma membranes studied by phosphorylation. J. Biol. Chem. 265: 3789-3792.

Adamo, H.P. and Penniston, J.T. (1991). New Ca^{2+} pump isoforms generated by alternative splicing of RPMCA2mRNA. Biochem. J. In press.

Al-Jobore, A. and Roufogalis, B.D. (1981). Phospholipid and calmodulin activation of solubilized calcium-transport ATPase from human erythrocytes: regulation by magnesium. Can. J. Biochem. 59: 880-885.

Allen, G., Trinnaman, B.J. and Green, N.M. (1980). Primary structure of the calcium ion-

transporting adenosine triphosphatase from rabbit skeletal sarcoplasmic reticulum. Peptides derived from digestion with cNBr, and the sequences of three long extra membranes segments. Biochem. J. 187: 591-616.

Anderson, J.M., Charbonneau, H., Jones, H.P., McCann, R.O. and Cormier, M.J. (1980). Characterisation of the plant nicotinamide adenine dinucleotide kinase activator protein and its identification as calmodulin. Biochemistry. 19: 3113-3120.

Androlles, P.J. and Reed, G.B. (1964). Confirmatory tests for aflatoxin B_1. J. Ass. Off. Agr. Chem. 47: 801-803.

Ansah, T.A., Moila, A. and Katz, S. (1984). Ca^{2+}-ATPase activity in pancreatic acinar plasma membranes. Regulation by calmodulin and acidic phospholipids. J. Biol. Chem. 259: 13442-13450.

Asao, T., Buchi, O., Abdel-Kader, M.M. Chang, S.B., Wick, E.L. and Wogan, G.N. (1965). The structures of aflatoxin B_1 and G_1. J. Am. Chem. Soc. 87: 882-886.

Au, K.S. (1986). Activation of erythrocyte membrane Ca^{2+}-ATPase by calpain. Biochim. Biophys. Acta. 905: 273-278.

Autric, F., Ferraz, C., Kilhoffer, M. and Demaille, J.G. (1980). Large-scale purification and characterization of calmodulin from ram testis: its metal-ion dependent conformers. Biochim. Biophys. Acta. 631: 139-147.

Balaz, E. and Von Euler, J. (1952). The hyaluronidase content of necrotic tumour and testis tissue. Cancer Res. 12, 326-329.

Bababunmi, E.A. and Bassir, O. (1976). Activation of DNP-induced ATPase of mitochondria by aflatoxin B 9th Int. Congr. Biochemistry. Stocklom abs. pp. 220.

Bachs, O.K., Famulski, S., Mirabelli, F. and Carafoli, E. (1985). ATP-dependent Ca^{2+} transport in vesicles isolated from the bile canalicular region of the hepatocyte plasma membrane. Eur. J. Biochem. 147: 1-7.

Barnes, J.M., and Butler, W.H. (1964). Carcinogenic activity of aflatoxins to rats. Nature, 202: 1016-1019.

Bassir, O. and Emerole, G.O. (1975). Species differences in the metabolism of palmotoxin B_o and G_o in vitro. Xenobiotica. 5: 649-655.

Bassir, O. and Emafo, P. (1970). Oxidative metabolism of aflatoxin B_1 by mammalian liver slices and microsomes. Biochem. Pharmacol. 19: 1681-1687.

Bassir, O. and Bababunmi, E.A. (1973). The binding of aflatoxin B_1 with serum albumin. Biochem. Pharmacol. 22: 132-134.

Bayerdoerffer, E., Streb, H., Eckhardt, L., Hasse, W. and Schulz, I. (1984). Characterization of calcium uptake in rough endoplasmic reticulum of rat pancreas. J. Membr. Biol. 81: 69-82.

Belt, J.A. and Campbell, T.C. (1973). Effect of aflatoxin B_1 on mitochondria incorporation of UTP (abstr). Fed. Proc. Fed. Am. Socs. Exp. Biol. 32: 277.

_____ (1975). Effect of aflatoxin on mitochondria protein synthesis (abstr). Fed. Proc. Fed. Am. Socs. Exp. Biol. 34: 226.

Benaim, G., Zurini, M., and Carafoli, E. (1984). Different conformation states of the purified Ca^{2+}-ATPase of the erythrocyte plasma membrane as revealed by controlled trypsin proteolysis. J. Biol. Chem. 259: 8471-8477.

Benaim, G., Clark, A. and Carafoli, E. (1986). ATPase activity and Ca^{2+}-transport by reconstituted tryptic fragments of the Ca^{2+}-pump of the erythrocyte plasma membrane. Cell Calcium. 7: 175-186.

Bernhard, W., Frayssinet, C., Lafarge, C. and Le Breton, E. (1965). Lessions Nucleolaines precoces provoquees par l'aflatoxin-lans les cellulas. Hepatiques du rat Compt. Red. 261: 1785-1788.

Blaustein, M.P. and Hodgkin, A. (1969). The effect of cyanide on the efflux of calcium from squid axons. J. Physiol. Lond. 200: 497-527.

Blount, W.P. (1961). Turkey 'X' disease. J. Br. Turkey Fed. 52: 55-58.

Blumenthal, D.K., Takio, A.M., Edelman, A.M., Charbonneau, H., Titani, K., Walsh, K.A. and Krebs, E. (1982).

the calmodulin-binding domain of skeletal muscle myosin, lightchain kinase.
Proc. Natl. Acad. Sci. USA 82: 3187-3191.

Bond, G.H. and Clough, D.L. (1972). A soluble protein activator of (Mg^{2+} + Ca^{2+})-dependent ATPase in human red cell membranes.
Biochim. Biophys. Acta 323: 592-599.

Brandl, C.J., Green, N.M., Korczak, B. and Maclennan, D.H. (1986). Two Ca^{2+}-ATPase genes: homologies and mechanistic implications of deduced amino acid sequences.
Cell. 44: 597-607.

Brandt, N.R., Caswell, A.H. and Brunschavg J.P. (1980). ATP-energized Ca^{2+} pump in isolated transverse tubules of skeletal muscle.
J. Biol. Chem. 255: 6290-6298.

Brodin, P., Thomas, V. and Carafoli, E. (1991). Identification of two domains which mediate the binding of activating phospholipids to the plasma membrane Ca^{2+} pump. In press.

Brostrom, C.O., Brostrom, M.A. and Wolf, D.J. (1971). Calcium-dependent adenylate cyclase from rat cerebral cortex. J. Biol. Chem. 252: 5677-5685.

Butler, W.H. (1964). Acute toxicity of aflatoxin B_1 in rats. Brit. J. Cancer 18: 756-762.

Butler, W.H. and Barnes, J.M. (1966). Carcinoma of the glandular stomach in Rats diets containing aflatoxin M. Nature. 209: 90.

Butler, W.H., Greenbatt, M. and Lijinsky, N. (1969). Carcinogenesis in Rats by aflatoxin B_1, G_1, and B_1. Cancer Res. 29: 2206-2211.

Burgin, H. and Schatzmann, H.J. (1979). The relation between net calcium, alkali cation, and chloride movements in red cells exposed to salicylate. J. Physiol. (London) 287: 15-32.

Burnside, J.E., Sippel, W.L., Forgacs, J., Caril, W.T., Atwood, M.B. and Doll, E.R. (1957). A disease of swine and cattle caused by eating mouldy corn. II. Experimental production with pure cultures of moulds. Am. J. Vet. Res. 18: 817-824.

Cameron, E. (1966). Hyaluronidase and Cancer. New York Pergamon Press. pp. 260-265.

Carafoli, E., Tiozzo, R., Lugli, G., Govetti, F. and Kratzing, C. (1974). The release of calcium from heart mitochondria by sodium. J. Mol. Cell Cardiol. 6: 361-371.

Carafoli, E. and Crompton, M. (1978). The regulation of intracellular calcium. Curr. Topics. Membr. Transp. 10: 151-216.

Carafoli, E. (1981). Calmodulin in the membrane transport of Ca^{++}. Cell Calcium 2: 353-363.

Carafoli, F. (1984). Plasma membrane Ca^{2+} transport and Ca^{2+} handling by intracellular stores: an integrated picture with emphasis on regulation: Mechanisms of intestinal

electrolyte transport and regulation pp. 121-134 (Liss. New York, 1984).

Carafoli, E., James, P. and Strehler, E.E. (1990). Structure function relationships in the calcium pump of plasma membrane. Molecular and cellular regulation of calcium and phosphate metabolism pp. 181-193, Alan R. Liss, Inc.

Carafoli, E. (1991). Calcium pump of the plasma membrane. Physiological Rev. 71: 129-153.

Caride, A.J., Gorski, J.P. and Penniston, J.T. (1988). Topology of the erythrocyte Ca^{2+} pump. A monoclonal antibody against the almost inaccesible extracellular face. Biochem. J. 255: 663-670.

Caroni, P. and Carafoli, E. (1980). An ATP-dependent Ca^{2+} transport system in dog heart sarcolemma. Nature Lond. 283: 765-767.

_____ (1981). The Ca^{2+} pumping ATPase of heart sarcolemma. Characterization,

calmodulin dependence, and partial purification.
J. Biol. Chem. 256: 3263-3270.

Chafouleas, J.G., Wade, E.B., Hiroyoshi, H., Aubrey, E.B. and Anthony, R.M. (1982). Calmodulin and the cell cycle: Involvement in regulation of cell cycle progression
Cell 28: 41-50.

Cham, K.M. and Junger, K.D. (1983). Calcium transport and phosphorylated intermediate of (Ca^{2+} + Mg^{2+})-ATPase in plasma membranes of rat liver.
J. Biol. Chem. 253: 4404-4410.

Cheung, W.Y. (1970). Cyclic 3',5'-nucleotide phosphodiesterase: Demonstration of an activator.
Biochem. Biophys. Res. Commun 38: 533-538

Clark, M.R. and Carafoli, E. (1983). The stochiometry of the Ca^{2+}-pumping ATPase of erythrocytes.
Cell Calcium 4: 83-88.

Cliford, J.I. and Rees, K.R. (1966). Aflatoxin A site of action in the rat liver cell. Nature (Lond) 209: 312-313.

_____ (1967). The interaction of aflatoxins with purines and purine nucleosides. Biochem. J. 103: 467-471.

Cohen, P., Burchell, A. and Foulkes, A. (1978). Identification of the Ca^{2+}-dependent modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase. FEBS Lett. 92: 287-293.

Cohen, P., Cohen, P.T., Shenolikai, S., Nairn, A. and Victor, P.S. (1979). The role of calmodulin in the structure and regulation of phosphorylase kinase of rabbit skeletal muscle. Eur. J. Biochem. 100: 329-337.

Condrescu, M., Asses, L. and Dipolo, R. (1984). Partial purification and characterization of the (Ca^{2+} + Mg^{2+})-ATPase from squid

optic nerve plasma membranes.
Biochim. Biophys. Acta. 769: 261-269.

Crisan, E.V. and Grefig, A.T. (1967). The formation of aflatoxin derivative.
Contrib. Boyce. Thompson Inst. 24: 3-8.

Crey, R.G., Essigman, J.M., Reinhold, V.N. and Wogan, G.N. (1978). Identification of the principal aflatoxin B_1 DNA adduct formed in vivo in rat liver.
75: 1745-1749.

Dayton, W.R., Goll, D., Zeece, M., Robson, R.M. and Reville, W.J. (1976). Calcium activated protease possibly involved in myofibrillar protein turnover, purification from porcine muscle.
Biochemistry. 15: 2150-2167.

Dodman, J.R., Jackson, R.L., Schreibor, W.E. and Means, A.R. (1978). Sequence homology of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+}-binding proteins.
J. Biol. Chem. 253: 343-346.

De Jaegere, S., Wuytack, F., Eggermont, J.A., Verboomen, H. and Casteels, R. (1990). Molecular cloning and sequencing of the plasma-membrane Ca^{2+}-pump of pig smooth muscle. Biochem. J. 271: 655-660.

DeJonge, H.R., Ghijsen, J.M. and Van Os, C.H. (1981). Phosphorylated intermediates of Ca^{2+} ATPase and alkaline phosphatase in plasma membranes from rat duodenal epithelium. Biochim. Biophys. Acta 647: 140-149.

Denny, J.B. and Blobel, G. (1984). ^{125}I -labeled cross linking reagent that is hydrophylic, photoactivatable, and clearable through an azo linkage. Proc. Natl. Acad. Sci. USA 81: 5286-5290.

Desmedt, H., Paris, J.B., Borghgraef, R. and Wuyatck (1981). Calmodulin stimulation of renal (Ca^{2+} + Mg^{2+}) -ATPase. FEBS Lett. 131: 60-62.

_____ (1983). Phosphorylated intermediates of (Ca^{2+} + Mg^{2+})-ATPase and alkaline Biophys. Acta. 728: 409-418.

_____ (1984). Calcium-induced phosphorylation and ^{125}I -calmodulin binding in renal membrane preparations. Biochim. Biophys. Acta 776: 122-132.

Detroy, R.W. and Hesseltine, C.W. (1970). Structure of a new transformational product of aflatoxin B_1. Can. J. Biochem. 48: 830-832.

Dieter, P. and Marme, D. (1981). A calmodulin-dependent microsomal ATPase from corn (Zea mays L). FEBS Lett. 125: 245-248,

Dickens, F. and Jones, H.E.H. (1964). The carcinogenic action of aflatoxin after its subcutaneous injection in the rat. Brit. J. Cancer. 17: 691-698.

Dipolo, R. (1979). Ca pump driven by ATP in squid axon. Nature Lond. 274: 390-392.

Dixon, D. and Haynes, D. (1989). Kinetic characterization of the Ca^{2+}-pumping ATPase of cardiac sarcolemma in four states of

activation.
J. Biol. Chem. 264: 13612-13622.

Dodge, J.T., Mictchell, C. and Hanahan, D.J. (1963). The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes.
Arch. Biochem. Biophys. 100: 119-128.

Doherty, W.P. and Campbell, T.C. (1973). Aflatoxin inhibition of rat liver mitochondria.
Chem. Biol. Interact. 7: 63-77

Dollear, F.G. and Mann, G.E. (1968). Elimination of aflatoxins from peanut meal.
J. Am, Oil Chem. Soc. 45: 862-865.

Dunhan, E.T. and Glynn, I.M. (1961). Adenosine-triphosphatase activity and the active movements of alkali metal ions.
J. Physiol. Lond. 156: 274-293.

Dutton, M.F. and Heathcote, J.G. (1963). Two new hydroxyl aflatoxin.
Biochemical Journal. 101: 21-22.

Dwarakanath, C.T., Rayner, E.T., Mann, G.E. and Dollear, F.G. (1968). Reduction of aflatoxin levels in cotton seed and peanuts by ozonization. J. Am. Oil Chem. Soc. 45: 93-95.

Ebashi, S. (1958). A granule-bound relaxation factor in skeletal muscle. Arch. Biochem. Biophys. 76: 410-423.

Enyedi, A., Sarkadi, B. Szasz, I., Bot, B. and Gardos, G. (1980). Molecular properties of the red cell calcium pump II. Effects of proteolysis, proteolytic digestion and drugs on the calcium-induced phosphorylation by ATP in inside/out red cell membrane vesicles. Cell Calcium 1: 299-310.

Enyedi, A., Flura, M., Sarkadi, B., Gardos G. and Carafoli, E. (1987). The maximal velocity and the calcium affinity of the red cell calcium pump may be regulated independently. J. Biol. Chem. 262: 6425-6430.

Enyedi, A., Vorherr, T., James, P., McCormick, D.J., Filoteo, A., Carafoli, E and Penniston, J.T. (1989). The calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts both with calmodulin and with another part of the pump. J. Biol. Chem. 264: 12313-12321.

Emerole, G.O. and Uwaifo, A.O. (1980). Aflatoxins: Carcinogenicity and Mutagenicity in "Toxicology in the Tropics" Edited by Smith, R.L. and Bababunmi E.A. published by Taylor and Francis (Lond) pp. 80-82.

Emerole, G.O., Uwaifo, A.O., Thabrew, M.I. and Bababunmi, E.A. (1982). The presence of aflatoxin and some poly cyclic aromatic hydrocarbons in human foods. Cancer Lett. 15, 123-129.

Evrain, C., Cittanova, N. and Jagle, M.F. (1978). Fluoresence studies of the interactions of serum albumin and rat alpha -Feto protein with aflatoxin B_1. Biochim. Biophys. Acta. 533: 408-414.

Farrance, M.L. and Vincenzi, F.F. (1977). Enhancement of (Ca^{2+} + Mg^{2+})-ATPase activity of human erythrocyte membranes by hemolysis in isoosmotic imizazole buffer. I. General properties of various membrane preparations and the mechanism of the isoosmotic imidazole effect. Biochim. Biophys. Acta. 471: 49-58.

Fatt, P. and Ginsborg, B. (1958). The ionic requirements for the production of action potentials in crustacean muscle J. Physiol. (Lond) 142: 516-543

Fiegelson, P. and Greongard, O. (1962). Regalation of liver tryptophan pyrrole activity. J. Biol. Chem. 237: 1908-1913.

Filoteo, A.G., Gorski, J.P. and Penniston J.T. (1987). The ATP-binding site of the erythrocyte membrane Ca^{2+} pump. J. Biol. Chem. 262: 5526-6530.

Fiske, C.H. and Subbarow, Y. (1925). The colorimetric determination of phosphorous. J. Biol. Chem. 66: 375-400.

Foster, P.L., Elsenstadt, E. and Miller, J.H. (1983). Base substitution mutations induced by metabolically activated aflatoxin B_1. Proc. Natl. Acad. Sci. 80: 2695-2698.

Frayssinet, C., Lafarge, C., De-Recondo, A.M. and Breton, E. (1964). Inhibition de l'hypertrophie compesatria du Folle. Chez le Rat parles Toxines d' Aspergillus flavus. Compt. Rend. Acad. Sci. Paris. 259: 2143-2146.

Friedman, M.A. and Wogan, G.N. (1966). Effects of aflatoxin B_1 on enzyme induction and nuclear RNA metabolism in Rat liver. Fed. Proc. 25: 662-665.

_____ (1967). Effects of aflatoxin B_1 on RNA polymerase activity and incorporation of cytidine into RNA of Rat liver Nucleic Fed. Proc. 26: 358-365.

Fiszer-Szarfar, B. and Szafard, D. (1973). Lysosomal hyaluronidase activity in normal rat liver and in chemically induced hepatoma. Cancer Res. 33: 1104-1108.

Fujimoto, S. and Ohba, Y. (1975). Interaction of aflatoxin B_1 with DNA. J. Biochem. 77: 187-195.

Garbers, D.C., Hansbrough, J.R., Radany, E.W., Hyne, R.V. and Kopf, G.S. (1980). Purification and characterization of calmodulin from sea urchin spermatozoa. R. Reprod. Fertil. 59: 377-381.

Garrahan, P.J. and Rega, A.F. (1978). Activation of partial reactions of the Ca^{2+}-ATPase from human red cells by Mg^{2+} and ATP. Biochim. Biophys. Acta. 513: 59-65.

Garvican, L. and Rees, U. (1974). The effect of aflatoxin B_1 on the maturation of ribosomal and transfer RNA in monkey kidney. (CV-1) cell and culture. Chem. Biol. Interact. 9: 429-434.

Gelboin, H.V., Wortham, J.S., Friedman, M. and Wogan, G.N. (1966). Rapid and marked inhibition of Rat liver RNA polymerase by aflatoxin B_1. Science 154: 1205-1206.

Ghijsen, W.P., Gmaj, and Murer, H. (1984). Ca^{2+}-stimulated Mg^{2+}-independent ATP-hydrolysis of the high affinity Ca^{2+}-pumping ATPase. Two different activities in rat kidney basolateral membranes. Biochim. Biophys. Acta. 778: 481-488

Gietzen, K., Tejcka, M. and Wolf, H.U. (1980). Calmodulin affinity chromatography yields a functional purified erythrocyte (Ca^{2+} + Mg^{2+})-dependent adenosine triphosphatase. Biochem. J. 189: 81-88.

Gietzen, K., Sadorf, I. and Badder, H. (1982). A model for regulation of the calmodulin independent enzyme erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase by activators and inhibitors. Biochem. J. 207: 541-545.

Gopalan, A.O. (1972). The relationship of hepatoma in rainbow trout to aflatoxin contamination and cottonseed meal. Cancer Res. 28, 987-991.

Gopinath, R.M. and Vincenzi, F.F. (1977). Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+} + Mg^{2+})-ATPase. Biochem. Biophys. Res. Commun. 77: 1203-1209.

Grab, D.J., Berzins, K., Cohen, R.S. and Slekevitz, P. (1979). Presence of calmodulin in postsynaptic densities isolated from canine cerebral cortex. J. Biol. Chem. 254: 8690-8696.

Graf, E., Filoteo, A.G. and Penniston, J.T. (1980). Preparation of ^{125}I-calmodulin and the Ca^{2+} ATPase from human erythrocyte membranes. Arch. Biochem. Biophys. 210: 257-262.

Gitelman, S.E., and Witman, G.B. (1980). Purification of calmodulin from chalmydomonas: Calmodulin occurs in cell bodies and flagella. Biochem. Biophys. Res. Commun. 98: 764-770.

Gmaj, P., Murer, H. and Kinne, R. (1979). Calcium on transport across plasma membranes isolated from rat kidney cortex. Biochem. J. 178: 549-557.

Gmaj, P., Murer, H. and Carafoli, E. (1982). Localization and properties of a high affinity (Ca^{2+}+ Mg^{2+})-ATPase in isolated kidney cortex plasma membranes. FEBS Lett. 131: 60-62.

Gmaj, P., Zurini, M., Murer, H. and Carafoli, E (1983). A high affinity, calmodulin-dependent Ca^{2+} pump in the baso -lateral plasma membranes of kidney cortex. Eur. J. Biochem. 136: 71-76.

Graf, E. and Penniston, J.T. (1981). The Substrate, at low ATP concentration of Ca^{2+}-ATPase from human erythrocyte membrane. J. Biol. Chem. 256: 1587-1589.

Graffi , A. and Krebsforch, Z. (1940). Differences of mitochondria protein synthesis in vitro between tumour and normal tissues. Biochem. Biophys. Res. Commun. 21: 418-423.

Greeb, J. and Shull, G.E. (1989). Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that expressed predominantly in Brain and Skeletal muscle. J. Biol. Chem. 264: 18569-18576.

Gross, J. and Marme, D. (1978). ATP-dependent Ca^{2+} uptake into plant membrane vesicles. Proc. Natl. Acad. Sci. USA. 75: 1232-1236

Guerini, D., Krebs, J. and Carafoli, E. (1984). Stimulatioh of the purified erythrocyte Ca^{2+}-ATPase by tryptic fragments of calmodulin. J. Biol. Chem. 259: 15172-15177.

Gurtoo, H.L, and Dahms, R. (1974). On the nature of the binding of aflatoxin B_{2a} to rat hepatic hucrosomes. Res. Commun. Chem. Pathol. Pharmacol. 9: 107-118.

Hakim, G., Itano, T., Verma, V.K. and Penniston, J.T. (1982). Purification of Ca^{2+} and Mg^{2+}-requiring ATPase from rat brain synaptic plasma membrane. Biochem. J. 207: 225-231.

Hanahan, D., Taverna, R.D., Flynn, D. and Ekholm, J. (1978). The interaction of Ca^{2+}/Mg^{2+} ATPase activator protein with human erythrocyte membranes. Biochim. Biophys. Res. Commun. 84: 1009-1015.

Hasselbach, W. and Makinose, M. (1961). Die calcium pumpe der, Erschlaff-ungsgrana muskels unde lhre Abhangigkeit von der ATP-spaltung. Biochem. 2333: 518-528.

Hartely, R.D., Nesbitt, B.F. and O'Kelly, J. (1963). Toxic metabolites of Aspergillus flavus. Nature. 198: 1056-1058.

Harris, R.A., Stephens, R.W., Ghoshi, P. and Taylor, A.T.P. (1977). Endo and exo-glycoxidases in an experimental rat oesteosarcoma. Anst. J. Exp. Biol. Med. Sci. 55: 363-370.

Hayes, L.C., Platt, F.V., Tilzer, L.L. and Chiza, M. (1975). Characterization of microsomes produced by aflatoxin B_1. Chem. Biol. Interact. 10: 343-347.

Heine, W.D. and Schabort, J.C. (1986). Aflatoxin B_1 transport in rat blood plasma binding to albumin *in vivo* and *in vitro* and spectrofluorimetric studies into the nature of the interaction. Biochim. Biophys. Acta. 881: 383-390.

Heilbrunn, L.V. and Wiercinski, F.J. (1947). The action of various cations on muscle protoplasm. J. Cell. Comp. Physiol. 29: 1-532

Hennessey, J.P. Jr and Scarborough, G.A. (1990). Direct evidence for the cytoplasmic location of the NH_2-and COOH-terminal J. Biol. Chem. 265: 532-537.

Hidaka, H., Sasaki, Y., Tanaka, T., Endo., T., Ohno, S., Fujii, Y. and Nagata, T. (1981). N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulfonamide, a calmodulin antagonist inhibits cell proliferation. Proc. Natl. Acad. Sci. (USA) 78: 4354-4357

Hidalgo, C., Gonzales, M.E. and Garcia, A.M. (1986). Calcium transport in transverse tubulis isolated from rabbit skeletal muscle. Biochim. Biophys. Acta. 854: 279-286.

Hildmann, B., Schmidt, A. and Murer, H. (1982). Ca^{2+}-transport across basolateral plasma membranes from rat small intestinal epithelial cells. J. Membr. Biol. 65: 55-62.

Hinds, T.R. and Andreasen, T.J. (1981). Photochemical cross-linking of azido calmodulin to the (Ca^{2+} + Mg^{2+})-ATPase at low Ca^{2+} concentration. J. Biol. Chem. 256: 7877-7882.

Hoffman, J.F. (1962). Cation transport and structure of the cell plasma membrane. Circulation 26: 1201-1213.

Hymel, L., Maurer, A., Berenski, C., Jung, C.Y. and Fleischer, S. (1984). Target size of calcium pump protein from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 259: 4890-4895.

Imamura, K. and Schulz, I. (1985). Phosphorylated intermediate of (Ca^{2+} + K^{+})-stimulated, Mg^{2+}-dependent transport ATPase in endoplasmic reticulum from rat pancreatic acinar cells. J. Biol. Chem. 260: 11339-11347.

Iwasa, Y.O., Iwasa, T., Higashi, K., Matsui, K. and Miyamoto, E. (1982). Demonstration of a high affinity Ca^{2+}-ATPase in rat liver plasma membranes. Biochem. Biophys. Res. Commun. 105: 448-494.

Iwasa, T., Iwasa, Y. and Krinshnaraj, R. (1983). A high-affinity (Ca^{2+} + Mg^{2+})-ATPase in plasma membranes of rat ascites hepatoma AH 109A cells. Biochim. Biophys. Acta. 731: 229-238.

Katz, S. and Blostein, R. (1975). Ca^{2+}-stimulated membrane phosphorylation and ATPase activity of the human erythrocyte. Biochim. Biophys. Acta. 389: 314-324.

Klaven, N.B., Pershadsing, H.A.O., Henius, G.U., Larris, P.C., Long, J.W. and McDonald, J.M. (1983). A high affinity calmodulin sensitive (Ca^{2+} + Mg^{2+})-ATPase and associated calcium transport pump in the Ehrlich ascites tumour cell plasma membranes. Arch. Biochem. Biophys. 226: 618-628.

Klee, C.B. (1980). In protein phosphorylation and Bioregulation (Thomas, G., Podesta, E.J. Gordon, E. Eds.) pp. 61-69, Karger, Basle.

Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974). Electrophoretic separation of different phosphoproteins associated with Ca^{2+}-ATPase and Na, K -ATPase in human red cell ghosts. J. Gen. Physiol. 63: 324-336.

Kotagal, N., Patker, C., Landt, M., McDonald, J.M. and Colca, P. (1982). Regulation of pancreatic islet-cell plasma membrane (Ca^{2+} + Mg^{2+})-ATPase by calmodulin. FEBS Lett. 137: 249-252.

Kosk-Kosicka, D., Bzdega, T. and Johnson, D.J. (1989). Fluorescence studies on calmodulin binding to erythrocyte Ca^{2+}-ATPase in different oligomerization states. Biochemistry. 29: 1875-1879.

Kraus-Freidmann, N., Biber, J., Murer, H. and Carafoli, E. (1982). Calcium uptake in isolated hepatic plasma membrane vesicles. Eur. J. Biochem. 129: 7-12.

Krebs, J., Vasak, M., Scarpa, A. and Carafoli, E. Conformational differences between the E_1 and E_2 states of the calcium adenosinetriphosphatase of the erythrocyte plasma membrane as revealed by circular dicroism and fluorescence spectroscopy. Biochemistry. 26: 3921-3926.

Kretsinger, R.H. (1975). Hypothesis: Ca^{2+} modulated proteins contain EF hands; in Carafoli, Clement, Drabikowski, Margreth. Calcium transport in contraction and secretion pp. 468-478 (North Holland Amsterdan).

Kretsinger, R.H. (1980). Structure and Evolution of calcium modulated protein. (CRC Crit. Rev Biochem. 8: 119-184.

Kulik, M.M. and Holaday, C.E. (1967). Aflatoxin a metabolic product of several fungi. Mycopathol. Mycol. Appl. 30: 137-140.

Kuwayama, H. (1988). The membrane potential modulates the ATP-dependent Ca^{2+} pump of cardiac sarcolemma. Biochim. Biophys. Acta. 940: 295-299.

Kuwayama, H. and Kanazawa, T. (1982). Purification of cardiac sarcolemma vesicles: high sodium pump content and ATP-dependent, calmodulin-activated calcium uptake. J. Biochem. 91: 1419-1426.

Kuznicki, J., Kuznicki, L. and Drabikowski, W. (1979). Ca^{2+}-binding modulator protein in protozoa and mycomycetes. Cell. Biol. Int. Rep. 3: 17-23.

Laemmli, U.K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

Lafarge, C., Frayssinet, C. and Simard, R. (1966). Inhibition preferentielle des syntheses de RNA nucleolaire provoquee par l'aflatoxine dansle cellules Hepatques du Rat. Compt. Rend. Acad. Sci. Paris. 263: 1011-1014.

Lamers, J.M. and Stinis, J.T. (1981). An electrogenic Na^+/Ca^{2+} antiporter in addition to the Ca^{2+}-pump in cardiac sarcolemma. Biochim. Biophys. Acta. 640: 521-534.

Lancaster, C.M., Jenkins, F.B. and Philip, J.M. (1961). Toxicity associated with certain samples of groundnuts. Nature 192: 125-129.

Lancaster, M.C. (1968). Comparative aspects of aflatoxin induced hepatic tumours. Cancer Res. 32: 2288-2292.

Lanzetta, P.A., Alvarey, L.J., Reenach, P.S. and Candia, O.A. (1979). An improved assay for namole amounts of inorganic phosphate. Analytical Biochem. 100: 95-97.

Larsen, F.L. and Vincenzi, F.F. (1979). Calcium transport across the plasma membrane: stimulation by calmodulin. Science Wash (DC) 204: 306-308.

Larsen, F.L., Katz, S., Roufogalis, B.D. (1981). Calmodulin regulation of Ca^{2+}-transport in human erythrocytes. Biochem. J. 200: 185.

Lew, P.D. and Stossel, T.P. (1980). Calcium transport by macrophage plasma membranes. J. Biol. Chem. 255: 5841-5846.

Lichtman, A.H., Segel, G.B. and Lichtman, M.A. (1981). Calcium transport and calcium-ATPase activity in human lymphocyte plasma membrane vesicles. J. Biol. Chem. 256: 6145-6154.

Linden, L.D., Dedman, J.R., Chaufouleas, J.G., Means, A.R. and Roth, T.F. (1981). Interactions of calmodulin and coated vesicles from brain. Proc. Natl. Acad. Sci. (USA). 78: 308-312.

Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974). Cyclic 3'.5'-nucleotide phosphodiesterase: Purification, characterization, and active form of an activator from bovine brain. J. Biol. Chem. 249: 4943-4954.

Locke, F.A. (1894). The role of calcium in the contraction of isolated frog heart muscle.
J. Physiol. 15: 43-52.

Lotersztajn, S., Hanoune, J. and Pecker, F. (1981). A high affinity calcium-stimulated, Mg^{2+}-dependent ATPase in rat liver plasma membranes. Dependence on an endogenous protein activator distinct from calmodulin.
J. Biol. Chem. 257: 11209-11215.

Lowry, O.H., Rosebrough, N.J., Fan, A.L. and Randall, R.J. (1951). Protein measurement with the Folin-phenol reagent.
J. Biol. Chem. 193: 265-275.

Luthra, H.G., Watts, R.P., Scherer, K.L. and Kim, H.D. (1980). Calmodulin. An Activator of human erythrocyte (Ca^{2+} + Mg^{2+}) ATPase phosphorylation.
Biochim. Biophys. Acta. 633: 299-304.

Maclennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985). Amino acid sequence of a Ca^{2+} + Mg^{2+} -dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature Lond. 316: 696-700.

Markwell, M.K., Haasl, S.M., Tolbert, N.E. and Bierber, L.L. (1981). Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol. 72: 296-303.

Marme, D. and Dieter, P. (1983). Role of Ca^{2+} and calmodulin in plants. In: Calcium and cell function, edited by W.Y. Cheung. New York Academic, pp. 264-312.

Mattes, W.B., Hatly, J.A., Kohn, K.W. and Matheson, D.W. (1988). Mechanism of DNA sequence selective alkylation of guanine N^7-positions by nitrogen mustards. Nucleic Acids Res. 45: 10531-10549.

Michalak, M., Famulski, R. and Carafoli, E. (1984). The calcium pumping ATPase in skeletal muscle sarcolemma. Calmodulin dependence, regulation by cAMP dependent phosphorylation, and purification. J. Biol. Chem. 259: 15540-15547.

Michelson, J.R., Beaudry, T.M. and Louis, C.F.(1985). Regulation of skeletal muscle sarcolemmal ATP-dependent calcium transport by calmodulin and cAMP-dependent protein kinase. Arch. Biochem. Biophys. 242: 127-136.

Moore, L., Fitzpatrick, D.F., Chen, T.S. and Landon, E.J. (1974). Calcium pump activity of renal plasma membranes and renal microsomes. Biochim. Biophys. Acta. 345: 405-418.

Morcos, N.C. and Drummond, G.I. (Ca^{2+}+ Mg^{2+})-ATPase in enriched sarcolemma from dog heart. Biochim. Biophys. Acta. 598: 27-39.

Morel, N., Wibo, N. and Godfraind, T. (1981). A calmodulin-stimulated Ca^{2+} pump in rat aorta plasma membranes. Biochim. Biophys. Acta. 644: 82-88.

Morimoto, S., Birge, S.J., Shen, V. and Avioli, L.V. (1985). (Ca^{2+} + Mg^{2+})-ATPase activity in plasma membrane of circulation mononuclear cells. J. Biol. Chem. 260: 14953-14957.

Muallem, S., and Karlish, S.J. (1979). Is the red cell calcium pump regulated by ATP? Nature Lond. 277: 238-240.

_____ (1980). Regulatory interaction between calmodulin and ATP in the red cell Ca^{2+} pump. Biochim. Biophys. Acta 597: 631-636.

_____ (1981). Studies on the mechanism of regulation of the red cell Ca^{2+} pump by calmodulin and ATP. Biochim. Biophys. Acta. 647: 73-86.

Muench, K.F., Mistra, R.P. and Huwayun, Z.H. (1983). Sequence specificity in aflatoxin. DNA Interactions. 80: 6-10.

Nairin, A.C. and Perry, S.V. (1979). Calmodulin and myosin light-chain kinase of fast skeletal muscle. J. Biochem. (Tokyo). 179: 89-87.

Nellans, H.N. and Popovich, J.E. (1981). Calmodulin-regulated, ATP-driven calcium transport by basolateral in membranes of rat small intestine. J. Biol. Chem. 256: 9932-9936.

Nelson, D.R. and Hanahan, D.J. (1985). Phospholipid and detergent effects on (Ca^{2+} + Mg^{2+})-ATPase purified from human erythrocytes. Arch. Biochem. Biophy. 236: 720-730.

Newberne, J.W. and Bailey, W.S. (1955). Notes on recent outbreak and experimental reproduction of hepatitis X in dogs. J. Am. Vet. Med. Assoc. 127: 59-64.

Newbern, J.W. (1965). Carcinogenicity of aflatoxin contaminated peanut meals. In: Wogan G.N. (ed) Mycotoxins in food stuffs pp. 187-208; Cambridge, Mass. Press.

Newton, D.L., Olderwurtel, M.D., Krinks, M.H. Shiloach, J. and Klee, C.B. (1984). Agonist and antagonist properties of calmodulin fragments. J. Biol. Chem. 259: 4419-4426.

Neyes, L., Reinlib, L. and Carafoli, E. (1985). Phosphorylation of the Ca^{2+}-pumping ATPase of heart sarcolemma and erythrocyte plasma membrane by the cAMP-dependent protein kinase. J. Biol. Chem. 260: 10283-10287.

Nguyen, T.D. and Siengenthaler, P.A. (1985). Purification and some properties of a Mg^{2+}-Ca^{2+} and calmodulin-stimulated ATPase from spinach chloroplast envelope membranes. Biochim. Biophys. Acta. 840: 99-106.

Niggli, V., Penniston, J.T. and Carafoli, E. Purified (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane: reconstitution and effect of calmodulin and phospholipids. J. Biol. Chem. 256: 395-401.

Niggli, V., Ronner, P., Carafoli, E. and Penniston, J.T. (1979). Effect of calmodulin on the (Ca^{2+} + Mg^{2+})-ATPase partially purified from erythrocyte membranes. Arch. Biochem. Biophys. 198: 124-130.

Niggli, V., Adunyah, E.S. and Carafoli, E. (1981). Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. J. Biol. Chem. 256: 8588-8592.

Niggli, V., Adunyah, E.S., Penniston, J.T. and Carafoli, E. (1981). Purified (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane:

reconstitution and effect of calmodulin and phospholipids.
J. Biol. Chem. 256: 395-401.

Niggli, V., Sigel, E. and Carafoli, E. (1982). The purified Ca^{2+} pump of human erythrocyte membranes catalyzes and electroneutral Ca^{2+}/H^{+} exchange in reconstituted liposomal systems.
J. Biol. Chem. 257: 2350-2356.

Niggli, V. Adunyah, E.S., Cameron, B.F., Bababunmi, E.A. and Carafoli, E. (1982). The Ca^{2+} pump of sickle cell plasma membranes. Purification and reconstitution of the ATPase enzyme.
Cell Calcium 3: 131-151.

Ochs, D.L. and Reed, P.W. (1983). ATP-dependent calcium transport in plasma membrane vesicles from neutrophil leucocytes.
J. Biol. Chem. 258: 10116-10122.

Ochinnikov, Yu. A., Luneva, N.M., Arystarkhova, E.A. Gerondyan, N.M., Arzamazova, N.M., Kozlich, A.T., Nesmeyanov, V.A. and Modiyanov, N.N.

(1988). Topology of Na^+ K^+ ATPase. Identification of the extra- and intracellular hydrophilic loops of the catalytic subunit by specific antibodies. FEBS Lett. 227: 230-234.

Olorunsogo, O.O., Okudolo, B.E., Lawal, S.O.A. and Falase, A.O. (1985). Erythrocyte membrane Ca^{2+}-pumping ATPase of hypertensive humans: Reduced stimulation by calmodulin. Biosience Reports. 5: 525-531.

Papazian, D.H., Rahamimoff, H. and Goldin, S.M. (1979). Reconstitution and purification by "transport specificity fraction" of an ATP-dependent calcium transport component from synaptosome-derived vesicles. Proc. Natl. Acad. Sci. (USA) 76: 3708-3712.

Papp, B., Sarkacli, B., Enyedi, A., Caride, A.J., Penrsiston, J.T. and Gardos, E. (1989). Functional domains of the in situ red

cell membrane calcium pump revealed by proteolysis and monoclonal antibodies. J. Biol. Chem. 264: 4577-4582.

Patterson, J.S. (1962). Groundnut toxicity as the cause of oxidative hepatitis oedema disease of guinea pigs. Vet. Res. 74: 639-640.

Pearson, R.B., Wettenhall, R.C., Means A.R., Hartshorne, D.J. and Kemp, B.E. (1988). Autoregulation of enzymes by pseudosubstrate prototypes: myosin light chain kinase. Science. 241: 970-973.

Pederson, P.L. and Carafoli, E. (1987a). Ion motive ATPases. Trends. Biochem. Sci. 12: 146-150.

_____ (1987b). Ion motive ATPases. Trends Biochem. Sci. 12: 186-189.

Pershandsingh, H.A., McDaniel, M.L., Landt, M., Bry, C.G., Lacy, P.E. and McDonald, J.M. Ca^{2+} activated ATPase and ATP-dependent

calmodulin-stimulated Ca^{2+}-transport in islet cell plasma membrane. Nature Lond. 288: 492-494.

Pershandsingh, H.A. and McDonald, J.M. Direct addition of insulin inhibits a high affinity Ca^{2+} ATPase in isolated adipocyte plasma membranes. Nature Lond. 281: 495-497.

_____ (1980). A high affinity, calcium-stimulated, Mg^{2+}-dependent adenosine triphosphatase in rat adipocyte plasma membranes. J. Biol. Chem. 255: 4087-4093.

Pontremoli, S., Melloni, E., Sparatore, S., Michetti, M. and Horecker, B.L. (1984). Activation by hemoglobin of the Ca^{2+} requiring neutral proteinase of human erythrocyte: Structural requirements. Biochim. Biophy. Res. Commun. 123: 331-337.

Pontremoli, S. and Melloni, E. (1986). Regulation of Ca^{2+}-dependent proteinase of human erythrocytes. In "Calcium and cell function". Vol.VI, pp. 159-181.

Pokrovsky, A.A., Krowchenko, L.V. and Tutlyan, V.A. (1972). The effect of aflatoxin on rat liver. Toxicol. 10: 25-30.

Popescu, L.M. and Ignat, P. (1983). Calmodulin-dependent Ca^{2+} pump ATPase of human smooth muscle sarcolemma. Cell Calcium. 4: 219-235.

Prentki, M., Wollheim, C.B. and Lew, P.D. (1984). Ca^{2+} homeostasis in permeabilized human neutrophiles. J. Biol. Chem. 259: 13777-13782.

Purchase, I.F.H. (1967). Acute toxicity of aflatoxins M_1 and M_2 in one day o duckling. Food Cosmet. Toxicol. 5, 339-342.

Rabin, B.R., Blyth, C.A., Doherty, D., Freedman, R.B. and Williams, D.J. (1974). The effects of steroid hormones and carcinogens on the interaction of membranes with polysomes. J. Clin. Pathol. 27: 51-59.

Raess, B.U. and Vincenzi, F.F. (1980). A semi-automated method for the determination of multiple membrane ATPase activities. J. Pharm. Methods 4: 273-283.

Rasmussen, G.A. and Goodman, D.B.P. (1977). Relationships between calcium and cyclic nucleotides in cell activation. Physiol. Rev. 57: 421-509.

Rega , A.F. and Garrahan, P.J. (1975). Calcium ion dependent phosphorylation of human erythrocyte membranes. J. Membr. Biol. 22: 313-327.

_____ (1986). The Ca^{2+}-pump of plasma membranes CRC Press Inc. Boca Raton, FL.

Richards, D.E., Rega, A.F. and Garrahan, P.J. (1978). Two classes of site for ATP in the Ca^{2+}-ATPase from human red cell membranes. Biochim. Biophys. Acta 511: 194-201.

Roelofsen, B. and Schatzmann, H.J. (1977). The lipid requirement of the (Ca^{2+} + Mg^{2+})-ATPase in the erythrocyte membrane as studied by various highly purified phospholipases. Biochim. Biophys. Acta 464: 17-36.

Rogers, A.E. and Newberne, P.M. (1967). The effects of aflatoxin B_1 and dimethylsulfoxide on thymidine ^3H uptake and mitosis in rat liver. Cancer Res. 27: 855-864.

Romero, P.J., and Oritz, C.E. (1988). Electrogenic behaviour of the human red cell Ca^{2+} pump revealed by disulfonic stilbenes. J. Membr. Biol. 101: 237-246.

Ronner, P. (1978). Further characterization and partial purification of the (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes. Ph.D. Thesis ETH, Zurich.

Ronner, P. , Gazzotti, P. and Carafoli, E. (1977). A lipid requirement for the (Ca^{2+} + Mg^{2+})-ATPase of erythrocyte membranes. Arch. Biochem. Biophys. 179: 578-383.

Roufogalis, B.D. (1979). Regulation of calcium translocation across the red blood cell membrane. Can. J. Phys. Pharmacol. 57: 1331-1333.

Sarkadi, B., Enyedi, A. and Gardos, G. (1980). Molecular properties of the red cell calcium pump. I. Effect of calmodulin, proteolytic digestion, and drugs on the kinetics of active calcium uptake in inside/out red cell membrane vesicles. Cell Calcium. 1: 287-298.

Sarkadi, B. and Tostenson, D.C. (1979). In: Transport across biological membranes (Giebisch, G., Tostenson, D.C. and

using NH eds.) Vol.2 pp. 117, Springer, Verlag Berlin, West Germany.

Scharff, O. (1973). The influence of Ca^{2+} ions on the preparation of the (Ca^{2+} + Mg^{2+})-activated membrane ATPase in human red cell. Scand. J. Chim. Lab. Invest. 39: 313-320.

Scharff, O. (1978). Stimulating effects of monovalent cations on activator dissociated and activator - associated states of Ca^{2+}-ATPase in human erythrocytes. Biochim. Biophys. Acta. 512: 309-317.

_____ (1981). Kinetics of calcium-dependent membrane ATPase in human erythrocytes. In Lassen HH, Using HH, Wieth J.O. (eds): "Membrane Transport in Erythrocytes", Alfred Benzon Symposium 14, Copenhagen: Munksgaard, p. 236.

Scharff, O. and Foder, B. (1977). Reversible shift between two states of Ca^{2+} ATPase in human erythrocytes mediated by Ca^{2+} and a membrane-bound activator. Biochim. Biophys. Acta. 50: 67-77.

Schatzmann, H.J. (1966). ATP-dependent Ca^{2+} extrusion from human red cells. Experientia Basel 22: 364-368.

_____ (1982). The calcium pump of erythrocytes and other animal cells In: Membrane Transport of calcium, edited by Carafoli, E. (Lon). Academic pp. 41-108.

Schatzmann, H.J. and Vincenzi, F.F. (1969). Calcium movements across the membrane of human red cells. J. Phys. 201: 369.

Schatzmann, H.J. and Burgin, H. (1978). Calcium in human blood red cells. Ann. N.Y. Acad. Sci. 307: 125-147.

Schneider, C., Mottola, C., and Romed, D. (1979). Phosphorylation intermediate in the Ca^{2+}-dependent ATPase reaction of macrophage plasma membrane. J. Supramol. Struct. 10: 433-441.

Schoental, R. (1970). Hepatotoxic activity of retrovirus, senkirkine, and hydroxy senkirkine in new born rats and the role of epoxides in carcinogenicity by pyrolizidine alkanoids of aflatoxin. Nature (Lon.) 227: 401-402.

Scully, S.P., Segel, G.B. and Lichtmann, M.A. (1982). Plasma membrane vesicles prepared from unadhered monocytes: Characterization of calcium transport and the calcium ATPase. Cell Calcium 3: 515-530.

Sergeant, K., Sheridan, A. and O'Kelly, J. (1961). Toxicity associated with certain samples of groundnuts. Nature (Lond.) 192: 1096-1097.

Shen, V., Kohler, G. and Peck, W.A. (1983). High affinity, calmodulin-responsive (Ca^{2+} + Mg^{2+})-ATPase in isolated bone cells. Biochim. Biophys. Acta. 727: 230-238.

Shull, G.E. and Greeb, J. (1988). Molecular cloning of a two isoforms of the plasma membrane Ca^{2+} transporting

ATPase from rat brain. Structural and functional domains exhibit similarity to Na^+ K^+-and other cation transport ATPases.
J. Biol. Chem. 263: 8647-8657.

Sinnhusber, R.O. and Wales, J.H. (1965). Aflatoxin in cotton seed meal and hepatoma in rainbow trout.
Fed. Proc. 24: 627.

Smallwood, J.I., Waisman, D.M., Lafreiniere, D. and Rasmussen, H. (1983). Evidence that the erythrocyte calcium pump catalyzes a Ca^{2+}; H^+ exchange.
J. Biol. Chem. 258: 11092-11097.

Smallwood, J.I., Gugi, B. and Rasmussen, H. (1988). Modulation of erythrocyte Ca^{2+} pump activity by protein kinase C.
J. Biol. Chem. 263: 2195-2202.

Smallwood, J.I. and Rasmussen, H. (1988). Regulation of erythrocyte Ca^{2+} pump activity by protein kinase C.
J. Biol. Chem. 263: 2195-2202.

Sorensen, R.G. and Mahler, H.R. (1981). Calcium-stimulated adenosine triphosphatases in synaptic membranes. J. Neuro. Chem. 37: 1407-1418.

Spensley, P.C. (1963). Aflatoxin, the active principle in Turkey 'X' disease. Endeavour 22: 75-79.

Spitzer, B., Bohmer, F.D. and Grosse, R. (1983). Identification of Ca^{2+}-pump related phosphoprotein in plasma membrane vesicles of Ehrlich ascites carcinoma cells. Biochim. Biophys. Acta. 728: 50-58.

Sporn, M.B., Dingman, H.L. and Wogan, G.N. (1966). Aflatoxin B_1: Binding to DNA in vitro and alternative of RNA metabolism in vivo. Science 151: 1539-1541.

Stieger, J., and Schatzmann, H.J. (1981). Metal requirement of isolated red cell Ca^{2+}-pump ATPase after elimination of calmodulin dependence by trypsin attack. Cell Calcium 2: 601-616.

Stoloff, L. (1967). Collaborative study of a method for the identification of aflatoxin B_1 by derivative formation. J. Ass. Off. Analyt. Chem. 50: 354-360.

Strehler, (1991). Recent advances in the molecular characterization of plasma membrane Ca^{2+} pumps. J. Mem. Biol. 120: 1-15.

Strehler, E.E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A., Penniston, J.T. and Carafoli, E. (1989). Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocyte membrane. J. Biol. Chem. 265: 2835-2842.

Strehler, E.E., Strehler-page, M.A., Vogel, G., and Carafoli, E. (1989). mRNAs for plasma membrane calcium pump isoforms differing in their regulatory domain are generated by alternative splicing involving two internal donor sites in a single exon. Proc. Natl. Acad. Sci. USA 86: 6908-6912.

Sulakhe, P.V., Drummond, G.I. and NG, D.C. (1973). Adenosine triphosphatase activities of muscle sarcolemma. J. Biol. Chem. 248: 4158-4162.

Suzuki, K. (1987). Calcium activated neutral protease: domain structure and activity regulation. TIBS. 12: 103-105.

Svododa, D. and Soga, J. (1966). Early effects of pyrrolizidine alkaloids on the fine structure of rat liver cells. Am. J. Pathol. 48: 347-373.

Swenson, D.H., Miller, J.A. and Miller, E.C. (1975). The reactivity and carcinogenecity of aflatoxin B_1 2,3 dichloride a model for the putative 2,3 oxide metabolite of aflatoxin B_1. Cancer Res. 35: 3811-3823.

Szasz, I., Hasitz, M., Sarkadi, B. and Gardos, G. (1978). Phosphorylation of the calcium pump intermediate in intact red cells,

Isolated membranes, and inside-out vesicles.
Mol. Cell. Biochem. 22: 147-152.

Tash, J.S. and Mann, A.R. (1973). Adenosine 3'5-cyclic monophosphate in relation to motility and senescence of spermatozoa. Proc. Roy. Soc. (Lon.) Ser. B. 184: 109-114.

Taylor, W.R. and Thornton, J.M. (1984). Recognition of super-secondary structure in proteins. J. Mol. Biol. 173: 487-512.

Taverna, R.D. and Hanahan, D.H. (1980). Modulation of human erythrocyte Ca^{2+}/Mg^{2+} ATPase activity by phospholipase A_2 and proteases. A comparison with calmodulin. Biochim. Biophys. Res. Commun. 94: 652-659.

Trager, W. and Stoloff, L. (1966). A study of possible reactions for aflatoxin detoxification. J. Ass. Off. Analyt. Chem. 49: 1268

Tuana, B.S., Dzurba, A., Panagla, V. and Dhalla, N.S. (1981). Stimulation of heart sarcolemmal calcium pumps by calmodulin. Biochem. Biophs. Res. Commun. 100: 1245-1250.

Tulpule, P.G., Madhavan, T.W. and Gopalahan, C. (1964) Effect of feeding aflatoxin in young monkeys. Lancet 1: 962-963.

Uwaifo, A.O. and Bassir, O. (1977). The effect of functional groups on the interaction of aflatoxin B_1 and G_1 with starch, cellulose, derivatives. Biochem. Pharmacol. 26: 863-866.

Vancorven, E.J., Roche, J.M. and Van Os, C.H. (1985). Distribution of Ca^{2+} ATPase, ATP-dependent Ca^{2+} transport calmodulin and vitamin D-dependent Ca^{2+} binding protein along the villus/crypt axis in rat Biochim. Biophys. Acta. 320: 274-282.

Vander Merwe, K.J., Fourie, L. and Scott, B. (1963). The structure of the aflatoxin. Chem. Ind. 41: 1660-1661.

Van der Zizden, A.S.M. and Barret, C.B. (1962). Isolation in crystalline form of a toxin responsible for Turkey 'X' disease Nature (Lond) 195: 1060-1062

Vasington, F.D. and Murphy, J.V. (1961). Ca^{2+} ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. J. Biol. Chem. 237: 2670-2677.

Verbist, J., Theodorus, W.J., Gadella, Jr., Raeymaekers, L., Wujtack, F., Karel, W.A. and Casteels, R. (1991). Phospho-inositid protein interactions of the plasma-membrane Ca^{2+}-transport ATPase as revealed by fluoresence energy transfer. Biochimica et Biophysica Acta 1063:

Verma, A.K., Filoteo, A.G., Standford, D.R., Wieben, E.D., Penniston, J.T., Strehler,

E.E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler-page, M.A., James, P., Vorherr, T., Krebs, J. and Carafoli, E. (1988). Complete primary structure of a human plasma membrane Ca^{2+} pump. J. Biol. Chem. 263: 14152-14159.

Verret, M., Marliac, J. and Maclaughlun (1964). Use of the chicken embryo in the assay of aflatoxin toxicity. J. Ass. Office Final. Chem. 47: 1003-1006.

Villalobo, A. and Roufogalis, B.D. (1986). Proton counter transport by the reconstituted erythrocyte Ca^{2+}-translocating ATPase: Evidence using ionophoretic compounds. J. Membr. Biol. 93: 249-258.

Villalobo, A., Brown, L. and Roufogalis, B.D. (1986). Kinetic properties of the purified Ca^{2+}-translocating ATPase from human erythrocyte plasma membrane. Biochim. Biophy. Acta. 854: 9-20.

Volpi, M., Naccahe, P.H. and Sha'afi, R.I. (1983). Calcium transport in inside-out membrane

vesicles prepared from rabbit neutrophills.
J. Biol. Chem. 258: 4153-4158.

Vorherr, T., Kessler, T., Hofmann, F. and Carafoli E. (1991). The calmodulin-binding domain mediates the self association of the plasma membrane Ca^{2+} pump.
J. Biol. Chem. 266: 22-27.

Waisman, D.M., Gimble, J.M., Goodman, D.,B.P. and Rasmussen, H. (1988). Studies of the Ca^{2+} transport mechanism of human erythrocyte inside-out vesicles. II. Stimulation of the Ca^{2+} pump by phosphate.
J. Biol. Chem. 256: 415-419.

Walsh, M.P., Cavadore, J.C., Vallet, B. and Denaill J.G. (1980). Calmodulin-dependent myosi light-chain kinase from cardiac and smooth muscle. A comparative study.
Can. J. Biochem. 58: 299-308.

Wang, J.H. and Desai, R. (1976). Modulator bindin protein. Bovine brain protein exhibitin

vesicles prepared from rabbit neutrophills.
J. Biol. Chem. 258: 4153-4158.

Vorherr, T., Kessler, T., Hofmann, F. and Carafoli, E. (1991). The calmodulin-binding domain mediates the self association of the plasma membrane Ca^{2+} pump.
J. Biol. Chem. 266: 22-27.

Waisman, D.M., Gimble, J.M., Goodman, D.,B.P. and Rasmussen, H. (1988). Studies of the Ca^{2+} transport mechanism of human erythrocyte inside-out vesicles. II. Stimulation of the Ca^{2+} pump by phosphate.
J. Biol. Chem. 256: 415-419.

Walsh, M.P., Cavadore, J.C., Vallet, B. and Denaille, J.G. (1980). Calmodulin-dependent myosin light-chain kinase from cardiac and smooth muscle. A comparative study.
Can. J. Biochem. 58: 299-308.

Wang, J.H. and Desai, R. (1976). Modulator binding protein. Bovine brain protein exhibiting

the Ca^{2+}-dependent association with the protein modulator of cyclic nucleotide phosphodiesterase. J. Biol. Chem. 252: 4175-4184.

Wang, K.K.W., Villalobo, A. and Roufogalis, B.D. (1988). Activation of the Ca^{2+} ATPase of human erythrocyte membrane by an endogenous Ca^{2+}-dependent neutral protease. Arch. Biochem. Biophys. 260: 696-704.

Wang, K.K.W., Roufogalis, B.D. and Villalobo, A. (1989). Calpain 1 activates Ca^{2+} transport by the reconstituted erythrocyte Ca^{2+} pump. J. Membr. Biol. 112: 233-245.

Wang, K.K.W., Wrught, L.C., Machan, L.L., Allen, B.G., Conigrave, A.D. and Roufogalis (1991). Protein kinase C phosphorylates the carboxyl-terminus of the plasma membrane Ca^{2+} ATPase from human erythrocytes. J. Biol. Chem. 266: 9078-9085.

Wannop, C.G. (1964). The histopathology of turkey 'X' disease in Great Britain. Avian Dis. 5: 37-38.

Weber, K. and Osborn, M. (1969). The reliability of molecular weight determinations by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

Welsh, M.J., Dodman, J.R., Brinkley, B.R. and Means, A.R. (1978). Calcium-dependent regulator protein. Localization in mitotic apparatus of eukaryotic cells. Proc. Natl. Acad. Sci. (USA) 75: 1867-1871.

_____ (1979). Tubulin and calmodulin. Effects of microtubule and microfilaments inhibitors on localization in the mitotic apparators. J. Cell. Biol. 81: 624-634.

Wetzker, R., Klinger, R. and Frunder, H. (1983). Effects of fatty acids on activity and calmodulin binding Ca^{2+} ATPase of human

erythrocyte membranes.
Biochim. Biophys. Acta. 730: 196-200.

Wogan, G.N. (1966). Chemical nature and biological effects of the aflatoxins.
Bateriol. Rev. 30: 460-470.

_____ (1968). Biochemical responses to aflatoxins.
Cancer Res. 28: 2282-2287.

_____ (1977). Mycotoxins and other naturally occuring carcinogens.
Adv. Mod. Toxicol. 3: 263-290.

Wogan, G.N. and Newberne, P.M. (1967). Dose response characteristics of aflatoxin B_1 carcinogenesis in the rat.
Cancer Res. 27: 2370-2376.

Wolff, S.P., Cook, G.H., Goldhammer, H.R. and Berkowtz, S.A. (1980). Calmodulin activates prokaryotic adenylate cyclase.
Proc. Natl. Acad. Sci. USA 77: 3841-3840.

Wong, P.Y.K. and Cheung, W.Y: (1979). Calmodulin stimulates human platele phospholipase A_2.
Biochem. Biophys. Res. Commun. 90: 473-480.

Wrzosek, A., Famulski, K.S., Lehotsky, J. and Pikula, S. (1989). Conformational charges of (Ca^{2+} + Mg^{2+})-ATPase of erythrocyte plasma membrane caused by calmodulin and phosphatidyl serine as revealed by circular dichroism and fluorescence studies. Biochim. Biophys. Acta 986: 263-270.

Wuytack, E., Deschutter, G. and Casteels, R. (1980). The effect of calmodulin on active calcium ion transport and (Ca^{2+} + Mg^{2+})-dependent ATPase in microsomal fractions. Biochem. J. 190: 827-831.

_____ (1985). Partial purification of (Ca^{2+} + Mg^{2+})-dependent ATPase from pig smooth muscle and reconstitution of an ATP-dependent Ca^{2+}-transport system. Biochim. J. 198: 265-271.

Yazawa, M., Sakuma, N. and Yagi, K. (1980). Calmodulin from muscle of marine invertebrates, scallop and sea anemone. Comparison with calmodulins from rabbit skeletal muscle

and pig brain.
J. Biochem. (Tokyo) 87: 1313-1320.

Yu, F.L. (1977). Selective inhibition of rat hepatic nuclear RNA polymerase II by actinomycin D. In vivo.
Fed. Proc. Fedn. Amer. Socs. Exp. Biol. 36: 807-810.

Zurini, M., Krebs, J., Penniston, J.T. and Carafoli, E (1984). Controlled proteolysis of the Ca^{2+} ATPase of the erythrocyte membrane. A correlation between the structure and the function of the enzyme.
J. Biol. Chem. 259: 618-627.

Zvavitch, E., James, P., Vorherr, T., Falchetto, R., Moynavov, N. and Carafoli, E. (1990). Mapping of functional domains in the plasma membrane Ca^{2+} pump using trypsin proteolysis.
Biochem. 29: 8070-8076.

Appendix 1

Codes and their meanings for the structure of Ca^{2+}-ATPase

ALA	A
ARG	R
ASN	N
ASP	D
ASX	B
CYS	C
GLN	Q
GLU	E
GlX	Z
GLY	G
HIS	H
ISO	I
LEU	L
LYS	K
MET	M
PHE	F
PRO	P
SER	S
THR	T
TRP	W
TYR	Y
VAL	V

ADDITIONAL REFERENCES

Anderson, J.P. (1989). Ca^{2+}-pump mediates self dimerasation.
Biochim. Biophys. Acta. 988: 47-72

Bensadoun, A. and Wunstein, D. (1976). Assay of proteins in the presence of interfering materials.
Annal. Biochem. 70: 241-250.

Bond, G.H. and Clough, D.A. (1973). A Soluble protein activator of (Mg^{2+} + Ca^{2+})-dependent ATPase in human red cell membranes.
Biochim. Biophys. Acta. 288: 423

Butter, W.H. (1965). Liver injury and aflatoxin.
In: G.N. Wogan (ed). Mycotoxins in Foodstuffs pp. 175-186 Cambridge, Mass, M.I.T. Press.

Carafoli, E., Fischer, R., James, P., Crebs, J., Maeda, M. Enyedi, A., Morelli, A. and De Flora, A. (1987). In calcium binding proteins in health and disease.
(Norman, A.W., Vanaman, T.C. and Means, A.R. eds). pp. 78-91. Academic Press, San Diego, CA.

Carafoli, E. (1991). Calcium pump of the plasma membrane.
Physiological Reviews. 72: 129-153.

Epel, D.E., Wallace, R.W. and Cheung, W.Y. (1981). Calmodulin activates NAD-Kinase of Sea Urchin eggs: an early event of fertilization.
Cell 23, 543-549.

Graffi, A. and Krebsforch, Z (1940). Differences of mitochondrial protein synthesis in vitro between tumor and normal tissues.
Biochem. Biophys. Res. Commun. 21: 418-423.

Ghijsen, W.E.J.M. and Van Os, C.H. (1979). Ca^{2+}-stimulated ATPase in brush border and basolateral membranes of rat duodenum with high affinity sites for Ca ions.
Nature Lond 279: 802-804.

Gopinath, R.M. and Vincenzi, F.F. (1977). Phosphodiesterase protein activator mimics red blood cell cytoplasmatic activator of the (Ca^{2+} + Mg^{2+})-ATPase.
Biochem. Biophys. Res. Commun. 72: 1203-1209.

Graf, E. and Penniston, J.T. (1981). Ca^{2+}-ATP: The substrate, at low ATP concentrations, of Ca^{2+}-ATPase from human erythrocyte membranes. J. Biol. Chem. 256: 1587.

James, P., Zvaritch, E., Hakhparanov, M.S., Penniston, J.T. and Carafoli, E. (1987). The amino acid sequence of the phosphorylation domain of the erythrocyte Ca^{2+}-ATPase. Biochem. Biophys. Res. Commun. 149: 7-12.

James, P., Maeda, M., Fischer, R., Verma, A.K., Krebs, J. and Carafoli, E. (1988). Identification and primary structure of a calmodulin binding domain of the Ca^{2+}-pump of human erythrocytes. J. Biol. Chem. 263: 2905-2910.

James, S.P., Vorherr, T., Krebs, J., Morell, A., Castello, G., McCormick, D.J., Penniston, J.T., De-Flora, A. and Carafoli, F. (1989). Modulation of erythrocyte Ca^{2+}-ATPase by selective calpain cleavage of the calmodulin binding domain. J. Biol. Chem. 264: 8289-8296.

James, P.H., Pruschy, M., Vorherr, T., Penniston, J.T. and Carafoli, E. (1989). Primary structure of the cAMP-dependent phosphorylation site of the plasma membrane calcium pump. Biochemistry 28: 4253-4258.

Jamienson, G.A. (Jr). Vanaman, T.C. and Blum, J.T. (1979). Presence of calmodulin in Tetrahymena. Proc. Natl. Acad. Sci. (USA) 76: 6471-6475.

Jarrett, H.W. and Penniston, J.T. (1977). Partial purification of the (Ca^{2+} + Mg^{2+})-ATPase activator from human erythrocytes: its similarity to the activator of 3'-5'-cyclic nucleotide phosphodiesterase. Biochem. Biophys. Res. Commun. 77: 1210-1216.

Jarret, H.W., and Penniston, J.T. (1978). Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes: its membership to the class of Ca^{2+}-binding modulator proteins. J. Biol. Chem. 253: 4676-4682.

Jeffery, B., Roufogalis, B.D. and Katz, S. (1981). The effect of calmodulin on the phosphoprotein intermediate of Mg^{2+}-dependent, Ca^{2+}-stimulated adenosine triphosphatase in human erythrocyte membranes. Biochem. J. 194: 481-486.

Jones, L.R., Besch, H.R. (Jr.), Fleming, J.W., Mc Connaughey, M.M. and Watanabe, A.M. (1979). Separation of vesicles of Cardiac Sarcolemma from vesicles of Cardiac Sarcoplasmic reticulum. J. Biol. Chem. 254: 530-539.

Kosk-Kosicka, D. and Bzdega, T. (1988). Activation of the erythrocyte Ca^{2+}-ATPase by either self-association or interaction with calmodulin. J. Biol. Chem. 263: 18184-18189.

Lang, D.A., Wickenden, C., Wynne, J. and Lucy, J.A. (1984). Proteolysis of ancyrin and of band 3 protein in chemically induced cell fusion. Biochem. J. 218: 295-305.

Larsen, F.L., Katz, S., Roufogalis, B.D., Brooks, E.E. (1981). Physiological shear stresses enhance the Ca^{2+}-permeability of human erythrocytes. Nature. 294: 667-668.

Mann, D.M., Brandt, P., Sisken, B. and Vannaman, T. (1990). Adv. Exp. Med. Biol. 255: 71-78.

Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. and Horecker, B.L. (1986). The involvement of calmpain in the activation of protein kinase C in Neutrophilis stimulated by phorbol myristic acid. J. Biol. Chem. 261: 4101-4105.

Michaelis, M.L., Kitos, T.E., Nunley, E.W., Lecluyse, Ed and Michaeli's, E.K. (1987). Characteristics of Mg^{2+}-dependent, ATP-activated Ca^{2+}-transport in synaptic and microsomal membranes and in permeabilized synaptosomes. J. Biol. Chem. 262: 4182-4189.

Reuter, H., and Seitz, N. (1968). The dependence of Ca^{2+} efflux from cardiac muscle on temperature and external ion composition. J. Physiol. Lond. 195: 451-470.

Richards, D.E., Vidal, J.C., Garrahan, P.J., and Rega, A.F. (1977). ATPase and phosphatase activities from human red cell membranes: II. The effects of phospholipases on Ca^{2+}-dependent enzymic activities. J. Membr. Biol. 35: 125.

Ringer, S. (1882). Regarding the action of hydrate of Soda, hydrate of ammonia, and hydrate of potash on the ventricles of the frogss heart. J. Physiol. Lond. 3: 195-202.

_____ (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. Lond. 4: 29-42.

Sarkadi, B., Enyedi, A., Myers, A.,and Gardos, G.(1982). The function and regulation of the calcium pump in the erythrocyte membrane. Ann. New York Acad. Sci. 402: 329-335.

Serrano, R. (1988). The Ca^{2+}-pump isoforms. Biochim. Biophys. Acta 947: 1-28.

Scharff, O. and Foder, B. (1975). Reversible shift between two states of Ca^{2+}-ATPase in human erythrocytes mediated by Ca^{2+} and a membrane bound activator. Biochim. Biophys Acta 509: 67

Schatzmann, H.J. (1973). Dependence on calcium concentration and stoichcometry of the calcium pump in human red cells. J. Physiol. 235: 551.

Silver, S., Nucifora, G., Chu, L. and Mistra, T.K. (1989). Comparative studies of the isoform of Ca^{2+}-pump. TIBS 14: 76-80.

Strehler, E.E., James, P., Fisher, R., Heim, R., Vorher, T., Filoteo, A.G., Penniston, J.T. and Carafoli, E. (1990). Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocytemembrane. J. Biol. Chem. 265: 2835-2842.

Uwaifo, A.O. and Bassir, O. (1978). Toxic effects of aflatoxin B_1 on Bacillus brevis. Biochem. and Expert. Biol. XIV: 4

Vorherr, T., James, P., Krebs, J., Enyed, A., McCormick, D.J., Penniston, J.T. and Carafoli, E. (1990). Interaction of calmodulin with the calmodulin binding domain of the plasma membrane Ca^{2+}-pump. Biochemistry 29: 355-365.

Waisman, R.T., Walters, R.F. and Weiser, M. (1988). Calcium transport by rat duodenal villus and cryot basolateral membranes. Am. J. Physiol. 252 (Gastrointest. Liver Physiol. 15): G170-G177.

Wang, K.K.W., Villalobo, A. and Roufocalis, B.D. (1989). Calmodulin-binding proteins as calpain substrates. Biochem. J. 262: 693-706

Watterson, M.D., Sharief, F. and Vanaman, T.C. (1980). The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. J. Biol. Chem. 255: 962-971.

Wuytack, F., Deschutter, G. and Casteels
Partial purification of (Ca^{2+} + Mg^{2+})-dependent ATPase from pig smooth muscle and reconstitution of an ATP-dependent Ca^{2+}-transport system.
Biochem. J. 198: 265-271.