EFFECTS OF THE LEAF DECOCTION OF *MOMORDICA CHARANTIA* (BITTER LEMON) ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE AND FERTILITY IN MALE ALBINO RATS

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ABSTRACT

Certain anti-cancer agents act by inducing the intrinsic pathway of apoptosis which is mediated by the release of cytochrome C when the Mitochondrial Membrane Permeability Transition (MMPT) pore opens. Several studies have authenticated the use of *Momordica charantia (M. charantia)* in the treatment of diabetes, breast cancer, skin tumor and prostatic cancer. This study was therefore carried out to determine whether the decoction of *M. charantia* will induce the opening of MMPT pore and the consequence of this on other parameters such as liver function and male fertility.

Different doses, 35, 45, 55 and 65mg/100g body weight of the aqueous decoction of the fresh leaves of *M. charantia* were orally administered to groups A,B,C and D of animals respectively, for 30 days. The control group was fed with only water and feed *ad libitum*. Opening of MMPT pore was assayed in Mannitol-Sucrose-HEPES (MSH) buffer (210mM Mannitol, 7mM Sucrose and 5mM HEPES). Three hundred micromolar $CaCl_2$/mg mitochondrial protein was used in inducing opening of MMPTP and changes in volume of liver mitochondria were measured quantitatively at 540nm in a Beckman UV spectrophotometer. Hepatic injury was assessed histologically and by the levels of Alanine Transaminase (ALT), Aspartate Transaminase (AST), γ-Glutamyl Transferase (GGT) and Alkaline Phosphatase (ALP) in serum. Epididymal sperm samples obtained from the animals were analyzed for motility, viability, sperm counts and morphology. Results were statistically analyzed using student's t-test and ANOVA at 0.05 level of significance.

There were significant increases in the extent to which the different doses of the decoction induced opening of the MMPT pore. Maximum induction ($-\Delta_{A540}$ -0.309) was obtained at 55mg/100g bw, which translates to about 11-fold increase when compared with the control value

($-\Delta_{A530}$ 0.029) though, the extent of por/e induction decreased to about 9-fold ($-\Delta_{A530}$ -0.249) at 65mg/100g bw. Significant reduction in sperm motility relative to control was observed for all treated animals. Sperm counts were also significantly reduced in this order: Group D< Group C< Group B< Group A< control with values ranging from 82.00±2.45x10^{6} cells/ml (control) to 6.25±8.26x10^{6} cells /ml (Group D). Likewise, significant reductions of 86.25±4.79%, 91.67±2.89% and 88.75±2.50% in percentage viability were observed in animals that received 45, 55 and 65mg/100g bw of decoction respectively, compared with control (96.5±1.73%). Morphological abnormalities of sperm above the proposed 10% (Group B=12.94%, Group C=13.84% and Group D = 13.02%) were also observed in animals that received 45mg/100g bw and above. There were significant dose-dependent increases in ALP and GGT levels for all groups relative to the control.

Dose-related toxic effects of orally administered leaf decoction of *M. charantia* was observed in albino rats and may be implicated in male infertility in individuals who rely on the decoction in treating various ailments.

KEY WORDS: *Momordica charantia*, Decoction, Mitochondrial Membrane Permeability Transition Pore, Liver Function, Male Fertility.

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My love goes to my dear and understanding Mummy- Mrs Dora Ehigie, You'll by His grace live long and see your desire come through for us in Jesus' name. Amen.

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Finally, it is of the Lord's mercies that I am not consumed, because His compassions fail not. They are new every morning, great is Thy faithfulness. AMEN.

CERTIFICATION

I certify that this work was carried out by ODEWUSI ADEOLA FOLASADE in the Department of Biochemistry, University of Ibadan, Nigeria.

24-6-11

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DEDICATION

I dedicate this piece to God Almighty, my refuge and strength, a very present help in trouble. He, who remembered me in my low estate and brought me up also out of a horrible pit, out of the miry clay, and set my feet upon the rock, and established my goings. He hath put a new song in my mouth, even praise unto our God; many shall see it and fear, and shall trust in the Lord.

The Lord has been mindful of us, He will bless us; He will bless the house of Israel; He will bless the house of Aaron. He will bless them that fear Him both small and great. I will say of the Lord, He is my refuge and my fortress, my God in Him will I trust.

Also I dedicate this work to my grandparents; Late Pa Oyebambi Ajala (To your sweet memories Baba Ladeoyo. It still hurts that you did not wait to see the completion of this work), and Ma Oyebola Oyedijo and also to my parents, Elder F.O. Odewusi, Mrs M.O. Odewusi and Mrs Dora Ehigie.

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CHAPTER ONE

1.1 INTRODUCTION

The mitochondrion is a central organelle which plays a prominent role in cell death because it is crucial for signal transduction and amplification of the apoptotic response (Green and Reed, 1998; Johnson and Boise, 1999). It is now well established that certain anti-cancer agents induce the intrinsic pathway of apoptosis which may be mediated by the release of cytochrome C when the mitochondrial membrane permeability transition (MMPT) pore is open (Schmitt and Lowe, 1999)

This pore is thought to be formed through a Ca^{2+} - triggered conformational change of the adenine nucleotidetranslocase (ANT) bound to matrix cyclophilin-D (CYP-D), a peptidyl –prolyl cis.trans isomerase (PPIase), unique to the mitochondria and so named because of its cyclosporine A (CSA)-binding properties (Schmitt and Lowe, 1999; Halestrap and Davidson, 1990). CYP-D catalyses the interconversion between cis and trans-conformation of peptide bonds adjacent to proline residues and as such, it is ideally suited for causing the conformational change in a membrane protein that would be required to induce formation of a pore. This membrane protein has been identified to be adenine nucleotide translocase (ANT), which transports ADP and ATP across the inner mitochondrial membrane (Halestrap, and Davidson, 1990).

It has been confirmed that the conformational state of the ANT greatly influenced the sensitivity of the MPTP to $[Ca^{2+}]$ which led to the proposal that in the presence of calcium, a cyclophilin D-mediated conformational change of the ANT was responsible for the formation of MMPT pore(Halestrap and Davidson, 1990; Halestrap, *et al.*, 2000).

It has been established that once released, cytochrome C binds to apoptotic protease activator factor-1 (APAF-1) in the presence of ATP or dATP and forms a complex that

processes and activates pro-caspase-3 and -7 (Salch, *et al.*,1999). The release of cytochrome C has been linked to a loss of mitochondrial membrane potential ($\Delta\varphi m$) and increase in permeability transition (Petit, *et al.*,1997; Shimizu, *et al.*, 1999; Marzo, *et al.*, 1998). The consistent observation of mitochondrial dysfunction prior to the nuclear changes associated with apoptotic cell death implies that it may be a critical regulator of the metabolic events involved in the apoptotic cascade (Deckwerth and Johnson, 1993; Jacobson, *et al.*; Newmeyer, *et al.*, 1994).

Furthermore, in cell-free systems, mitochondria are a necessary component of the cytosolic fraction to produce apoptotic features in isolated nucleic (Newmeyer, *et al.*, 1994)). Therefore, the opening of the pore is *sine qua non* to the initiation of cell death and a putative target for the design of novel strategies for blocking pathological cell loss or for killing unwanted cells.

It is now abundantly clear that a number of phytochemicals can act as chemopreventives in the development of cancers and the diseases arising from dysregulated apoptosis (Martin, 2006). Bitter melon or *Momordica charantia* (*M. charantia*) of the family Cucurbitaceae is a medicinal plant known as "Ejinrin wewe" by the Yorubas of South Western Nigeria. Although its country of origin is uncertain, the plant is commonly cultivated for its fruit in tropical regions of India, China, East Africa and Central and South America. It is herbaceous, climbing or having prostate vines with simple or forked tendrils (Garau, *et al.*, 2003; Rivera, 1941). It has lobed leaves, yellow flowers and edible and bitter-tasting orange - yellow fruits. The unripe fruit is green and is cucumber -shaped with a bumpy looking surface. The parts of the plant used for medicinal purpose include the fruits, leaves, seeds, whole plant and seed oil. (Anon, 1999; Cunnick and Takemoto, 1993).

Interestingly, the plant has a long history of use by the indigenous people of the Amazon who grow it in their gardens for food and medicine. They add the fruit and/or leaves to

beans and soup for a bitter or sour flavour. A leaf tea is used for diabetes, to expel intestinal gas, and as an antiviral for measles, hepatitis and feverish conditions. *M. charantia* is used topically for sores, wounds, and infections and either internally or externally for worms and parasites (Tropical plant database, 2007). Bitter melon has also been used as both a food and medicine throughout Asia as a therapeutic remedy in a variety of illnesses such as leukemia, diabetes, asthma, insect bites, menstrual cycle problems, stomach problems, as well as many other maladies. In Brazilian herbal medicine, bitter melon is used for tumors, wounds, rheumatism, malaria, vaginal discharge, inflammation, menstrual problems, diabetes, colic, fevers, and worms. It is also used to induce abortions and as an aphrodisiac (Tropical plant database, 2007). It is prepared into a topical remedy for the skin to treat vaginitis, hemorrhoids, scabies, itchy rashes, eczema, leprosy and other skin problems.

In Mexico, the entire plant is used for diabetes and dysentery, the root is a reputed aphrodisiac. In Peruvian herbal medicine, the leaf or aerial parts of the plant are used to treat measles, malaria, and all types of inflammation. In Nicaragua, the leaf is commonly used for stomach pain, diabetes, fevers, colds, coughs, headaches, malaria, skin complaints, menstrual disorders, aches and pains, hypertension, infections, and as an aid in childbirth (Tropical plant database, 2007). In Nigeria, the aqueous extracts of the leaves and fruits are used for similar ailments listed above (Sofowora, 1984). The popularity of *Momordica charantia* in traditional medicine suggests that it contains bioactive agents that will be potentially useful in drug development. Over 100 studies using modern techniques have authenticated its use in diabetes and its complications. Most importantly, some of these studies have shown its efficacy in various cancers such as lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of tongue and larynx, human bladder carcinomas and Hodgkin's disease (Grover and Yadav 2004).Previous reports (Singh, *et al.*, 1989;

Srivastava, *et al* 1993; Ng, *et al*., 1994; Patel and Srinivasan, 1997; Naseem, *et al*., 1998), have shown the effects of various parts of the plant (seed, fruit and even the whole plant) in different extraction media such as benzene, alcohol, petroleum ether, acetone, water and as crude extracts and juices in the treatment of many ailments. However, there is paucity of information on the biochemical basis of some of the pharmacological effects of the bioactive components of *M.charantia* especially with respect to the apoptotic machinery of the cell.

This study therefore was carried out in order to determine whether the decoction of *M. charantia* will induce the opening of MMPT pore and the possible consequences of this opening on other parameters such as liver function and male fertility.

1.1 OBJECTIVE

Apoptosis is a type of cell death that plays an important role in early development and growth of normal adult tissues. It is regulated by physiological stimuli and present in many species and tissues (Kerr *et al.*, 1987). Apoptosis occurs following a moderate insult, insufficient to kill the cell outrightly but enough to cause significant damage. Examples include a short period of hypoxia and exposure to low doses of a chemical toxin. In contrast, if the initial damage to a cell is too severe, the precisely regulated process of apoptosis is not possible and cell death occurs via necrosis (Halestrap, *et al.*, 1998). In recent years, it has become apparent that mitochondria play a critical role in the mechanism of both apoptotic and necrotic cell death through the opening of the mitochondrial membrane permeability transition pore (MMPTP) (Halestrap *et al.*, 2000). The MMPTP opens when mitochondria are exposed to high calcium concentrations, oxidative stress, e.t.c., uncoupling oxidative phosphorylation and hydrolyzing ATP rather than synthesizing it. If left unrestrained, this situation would lead to cell death. The inhibition of MMPTP constitutes an important strategy for the pharmaceutical prevention of unwarranted cell death. Conversely, induction of MMPT in tumor cells constitutes the goal of anticancer chemotherapy (Kroemer *et al.*, 2007) and it is now abundantly clear that a number of phytochemicals can act as chemopreventives in the development of cancers and the diseases arising from dysregulated apoptosis (Martin, 2006).

Several studies have authenticated the use of *Momordica charantia* in the treatment of various ailments, most importantly, cancers such as lymphoid, leukemia, lymphoma, breast cancer, skin tumor, prostatic cancer e.t.c. (Grover and Yadav, 2004). This study was therefore carried out to determine whether the decoction of *M. charantia* will induce the opening of the MMPT pore as well as to investigate the consequence of this opening on other parameters such as liver function and male fertility.

CHAPTER TWO

LITERATURE REVIEW

2.1. THE MITOCHONDRION

Mitochondria are organelles of eukaryotic cells, believed to have arisen during evolution, when aerobic bacteria capable of oxidative phosphorylation took up symbiotic residence within primitive, anaerobic eukaryotic host cells (Margulis and Schwartz, 1972). Mitochondria are very conspicuous in the cytoplasm of most eukaryotic cells. They are membrane-bound organelles of varying sizes but they typically have a diameter of about 1μm, similar to that of bacteria cells. They vary widely in shape, number and location, depending on the cell type or tissue function (Lehninger, 1964).

Most plant and animal cells contain several hundreds to a thousand mitochondria. Generally, cells in more metabolically active tissues devote larger proportion of their volumes to mitochondria. Mitochondria are found mostly in eukaryotic cells (Henze and Martin, 2003). Often the mitochondrion is referred to as the power house of the cell and rightly so, because the inner membrane bears the components of the respiratory chain and the enzyme complex responsible for ATP (Adenosine triphosphate) synthesis. ATP is used as a source of chemical energy in the cell. This singular function stands out the mitochondrion as the power house of the cell as it is known today (Mc Bride, *et al.*, 2006). Many cells possess only a single mitochondrion while others can have several millions. Mitochondria have their own independent genomes with the mitochondrial DNA coding for certain proteins specific to the inner membrane, but other mitochondrial proteins are encoded in nuclear DNA (Andersson, *et al.*, 2003).

The number of mitochondria per cell appears to be relatively constant and characteristic for any given cell type. A rat liver cell for example, contains about 800 mitochondria. Unlike other membranous structures such as lysosomes, Golgi complexes, and the nuclear envelope, mitochondria are produced only by division of previously existing mitochondria (i.e they undergo fission when they become too large) (Nelson and Cox, 2005). The ATP molecules formed by mitochondria diffuse only a distance to the ATP-requiring contractile elements. Mitochondria are also frequently located adjacent to cytoplasmic fat droplets, which serve as a source of fuel for oxidation (Fiskum, 2000).

Each mitochondrion, as shown (Fig.2.1) has two membranes, viz:

The outer membrane which is unwrinkled and completely surrounds the organelle. This membrane is readily permeable to small molecules and concentrated ions; transmembrane channels composed of the protein, porin allow molecules of molecular weighs less than 5,000 Daltons to pass easily (Nelson and Cox, 2005) and the inner membrane which is impermeable to most small molecules and ions, including protons (H^+); the only species that cross it being those for which there are specific transporter proteins (Mannella, 2006).

The matrix contains the enzymes that are responsible for citric acid cycle reactions. It also contains dissolved oxygen, water, carbon dioxide, the recyclable intermediates that serve as energy shuttle, and much more. Because of the folds of the cristae, no part of the matrix is far from the inner membrane. Therefore, matrix components can diffuse to inner membrane complexes and transport proteins within a relatively short time (Rappaport *et al*. 1998).

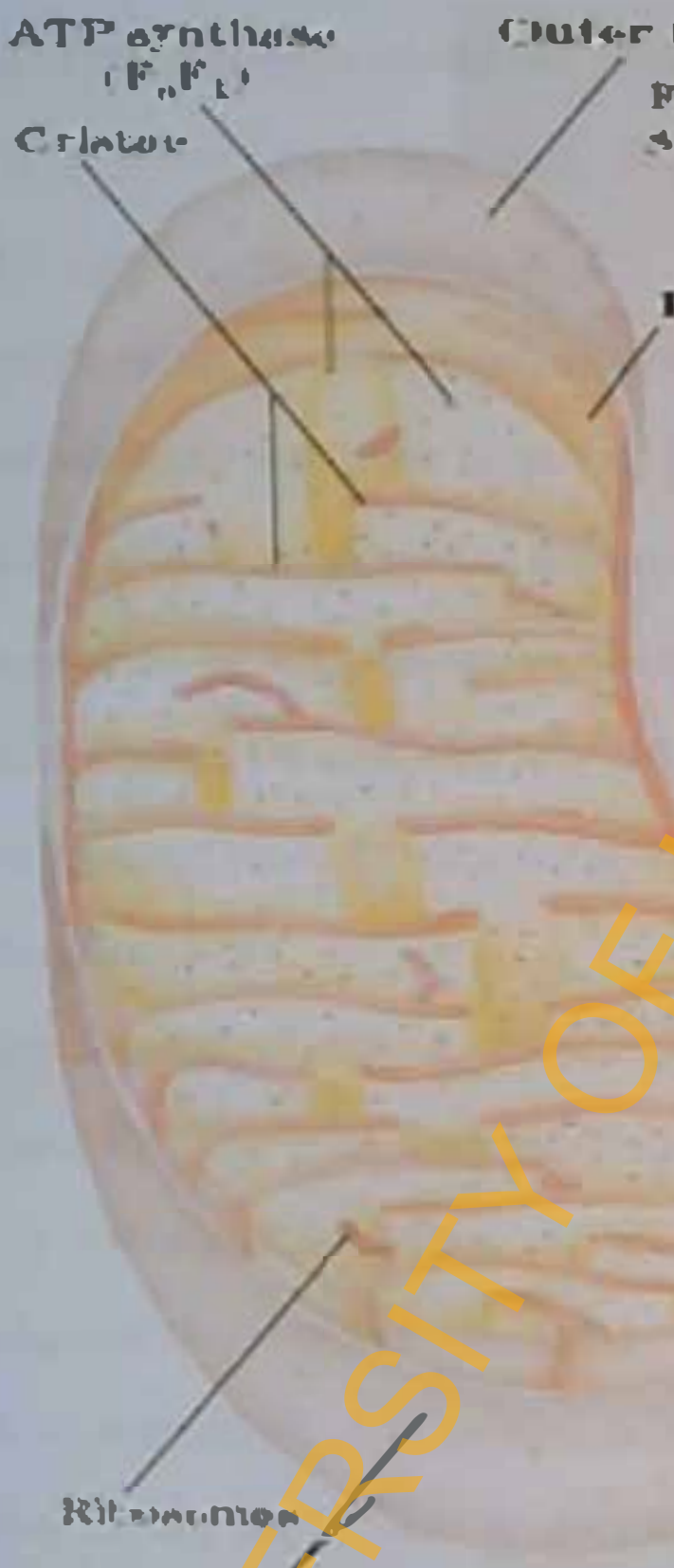

Fig. 2.1: Biochemical Anatomy of Mitochondrion (Scheffler, 1999).

2.1.1. INNER MITOCHONDRIAL MEMBRANE

The inner mitochondrial membrane has more than 100 polypeptides and a very high protein to phospholipids ratio (3:1). It is rich in an unusual phospholipid called cardiolipin. Unlike the outer membrane the inner membrane does not contain Porin, and it is highly impermeable; almost all ions and molecules require special membrane transporters to enter or exit the matrix. The inner membrane has numerous cristae that expand its surface area, enhancing its ability to generate ATP (Mannella, 2006). In the liver mitochondria, the surface area is about 5 times that of the outer membrane. The inner mitochondrial membrane consists of regions of inner boundary membrane that are parallel to the outer membrane and regions invaginating into the matrix as cristae. These infoldings of the inner membrane are of various shapes, some are tubular, and in some other mitochondria, the cristae are longitudinal rather than lateral. The cristae include the electron transport chain, the phosphorylation apparatus, and transporter proteins (Hatefi and Thompson, 2000).

The inner boundary membrane participates in transport reactions including the formation of contact sites, which are dynamic structures that involve fusion of the inner and the outer mitochondrial membranes and are key participants in protein import, energy coupling with the cytosol via formation of creatine phosphate and uptake of fatty acids of oxidative metabolism. The number of cristae varies according to the intensity of oxidative metabolism, with an increased number present in actively respiring mitochondria (Skarka and Ostadal, 2002). The main functions of proteins in the inner membrane include oxidation reactions, ATP synthesis and transportation (Rich, 2003).

2.1.1.1 THE ELECTRON TRANSPORT CHAIN (ETC).

Mitochondria, the power house of eukaryotic cells, oxidize substrates (amino acids, carbohydrates, fatty acids) and reduce co-enzymes NAD^+ and FAD^+ to the energy-rich molecules, NADH and $FADH_2$. The redox energy from NADH and $FADH_2$ is transformed

to oxygen in several steps via reoxidatnin in the electron transport chain The ETC is composed of the mitochondrial enzymes (known as complexes I, II, III and IV) that transfer electrons from one complex to another. Electron flow between the major complexes of this chain drives the extrusion of protons, establishing a steep electrochemical gradient across the inner mitochondrial membrane. The gradient ultimately powers most cellular functions, particularly by allowing the formation of ATP via ATP synthase (Fig. 2.2) (Scheffler, 1999).

Components of the Electron Transport Chain

- Complex I which is also known as NADH dehydrogenase complex is a large flavoprotein complex containing more than 25 polypeptide chains. The entire complex is embedded in the inner mitochondrial membrane, oriented with its NADH-binding site facing the matrix such that it can interact with NADH produced by any of the several matrix dehydrogenases. The overall reaction catalyzed by complex I is:

$$NADH + H^{+} + UQ \longrightarrow NAD^{+} + UQH_2,$$

in which oxidized ubiquinone (UQ) accepts a hydrogen ion (two electrons and a proton) from NADH and a proton from the solvent water in the matrix.

The enzyme complex first transfers a pair of reducing equivalent from NADH to its prosthetic group, FMN. The complex also contains seven Fe-S (iron-sulphur) centres of at least two different types, through which electrons pass on their way from FMN to ubiquinone. Rotenone (a plant product commonly used as an insecticide) and the antibiotic Piericidin A, all inhibit electron flow from these Fe-S centres to ubiquinone. Ubiquinol (UQH_2) diffuses in the membrane from complex I to complex III, where it is oxidized to UQ. The flow of electrons from complex I to UQ to complex III is accompanied by the movement of protons from the mitochondrial matrix to the outer (cytosolic) side of the inner mitochondrial membrane (the inter-membrane space)

- Complex II is also called succinate dehydrogenase and it is the only membrane-bound enzyme in the citric acid cycle. Although smaller and simpler than complex I, it contains two types of prosthetic groups and at least four different proteins. One protein has a covalently bound FAD and an Fe-S centre, with four Fe atoms; a second iron-sulfur protein is also present. Electrons are believed to pass from succinate to FAD, then through the Fe-S centres to ubiquinone. Other substrates for mitochondrial dehydrogenase also pass electrons into the respiratory chain at the level of ubiquinone, but not through complex II.
- Complex III, also known as cytochrome bc1 complex or ubiquinone-cyrochrome C oxidoreductase contains cytochromes b_{562} and b_{566}, cytochrome C_1, an iron-sulphur protein, and at least six other protein subunits. These proteins are asymmetrically disposed in the inner mitochondrial membrane; cytochrome b spans the membrane, and both cytochrome C_1 and the Fe-S protein are on the outer surface. The switch between the two-electron carrier ubiquinone and the one-electron carriers (cytochromes b562, b566, C_1 and C) is accomplished in a series of reactions called the Q cycle. Although the path of electron flow through this segment of the respiratory chain is complicated, the net effect of the transfer is simple; UQH_2 is oxidized to UQ and cytochrome C becomes reduced. Complex III functions as a proton pump; as a result of the asymmetric orientation of the complex, protons produced when UQH_2 is oxidized to UQ are released to the inter membrane space, producing a trans membrane difference. The complex bc1 is inhibited by antimycin, which blocks electron transfer from Cyt.b to Cyt.C_1
- Complex IV: Also known as cytochrome oxidase. This complex contains cytochromes a and a_3. These cytochromes consists of two-heme groups bound to different regions of the same large protein that are therefore spectrally and functionally distinct. Cytochrome oxidase also contains two copper ions, CuA and CuB that are crucial to the transfer of electrons to O_2 (Reduction of oxygen). This complex enzyme has evolved to carry out

the four-electron reduction of O_2 without generating incompletely reduced intermediates such as hydrogen peroxide or hydroxyl free radicals, very reactive species that would damage cellular components. The flow of electrons from cytochrome C to O_2 through complex IV causes a net movement of protons from the matrix to the inter membrane space; complex IV functions as a proton pump that contributes to the proton-motive force. Cytochrome oxidase could be inhibited by cyanide and carbon monoxide.

- Complex V: ATP synthase: This is the ATP-synthesizing enzyme complex of the inner mitochondrial membrane, it has two major components (or factors), F_1 and Fo. The subscript letter O in Fo denotes that it is the portion of the ATP synthase that confers sensitivity to oligomycin, a potent inhibitor of this enzyme complex and thus, of oxidative phosphorylation. The other factor or component, F_1 is the ATP-synthesizing component. It consists of six subunits in all aerobic organisms and is made up of several binding sites for ATP and ADP, including catalytic site for ATP synthesis. It is a peripheral membrane protein complex, held to the membrane by its interaction with Fo, an integral membrane complex of four different polypeptides that forms a transmembrane channel through which protons can cross the membrane (Scheffler, *et al.* 1999). As proton concentration increases in the intermembrane space, a strong electrochemical gradient is established across the inner membrane. Whenever protons return to the matrix through the Fo component of the ATP synthase complex, their potential energy is used to synthesize ATP from ADP and P_i in a process called CHEMIOSMOSIS (Mitchell, 1961; Logan, *et al*., 2005).

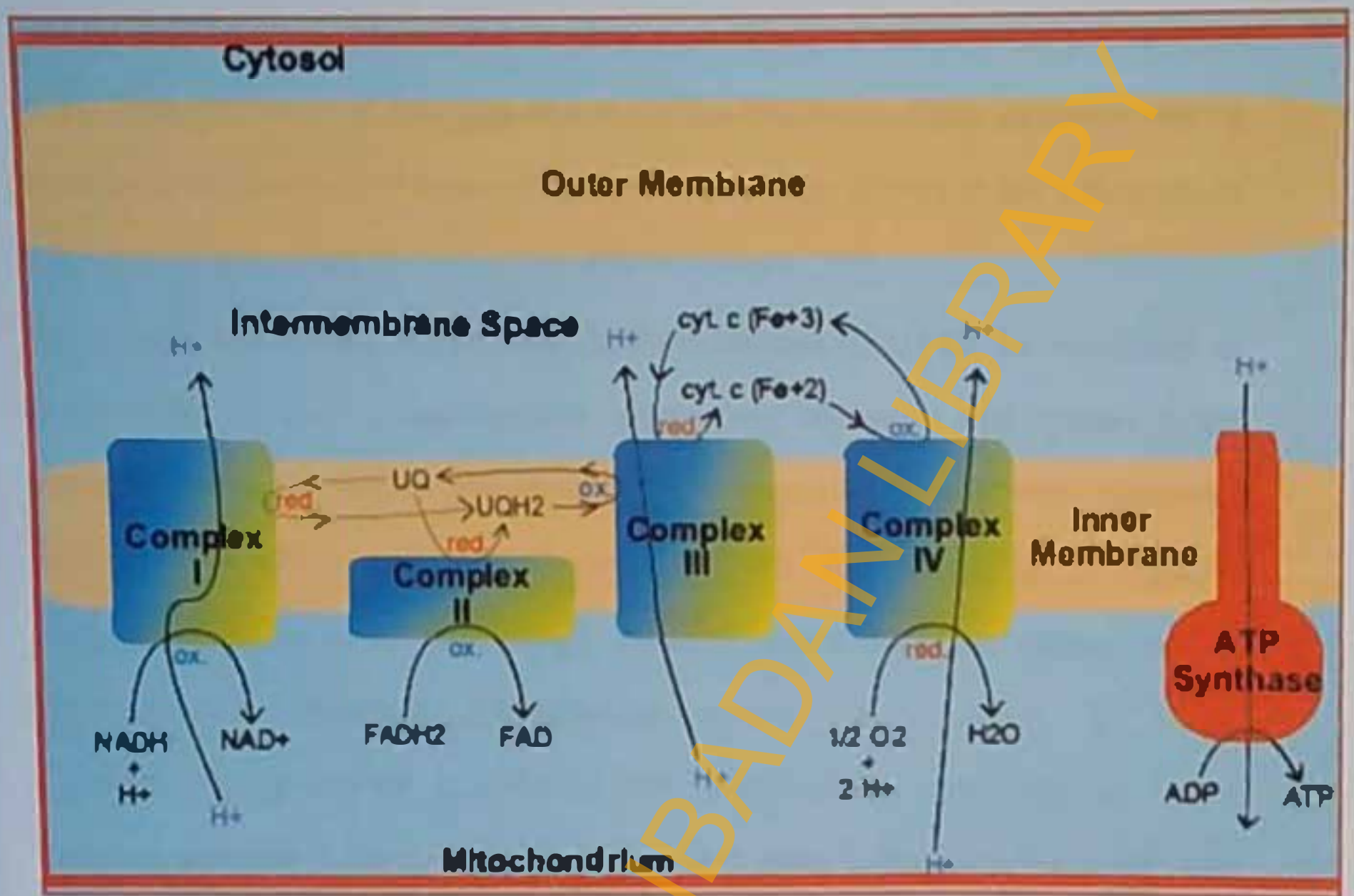

Fig. 2.2: Schemata of the mitochondrial electron transport chain, showing Complexes I-V, Coenzyme Q and Cytochrome C (Nicholls and Ferguson, 2002).

2.1.2. CHEMIOSMOSIS

This is the diffusion of ions across a membrane specifically, it relates to the generation of ATP by the movement of hydrogen ions across the membrane. These hydrogen ions i.e protons will diffuse from all areas of high concentrations to an area of low concentration helping in the generation of ATP as they diffuse (Cooper, 2000)

The chemiosmotic model was proposed by Peter Mitchell (1961) for the mechanism of mitochondrial oxidative phosphorylation when it was discovered that contrary to the earlier investigators' belief, phosphorylation is always associated with membrane structure. The earlier investigators' conclusion was that mitochondrial oxidative phosphorylation involved high-energy chemical intermediate as obtains in the glyceraldehydes-3-phosphate dehydrogenase reaction in glycolysis. In which glyceraldelyde-3-phosphate is oxidized and simultaneously converted to 1,3 – biphosphoglycerate, a compound with a high-energy group at the site of oxidation ATP is formed when 1,3-biphosphoglycerate transfers its activated Pi to ADP. Thus, it was believed that as the ATP synthesis is driven by this high energy intermediate, 1,3-biphosphoglycerate, ATP synthesis in the mitochondria is also driven by a putative chemical intermediate. However, this conclusion was proved wrong by the inability to isolate such (the putative) chemical intermediate during mitochondrial oxidative phosphorylation. This led to the chemiosmotic model

The general idea of the hypothesis (Mitchell, 1961, 1969) is that the coupling of oxidative phosphorylation to electron transport is not brought about by chemical high-energy intermediates but it was suggested that the transport of electrons along the carriers in the respiratory chain generates a gradient of H^+ (protons) across the mitochondrial membrane. This gradient was also postulated to be achieved by the extraction of H^+ ions from the mitochondrial matrix and their ejection into the surrounding medium such that there is a separation between H^+ and OH^- ions on the opposite sides of the inner

membrane (Mitchell, 1961; Mitchell, 1969). In this simple version of the chemiosmotic theory applied to mitochondria (Fig. 2.3), electrons from NADH and other oxidizable substrates pass through a chain of carriers (cytochromes e.t.c) arranged asymmetrically in the membrane (Mitchell and Moyle, 1968). Electron flow is accompanied by proton transfer across the mitochondrial membrane, producing both a chemical (ΔpH) and an electrical ($\Delta\varphi m$) gradient. (The electrical potential inside is negative and for chemical potential, pH is alkaline inside) (Mitchell, 1969).

$$ADP+Pi + [H^+]\ out \longrightarrow ATP+H_2O + [H^+]\ in.$$

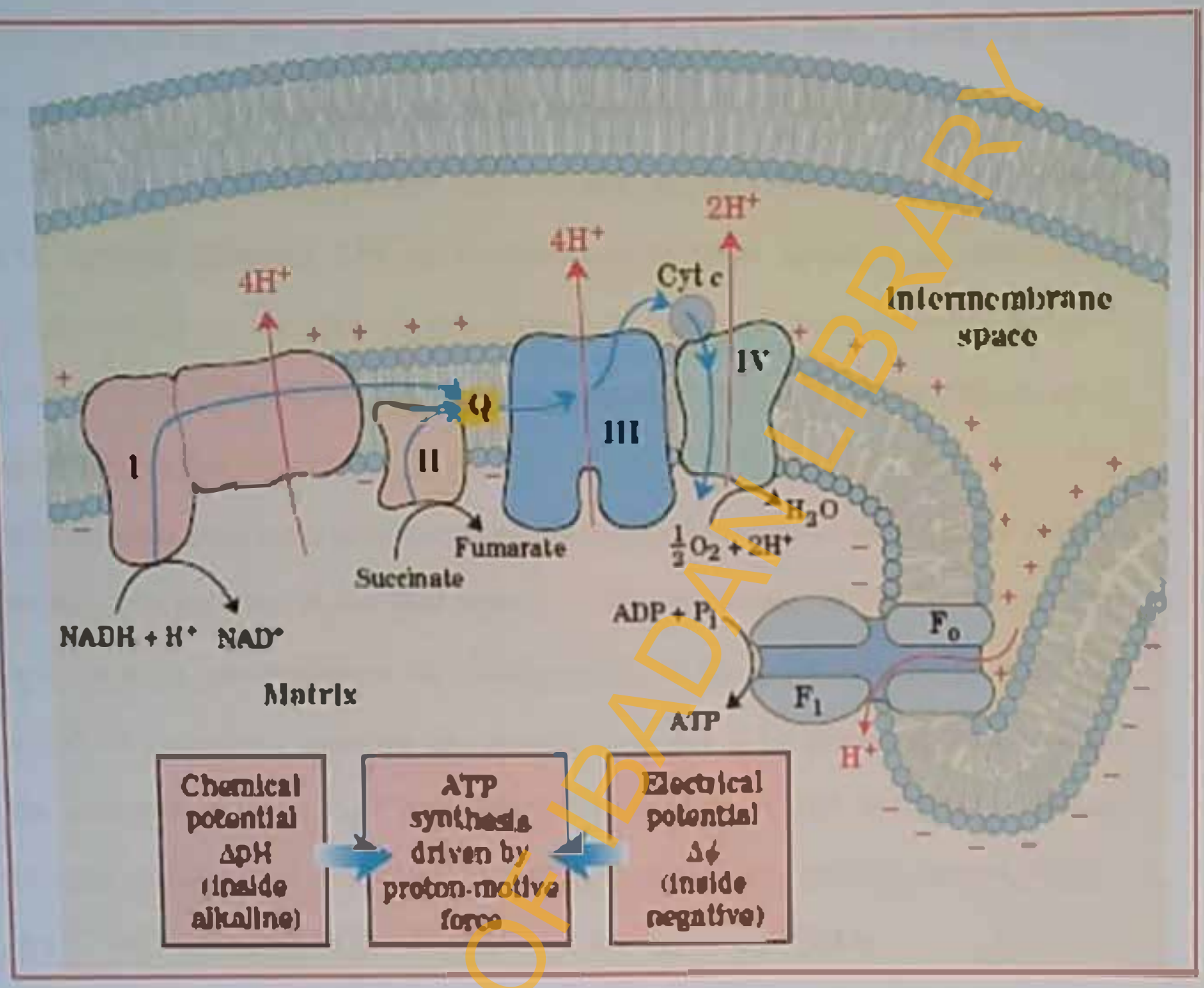

Fig. 2.3: Chemiosmotic model (Mitchell, 1979).

The inner mitochondrial membrane is impermeable to protons; protons can reenter the matrix only through proton-specific channels (Fo). The proton-motive force that drives protons back into the matrix provides the energy ($\Delta\varphi m$) for ATP synthesis, catalyzed by the F_1 component of the synthase. (Mitchell and Moyle, 1968). The process whereby ATP synthase generates ATP by chemiosmosis is called oxidative phosphorylation because oxygen is the final electron acceptor in the mitochondrial electron transport chain. Oxidative phosphorylation is the culmination of energy – yielding metabolism in aerobic organisms.

All enzymatic steps in the oxidative degradation of carbohydrates, fats and amino acids in aerobic cells converge at this final stage of cellular respiration, in which electrons flow from catabolic intermediates to O_2, yielding energy for the generation of ATP from ADP and Pi. In eukaryotes, oxidative phosphorylation occurs in mitochondria and it involves the reduction of O_2 to H_2O with electrons donated by NADH and $FADH_2$, (Redox coenzymes) and occurs equally well in both light and darkness. (Nelson and Cox, 2005).

2.1.3. MITOCHONDRIAL MEMBRANE POTENTIAL (MMP).

Of the two components of the proton-motive force: the membrane potential ($\Delta\varphi m$) which arises from the net movement of positive charge across the inner membrane contributes most of the energy stored in the gradient, typically -150mV. Hence, for practical purposes, MMP may be used on its own as an indicator of energization state of mitochondria (Mathur *et al*, 2000). The proton pump is localized on the inner membrane, it pumps protons from groundmass to the intermembrane in order to form the transmembrane potential, ($\Delta\varphi m$) between outer and inner membranes of the mathochondrion (Zamzami, *et al*; 1996a).

When protons return, they pass energy to ADP and Pi to generate ATP. So, $\Delta\Psi m$ plays a crucial role in keeping the function of mitochondria and mitochondrial depolarization, which leads to $\Delta\psi m$ alteration and has been implicated as an early-onset, and even a

crucial event in cell death (Yan, *et al*, 2007). Changes in MMP are integral to cell life-death transition although the answer whether as a primary cause or a secondary event is yet unknown. In normal cell function, the maintenance of MMP is essential for ATP synthesis. MMP is highly negative, approximately -180mV, due to the chemiosmotic gradient of protons across the inner mitochondrial membrane. the energy of which is used for ATP synthesis by the respiratory chain. MMP also provides the driving force for Ca^{2+} uptake into the mitochondrion by the Ca^{2+} uniporter and it is now generally accepted that it is the Ca^{2+} signal in the mitochondrion that stimulates ATP production in response to an increased energy demand by the cell (Hansford and Zorov, 1998). The consistent observation of a reduced or disrupted ($\Delta\varphi m$) in preapoptosis indicates maintenance of the ($\Delta\varphi m$) is necessary for cell survival (Deckwerth and Johnson, 1993; Vayssiere *et al*., 1995; Zamzami *et al*; 1995a; 1996b).

Further studies of apoptosis indicate that there is mitochondrial depolarization and ($\Delta\varphi m$) disruption in injured cells induced by apoptosis repulsion. moreover. these changes take place before alteration due to apoptosis in cells. All of these indicate that ($\Delta\varphi m$) alteration is the early stage of apoptosis. (Petit *et al*, 1995; Zamzami *et al*., 1995b). Isolation and subsequent culture of a population of cells with a sub normal (low) $\Delta\varphi m$ indicated that they proceeded to an apoptotic morphology rapidly when compared to cells with a normal (high) $\Delta\varphi m$ (Zamzami *et al*; 1955a) Disruption of the $\Delta\varphi m$ has also been shown to be involved in apoptosis mediated by a variety of apoptogens, including etoposide, ceramide e.t.c (Zamzami *et al*., 1996b; De Caudin *et al*., 1997). It has also been shown that the reduction or collapse of the $\Delta\varphi m$ is involved in the induction and is the consequence of the membrane permeability transiton (MPT) (Zoratti and Szabo, 1994; Kroemer *et al*., 1995). Based mainly on *in vitro* observations, one currently popular model consistent with excitotoxic apoptosis proposes that mithcondrial Ca^{2+} overload triggers an injury response, possibly through a mitochondrial permeability transition

(MPT), that leads to the loss of Δφm permeabilization of the inner mitochondrial membrane, swelling of the matrix and outer membrane rupture, followed by release of apoptogenic proteins (Kroemer and Reed, 2000; Bernardi *et al.* 2001; Friberg and Wieloch, 2002; Mattson and Kroemer, 2003).

Therefore, the observation of mitochondria with reduced or diminished Δφm appears to be associated with dysfunctional mitochondria in preapoptotic cells (Isenberg and Klaunig, 2000).

2.1.4. MITOCHONDRIAL PERMEABILITY TRANSITION (MPT)

Mitochondrial permeability transition (MPT), is an increase in the permeability of the mitochondrial inner membrane to molecules/solutes of less than 1,500Daltons. (<1,500 Daltons) including protons and it is favored by Ca^{2+} uptake. When respiring mitochondria take up Ca^{2+} in the presence of inorganic phosphate (Pi) and external adenine nucleotides, the accumulated Ca^{2+} is retained indefinitely causing a damage which has been identified as the Ca^{2+} -dependent pore opening in the inner mitochondrial membrane (i.e MPTP) The MPTP is a non-specific protein pore that is formed in the membranes of mitochondria under certain pathological condition such as stroke. Induction of MPTP can lead to mitochondrial swelling and cell death and it plays a major role in some types of apoptosis (Gunter and Pfeiffer, 1990).

The inner mitochondrial membrane is highly impermeable to tiny solutes and ions in order to enable efficient energy conversion. However, in the presence of certain triggers such as Ca^{2+}, the inner mitochondrial membrane is known to become highly permeable to such molecules. This transition in permeability is what is known these days as MPT and it is believed to reflect the opening of the proteinaceous pores (Zoratti, and Szabo, 1995; and Bernardi, 1999). The mechanism of oxidative phosphorylation requires that the mitochondrial inner membrane be impermeable to all but a few selected metabolites and ions. If this permeability barrier is lost, mitochondria become uncoupled and hydrolyze

ATP rather than synthesize it, left unrestrained, this would inevitably lead to cell death. It is now recognized that if the latent non-specific protein pore (MPTP) in the mitochondrial inner membrane gets activated, it causes just the increase in membrane permeability needed to uncouple the mitochondria. MPTP opens when mitochondria are exposed to high Ca^{2+} concentrations, especially, when this is associated with adenine nucleotide depletion and oxidative stress (Zoratti and Szabo, 1995; Halestrap *et al*, 1997b; Crompton, 1999). Not only does the opening of the MPTP prevent ATP synthesis, it also causes the loss of ions and metabolites from the mitochondrial matrix and induces extensive swelling of the mitochondria as a result of the colloidal osmotic pressure exerted by the matrix proteins (Lemaster *et al*, 1998;).

The MPT was first described several years ago, although, originally, it was thought to occur as the result of phospholipase A_2 degradation of the inner membrane. However, a major breakthrough came in 1988, when Crompton and colleagues demonstrated that the process could be inhibited specifically by sub-micromolar concentrations of the immunosuppressive drug cyclosporin A (CSA). (Crompton *et al*, 1988). Both CSA and Bongkrekic acid (BKA) are now known as effective inhibitors of the MPT, and these have been used to judge induction of the MPT (Bernardi, 1996; Halestrap, *et al*., 1997a). However recent studies indicated that an MPT insensitive to these inhibitors could also be induced (Gudz *et al*; 1997; Malkevitch, *et al*; 1997 and Sultan and Sokolove, 2001).

MPT is frequently studied in liver cells which have especially large numbers of mitochondria (Fiskum, 2000). It causes the release of apoptosis-inducible mitochondrial proteins such as cytochrome C into the cytosol, and these proteins trigger the subsequent reactions that executive apoptosis. The MPT involves the formation of a non-specific pore across the inner mitochondrial membrane permitting the free distribution of ions, solutes and small – molecular-weight molecules (≤1500 Dalton) across the membrane (Bernardi *et al*, 1994). The collapse of the mitochondrial membrane potential ($\Delta\psi m$) and

uncoupling of the electron transport chain from ATP production have been shown to promote MPT (Kroemer *et al*, 1995). The physiological roles of mitochondria MPT are still obscure; however, some studies revealed that MPT is a key event during the process of programmed cell death, also known as apoptosis. (Skulachev, 1996; Kroemer and Reed, 2000; Bernardi *et al*., 2001) and it is believed to be accompanied by:

- **Collapse of membrane potential and Uncoupling of the electron transport chain from ATP production.**

  Induction of MPT, which increases mitochondrial membrane permeability, causes mitochondria to become further depolarized, meaning that $\Delta\psi m$ is abolished. When $\Delta\psi m$ is lost, protons and some molecules are able to flow across the inner mitochondrial membrane unhibited. (Schinder *et al*; 1996 and White *et al*; 1996). Loss of $\Delta\psi m$ interferes with the production of ATP, the cell's main source of energy, because mithocondria must have an electrochemical gradient to provide the driving force for ATP production. (Stavrovkaya and Kristal, 2005)

- **Loss of small matrix solutes, including Ca^{2+}.**

  MPT allows Ca^{2+} to leave the mitochondrion, which can place further stress on nearby mitochondria, and which can activate harmful calcium-dependent proteases such as calpain (Kristian and Siesjo, 1998).

- **Oxidation of pyridine nucleotides.**

  Reactive oxygen species (ROS) are also produced as a result of MPT. MPT can allow antioxidant molecules such as glutathione to exit mitochondria, reducing the organelles' ability to neutralize Reactive Oxygen Species. In addition, the electron transport chain (ETC) may produce more free radicals due to loss of components of the electron transport chain, such as Cytochrome c, which in turn leads to escape of electrons from the chain through the Mitochondrial Permeability Transition Pore (Leutjens *et al*., 2000)

- **Extensive Swelling.**

Isolated mitochondria undergoing MPT show colloidosmotic, so called large amplitude swelling, which results in the decrease of photometric absorption at 540nm (Haworth and Hunter, 1979). MPT causes mithocondria to become permeable to molecules smaller than 1500 Dalton, which, once inside, draw water in by increasing the organelle's osmolar load (Buki *et al*; 2000). This event may lead mitochondria to swell and may cause the outer membrane to rupture, releasing cytochrome C (Buki *et al*; 2000). Release of Cytochrome c can in turn cause the cell to go through apoptosis ("commit suicide") by activating pro-apoptotic factors. Other researchers contend that it is not mitochondrial membrane rupture that leads to cytochrome C release, but rather another mechanism, such as translocation of the molecule through channels in the outer membrane, which does not involve the MPTP (Priault, *et al*., 1999).

Much research has found that the fate of the cell after an insult depends on the extent of MPT. If MPT occurs to only a slight extent, the cell may recover, whereas, if it occurs more it may undergo apoptosis. If it occurs to an even larger degree, the cell is likely to undergo necrotic cell death. (Haworth and Hunter, 2001; Honda *et al*., 2006).

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2.1.5. THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE (MPTP).

The MPTP is a non selective, high conductance channel with multiple macromolecular components (Alano *et al*., 2002, Haworth and Hunter, 1979). It forms at sites where the inner and outer membranes of the mitochondrion meet (Crompton, 1999). Though the exact structure of the MPTP is still unknown, several proteins probably come together to

form the pore, including adenine nucleotide translocase (ANT) (Halestrap and Brenner, 2003), the mitochondrial inner membrane transporter (Tim), the protein transporter at the outer membrane (Tom), the outer membrane voltage-depended anion channel (VDAC) and cyclophilin D (Fiskum, 2001).

Cyclosporin A blocks the formation of the MPT pore by interacting with Cyclophilin D from the mitochondrial matrix and preventing its joining the pore (Sullivan *et al*., 2000). MPTP open-close transitions are modulated by the transmembrane electrical potential, by matrix pH, by redox potential, by adenine nucleotides and by Mg^{2+} (Bernardi, 1996). Opening of the MPTP is induced when the mitochondria are exposed to high calcium concentrations, especially when this is associated with adenine nucleotide depletion and oxidative stress (Halestrap, *et al*; 1997b; Crompton, 1999). Opening of the MPTP allows free entry into the mitochondria of any small molecule (<1500 Daltons) including protons (Halestrap, *et al*., 2000). An important consequence of opening of the MPTP is uncoupling of oxidative phosphorylation (Olorunsogo and Bababunmi, 1980).

Loss of $\Delta\psi m$ interferes with the production of ATP, the cells main source of energy, because mitochondria must have an electrochemical gradient to provide the driving force for ATP production. In cell damage resulting from conditions such as neurodegenerative diseases and head injury, opening of mitochondrial permeability transition pore can greatly reduce ATP production, and can cause ATP Synthase (through its reversal) to begin hydrolyzing, rather than producing ATP (Stavrovskaya, *et al*., 2005). Thus, should the pore remain open, depletion of intracellular ATP will occur and inevitably lead to necrosis. Opening of the MPTP generates a colloidal osmotic pressure across the inner mitochondrial membrane. This drives water into the matrix and causes swelling.

The inner membrane being extensively folded into cristae can expand to compensate but the outer membrane cannot and this ruptures, releasing intermembrane proteins. It is the release of these proteins such as Cytochrome c that enables the mitochondria play a role

in apoptosis i.e the release of Cytochrome c causes cells to go through apoptosis by activating pro-apoptotic factors (Halestrap *et al.*, 1998a; Lemasters, *et al* 1998; Buki *et al*., 2000). MPTP also allows Ca^{2+} to leave the mitochondrion, which can place further stress on nearby mitochondria, and which can activate harmful calcium dependent proteases such as calpain. Reactive oxygen species (ROS) are also produced as a result of opening of the MPT pore. MPT can allow anti-oxidant molecules such as glutathione to exit mitochondria, reducing the organelle's ability to neutralize ROS.

In addition the electron transport chain (ETC), may produce more free radicals due to loss of components of the ETC, such as Cytochrome c through the MPTP. (Lueljens *et al*., 2000). Loss of the components of ETC can lead to escape of electrons from the chain, which can then reduce molecules and form free radicals.

2.1.5.1. POSSIBLE EVOLUTIONARY PURPOSE OF THE MPTP.

The existence of a pore that causes death led to speculation about its possible evolutionary benefit. Some have speculated that the MPT pore may minimize injury by causing badly injured cells to die quickly and by preventing cells from oxidizing substances that could be used elsewhere (Haworth and Hunter *et al*., 2001). There is controversy about the question of whether the MPTP is able to exist in a harmless, "Low -conductance" state. This low-conductance state would not induce MPT (Ichas and Mazat, 1998) and would allow certain molecules and ions to cross the mitochondrial membranes. The low -conductance state may allow small molecules like Ca^{2+} to leave mitochondria quickly in order to aid the cycling of Ca^{2+} in healthy cells (Hunter and Haworth, 1979b; Altschuld *et al*., 1992). If this is the case, MPT may be a harmful side effect of abnormal activity of a usually beneficial MPTP

2.1.5.2. THE MOLECULAR IDENTITY OF THE PORE

The MPTP was first described several decades ago, but was initially thought to represent phospholipase A2-mediated damage to the inner membrane. However, pioneering studies

by Haworth and Hunter in the late seventies (1979) suggested otherwise, since the pore has a specific molecular weight cut-off and could be rapidly closed by chelating calcium. Their data were confirmed in the late eighties by Martin Crompton and Colleagues (Crompton. et al., 1988) who went on to make a key discovery: opening of the MPTP could be inhibited specifically by sub-micromolar concentrations of the immunosuppressive drug cyclosporin A (CSA) (Compton 1999).

It's been demonstrated by Halestrap and Davidson (1990) and Connern and Halestrap (1992) that the effect of CSA was exerted through inhibition of a peptidyl-prolyl cis-trans isomerase (PPIase), unique to the mitochondria, otherwise known as Cyclophilin D. (CYP-D) because of its cyclosporin A (CSA)-binding properties. (CYP-D is petidyl-prolyl-cis- tans- isomerse PPIase wluch catalyses the interconversion between cis and trans conformation of peptide bonds adjacent to proline residue, as such, it is ideally suited for causing the conformational change in a membrane protein that would be required to induce formation of a pore. This membrane protein has been identified to be adenine nucleotide translocase (ANT), whose normal function it to transport ADP and ATP across the inner mitochondrial membrane. It has been confirmed by data generated by Halestrap and other scientists that the conformational state of the ANT greatly influenced the sensitivity of the MPTP to $[Ca^{2+}]$. This led to the proposal by Halestrap and Davidson (1990) that in the presence of calcium, a cyclophilin D-mediated conformational change of the ANT was responsible for the formation of MPTP (Halestrap and Davidson, 1990).

Subsequent data have shown that oxidative stress greatly enhances the calcium-sensitivity of the pore by two mechanisms (Halestrap *et al.* 1997b). First, by increasing Cyp-D's binding to the ANT (This is enhanced when the thiol groups on ANT are modified by oxidative stress and second, by greatly reducing the affinity of the intra-mitochondrial adenine nucleotide-binding site on the ANT; binding of Adenine nucleotides to this site

was shown to inhibit the MPTP competitively with respect to [Ca^{2+}]. The evidence in support of this hypothesis has been mounting steadily (Halestrap 1999; Crompton, 1999) and Halestrap et al have been able to demonstrate directly that CYP-D binds very tightly and specifically to the ANT (Halestrap, *et al.*, 1998b). Binding was prevented but not reversed (Woodsfield *et al.*, 1998). Crompton and Colleagues have reported similar data but with two important differences probably, because of the distinct detergent used (Crompton *et al.*, 1998). First, the binding they observed was not prevented by CSA treatment and second, they observed that both porin Voltage-Dependent Anion Channel [VDAC] and ANT bound tightly to CYP-D, whereas, according to Halestrap *et al.*, no other protein apart from ANT was bound. Porin is known to be associated with the ANT at contact sites between the mitochondrial outer and inner membranes and its involvement in the MPTP is a matter of controversy (Halestrap, *et al.*, 1998b).

Final proof that the MPTP is composed of just the ANT and CYP-D would require reconstitution of the pure proteins into proteoliposomes to form a CSA-Sensitive calcium-activated pore. Earlier data had shown that the ANT alone could produce such a pore either when critical thiol groups were modified or when exposed to high [Ca^{2+}] (>1mM) (Brustovetsky and Klingenberg, 1996). Crompton and colleagues were able to reconstitute their ANT/Porin/CYP-D complex into proteoliposomes to produce a CSA-inhibitable pore that opened at 100μM [Ca^{2+}] (Crompton *et al.* 1998) and Halestrap *et al* have also succeeded in doing the same with pure ANT and CYP-D (in the absence of porin). (Halestrap *et al*, 2000). Thus, it now seems that the controversy over the identity of the MPTP may be resolved; the components are the ANT and CYP-D as originally proposed (Halestrap and Davidson 1990). However, this does not exclude a role for other proteins such as porin in the regulation of the MPTP (Halestrap, *et al.*, 2000). Figure 4 shows the proposed scheme for the mechanism of pore opening by Halestrap and Davidson (1990). AMP-P, AMP-PP, P and PP represent ADP, ATP, P_i and PP_i respectively. In all cases, the carrier is assumed to be in the 'c' conformation (conformation in which the ADP/ATP binding site in ANT is on the cytosolic side).

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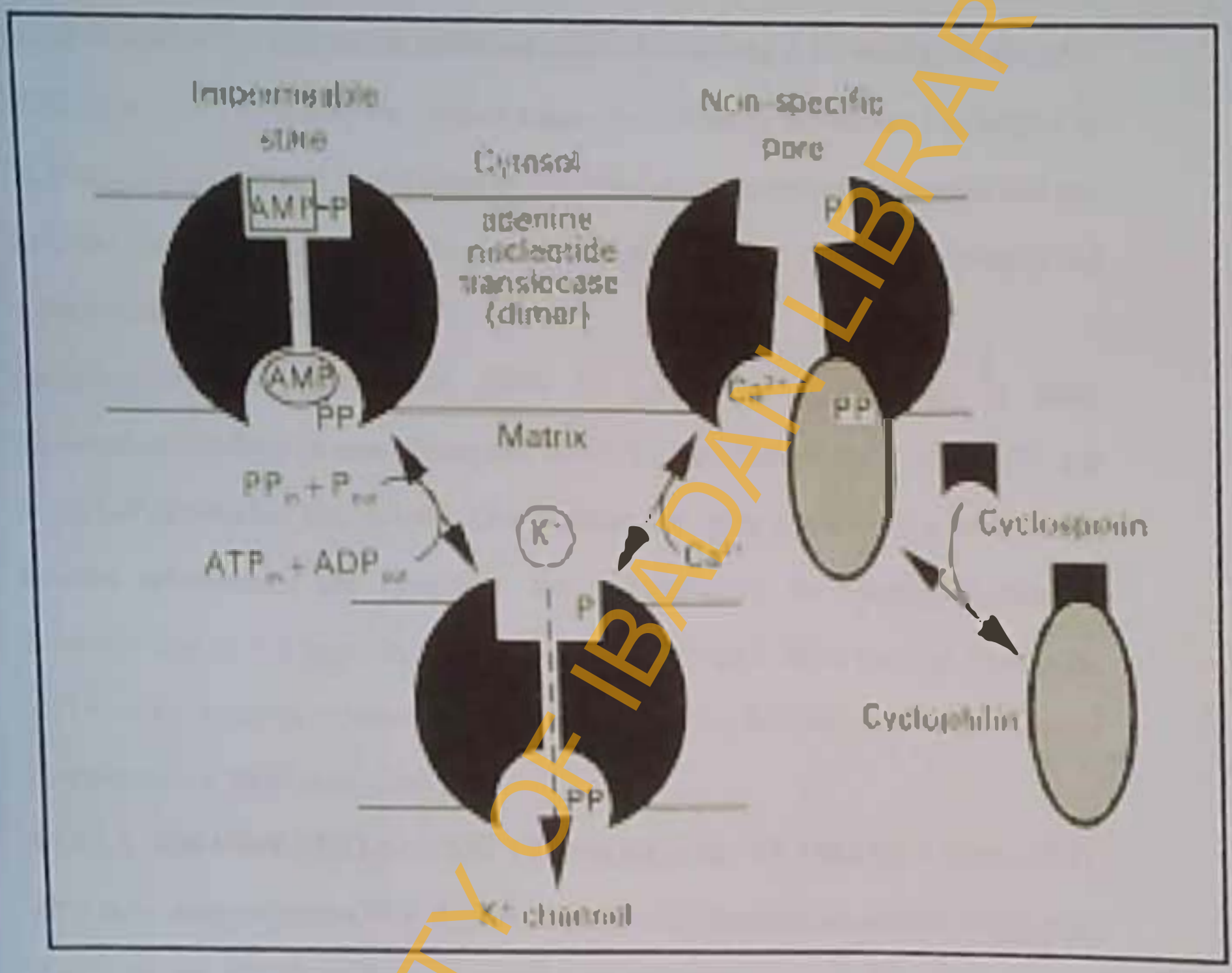


Fig. 2.4: Proposed scheme for the mechanism of pore opening by Halestrap and Davidson (1990).

The above model is probably the most widely held and in outline, it is proposed that calcium triggers a conformational change. The process is greatly facilitated by the binding of CYP-D which is enhanced when thiol groups on the ANT are modified by oxidative stress. This also has an additional effect of inhibiting ATP-binding to the ANT which competitively inhibits the calcium trigger site. Binding of adenine nucleotides to the matrix side of the ANT is enhanced by the mitochondrial membrane potential and this provides an explanation for why the pore opens more readily in deenergized mitochondria (Halestrap, 1999).

Physiologically, the diameter of MPTP is 0.2-0.3μm, large enough to allow micromolecule solutes to pass (Crompton, 2000). By the effect of Ca^{2+}, ADP, ATP, and so on, MPTP switches alternatively. This condition may play a role in material exchange between mitochondria and cytoplasm. But pathologically, the opening diameter is obviously large (1.8-2.6μm), so, that the solute smaller than 1.5kDa can pass through the MPTP to the cytoplasm, causing mitochondrial swelling and transmembrane potential disappearance (Chelli, *et al*., 2001).

2.1.5.2.1. ADENINE NUCLEOTIDE TRANSLOCASE / TRANSLOCATOR (ANT).

ANT is an integral protein (Fig. 2.5) of the inner mitochondrial membrane which binds ADP^{3-} on the outside (Cytosolic surface) of the inner membrane and transports it inwardly in exchange for an ATP^{4-} molecule simultaneously transported outward (Halestrap, 1999). Because this antiporter moves four negative charges out for every three moved in, its activity is favoured by the trans-membrane electrochemical gradients which gives the matrix a net negative charge. The proton motive force drives ATP-ADP exchange (Slater, 1987).

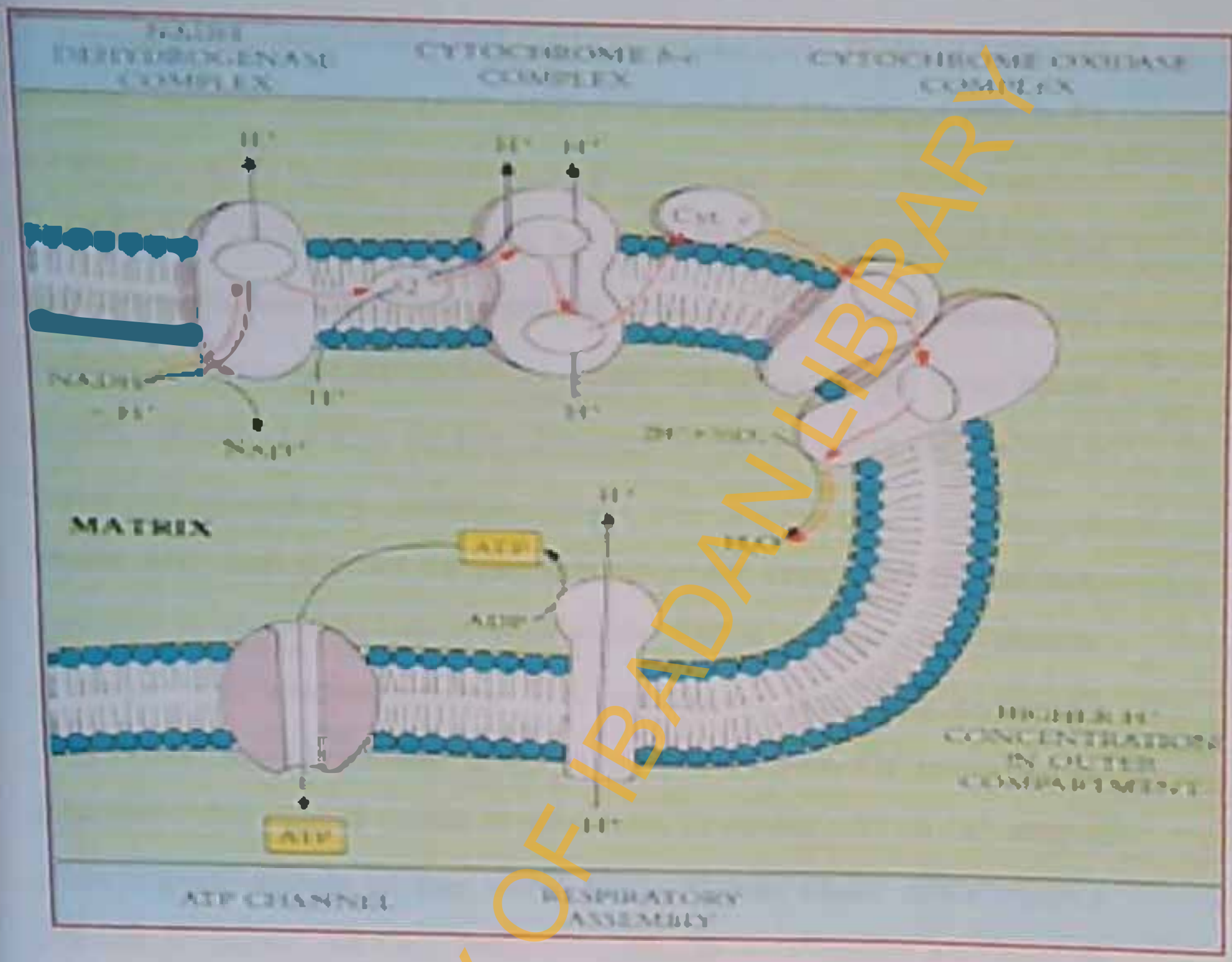


Fig. 2.5: Location of ANT on the mitochondrial membrane (Slater, 1987).

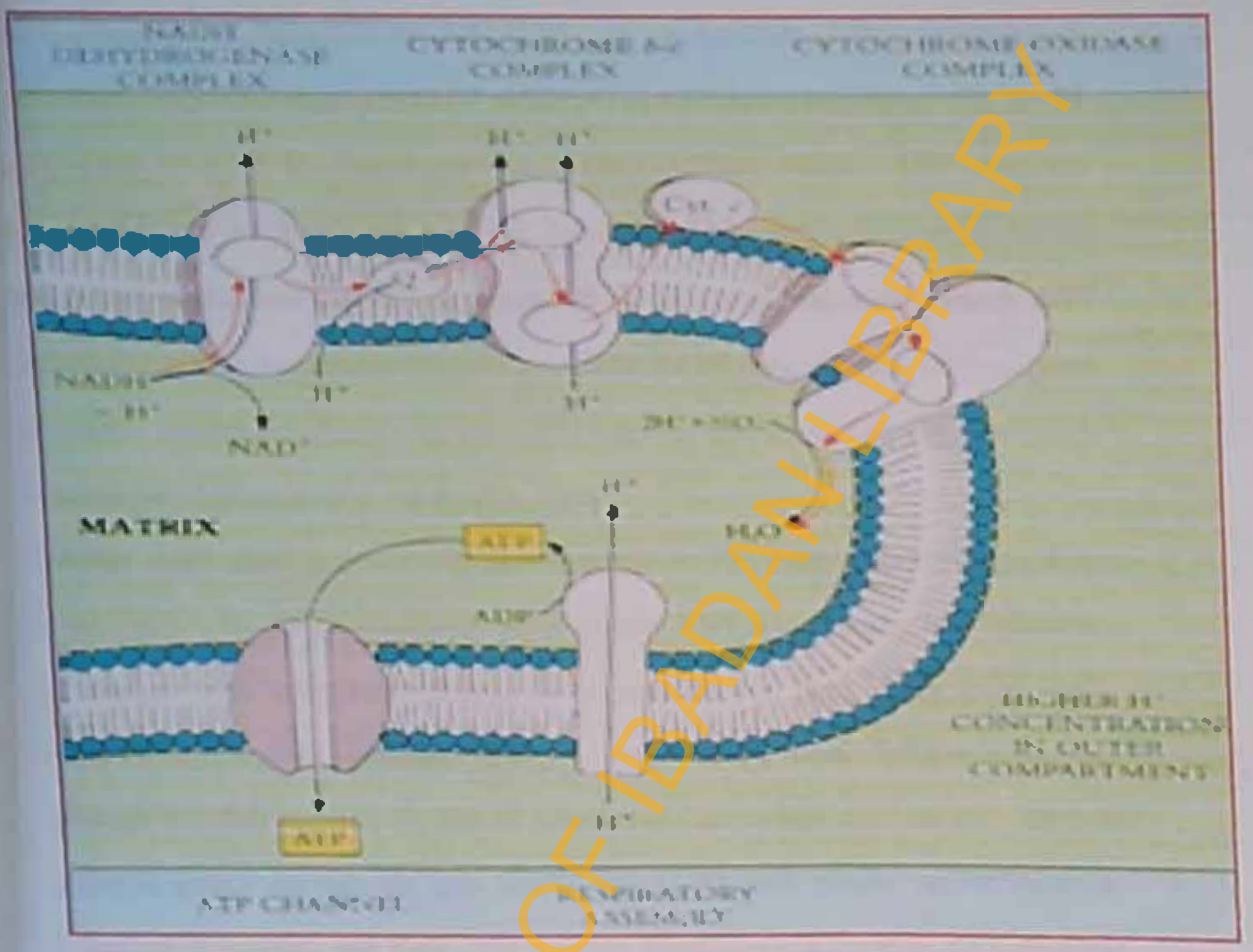


Fig. 2.5: Location of ANT on the mitochondrial membrane (Slater, 1987).

ANT operates as a gated pore and it has long been recognized that PT pore opening is highly susceptible to ligands of the ANT (Le Quoc, and Le Quoc, 1988). Of a range of nucleotides, only ANT substrates (ADP, dADP,ATP) were found to interact with the PT pore when occupied by transportable substrates(Halestrap, 1998b). ANT alternates between the two conformations in which the ADP/ATP-binding site is either on the matrix side of the inner membrane (m-state) or on the cytoplasmic side (c-state). ANT ligands that bind to m-state (i.e bongkrekate) inhibit the PT pore, whereas, c-state ligands (pyridoxal phosphate) activate the pore. This suggests that the c-state conformation is required for PT pore opening (Crompton, 1999).

Whether ANT itself provides the pore structure in the inner membrane has been investigated in reconstituted systems. When purified ANT is incorporate into liposomes, it changes from selective antiporter to a non-selective pore under high [Ca^{2+}] (Brustovetsky, and Klingenberg, 1996). As with the PT pore, Ca^{2+} acts reversibly, although the time required for loss of pore activity of purified ANT on Ca^{2+} removal (20mins) greatly exceeds the time needed for the PT pore closure on Ca^{2+}chelation (<50secs) (Crompton, and Costi, 1990). Other features of Ca^{2+}-treated ANT resemble those of the PT pore In planar lipid membranes, the conductance of the ANT-derived pore was inhibited at low pH, with half-maximal activity at pH 6.2 which is similar to that of the PT pore. (Al Nasser, and Crompton, 1986).

The Current-Voltage relationship showed a pronounced reversal of conductance at 150mv to 180mv of both signs, (+or-) reminiscent of the dependency of the PT pore on inner – membrane potential (Brustovetsky and Klingenberg, 1996). Taken as a whole, these data suggest that the c-state conformation of ANT may be deformed into a non-selective pore by high [Ca^{2+}], in line with original proposals. (Le Quoc, and Le Quoc, 1988) But any deformation will need to occur in a highly reversible manner as is shown from pulsed-flow analyses of EGTA-induced pore closure (Crompton and Costi, 1990)

ANT is only able to change conformations between the m-and the c-states in the presence of transportable substrates. This ensures strict antiport. In essence, the complimentarily between the transported solute and the intermediate (between m-and c-) states of the carrier provides the binding energy for the conformational change to occur.

Thus, ADP catalysis of PT pore flicker means that ANT must be in its native state between flickers. If ANT were in a deformed state (but closed) between flickers, then, the binding energy between ATP and ANT would not be available for the conformational change to the c-state to take place, and an open PT pore would not be produced. Pore flicker presumably allows loss of matrix Ca^{2+} and rapid reversion of ANT to its native state (Altschuld, *et al*., 1992; Ichas and Mazat, 1998).

2.1.5.2.2. CYCLOPHYLIN – D (CYP-D)

It is clear that ANT, by itself, does not provide the PT pore. Thus, treatment of solute-loaded submitochandrial particules with high $[Ca^{2+}]$ with or without Ca^{2+} ionophore to allow Ca^{2+} access to both faces of inner membrane does not lead to solute release. (Compton, *et al*., 1992; Mc Guiness, *et al*., 1990) As with ANT, the participation of a further component was first suggested from the effect of the respective ligands. PT pore opening is blocked by CSA at a concentration (approximately 50 pmol/mg of mitochondrial protein) much less than that of ANT (Crompton, et al., 1988).

Cyclophilin-D (CYP-D)'s involvement was suggested from the similar amount of bound CSA needed to block the pore and to inhibit the enzymic activity of mitochondrial CYP-D (Mc Guinness, *et al*., 1990; Halestrap, and Davidson, 1990) and from the similar relative sensitivities of the PT pore and mitochondrial CYP-D to CSA analogues (Griffiths and Halestrap, 1991; Nicolli, *et al*., 1996). In a further approach, a photoactive radiolabelled CSA derivative was used to tag the CSA "receptor" (Andreeva, *et al*., 1995; Tanveer, *et al*., 1996).

Two pore ligands, Ca^{2+} and ADP, were used in conjunction with the derivative to pinpoint the relevant component These ligands were chosen because they were known to influence CSA interactions with the pore Intramitochondrial Ca^{2+} not only activates the PT pore, but also depresses CSA binding to its "receptor" on the pore (Mc Guinness, *et al*, 1990, Crompton and Andreeva, 1994) Conversely, ADP promotes CSA binding (Andreeva and Crompton, 1994) When photolabelling was carried out in the presence and absence of these ligands, a number of mitochondrial components became covalently labeled by the the CSA derivative, but only photolabelling of CYP-D was promoted by ADP and abolished by Ca^{2+} (Andreeva *et al*, 1995, Tanveer, *et al*, 1996) Thereby identifying CYP-D as the pore-associated CSA – binding component

It is generally assumed that CYP-D associates with ANT via the active site and that CSA blocks the pore by preventing this association (CYP-D serves as a CSA receptor on the PT pore) In line with this, the binding of CYP-D to ANT in Triton – X-100 solubilized membranes was blocked by CSA

2.1.5.3. FACTORS INDUCING MPTP OPENING.

Various factors enhance the likelihood of MPTP opening In some mitochondria, such as those in the central nervous system, high levels of Ca^{2+} within the mitochondria can cause the opening of the pore (Hunter and Haworth, 1979a, Brustovetsky, *et al*, 2002) This is possibly because Ca^{2+} binds to and activates Ca^{2+} - binding sites on the matrix side of the MPTP (Haworth and Hunter, 1979, Ichas and Mazat, 1998) The presence of free radicals, another result of excessive intracellular calcium concentrations can also cause the MPTP to open (Fiskum, 2001, Brustovetsky, *et al*, 2003) Other factors that increase the likehood that the MPTP will be induced include the presence of certain fatty acids (Garcia-Ruiz, *et al*, 2000) and inorganic phosphate (Nicholls and Brand, 1980) However, these factors cannot open the pore without Ca^{2+}, though at high enough concentrations, Ca^{2+} alone can induce MPT (Gunter, *et al*, 1994) Stress in the endoplasmic reticulum can be a factor in triggering MPT (Deniaud *et al*, 2007)

things that can cause the pore to close or remain closed include acidic conditions (Friberg and Wieloch, 2002), high concentrations of ADP (Hunter and Haworth, 1979b, Bristovetsky *et al*., 2003), high concentrations of ATP (Beutner *et al*., 1998) and high concentrations of NADH (Hunter and Haworth 1979a) Divalent cations like Mg^{2+} also inhibit MPT, because they can compete with Ca^{2+} for the Ca^{2+}-binding sites on the matrix side of the MPTP, (Haworth and Hunter, 1979)

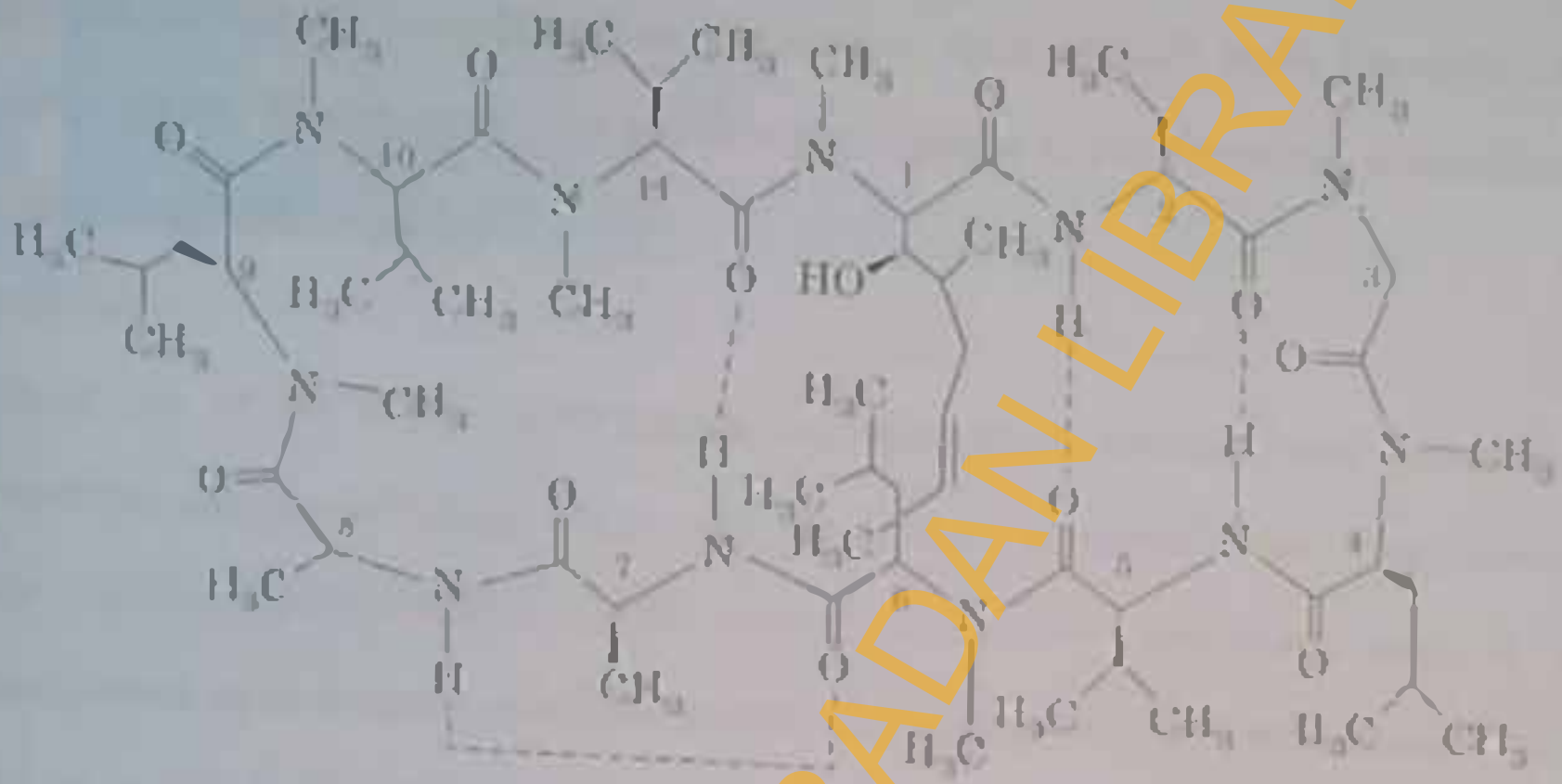
In conclusion, the existence of a pore that causes cell death has led to speculation about its possible evolutionary benefit Some have speculated that the MPT pore may minimize injury by causing badly injured cells to die quickly and by preventing cells from oxidizing substances that could be used elsewhere (Haworth and Hunter, 2001)

2.1.5.4. INHIBITORS OF THE MPTP

- **Cyclosporin A (CSA).**

In 1988, Crompton and his colleagues demonstrated that the process of membrane permeability transition could be superficially inhibited by sub-micromolar concentrations of CSA (Cronipton *et al*., 1988) This was a major breakthrough in the understanding of the molecular mechanism of the MPT because it led to the discovery that CSA exerted its inhibitory effect through binding to a specific Cyclophilin (CYP-D) within the mitochondrial matrix (Connern, and Halestrap, 1992) CSA is a neutral lipophylic, fungally produced, 11-residue cyclic peptide (Fig 2.6) extracted from the fungus "*Tolypocladium inflatum*"

It is a potent immunosuppressive agent which is used principally to suppress graft rejection after organ (e.g kidney, heart, lung, liver, pancreas and bone marrow) transplants (Kahan *et al*., 1983) It is a highly effective agent for the treatment of autoimmune disorders and for preventing organ-transplant rejection Indeed, until the advent of CSA in the early 1980's the long-term survival of a transplanted organ and its recipient was a rare occurrence (Voet and Voet, 2004)



Cyclosporin A

Fig. 2.6: STRUCTURE OF CSA (Voet and Voet, 2004).

Cyclosporin A specifically binds cyclophylin-D (So named because they are specifically inhibited by CSA) and it had been proposed that when bond by CSA, Cyclophylin-D remains inactive thereby maintaining the MPT pore in a closed state (Brockemeier *et al*., 1989, Halestrap and Davidson, 1990) Therefore, inhibition of MPT by cyclosporin A appears to prevent the cascade of events leading to apoptotic cell death (Isenberg and Klaunig, 2000) The structure of CSA strongly suggests a non-ribosomal biosynthetic mechanism (Zocher *et al*, 1986)

- **Spermine**

This is one of the three known polyamines; spermidine, spermine and putrescine Polyamines are responsible for the growth and function of normal cells They interact with various macromolecules, both electrostatically and covalently and, as a consequence, have a variety of cellular effects The complexity of polyamine metabolism and the multitude of compensatory mechanisms that are invoked to maintain polyamine homeostasis argue that these amines are critical to cell survival (Wallace *et al*, 2003)

The initial discovery of the polyamines dates back to 1678 when Antonie van Leeuwenhoek isolated some three-sided crystals from human semen (Van Leeuwenhoek, 1678) However, it was not until 1924 that the empirical formulae of the crystals were deduced (Dudley *et al*., 1924), and it was a further 2 years before the products were synthesized chemically (Dudley *et al*., 1926) The name spermidine and spermine therefore reflect the original discovery

Polyamines are found in all living species, except two orders to Archaea, Methanobacteriales and Halobacteriales (Hamana and Matsuzaki, 1992) This conservation across evolution is a positive feature in that it argues for their importance in cell survival but it may also be a drawback in that it implies a lack of specific function (Wallace, 1998) Polyamines such as spermine and spermidine are used in DNA packaging, this is because their positive charge which they carry on each nitrogen atom at

physiological pH enables them to interact electrostatically with polyanionic macromolecules within the cell Spermidine and spermine can bridge the major and minor groves of DNA, acting as a clamp holding together either two different molecules or two distant parts of the same molecule (Matthews, 1993)

Structural studies indicate that polyamines interact with individual rather than multiple DNA molecules (Tabor and Tabor, 1984) In addition to interacting with DNA and RNA, polyamines can also interact with acidic phospholipids in membranes (Schuber, 1989) In general, spermidine and spermine increase the rigidity of the membrane by forming complexes with phospholipids and proteins They may also have antioxidant role, preventing lipid peroxidation (Tadolini, 1988) Polyamines have been implicated in the regulation of several membrane-bound enzymes, including adenylate cyclase (Wright, *et al*, 1978), tissue transglutaminase (Beninati et al, 1998) and Voltage-activated Ca^{2+} channels (Nichols and Lopatin, 1997, Williams, 1997)

If the defining feature of the polyamines therefore were charge, then surely, one polycation would be sufficient? The most obvious choice would be spermine, as it has the greatest charge, largest length and most flexibility The sheer complexity of the regulation and metabolism used by the polyamines argues that they, or their associated enzyme activities, have other critical functions within the cell not based solely on direct charge-charge interactions (Wallace, *et al*, 2003)

The polyamine, spermine has been worked on by Sokolove and Lapidus and has been identified as an inhibitor of the MPT of isolated rat liver and heart mitochondria (Lapidus and Sokolove, 1993) Spermine, in combination with a series of triggering agents was used to clarify several mechanistic details of the transition process in isolated rat liver mitochondria Mitochondrial swelling was monitored as an indicator of transition occurrence

Their results indicated that,

1 Spermine inhibits the permeability transition of isolated rat liver mitochondria

2 The sensitivity of the permeability of liver mitochondria to spermine is highly dependent on the ionic composition of the assay medium

3 K^+ interacts with a site outside the mitochondria to decrease spermine effectiveness

4 Spermine likewise acts at an external site, and

5 The Ca^{2+} uniporter in its inactive form is not the protein responsible for mediating the permeability transition (Lapidus and Sokolove, 1993)

Spermine and spermidine are derived from methionine and ornithine by the pathway shown below (Fig 2 7) The first step is the decarboxylation of ornithine a component of the urea cycle and a precursor of arginine Ornithine decarboxylase is a PLP (pyridoxal phosphate) – requiring enzyme and is the target of several powerful inhibitors developed commercially as pharmaceutical agents (Nelson and Cox, 2005) The MPT is also potentially inhibited by proton (H^+), the effect of which is exerted from the matrix side of the inner membrane and is linked to reversible protonation of histidyl residues (Hunter and Haruoith, 1979a)

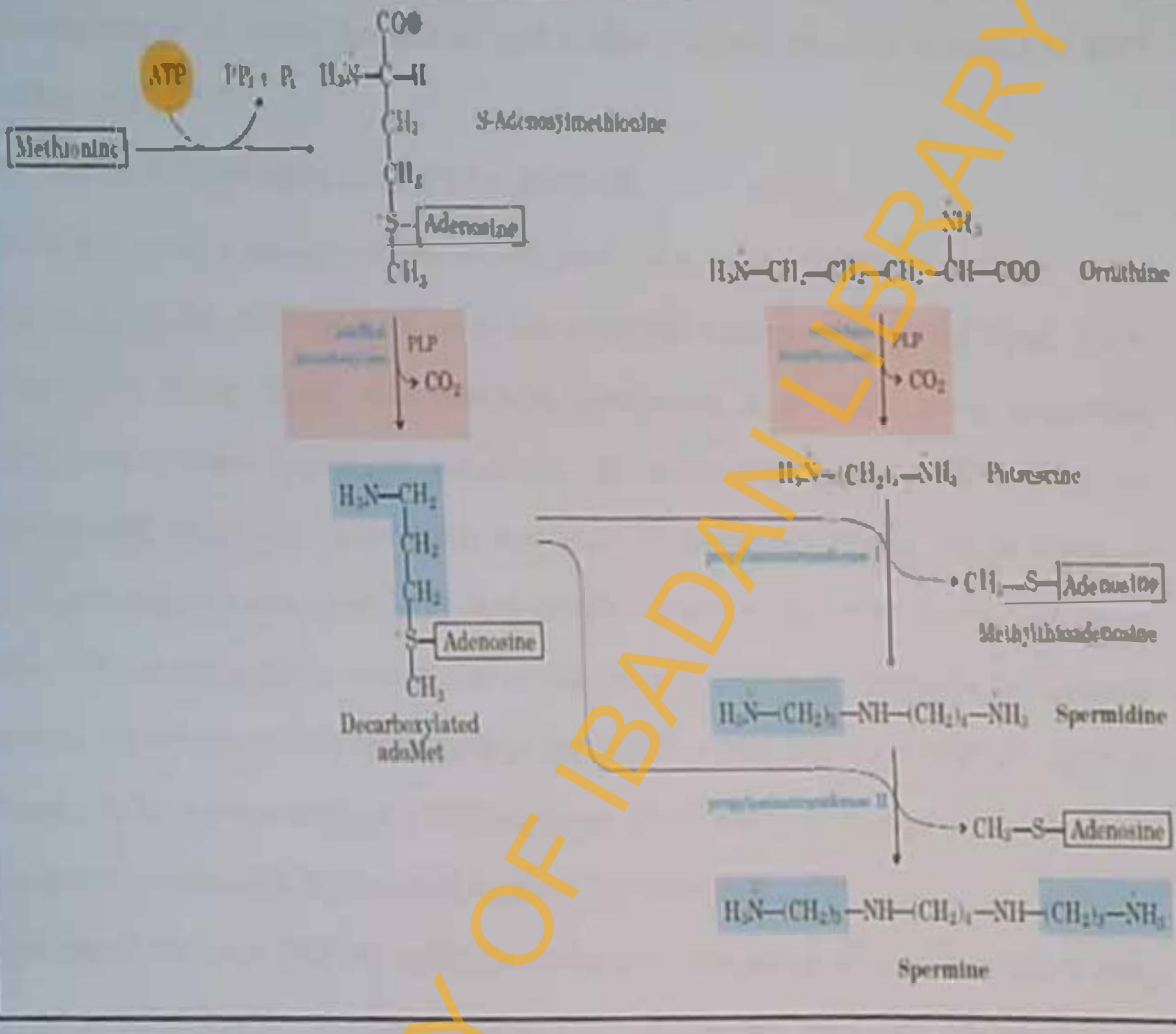


Fig. 2.7. Synthesis of Polyamines (Spermine) (Nelson and Cox, 2005).

Other inhibitors of MPTP are N-methyl-Val-4-cyclosporin A (MevalCSA), a non-immunosuppresant derivative of CSA, 2 – amino ethoxydiphenyl borate (2-APB) (Chinopoulous *et al*, 2003) Bongkekic acid is also a known effective inhibitor of MPT pore (Bernardi, 1996)

2.2 MITOCHONDRIA AND CELL DEATH.

Mitochondria play a prominent role in cell death as a central organelle involved in the signal transduction and amplification of the apoptotic response (Green and Reed, 1998, Johnson and Boise, 1999) Mitochondrial dysfunction is an early event preceeding nuclear and plasma membrane alterations It is characterized by an increase in mitochondrial membrane permeability and loss of membrane potential that is regulated by the permeability transition (PT) pore complex (Petit *et al*, 1997, Green and Reed, 1998) Mitochondria play a central role in both types of cell death, apoptosis and necrosis through the opening of the mitochondrial permeability transition pore (MPTP) which is thought to be formed through a Ca^{2+}-triggered conformational change of the Adenine nucleotide translocase (ANT) bound to matrix cyclophilin-D

Opening of the pore (MPTP) causes swelling and uncoupling of mitochondria, which, unrestrained, leads to necrosis Transient MPTP opening may also be involved in apoptosis by initially causing swelling and rupture of the outer membrane to release Cytochrome c (Cyt c), which then activates the caspase cascade and sets apoptosis in motion Subsequent MPTP closure allows ATP levels to be maintained, ensuring that cell death remains apoptotic rather than necrotic Thus, in addition to their more widely recognized function in the provision of ATP, mitochondria also play a critical role in the mechanism of cell death, both apoptotic and necrotic (Ankarcrona *et al*, 2000) An important role of mitochondria in apoptotic signaling is the translocation of Cytochrome c from the mitochondrial intermembrane compartment into the cytosol Once released, cytochrome C binds to APAF-1 in the presence of ATP or dATP and forms a complex

that processes and activates pro-caspase-3 and -7 (Salch *et al*., 1999). The release of cytochrome C has been linked to loss of mitochondrial membrane potential ($\Delta\psi m$) and increase in permeability transition (Petit, *et al*., 1997, Shimizu, *et al*., 1999, Marzo *et al*., 1998), although there are also reports providing evidence that these are independent events (Bossy-Wetzel *et al*., 1998). Probably $\Delta\psi m$ – dependent and independent mechanisms exist, differing with specific apoptotic stimuli (Zoratti and Szabo, 1995, Bossy-Wetzel *et al*., 1998).

The consistent observation of mitochondrial dysfunction prior to the nuclear changes associated with apoptotic cell death implies that it may be a critical regulator of the metabolic events involved in the apoptotic cascade (Deckwerth and Jonhson, 1993, Jacobson *et al*., 1994, Schulze-Osthoff *et al*., 1994, Petit *et al*., 1995, Vayssiere *et al*., 1995, Zamzami *et al*., 1995a, b, 1996a). Furthermore, in cell-free systems, mitochondria are a necessary component of the cytosolic fraction to produce apoptotic features in isolated nuclei (Newmeyer *et al*., 1994) and subsequent evidence revealed that only mitochondria undergoing the mitochondrial membrane permeability transition (MPT) are pro-apoptotic in this system (Zamzami *et al*., 1996b).

2.2.1. APOPTOSIS

Early pioneering studies of cell death delineated two major, morphologically distinct types, apoptosis and necrosis (Walker *et al*, 1988). Apoptosis, or programmed cell death, is an integral part of development and homeostasis, and hardwired into the genetic materials of cell that are destined to die. Often under pathological circumstances, such as in some neuro degenerative diseases and in stroke, the apoptotic program can be inappropriately implemented, resulting in detrimental cellular destruction (Ferri and Kroemer, 2001, Leist and Jaattela, 2001). This process requires energy and often even de novo macromolecular synthesis, and the specific biochemical steps involved in triggering

and executing apoptosis, as well as in removing the dead cell remnants generated by this process have been described in great detail (Hengartner, 2000, 2001).

Apoptosis is a term derived from the Greek word which means falling (or dropping) off in relation to petals from flowers or leaves from trees and it was first coined and used by Kerr *et al* in 1972. Apoptosis has been known to occur in multicellular organisms as a form of cell elimination occuring during development as well as in many physiologic and pathologic processes. A particular dramatic example of the physiologic role of apoptosis is seen in tissue remodeling during embryo development or metamorphosis such as when the tad pole loses its tail (Halestrap, *et al*, 2000). Apoptosis is also the mode of death for elimination of potentially self-reactive T-cells in the thymus (Nagata, 1997), and the common mechanism by which human tumor cells die, either spontaneously or in response to therapeutic agents (Kerr *et al*., 1994), or cell mediators such as nitric oxide (Cui *et al*, 1994).

Apoptosis has been proved as a genetically programmed process for cells to commit suicide in certain cases, it is an energy requiring process which plays a crucial role in tissue homeostasis often as a counter balance for cell proliferation and thus, must be strictly controlled/regulated as its deregulation often leads to malformations and diseases (Kerr *et al*, 1972). In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy (Thompson, 1995). Since apoptotic programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential drug targets. It is now well established that cancer agents induce apoptosis, and that distruption of apoptotic programs can reduce treatment sensitivity (Schmitt and Lowe, 1999). Anticancer agents induce apoptosis in normal tissue as well as in tumors. Pathologists who identified apoptosis in tumors have realized that apoptotic cell death was induced in a subset of normal tissues (e.g bone marrow and intestine) and it was suggested that the process might contribute to toxicity associated with chemotherapy (Searle *et al*., 1975).

Apoptosis occurs in cells destined for removal in a variety of normal situations, including larval development of the nematode C elegans, insect metamorphosis, development in mammalian embryos including the nephrogenic zone in the developing kidney, and regression or atrophy (e g in the prostate after castration) It follows the withdrawal of growth and trophic factors in many cells, nutritional deprivation, hormone treatment, ultraviolet irradiation, and exposure to toxic and infectious agents including reactive oxygen species and phosphate inhibitors e g Okadaic acid, calcium ionophores and a number of cancer chemotherapeutic agents In some cases, it appears that high concentrations of growth factors can counteract the effects of toxins (Wyllie, 1981, Ken *et al*, 1994, Raff *et al*, 1993)

2.2.1.1. THE MECHANISMS OF APOPTOSIS

There is more than a pathway to apoptosis, and these include its induction via 2 principal pathways One involving the ligation of death receptors, such as CD 95 (also known as the Fas/Apo-1) and tumor necrosis factor-receptor (TNF-R1), which on binding to the adapter protein FADD, (Fas-activated protein with death domain) recruit procaspase-8 into the death-inducing signaling complex (the extrinsic pathway) Another pathway that is triggered by a number of apoptotic stimuli (which may include moderate insult insufficient to kill the cell outrightly, but enough to cause significant cell damage such as anticancer drugs or irradiation) is essentially controlled at the mitochondrion (the intrinsic pathway) (Andrea Ren *et al*, 2001)

THE INTRINSIC/MITOCHONDRIAL PATHWAY

This is apoptosis triggered by internal signals The outer mitochondrial membranes of a healthy cell display the protein Bcl-2 on their surfaces. Bcl-2 inhibits apoptosis Internal damage to the cell (e g from ROS) causes related proteins, Bad and Bax, to migrate to the surface of the mitochondrion where they bind Bcl-2, blocking its protective effect and causing Cytochrome c release from the intermembrane space (Korsmeyer *et al*, , 2000, Alirnonti *et al*, 2001) The released cyt C binds to the protein Apaf-1 (Apoptotic protease activating factor-1) Using the energy provided by, ATP, these complexes aggregate to

form apoptosomes The apoptosomes bind to and activate caspase9 which is one of a family of over a dozen caspases (Li, *et al* , 1997, Green and Kroemer, 2005)

Caspases are all proteases which get their names because they cleave proteins, mostly each other at aspartic acid (Asp) residues (Thornberry and Lazebrik, 1998) Caspase 9 cleaves and, in so doing, activates other caspases (caspases-3 and -7), the executioner caspases creating an expanding cascade of proteolytic activity which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell (Alimonti, *et al* , 2003)

Shown below (Fig 8) is the classical apoptotic pathway Cells receive either a receptor-mediated or a non-receptor-mediated death signal to initiate the apoptotic pathway Constitutive upstream caspases (i e caspase 8) and pro-apoptotic Bcl-2 family proteins (i e Bid, Bax) are activated, resulting in a cascade of molecular events that act at the mitochondrion They can induce a loss of mitochondrial membrane potential ($\Delta\Psi m$) production of reactive oxygen species (ROS), permeability transition (PT) due to opening of the permeability transition pore, mitochondrial swelling and ultimately release of apoptosis – inducing factor (AIF) and cytochrome C Release of cytochrome C is a point of no return as cytochromic C forms a complex with caspase 9, Apaf-1 and dATP resuhing in the auto activation of caspase 9

Caspase 9 proceeds to cleave the downstream effector caspases (caspases 3, 6, e t c) that in turn act on many cellular proteins to give the typical biochemical and morphological features such as membrane blebbing and DNA fragmentation Some apoptotic pathways are able to induce cell death in a mitochondrial- independent manner that is not inhibited by Bcl-2 In these cases, proapoptotic upstream molecules can activate caspase-3 directly However, there is a feedback loop in which the activated caspase-3 acts on the mitochondrion to induce dysfunction at later stages of apoptosis (Alimonti *et al* , 2003)

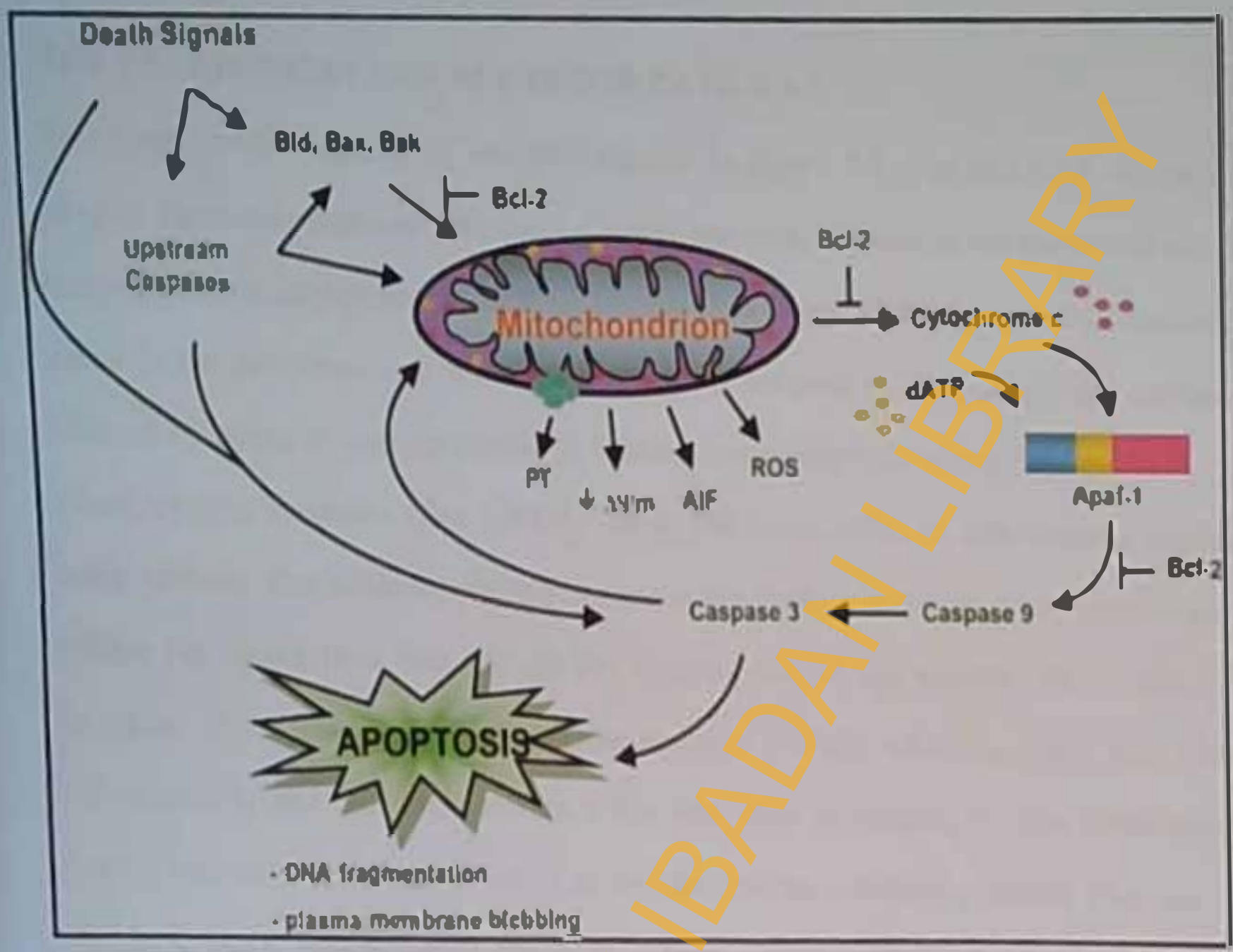


Fig. 2.8: The intrinsic apopoptotic pathway (Alimonti *et al.*, 2003).

THE EXTRINSIC/DEATH RECEPTOR PATHWAY

This is apoptosis triggered by external signals. In figure 2.9, Fas and TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell, binding of the complementary death activators (Fas L and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to phagocytosis of the cell.

There are five receptors (Fas, $TNFR_1$, DR3, DR4 and DR5) in the receptor-mediated death pathway. Fas-induced cell death requires the binding of either membrane-bound or soluble Fas ligand (m/s FasL) to the Fas receptor on the cell surface. This initiates the formation of the death-inducing signaling complex (DISC), which includes, Fas, FADD and caspase 8, and ultimately results in the activation of caspase 8. The interaction of FADD with caspase 8 can be blocked by the cellular inhibitory protein Flip and can therefore, block Fas-mediated apoptosis. There are two types of Fas-mediated death pathways. Type I is mitochondrial-independent and therefore not inhibited by the antiapoptotic protein Bcl-2. It involves the direct activation of effector caspase 3 by the activated caspase 8. In contrast, type 2 proceeds via the mithocondria, resulting in mitochondrial dysfunction and cytochrome C release, to eventually activate caspase 3. Since Bcl-2 functions primarily at the mitochondria, this pathway can be inhibited by Bcl_2 (Almonti *et al*., 2003).

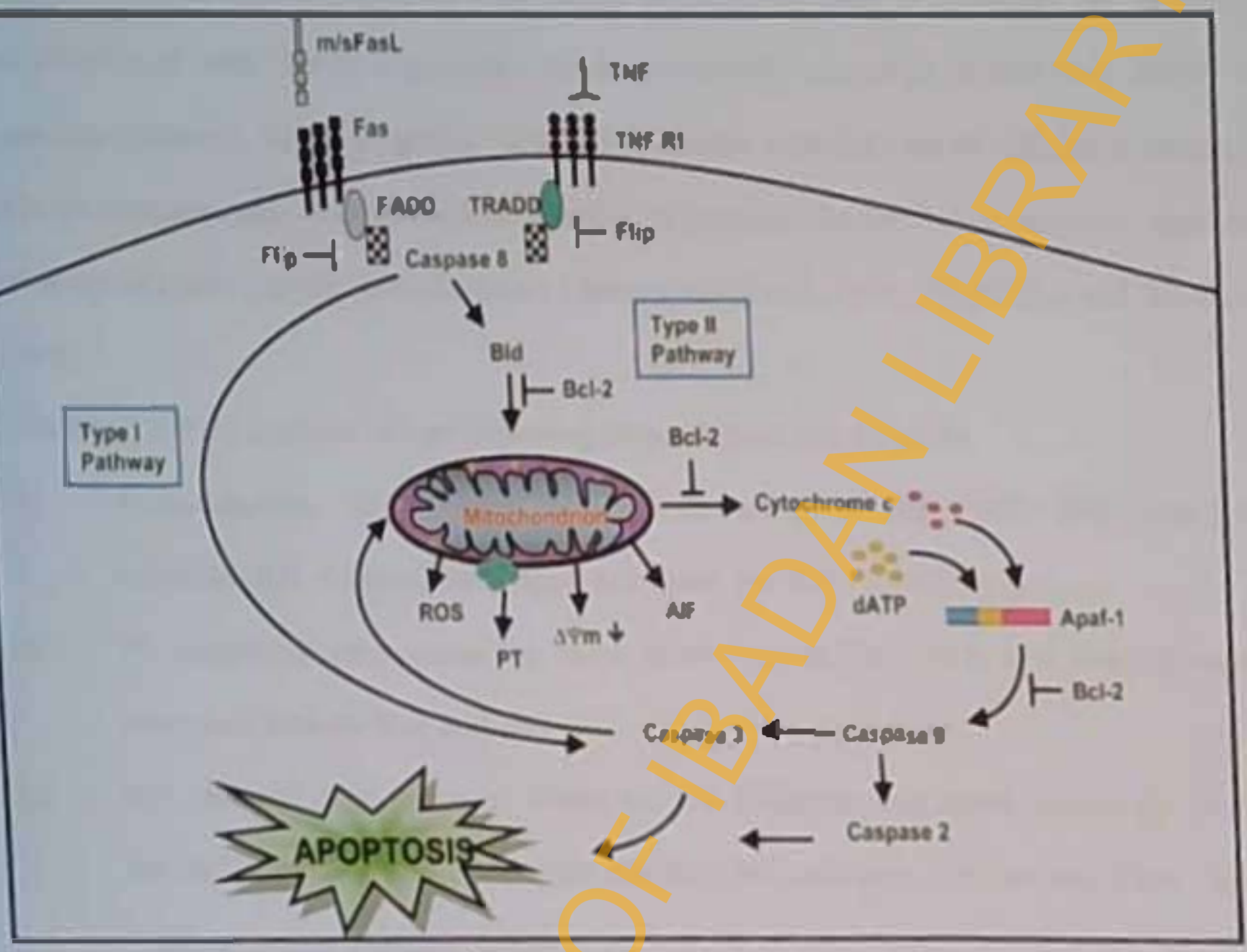


Fig. 2.9: The extrinsic apoptotic pathway (Alimonti *et al.*, 2003).

2.2.1.2. **PROTEIN EFFECTORS OF APOPTOSIS**

❖ **Bcl-2 family members**

Apoptosis is a highly regulated cell death mechanism that is required for selective elimination of cells. Various apoptotic signals eventually converge to activate a family of cysteine proteases called caspases, which then cleave a critical set of cellular proteins to initiate apoptotic cell death. The Bcl-2 family of proteins is a well characterized regulator of apoptosis that interact with caspases (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000).

The Bcl-2 family consists of the following three distinct sub-families;

(a) Anti-apoptotic. These share sequence homology at BH_1, BH_2, BH_3, and BH_4 domains (BH = Bcl-2 homology), examples are Bcl-2 itself and Bcl-xl.

(b) Pro-apoptotic. they share the same homology at BH_1, BH_2 and BH_3 domains, examples include Bax and Bak.

(c) BH_3 domain only proteins. These are pro-apoptotic, but share homology only at the BH_3 domain only. Examples are Bid, Bik and Bim (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000).

It has been shown that in addition to BH_1 and BH_2, the BH_4 domain is required for anti-apoptotic activity of Bcl-2 and Bcl-xl and that the BH3 domain of the pro-apoptotic members is essential and, itself, sufficient for pro-apoptotic activity (Adams and Cory, 1998; Green and Reed, 1998; Tsujimoto and Shimizu, 2000). Members of the Bcl-2 family form a group of proteins that play important roles in the regulation of cell death under both physiological and pathological conditions. Members of this family promote either cell survival, as in Bcl-2 and Bcl-xL, or cell death, as in the case of Bax and Bid (Smaili *et al*., 2000). In living cells Bax and Bid are predominantly soluble proteins (Hsu and Molday, 1994) and Bcl-2 is associated with the membranes of various organelles including endoplasmic reticulum, mitochondria, and nuclei (Krajewski *et al*, 1997) and

Bcl-xL exists in both soluble and membrane-bound forms (Hsu *et al*., 1997). During apoptosis, Bcl-2 remains bound to the membranes (Fig. 2.10) but the cytosolic forms of Bax, Bid and Bcl-xL have been found to redistribute from the cytosol into membranes, in particular mitochondrial membranes (Hsu *et al*., 1997, Li *et al*., 1998). The mechanism leading to Bax and Bcl-xL redistribution into the mitochondria are still unknown. Bid is believed to be post-translationally cleaved by caspase-8 to enable its translocation into mitochondria (Li *et al*, 1998).

X-ray crystallography and solution NMR analyses of the recombinant Bcl-xL have indicated that this protein shares a high degree of structural similarity to the translocation domain of diphtheria toxin (Muchmore *et al*., 1996) which can insert into bilayers (Kagan *et al*., 1981). This suggests that Bcl-2, Bcl-xL and Bax may form ion channels (Schlesinger *et al* 1997). *In vitro* studies have shown that the insertion of Bax causes the release of cytochrome c from mitochondria (Jurgensmeier *et al*., 1998). Cytochrome c has been proposed to interact with Apaf-1 (Zou *et al*, 1997). This complex in the presence of dATP, can catalyze the activation of caspases to proteolyze cellular constituents.

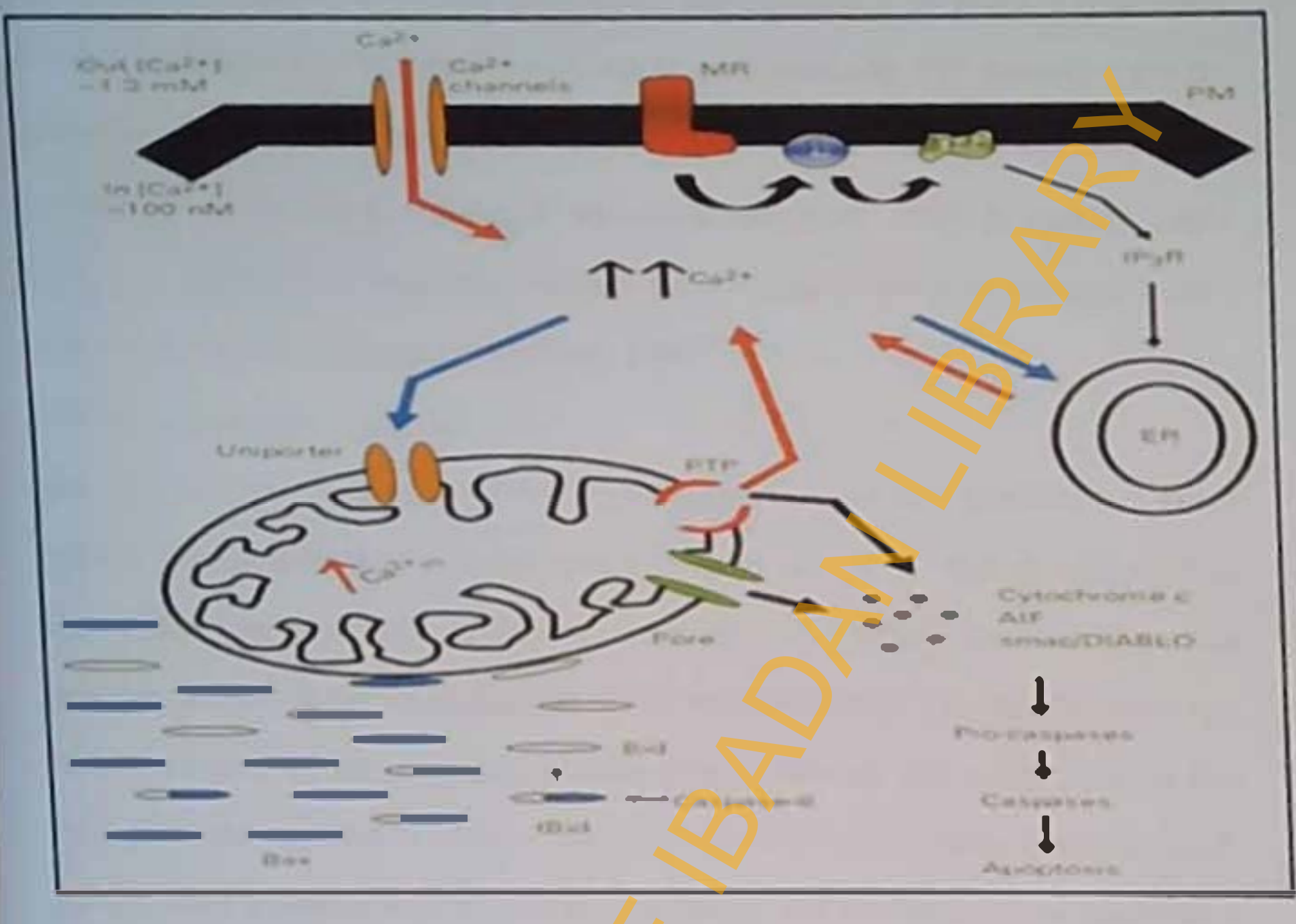


Fig. 2.10: Schematic representation of the apoptotic cascade and the interaction of Bax and Bid with mitochondrion (Smaili *et al.*, 2003).

Some reports have suggested that the release of cytochrome C is associated with Bax interaction with adenine nucleotide translocase (ANT) (Marzo *et al*, 1998), a component of the PTP, thus, release of cytochrome C can be associated with PTP activation and the attending loss of $\Delta\varphi m$ However, cytochrome C release can occur in the absence of permeability transition and collapse of $\Delta\varphi m$ (Goldstein *et al*, 2000) In addition, under special circumstances, permeability transition has been shown not to be involved in Bax-induced cytochrome C release (Eskes *et al*, 1998)

- **Bax as a proapoptotic protein**

Bax is a pro-apoptotic member of the Bcl-2 family and it was first identified as a Bcl-2 binding partner by immuno precipitation (Oltvai *et al*, 1993) Subsequently, it was shown that over expression of Bax can accelerate cell death in response to various apoptosis stimuli (Yang & Korsmeyer, 1996) Physiologically, Bax plays an important role in neuronal development and spermatogenesis Animals that are deficient in Bax have increased numbers of neurons and males are known to be sterile (Knudson *et al*, 1995) Under pathological conditions such as cerebral and cardiac ischemia, upregulation of Bax has been reported in the afflicted area of the tissues, implicating the participation of this protein in the promotion of neuronal and cardiomyocytic cell death (Krajewski *et al* 1997)

In certain cases of human colorectal cancer, mutations were found in the gene encoding Bax, suggesting that inactivation of Bax promotes tumorigenesis by rendering the tumor cells less susceptible to cell death (Le Blanc *et al*, 2002) Bax shares with other members of the Bcl-2 family the feature of having three conserved regions named BH domains 1-3 Several lines of evidence show that these domains can be important for the regulatory functions of these Bcl-2 family proteins In addition, Bax and a number of Bcl-2 family members also possess a hydrophobic segment at their C-terminal ends For Bcl-2, this hydrophobic segment is required to anchor the protein to various organelles.

including endoplasmic reticulum, mitochondria and nuclear outer membranes (Zha *et al* 1996). The 3-D structures of the Bax and its pro-survival antagonist Bcl-xL have recently been deciphered (Suzuki *et al*., 2000). These two proteins appear to share a significant structural homology with the translocation domain of diphtheria toxin, especially in a helical loop domain formed by α-helices 5 and 6. This particular domain of diphtheria toxin has been shown to penetrate the lipid bilayer to form pores (Muchmore *et al*., 1996, Kagan *et al*., 1981).

In healthy cells, Bax is predominantly a soluble monomeric protein (Hsu *et al*, 1997) despite the fact that it possesses a C-terminal hydrophobic segment which unlike those of Bcl-2 and Bcl-xL is sequestered inside a hydrophobic cleft (Suzuki *et al*., 2000). Upon induction of apoptosis by a variety of agents a significant fraction of Bax has been observed to translocate from the cytosol to the membrane fractions, in particular, the mitochondria (Hsu *et al*, 1997, Smaili *et al*, 2001). This translocation process appears to involve a conformational change in Bax, leading to the exposure of its C-terminal hydrophobic domain (Nechushtan *et al*., 1999). After translocation, Bax may form large oligomers once they reach the membrane.

These oligomers are associated with cytotoxic activities and have not been seen in other proteins such as Bid and Bad (Nechushtan *et al*., 2001). Deletion of the Bax C-terminal hydrophobic domain abrogated the ability of the mutant protein to translocate to mitochondria and greatly attenuated its ability to promote cell death. On the other hand, point mutations of Bax that target the expressed proteins to mitochondria greatly increased Bax toxicity (Nechushtan *et al*., 1999 & Nechushtan *et al*., 2001). The translocation of Bax to mitochondria is associated with the release of cytochrome C and the loss of Δψm (Jurgensmeier *et al*., 1998, Goldstein *et al*., 2000). These phenomena may be related to the observations that Bax can form ion channels or pores in mitochondrial membranes (Antonsson *et al*., 2000).

Cytochrome C activates caspase-3 leading to the proteolysis of the cell while the loss of Δφm corresponds to a decrease in cellular energy production. The pro-apoptotic activity of Bax, however, can be counteracted by co-expression with pro-survival factors Bcl-2 and Bcl-XL, which can block Bax translocation to the mitochondria during apoptosis (Vander Heiden *et al*., 1997). Smaili *et al*, (2003) using Cos-7 cells transfected with a GFP-Bax construct showed that it is possible to visualize that Bax is soluble in the cytosol of healthy cells and translocates to membranes upon apoptotic stimuli like staurosporine. It was also observed that the collapse of Δφm occurred just before the complete translocation of Bax to mitochondrial membranes (Smaili *et al*, 2001). In cells co-expressing Bax and Bcl-xL, staurosporine was not able to induce either Δφm collapse or Bax translocation.

- **BH3 domain proteins and cell death.**

The BH3 domain only proteins such as Bid, Bad and Bik are critical for heterodimerization with other family members. This interaction may be critical for death-regulatory activity. Caspase cleavage of truncated Bid (tBid) exposes the BH3 domain, causing its translocation to mitochondria and promoting cytochrome c release (Gross, *et al*., 1999). In neuronal cells, it has been shown that the BH3 domain causes a permeabilization of the outer membrane, leading to cytochrome c release without affecting inner membrane integrity or permeability transition. These effects may be dependent on the presence of Bax (Polster *et al*., 2001). The caspase-activated tBid is believed to trigger the homo-oligomerization of the pro-apoptotic family members such as Bak and Bax (Korsmeyer *et al*., 2000). This oligomerization can induce the release of cytochrome c (Wei *et al*., 2001). The precise mechanism by which cytochrome c is released is not understood, however, to a certain extent, release can be rescued by exogenous cytochrome c, showing a reversible component of mitochondrial respiratory dysfunction during apoptosis (Mootha *et al*., 2001).

Caspases

The process of apoptosis must be carefully controlled The characteristic morphological changes are downstream of interactions between two main groups of molecules (Caspases and the Bcl-2 family members) As there are interactions between the 2 groups, one group of molecules cannot be said to be downstream of the other

The morphological and cellular changes of apoptosis are due directly or indirectly to the action of a well conserved, highly specific and efficient family of cysteinyl aspartate-specific proteases called caspases (Thornberry and Lazebnik, 1998) Caspases are expressed as zymogens, the active enzymes are thought to be tetramers derived from 2-pro-caspase molecules, containing the large and small subunits (Cohen, 1997)

Cytochrome c (Cyt. c)

Cytochrome c is a peripheral membrane protein of known crystal structure that is loosely bound to the outer surface of the inner mitochondrial membrane It alternately binds to cytochrome c_1 of complex III and to cytochrome c oxidase (Complex IV) and thereby functions to shuttle electrons between them Cytochrome c's binding site contains several invariant Lys residues that lie in a ring around the exposed edge of its otherwise buried heme group Evidently, cytochrome c has negatively charged sites that are complementary to the ring of positively charged Lys residues on it (Voet and Voet, 2004) In 1996, Liu *et al*, made the critical observation that caspase activation by Apaf-1 in a cell-free system required dATP and cytochrome c Cytochrome c binds to Apaf-1, possibly at its c-terminal end, since a c-terminally truncated form of Apaf-1 no longer requires the cytochrome (Srinivasula *et al*, 1998)

The complex self-associates and recruits procaspases, which are then processed to their active forms These include caspases-4, 8, and 9, the later being recruited most strongly (Pan *et al*, 1998) The requirement of cytochrome c by an apoptotic pathway was the first incontrovertible evidence for the involvement of mitochondria in apoptosis Subsequent

work revealed that cytochrome c translocates from the mitochondria into the cytosol just a few hours into the apoptotic programme, e g after stimulation with Fas ligand, Tumor Necrosis Factor, staurosporine or withdrawal of growth factor (Reed, 1997, Luce *et al*, 1998)

Like most mitochondrial proteins, cytochrome c is encoded in the nucleus Apo-cytochrome c synthesized in the cytoplasm is imported in an unfolded state into the mitochondrial intermembrane space, here, the haem group is covalently attached, and the holoenzyme assumes its mature conformation Apo-cytochrome c is apoptoctically inactive Cytochrome c is the sole water-soluble cytochrome and acts as a mobile carrier of electrons between the bc1 complex and cytochrome oxidase It binds electrostatically to negatively charged surfaces of these complexes at the outer face of the inner membrane (Kannt *et al*, 1998) Since electrons flow rapidly down the respiratory chain, cytochrome c can associate and dissociate rapidly with each complex, and is not tightly bound to either It is normally restricted to the intermembrane space by the intergrity of the outer membrane It appears mitochondria are persuaded to lose their cyt c by the acuon of Bid, the BH3-only Bax interacting protein, which is a substrate of caspase-8 Wang's group showed that the 26,000 Mr cytosolic protein is cleaved in-vitro to produce a 15,000-Mr c-terminal fragment which binds to isolated mitochondria and brings about the release of cyt c (Luce *et al*, 1998) Bid's cleavage occurs in-vivo early in apoptosis (Li *et al*, 1998) It appears therefore, that caspase-8 activation of the DISC (Death-inducing signaling complex) can lead to Bid's cleavage and release of cyt c to the cytosol The mechanism by which cyt c translocates to the cytosol during apoptosis has not been elucidated in detail and is still a matter of debate

Much of the controversy has focused on the mode of action of the proApoptotic Bcl-2 family members such as Bid, Bak, and Bax which cause the release of cyt c The functions of the pro-apoptotic Bcl-2 members have been proposed to involve the

formation of pores in the outer mitochondrial membrane through which cytochrome c diffuses Other models suggest that these proteins affect channels in the outer or inner mitochondrial membranes, such as the permeability transition pore, thereby inducing hyperpolarization and permeability transition (Marchetti *et al*, 1996. Luo *et al* , 1998, Desagher *et al* , 1999, Jurgensmeier *et al* 1998, Eskes *et al* 2000) These events have been proposed to cause the entry of water and solutes, matrix swelling, and rupture of the outer membrane, which allows the passive release of cytochrome c However, it has been observed that in many cell types the release of cytochrome c occurs before or in the absence of a change in mitochondrial permeability (Goldstein *et al* , 2000), suggesting that this process involves additional or other mechanisms than opening of the permeability transition pore Probably, Aom-dependent and-independent mechanisms exist, differing with specific apoptotic stimuli (Zoratti and Szabo, 1995, Bossy-Wetzel *et al* , 1998)

PT pores are controlled by pro-and anti-apoptotic members of the Bcl-2 family of proteins, which can bind to these channels and regulate the release of cyt c into the cytosol (Shimizu *et al* , 1999, Marzo *et al* , 1998) The externalization of cyt c is a rapid and apoptosis-specific process because it was not observed in necrosis induced by diverse triggers (Andrea Renz *et al* , 2001)

- **Apoptosis Inducing Factor (AIF)**

The finding of other major pro-apoptotic factors in the intermembrane space has recently consolidated mitochondrial involvement in apoptosis These comprise a proportion of certain procaspases including procaspase-9 (Mancini *et al*, 1998, Susin *et al* , 1999a) and a 57,000 Mr apoptosis-inducing factor, AIF (Susin *et al* , 1996, Marchetti *et al* , 1996) Kroemer's group has identified AIF as a flavoprotein, showing sequence similarity to bacterial ferredoxin/NADH oxidoreductases, but its role as an oxidoreductase is obscure (Susin *et al* , 1999b)

A number of key observations establish the importance of AIF in apoptosis. Thus, in normal cells, AIF is restricted to mitochondria (immunofluorescene studies), but induction of apoptosis leads to AIF translocation to the cytosol and to the nucleus. When added to isolated nuclei, AIF brought about chromation condensation and DNA cleavage into large fragments (Susin *et al*, 1999a).

Recombinant AIF, without the flavin prosthetic group was similarly active, when injected into the cytoplasm of cells, AIF induced nuclear chromatin condensation. It also caused exposure of phosphatidylserine on the outside of the plasma membrane, a feature of apoptosing cells. When added to isolated mitochondria, AIF induced the release of cyt C and caspase-9. The latter finding suggests the possibility of a positive feedback loop (Susin *et al*., 1999b). Thus, like Cyt c, AIF seems to be bifunctional, with both oxidoreductase and apoptogenic functions (Crompton, 1999).

2.2.1.3. NON-PROTEIN EFFECTORS OF APOPTOSIS

- **Calcium ion (Ca^{2+})**

Calcium has the largest gradient of any chemical across the plasma membrane of all living cells. The concentration of calcium ions in extracellular fluids is in the millimolar range (10^{-3}M), by contrast, the calcium ion concentration in the cytosol is some 10,000-fold lower on the order of 10^{-7}M. This large concentration gradient is maintained by both the passive impermeability of the plasma membrane to calcium ions and by the active extrusion of calcium from the cell (Farber, 1990). A marked and prolonged increase in Ca^{2+} is harmful to cells because it leads to activation of calcium-dependent enzymes having potentially adverse effects, such as lipases, proteases, endonucleases and phosphatases. Also, cell calcium over load can cause mitochondrial failure which if irreversible can lead to cell death (Kristian and Siesjo, 1998).

Experiments utilizing nominally Ca^{2+}-free (2-5μM) medium and/or intra- or extra-cellular Ca^{2+} chelators showed that increases of $[Ca^{2+}]_i$ could result primarily from influx

from the extracellular space, redistribution from intracellular compartments, or from both influx and redistribution (Swann *et al*., 1991, Smith *et al*., 1992) Normally, $[Ca^{2+}]_i$ is tightly controlled in the range of 100nM and is regulated by transport systems in the plasmalemma (Carafoli, 1991), the mitochondria (Gunter and Pfeiffer, 1990) and the endoplasmic reticulum (Gill *et al*., 1989) Regulation of $[Ca^{2+}]_i$ can be affected by primary or secondary interactions involving each of these three main regulatory systems (Carafoli, 1987) and elevation of $[Ca^{2+}]_i$ are related to further influx (Putney and Bird, 1994, Randriamampita and Tsien, 1993, Berridge, 1993)

Mitochondria modulate the free cytosolic calcium concentration during and following intense activation of calcium conductances in plasma membranes (Friel and Tsien, 1994, White and Reynolds, 1996, White and Reynolds, 1997, Wang and Thayer, 1997) At steady state, there is a balance between influx of Ca^{2+} across the mitochondrial membrane Mitochondria start to accumulate calcium when the cytosolic calcium concentration rises over a "set point" (about 500nM) (Gunter and Pfeiffer, 1990) Each increament (Maintained) in resting cytosolic $[Ca^{2+}]_i$ gives rise to a proportionally greater increase in mitochondrial $[Ca^{2+}]$ until, at about 1 - 3μM cytosolic Ca^{2+}, mitochondrial Ca^{2+} overload occurs (the mitochondrial Ca^{2+} content tends towards infinity) (Nicholls, 1978)

The mitochondrial outer membrane is believed to be freely permeable to ions and molecules up to 5000Da, while the inner membrane is tightly sealed to all ions but for the presence of specific transporters The uptake of Ca^{2+} into the mitochondrial matrix under physiological conditions does not depend on ATP hydrolysis but rather on the presence of a so called "Ca^{2+} uniporter" (presumably, a gated channel) and the driving force is provided by the negative (in the matrix) membrane potential generated by the respiratory chain (Pozzan, and Rizzuto, 2000) This uptake mechanism serves to support Ca^{2+}-sensitive mitochondrial enzymes such as the key regulatory enzymes of oxidative

metabolism, namely pyruvate dehydrogenase, oxoglutarate dehydrogenase and isocitrate dehydrogenase (Goldstone *et al*., 1987) and acts as a mechanism for removal of $[Ca^{2+}]_i$ from local areas of the cell. Ca^{2+} efflux from mitochondria into the cytosol occurs via at least two different mechanisms

1. The Na^+ - dependent Ca^{2+} efflux which probably has a stoichiometry of $3Na^+ : 1\ Ca^{2+}$ (Pfeiffer *et al*., 2001) and is therefore electrogenic and favoured by the transmitochondrial membrane potential. This seems to be a dominant Ca^{2+} efflux mechanism in skeletal muscle, nervous system, heart and endothelial cells (Sedova & Blatter, 2000, Thayer *et al*., 2002). The Ca^{2+} extrusion through the Na^+ Ca^{2+} exchanger is accompanied by the accumulation of matrix Na^+ which is then extruded by the Na^+-H^+ exchanger thus completing the Ca^{2+} cycle and restoring ionic gradients (Kaftan *et al*., 2000)

2. Na^+ - independent Ca^{2+} efflux is probably directly coupled to H^+ entry with the stoichiometry of $nH^+ : Ca^{2+}$, where n is probably >2 (Pfeiffer *et al*., 2001). It was demonstrated that this exchange plays a significant role in liver and smooth muscle mitochondria (Bernardi, 1999). It is a non-electrogenic exchanger present also in kidney mitochondria, which behaves as an active $Ca^{2+}/2H^+$ exchanger (Gunter *et al*., 1998). Ca^{2+} could also be released from mitochondria through the permeability transition pore (PTP) (Rizzuto *et al*., 2000). The PTP is a channel located in the inner membrane which could be a pathway for Ca^{2+} efflux from the mitochondria (Gunter *et al*., 2000, Smaili *et al*., 2000). It is believed to have both low-and high-conductance states (Bernardi, 1999). Opening of the large-conductance PTP channel is enhanced by a reduction in $\Delta\varphi_m$, and is stimulated by elevated matrix $[Ca^{2+}]$ and increased matrix pH. Since this happens during Ca^{2+} overload, a large conductance mode of the PTP channel is therefore, thought to contribute to the collapse of $\Delta\varphi_m$ and release of mitochondrial factors that trigger apoptosis. Cytosolic Ca^{2+} has been proposed to play an important role in the triggering of

apoptotic signals in the regulation of cell death-specific enzymes such as endonucleases (McConkey & Orrenius, 1997)

Recent studies have shown that Ca^{2+} stores in the endoplasmic reticulum may play a role in apoptotic induction, since a reduction of Ca^{2+} release from the endoplasmic reticulum can prevent mitochondrial damage Endoplasmic reticulum Ca^{2+} release can also sensitize mitochondria to release cytochrome C, and pro-apoptotic proteins such as Bax and Bak have been suggested to play a role in this process (Nutt *et al*., 2002) Calcium signals have been identified as one of the major signals which converge on mitochondria to trigger the mitochondrial-dependent pathway of cardiac apoptotic cell death (Pacher *et al*., 2001) Currently, it is not clear what roles Bcl-2 family members play in Ca^{2+} signaling during cell death Some investigators have shown that they play a modulatory role, since over expression of these proteins can change Ca^{2+} concentration in the cytosol or in the intracellular stores

Overexpression of Bcl-2 has been reported to prevent Ca^{2+} release from the endoplasmic reticulum in response to apoptotic inducers such as thapsigargin (Baffy *et al*., 1993) Also, it has been shown that over expression of Bax in cultured cells caused a loss of endoplasmic reticulum content (Pan *et al*, 2001) tBid has also been associated with Ca^{2+} signaling and propagation to mitochondria It is possible that tBid induces a selective permeabilization of the outer mitochondrial membrane to Inositol triphosphate-induced mitochondrial Ca^{2+} - signaling (Csordas *et al*., 2002) This change in mitochondrial permeability might be related to the tBid-induced remodeling of mitochondrial structure that evokes a mobilization of the cytochrome C stores (Scorrano *et al*., 2002)

Ca^{2+} is the fundamental PT pore activator in almost all reports in the literature, with the only exception being PT pore opening in single immobilized mitochondria induced by intramitochondrially generated reactive oxygen species (Huser *et al*., 1998) In tramitochondrial Ca^{2+} activates the PT pore by binding to low affinity sites, i e Kd=

25μM (Al Nasser and Crompton, 1986), and increasing to Kd > 200μM in the presence of ADP (Halestrap *et al*., 1997). Since intramitochondrial free Ca^{2+} is normally maintained below 10μM, it is clear that severe mitochondrial Ca^{2+} overload is needed for pore activation. When basal (resting) cytosolic free Ca^{2+} rises, the mitochondrial Ca^{2+} cycle would be expected to produce mitochondrial Ca^{2+} overload.

Resting cytosolic [Ca^{2+}] increases during ischaemia, and increases further and more abruptly on reperfusion, when this is associated with injury. These changes have been extensively documented in particular in heart and brain (Chein and Engler, 1990, Piper *et al*., 1993). Cytosolic free [Ca^{2+}] in whole organs, such as heart can be measured by ^{19}F-NMR of the Ca^{2+} indicator, 5-fluorobis-(O-aminophenoxy) ethane- N,N, N^1,N^1, - tetra-acetic acid ("5F-BAPTA") (Steenbergen *et al*., 1990).

In perfused hearts, the rise in Ca^{2+} begins when about two third of cell ATP has been depleted, and reflects the failure of Ca^{2+} pumps in the plasma membrane and sarcoplasmic reticulum as the cytosolic phosphorylation potential falls. Intracellular acidification (from lactate) also contributes, by leading to increased intracellular Na^+ (plasma membrane Na^+/H^+ exchange)and consequently, impaired plasma membrane Na^+/Ca^{2+} exchange. This becomes pronounced on reperfusion, when the acidic extracellular fluid is washed out, leading to high pH gradient across the plasma membrane (Scholz, and Albus, 1993).

In early stages, the rise in the resting cytosolic free [Ca^{2+}] in ischaemia (heart) or anoxia (isolated myocytes) is promptly restored to low physiological levels on reperfusion/reoxygenation, indicating that the rise in [Ca^{2+}] precedes cell death (Allshire *et al*., 1987, Steenbergen *et al*., 1990, Miyata *et al*., 1992).

Cobbold and co-workers (Allshire *et al*., 1987), using cardiomyocytes loaded with acquorin as Ca^{2+} indicator, made the important observation that reoxygenation only restores low resting cytosolic [Ca^{2+}] if a critical limit of 1-2μM Ca^{2+} is not exceeded

When cytosolic free [Ca^{2+}] rose above this limit during anoxia, then reoxygenation failed to re-establish Ca^{2+} homeostasis, and cell death ensued This limit is remarkably close to the set point the resting cytosolic [Ca^{2+}] that produces mitochondrial Ca^{2+} overload, suggesting that mitochondrial Ca^{2+} overload and PT pore activation may be a precondition of this form of cell death (Crompton, 1999)

Oxidative Stress

It has been suggested that reactive oxygen species (ROS) play an important role in apoptosis, and several groups have shown that molecules that stimulate formation of ROS can result in apoptosis (Hensler *et al*., 1999, Kelso *et al*., 2001) and a process inhibited by antioxidants (Koren *et al*., 2001, Chrestensc *et al*., 2000) Others reported production of ROS by a wide range of apoptotic stimuli (Ruiz *et al*., 1997, Cai and Jones, 1998, Gottlieb *et al*., 2000 and Shaulian *et al*., 2000)

Under normal conditions, electron transport in the mitochondrial respiratory chain creates both a H^+ gradient across the inner mitochondrial membrane and an electrical potential, the inside of the mitochondria being negative During this process, ROS are produced up to 5% of the oxygen reduced is converted by complex I to superoxide (O^-_2) (Cadenas, 1989) It is known that the PTP can be induced by ROS probably due to dithiol cross-linking (Kowaltowski, 2001) Thus, increased production of superoxide favors the activation of a mitochondrial PT pore

It appears that this pore can act at least in two different levels of conductance and reversibility At low level of conductance, the MPT pore opening is reversible and does not entail a large amplitude swelling of mitochonorial matrix, although it does cause a collapse of the Δψm (Ichas and Mazat, 1998) At high level of conductance, the MPT pore opening is irreversible and leads to large amplitude swelling of the mitochondrial matrix During this event, cyt c and the apoptosis inducing factor (AIF) are released from

mitochondria (Kantrow and Piantados. 1997, Susin *et al*., 1997, Zanizani *et al*., 1997, and Kluck *et al*., 1997)

At high oxygen concentrations, the diminished availability of reduced cofactors of the respiratory chain and a high $\Delta\psi m$ tend to increase the mitochondrial radical formation, which is substantially enhanced in the presence of defects within the respiratory chain (Turrens, 1997) Superoxide is produced by mitochondria due to a switch from the normal four-electron reduction of oxygen to a one-electron reduction (Cai and Jones, 1998, Skulachev, 1998) The mitochondrial-derived ROS are vital not only because mitochondrial respiratory chain components are present in almost all eukayotic cells, but also because ROS produced in mitochondria can readily influence mitochondrial function without having to cope with long diffusion times from the cytosol (Li *et al*., 2003) Two sites in the respiratory chain complex I and complex III, have been suggested to be the major ROS sources (Turrens and Boverris, 1980, Turrens, *et al*., 1985, Turrens, 1997) Based on stoichiometrical calculations, superoxide was suggested as the primary product with hydrogen peroxide as the secondary product (Cadenas *et al*., 1977)

Superoxide is converted to H_2O_2 by superoxide dismutase resident in both intracellular and extracellular components (Marklund, 1988) H_2O_2 readily diffuses across cell membranes It has been shown that peroxides induce PT pore opening in isolated mitochondria provided Ca^{2+} is present, peroxides alone are ineffective (Crompton *et al*., 1987, Crompton and Costi, 1988) The requirement for both oxidant stress and Ca^{2+} for PT pore opening in isolated mitochondria implies a similar dual requirement in-vivo This question has not been addressed, but peroxide-induced oxidative stress is typically associated with Ca^{2+} over load (Nicotera *et al*. 1992)

Oxidant-stress-induced pore opening is readily reversible Thus, pore is fully reversed on restoration of normoxia (Crompton and Andreeva, 1993) Mitochondria lack catalase and the H_2O_2 is reduced by GSH (glutathione peroxidase) (Olafsdottir and Reed, 1988)

Oxidative stress could be enhanced by the oxidation of GSH and pyridine nucleotides through the sequential actions of glutathione peroxidase, glutathione reductase and pyridine nucleotide transhydrogenase (Niemenen *et al*., 1997). From this, it appears that pore activation is probably mediated via oxidation of the GSH, NADPH or NADH pools. Early reports showed that the complex I inhibitor rotenone and the complex bc1 inhibitor, antimycin could stimulate superoxide and hydrogen peroxide formation on submitochondrial particles (Turrens & Boveris, 1980, Turrens *et al*, 1982).

Mitochondria respond to this radical –induced oxidative stress with a defined antioxidant defense system, enzymatic antioxidant systems (mitochondrial super oxide dismutase and the glutathione redox system) (Radi *et al*, 1991). If the enzymatic scavengers are exhausted, another more potent mechanism takes place, mild uncoupling mediated by uncoupling proteins (Skulachev, 1998). If this still does not work and there is prolonged oxidative stress, such that a certain $\Delta\varphi_m$ decline is reached due to mild uncoupling, a reversible opening of the permeability transition pore (PTP) can occur.

This process increases the permeability of the inner mitochondrial membrane for solutes up to 1500 Da and results in a much greater decrease in $\Delta\varphi_m$ than during the previous steps. Further prolongation of oxidative stress usually result in an irreversible PT pore opening when $\Delta\varphi_m$ is completely dissipated and oxidation uncoupled from phosphorylation (ATP synthesis stops). The next step is the starting point of apoptotic process, if a sufficient number of mitochondria follow this path and release Cyt c (Skarka and Ostadal, 2002).

2.1.4. MORPHOLOGICAL CHANGES DURING APOPTOSIS

The process of apoptosis involves some morphological changes (Fig. 2.11), and though it was thought initially that only nuclei but not cytoplasmic organelles undergo major modifications (Kerr *et al*., 1972), large evidences have been accumulated indicating that all cellular compartments are concerned, with the mitochondria being early affected (Ferri and Kroemer, 2001).

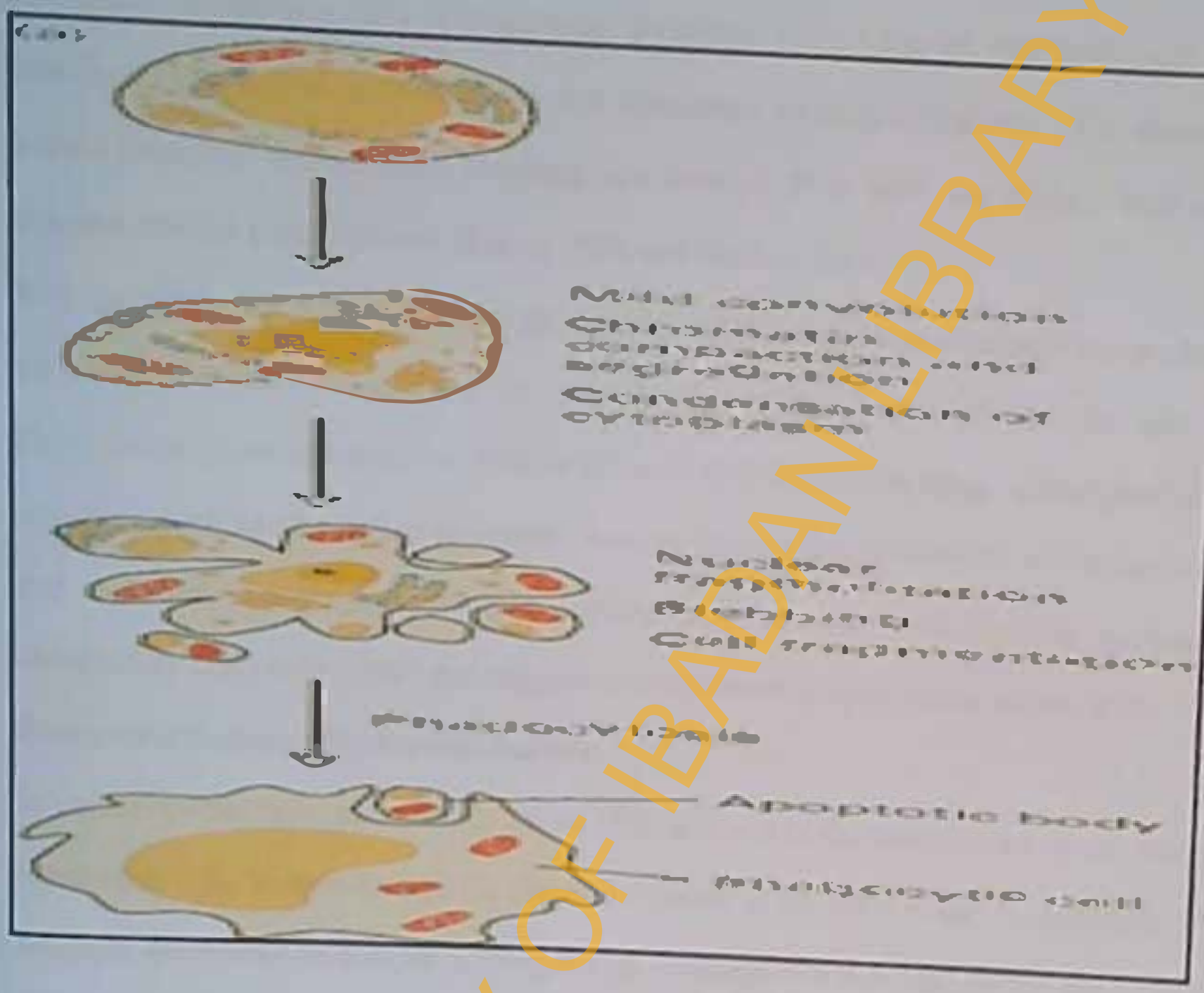


Fig 2.11: Schematic diagram of the morphological changes in apoptosis (www. aibas. org, retrieved March 27, 2008).

Some of the morphological changes characterized during apoptosis are listed below (I) Phosphatidyl serine externalization (II) Aggregation of compacted chromatin to the wall of nuclear membrane (III) Cytoplasmic shrinkage (IV) Loss of microvilli and gap junctions (V) Nuclear fragmentation and dispersion through cytoplasm (VI) apoptotic bodies (vesicles) (cytoplasmic blebbing) are formed If *in vivo*, the bodies "bud off," disperse and are phagocytosed (Lincz, 1998 and Hacker, 2000)

It is important to note that although apoptosis refers to characteristic morphology, variation can occur, depending on stimuli, the energy status of the cell and its environment (most extremely *in vivo* or *in vitro*) and though the range of morphological changes which can be called apoptosis must be limited (by definition), it is a spectrum and is broad enough to encompass observations such as mitochondrial exclusion, observed by Chiu *et al*, 2000 and suggested to represent a novel mode of cell death

1. **Phosphatidylserine (PS) Externalization:**

In non-apoptotic cells, phosphatidylserine (PS) is found in the inner leaflet of the plasma membrane The externalization to the outer leaflet is an early event in apoptosis PS externalization may be a signal to phagocytes, although this has been shown not to be an absolute requirement for macrophage recognition PS acts as a pro co-agulant, an effect that is reversed when it is bound to annexin V Annexin V is used to identify cells (Lincz, 1998)

2. **Cytoplasmic Shrinkage:** The shrinkage/condensation of the cytosol is presumed to result from water efflux, possibly accompanying K^+ and Cl^- loss through activated channels in the plasma membrane, although the details of this mechanism have not been elucidated (Trump and Berezesky, 1995) One possibility is that increased $[Ca^{2+}]$ induce activation of Ca^{2+} - dependent chloride channels, established as an important mechanism for cell volume regulation

3. **Cell junction changes:** these include separation of gap junctions leading to decreased or absent cell-cell communication and modification of desmosomes resulting from modification of intermediate filaments, all due to increased $[Ca^{2+}]_i$. Such disruption of junctions often results in remarkable shape changes in the cell as well as modifying trans-epithelial transport properties (Trump and Berezesky, 1995)

4. **Cytoplasmic blebbing:** In apoptosis, the morphological changes involve cytoplasmic blebbing, nuclear chromatin clumping and condensed mitochondria in the early phase. In the early reversible phase of apoptosis, one of the most obvious cellular changes is the formation of cytoplasmic blebs that often form rapidly at the cell surface (Nicotera, *et al*., 1986, Lennosters *et al*., 1987, Phelps *et al*., 1989). Bleb formation involves altered interactions of cytoskeletal elements with the plasmalemma. In several cell injury models, it has been observed that bleb formation occurs at a threshold of approximately 300-400nM $[Ca^{2+}]_i$ (Phelps *et al*., 1989, Smith *et al*., 1992).

Blebs in apoptosis contain a variety of organelles, such as mitochondria, ER, and peroxisomes. The blebs commonly pinch off and detach and are later phagocytosed by adjacent cells or sloughed off into extracellular space. It has been speculated that the disruption of the cytoskeletal-membrane interactions induced by bleb formation is due to Ca^{2+}-activated neutral protease degradation of cytoskeletal membrane-associated proteins (Nicotera *et al*., 1986, Elliget *et al*., 1991), or to Ca^{2+}-activated phospholipase activity and release of fatty acids (Trover *et al*., 1986). The calpains comprise a large group of isoenzymes with at least six distinct members. These proteins act as mediators of Ca^{2+} signals and may activate or modify other proteins including those of the cytoskeleton and protein kinases (Saido *et al*., 1994). Some of the alterations in the conformation of the cytoskeleton e.g. depolymerization of microtubules, result directly from increases of $[Ca^{2+}]_i$ while others may result from Ca^{2+}-activated protease

degradation of actin-binding proteins, which link actin to the plasma membrane (Bronk and Gores, 1993)

5. Nuclear changes In early stages of apoptosis, the cell undergoes marked cytosolic shrinkage and densification, widespread blebbing of the cytoplasm and nucleus (Hawthrop *et al*, 1991) and some mitochondrial condensation These changes occur early and are associated with clumping and densification of the nuclear chromatin This is associated with changes in the nuclear matrix (i e DNA condenses and marginates to the inside of the nuclear membrane The mechanism for these changes is not known but may be due to cleavage of nuclear lamina and nuclear scaffold attachment factor (Lincz, 1998), which normally appears to serve as an active site for regulating many genomic functions including DNA replication, gene transcription and RNA processing

Changes in the nuclear matrix can be detected by several monoclonal antibodies including those to (NUMP) Nuclear matrix protein and (NUMA) Nuclear Mitotic apparatus protein (Cohen *et al*, 1992, Yang *et al*, 1992) Chromatin fragment and depending on the cell type and interval after initiation, a "DNA ladder" pattern may be seen in acrylamide gels, resulting from double-stranded internucleosomal breaks (Activation of endonucleases that preferentially degrade DNA at the internucleosomal section is a very characteristic event of apoptosis (Arends *et al*, 1990 and Cromption, 1992)) While this pattern is readily induced in thymocytes after various stimuli, studies have shown that DNA breakdown may follow different patterns (Arends *et al*, 1990, Hawthrop *et al*, 1991, Gerschenson and Rotello, 1992, Evans, 1993)

In some cases the process proceeds much more slowly, with an ordered series of changes commencing with the formation of DNA fragment of 300kbp that are then degraded to 50kbp and finally to 10-10kbp Inhibitors of the serine proteases block the degradation of the large fragments In still other cases, other forms of DNA degradation not involving strand breaks have been shown to occur In spite of the prominence of DNA damage

during apoptosis, there is mounting evidence that this may be a later event because it is preceeded by the morphologic changes Moreover, inhibition of DNA fragmentation does not prevent cell death (Cohen, *et al*., 1992), and identical changes can be induced in nucleus-free cytoplasts (Raff, *et al*., 1993) Thus, while DNA fragmentation is a common feature of apoptosis, cell death by this mechanism can and does occur without it (Nagata, 2000)

2.2.2 NECROSIS

Necrosis, the second type of cell death is radically different from apoptosis in almost every respect The term derives from the Greek kernel "Necros", meaning 'dead' (with a sense of dismay), and refers to the accidental death of cells exposed to extreme environmental or genetically encoded insults (Walker, *et al*., 1988) Injured cells undergoing necrosis display gross morphological and ultrastructural features that contrast sharply with those exhibited by cells undergoing apoptosis Death is accompanied by extensive swelling of the cell, distension of various cellular organelles, clumping and random degradation of nuclear DNA, extensive plasma membrane endocytosis and autophagy (Hall, *et al*., 1997, Ferri & Kroemer, 2001)

Necrosis is generally considered to be a passive process because it does not require new protein synthesis, has only minimal energy requirements, and is not regulated by any homestatic mechanism In humans, necrotic cell death occurs generally in response to severe changes in physiological conditions, including hypoxia, ischemia, hypoglycemia, toxin exposure, to reactive oxygen metabolites, extreme temperature changes and nutrient deprivation (Walker *et al* 1988, Nicotera *et al* 1999) Several neurodegenerative syndromes and diseases, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (Price *et al*., 1998) and epilepsy, also involve necrosis Necrosis affects a group of cells rather than individuals as is the case during apoptosis (Majno and Joris, 1995, Levin, 1998)

It is characterized by a disruption of the cellular membrane and a swelling of the cytoplasm and mitochondria early on which culminate in the complete disintegration of organelles The process ends with total cell lysis Biochemical features of necrosis include loss of regulation of ion homeostasis, random digestion of DNA and DNA fragmentation after lysis Severely damaged cells do not form membrane-bound vesicles (Apoptotic bodies) and thus release their cellular contents which include proteolytic enzymes This normally results in inflammatory reactions with oedema and damage to surrounding cells (Aderem and Underhill, 1999)

2.2. DIFFERENCES BETWEEN APOPTOSIS AND NECROSIS

While an active participation of the affected cell, often involving *de novo* protein synthesis, characterizes apoptosis, necrosis is a passive and degenerative process Necrosis generally represents a cell's response to gross injury and is frequently induced by an overdose of cytotoxic agents The early event of necrosis is mitochondrial swelling followed by rupture of the plasma membrane and release of cytoplasmic constituents, which include proteolytic enzymes This process triggers an inflammatory reaction in the tissue and often results in scar formation DNA degradation is not so extensive during necrosis as in the case of apoptosis, and the products of degradation are heterogenous in size, not forming any discrete bands on electrophoretic gels as do those of apoptosis (Wyllie, 1992)

The main differences between Apoptosis and Necrosis are summarized below in table (2 1) and figure (2 12) below

TABLE 2.1: TABLE SHOWING THE DIFFERENCES IN APOPTOSIS AND NECROSIS.

Necrosis	Apoptosis
Morphological features	
• Loss of membrane asymmetry	• Membrane blebbing, but no loss of integrity
	• Segregation of chromatin of the nuclear membrane
• Begins with swelling of cytoplasm and membranes	• Begins with shrinking of cytoplasm and condensation of nucleus
• Ends with total cell lysis	• Ends with fragmentation of cell into smaller bodies
• No vesicle formation, complete lysis	• Formation of membrane-bound vesicles (apoptotic bodies)
• Disintegration into many irregular sized bodies	• Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family
Biochemical features	
• Loss of regulation of ion homeostasis	• Highly regulated process involving activation and enzymatic steps
• No energy requirement (passive process also occurs at 4°C)	• Energy (ATP)-dependent (active process does not occur at 4°C)
• Random digestion of DNA (smear of DNA after agarose gel electrophoresis)	• Non-random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis)
• Pathologic DNA fragmentation is the result of death	• Preapoptotic DNA fragmentation
	• Release of various factors (e.g. cytochrome C, AIF) into cytosol from mitochondria
	• Activation of caspase cascade
	• Rapid transmembrane asymmetry (i.e. translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)
Physiological significance	
• Affects groups of contiguous cells	• Affects individual cells
• Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemia, metabolic poisons)	• Induced by physiological stimuli (lack of growth factors, changes in hormonal environment)
• Phagocytosis by macrophages	• Phagocytosis by adjacent cells or macrophages
• Significant inflammatory response	• No inflammatory response

www.pathguy.com., retrieved March 27, 2008.

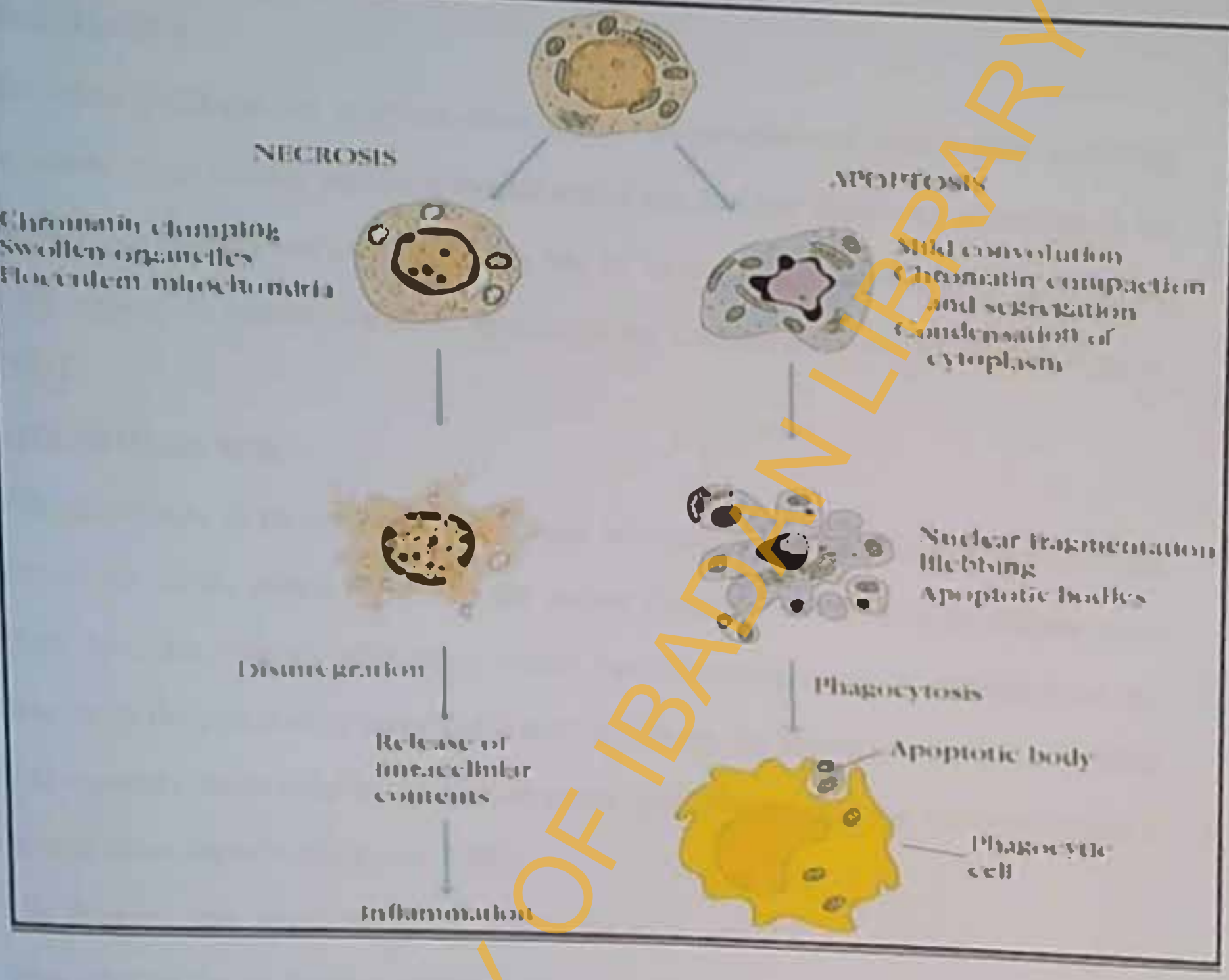


Fig. 2.12: Comparism of the Morphorlogical Changes between Apoptosis and Necrosis (www. aibns. org., retrieved, March 27, 2008).

3. DESCRIPTION OF THE REPRODUCTIVE SYSTEM.

3.1. THE TESTES

The testes (male glands) in all mammals are paired encapsulated, void organs consisting of seminiferous tubules separated by interstitial tissue Their sizes vary according to the species In rodents and ungulates, they can be as much as one percent or more of the body weight In human and some apes, they are considerably smaller (Harcourt *et al*, 1981)

3.2. THE EPIDIDYMIS.

The epididymis is an extremely convoluted structure, which is closely attached to the dorsal part of the lateral surface of the testicle (Setchell, 1977) It is an efferent route from the testis (ductuli afferentes), which has a tortuous canal It extends from the anterior to the posterior of testis and is held to it firmly by connective tissues It is filled into segments demarcated by connective tissue septa and the organ is contained within a fibrous tissue capsule (Oyeyemi. 2000)

The segment into which ductuli efferentes empty is called the initial segment and the remainder of the epididymis is loosely defined into three parts viz. The caput (Head), corpus (Body) and caudal (Tail) epididymis An alternative subdivision based on histological and functional criteria has been proposed (Glover and Nicander, 1971) The epididymis is divided into three parts, initial, middle and terminal segments The initial and middle segments are primarily concerned with sperm maturation while the terminal segment is where the matured sperm are stored prior to ejaculation or voidance into the urine In certain rodents such as rat, large accumulation of fat surrounds the proximal regions of the epididymis (Glover and Nicander) Changes occurring in sperm during epididymal migration have been correlated with the function and integrity of the testes and the epididymis (Rao, 1971)

3.3. THE SEMINAL VESICLES

The seminal vesicles are paired, bag shaped glands in man, stallion, rat and guinea pig, although the internal surface may be thrown into an intricate system of fold to form irregular diverticula In other mammals such as the bull, ram, and boar, the seminal vesicles consist of component glandular tissue arranged in multiple lobes and containing a system of ramified secretory ducts (Mann and Wilson, 1949)

3.4. HISTOLOGY OF THE MALE REPRODUCTIVE SYSTEM.

2.3.4.1. HISTOLOGY OF THE TESTIS

The Testis is a double gland organ that has both exocrine and endocrine function The exocrine product is chiefly the sex cells, and thus the testis may be referred to as a cytogenic gland, while the endocrine product is an internal secretion by certain specialized cells The testis is suspended within the scrotum and is immediately surrounded by the testicular capsule, which is composed of three layers, viz

- The outer layer, or tunica vaginalis
- The middle layer or tunica albuginea
- The innermost layer or tunica vasculosa

The histology of normal testis is shown in Fig 2 13

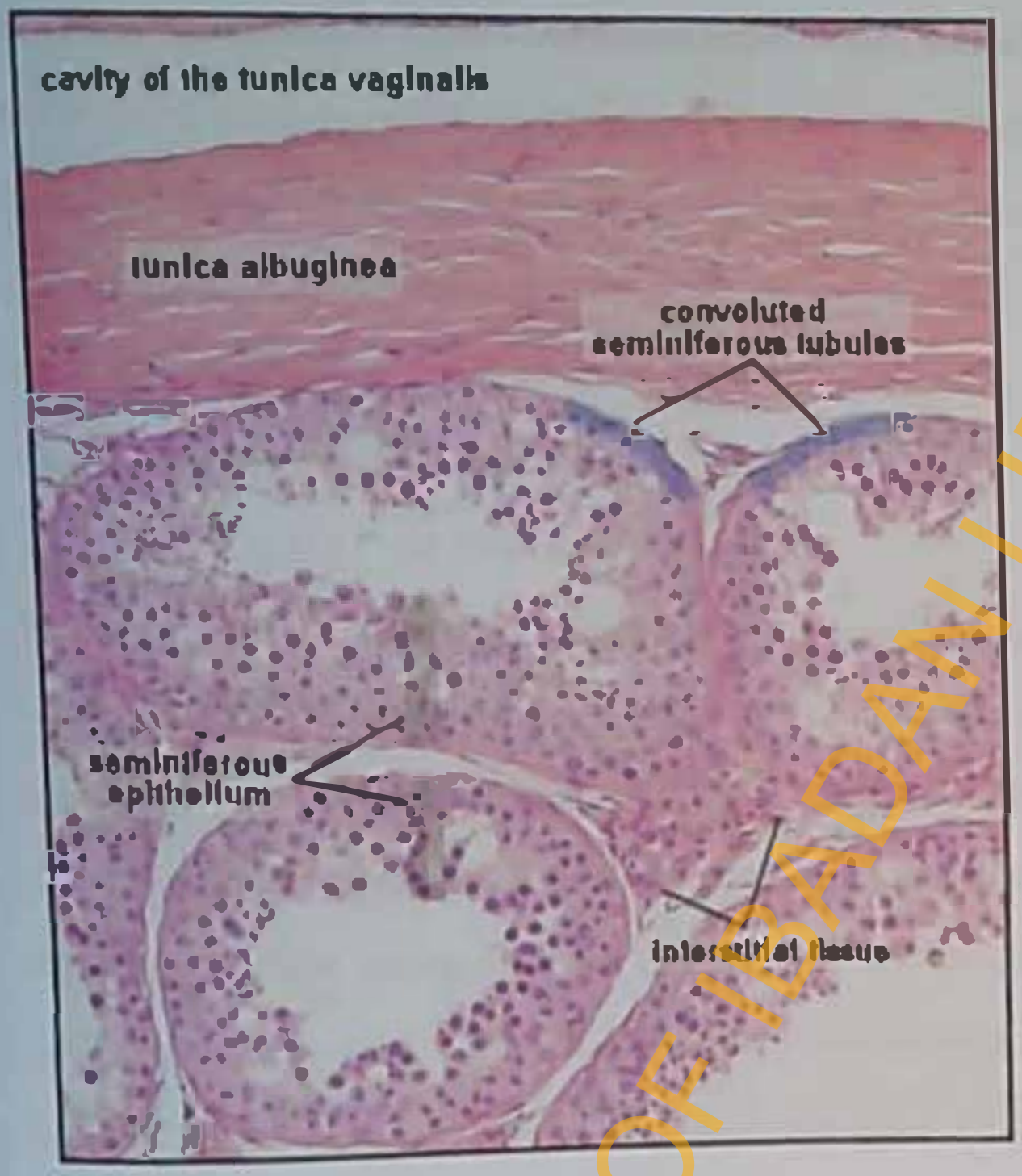


Fig. 2.13: Histology of Normal testis (Wheater's Functional Histology, 2006).

The tunica vasculosa is an extension of the interstitial tissue consisting of blood vessels and some leydig cells in a loose connective tissue, which extends over the internal aspect of the tunica albuginea and covers the septa and therefore, all the lobules. The albuginea itself consists of fibroblast and bundles of collagens but in some species, there is an appreciable number of smooth music cells. In the region of the testicular artery, there are a lot of moist cells, which may release vasoactive materials that influence the blood vessels of the testis (Nistral *et al.*, 1984).

3.4.2. HISTOLOGY OF THE EPIDIDYMIS.

The comprehensive histological description of the epididymis has been carried out in the rat (Fig. 2.14). This description subdivided the epididymis into between six and eight histological zones. The epididymal epithelium is complex in that it contains a variety of cell types each cell type varying as a proportion of the total population at different points along the duct. The predominant cell type is apical sterocilia, other cell types are apical cells, basal cells, clear cells and halo cells (intraepithelial lymphocyte). (Reid, and Cleland, 1957).

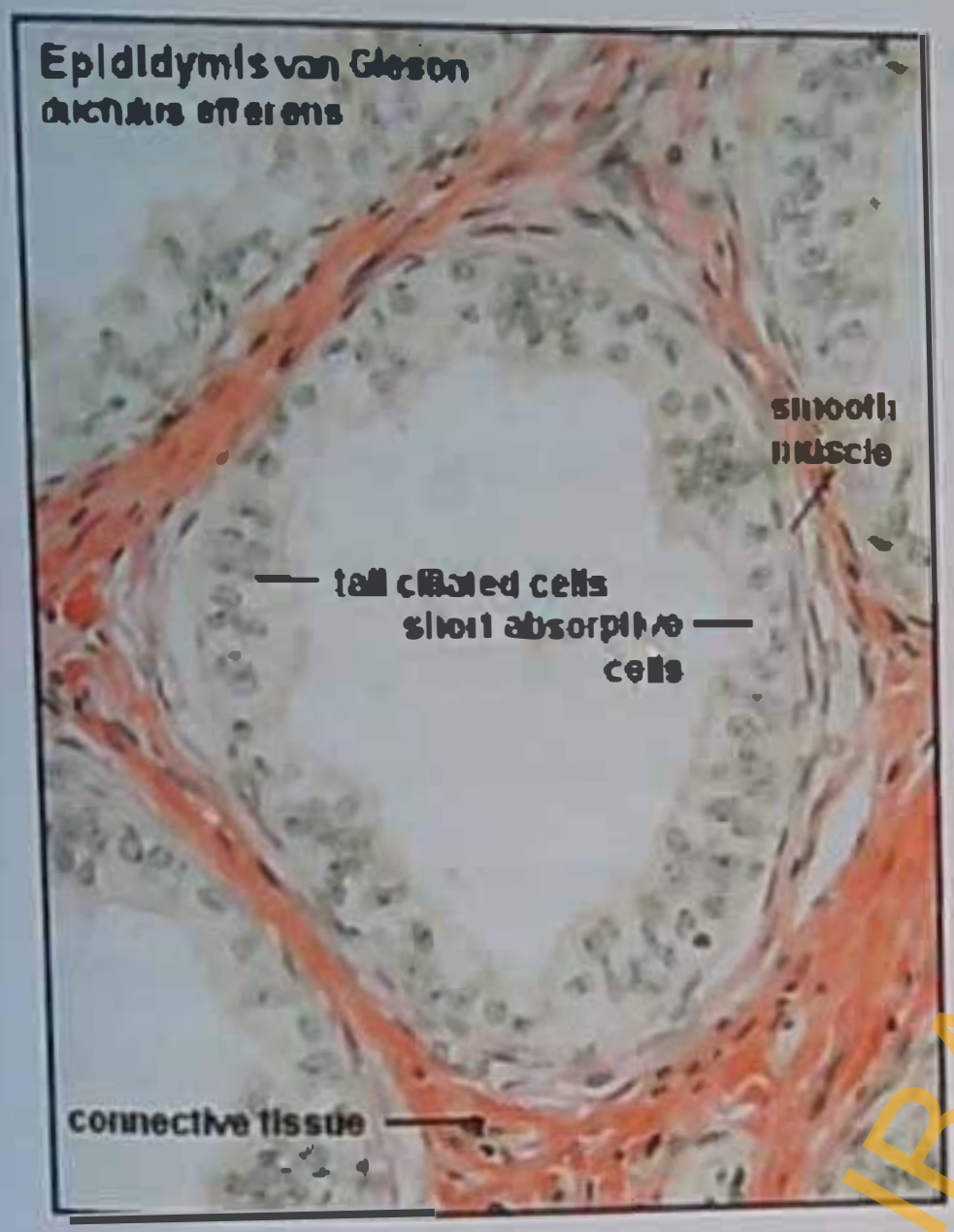


Fig. 2.14: Profile of the ductus epididymidis (Wheater's Functional Histology, 2006).

2.3.5 SPERM MORPHOLOGY

2.3.5.1. NORMAL SPERMATOZOA IN ANIMALS

Morphology refers to the shape and structure of the sperm. A normal-looking sperm has an oval head and a tail seven to fifteen times longer than the head (Fig. 2.15). On a semen analysis, in which sperm is looked at under the microscope, defective sperm can be identified by their large heads or strange tails (kinked, doubled, or coiled). Sperm morphology is routinely evaluated as part of a standard semen analysis. The results of sperm morphology indicate the percentage of sperm that appear normal when semen is viewed under the microscope. Abnormal sperm morphology may be a contributing factor in infertility. Morphology may be even more important than count or motility in determining potential fertility as abnormally shaped sperm cells cannot fertilize an egg. About 60% of sperm should be normal in size and shape for adequate fertility. The perfect structure is an oval head and long tail (www.healthcaremagic.com, 2009). 10% abnormality is allowed for breeding animals, values above this may indicate infertility in animals (Reece, 1997).

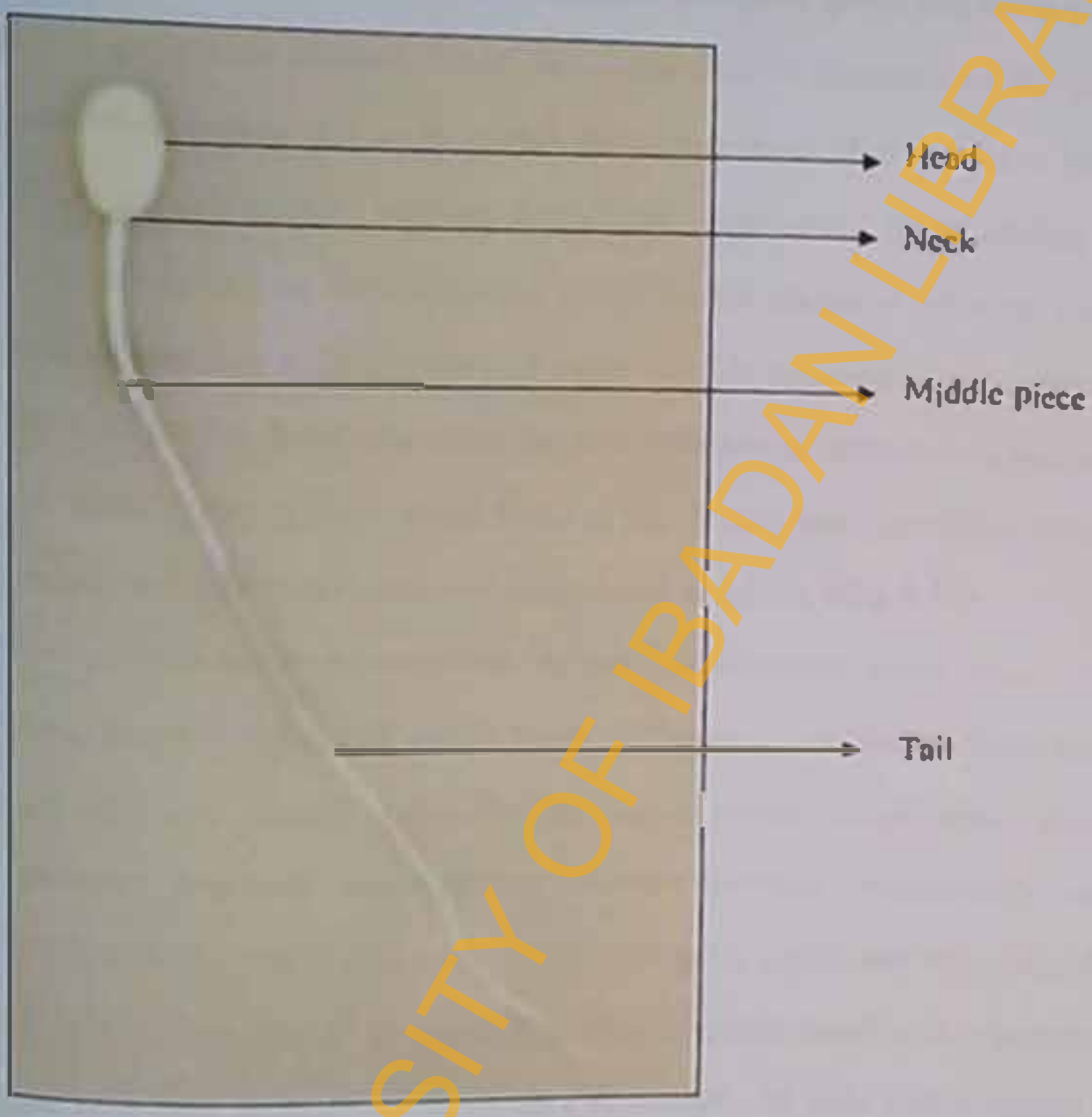


Fig. 2.15: Normal Rat spermatozoa
http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/morph.html. retrieved June 13, 2011.

2.3.5.2. SPERMATOZOAN ABNORMALITIES IN ANIMALS.

Spermatozoan abnormalities may be classified as head, body and tail defects. Blom (1950) have classified these three anatomical groups of sperm cell abnormalities into two further groups, as primary forms that occur due to disorders of the seminiferous or germinal epithelium and as secondary forms that occur after they have left the germinal epithelium, during their passage through the mesonephric ducts, during ejaculation or during manipulations of the ejaculate which include excessive agitation, over-heating, too rapid cooling, due to the presence of water, urine or antiseptics in the semen e.t.c. (Blom, 1950). In (1973), Blom classified the abnormalities as primary and secondary or major and minor sperm defects while Moss *et al*., (1988) have classified these defects into primary, secondary and tertiary or miscellaneous defects (Fig. 2.16).

Spermatozoan head abnormalities include microcephalic heads, macrocephalic heads, double heads, elongated or narrow heads, pyriform or pear-shaped heads with a narrow or tapering base, twisted and irregular-shaped heads, round short heads, abnormal acrosomes (knobbed spermatozoa), nuclear envelope invaginations (near equator), detached or free heads (Guernseys), detached galea capitis and acrosome. The latter two are possible "secondary" abnormalities. Macrocephalic heads with a broader than normal base have been shown by various tests to probably be cells with a diploid chromosome content (Gledhill, 1965; Salisbury and Baker, 1966).

Abnormal spermatozoan morphologies may be caused by the following:

- Testicular abnormalities that are present at birth (congenital).
- High fever.
- Illicit drug use.
- Infections (www.healthcaremagic.com, 2009).

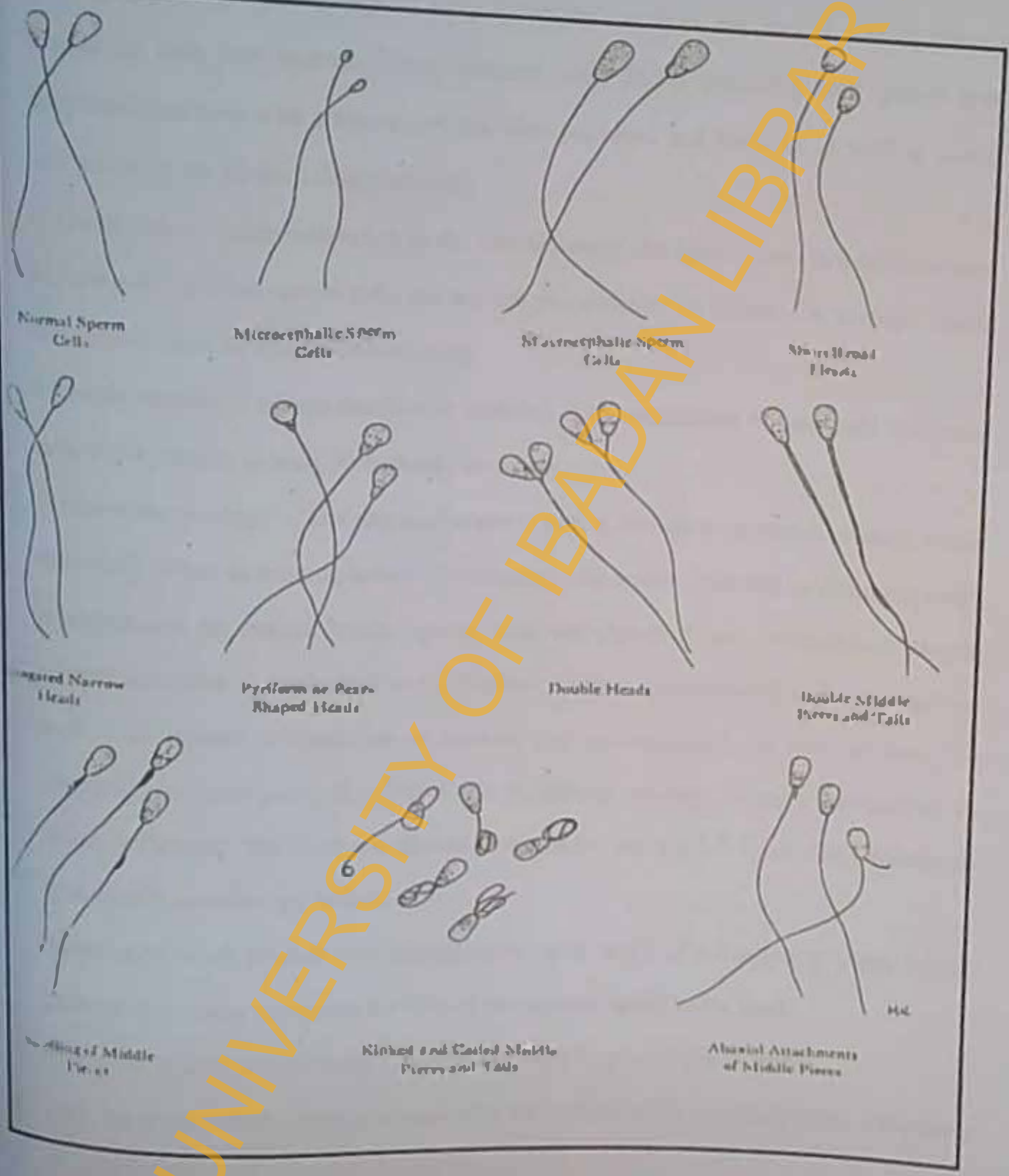


Fig. 2.16: Diagram of spermatozoan abnormalities (Blom, 1973).

2.3.6. MALE INFERTILITY TESTING.

2.3.6.1. SEMEN ANALYSIS.

Investigation of the possibility of male infertility involves the use of results from a number of tests and factors. Some common male factor infertility tests which give information on how well a sperm cell has been produced and matured, as well as how it interacts with the seminal fluids include:

1. *Sperm count:* - Also referred to as the concentration, the sperm count is a measurement of how many million sperm cells are present per milliliter of semen. On average, sperm cell count should be above 60 million/ml.

2. *Sperm motility:* - Sperm motility or mobility is an assessment of how well the sperm cells move; ideally at least 50% should be active.

3. *Sperm morphology:* - The shape of a sperm cell is also quite important when it comes to fertility. When examining sperm's morphology, the sperm cells will be examined under a microscope for certain traits. Sperm cells are classified into Normaloval shaped tapered, amorphous, duplicated and immature. Normal spermatozoid must have an oval form with smooth contour, an acrosomal cap encompassing 40-70% of head, no abnormality of mid piece, or tail and no cytoplasmic vacuoles of more than half of the sperm cell's head. The head size should be about 5-6 µm x 2.5-7.5 µm. Strict criteria for sperm cell's morphology include:

- Sperm cell's head: smooth oval configuration, with length of 5-6 microns, width, 2.5-3.5 microns. Acrosome composes 40-70% of the anterior sperm cell's head
- Mid piece: axially attached and 1.5 times the head length, <1 µm in width
- Tail: straight, uniform, slightly thinner than the midpiece and uncoiled (WHO Laboratory Manual, 1992).

2.3.6.2. SPECIALIZED TESTS.

It may also be necessary to carry out some or all of these specialized tests;

1. *Viability:* This technique affords the analyst to see which sperm cells are actually alive and kicking (literally).

2. *Post Ejaculatory Volume (PEV):* This test is done to see whether or not some or all of the sperm is ejaculated backwards to the bladder, a condition known as retrograde ejaculation.

3. *Fructose:* This test is carried out to determine azoospermia conditions, whether sperm is being blocked or just not produced.

4. *Spun specimen:* In some cases, there could be low sperm count that no sperm cells are noticed on the initial test slide. However, sperm cells may still be present in the ejaculate. This test helps to determines if there is any sperm by spinning down the ejaculate sample allowing all the sperm cells that may be present to separate and gather at the bottom of the tube.

2.3.6.3. SUPPLEMENTARY TESTING.

1. *Anti-sperm antibodies:* Some instances of male infertility can be attributed to anti-sperm antibodies. This is an immunological response whereby the male's body attacks the sperm cells, impeding fertility in a variety of ways. The antibodies may prevent the sperm cells from being able to properly travel through a female's cervical mucus.

2. *Kruger morphology:* If morphology of sperm cells is found to be a problem, a Kruger morphology test may be done, it allows for close examination of a sperm cell's shape This evaluation involves examining a sperm cell's head, mid piece and tail.

3. *White blood cells:* If a semen sample contains a higher than usual number of white blood cells, it may indicate a past infection or possibly inflammation, while some white

blood cells are expected to be found, a sample containing more than a million WBCs is considered to be problematic.

4. *Forward progression.* The test is designed to evaluate how much progress motile sperm are able to make (www.sharedjourney.com). World Health Organization (1992) classified it as follows.

0	No movement
1	Movement, non forward,
1+	Occasional movement of a few sperm.
2	Slow undirected
2+	Slow directly, forward movement.
3-	Fast, but undirected movement.
3	Fast, directed forward movement
3+	Very fast forward movement.
4	Extremely fast forward movement.

(WHO Laboratory Manual, 1992).

2.4. LIVER

2.4.1. ANATOMY OF THE LIVER

The liver is the largest gland of the body. It normally weighs about 1.5kg. It is an organ in the upper abdomen and fitting under the diaphragm. It has two main lobes, the right lobe being much larger than the left (Fig. 2.17) (Chopra, 2002).

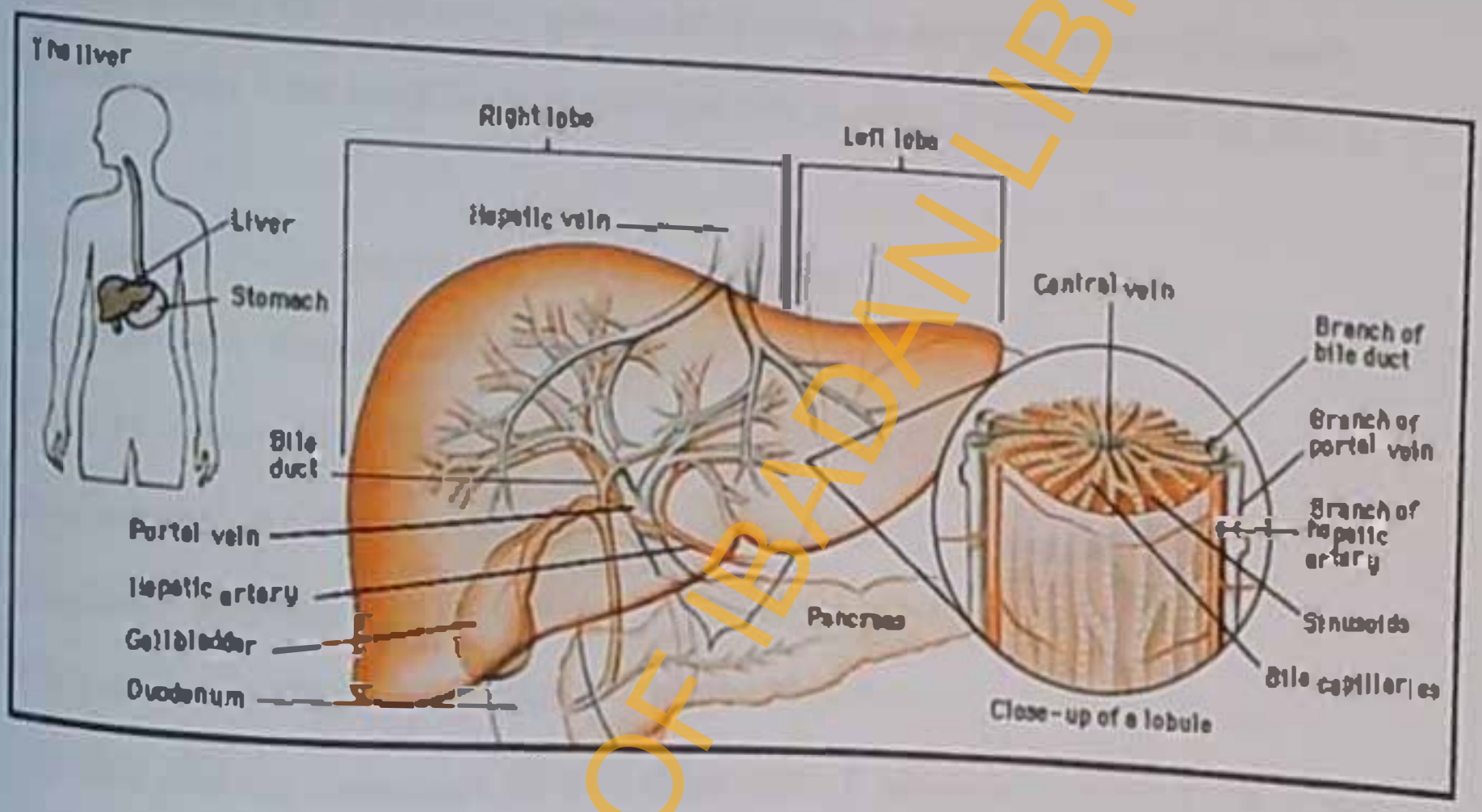


Fig. 2.17: Anatomy of the Liver (Liver-wikipedia, 2008).

2.4.2. FUNCTIONS OF THE LIVER

The various functions of the liver are carried out by the liver cells or hepatocytes.

The liver produce and excretes bile (a greenish liquid) required for emulsifying fats. Some of the bile drains directly into the duodenum, and some is stored in the gallbladder.

- The liver performs several roles in carbohydrate metabolism;

- *Gluconeogenesis* (the synthesis of glucose from certain amino acids, lactate or glycerol).
- Glycogenolysis (the breakdown of glycogen into glucose) (muscle tisseus can also do this)
- Glycogenesis (the formation of glycogen from glucose)

The breakdown of insulin and other hormones

The liver is responsible for the mainstay of protein metabolism

The liver also performs several roles in lipid metabolism:

- Cholesterol synthesis
- The production of triglycerides (fats)

The liver produces coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin.

The liver breaks down haemoglobin, creating metabolites that are added to bile as pigment (bilirubin and biliverdin)

The liver breaks down toxic substances and most medicinal products in a process called drug metabolism. This sometimes results in toxication, when the metabolite is more toxic than its precursor

The liver converts ammonia to urea.

The liver stores a multitude of substances, including glucose (in the form of glycogen), vitamin B_{12}, iron, and copper

- In the first trimester fetus, the liver is the main site of red blood cell production. By the 32nd week of gestation, the bone marrow has almost completely taken over that task.
- The liver is responsible for immunological effects- the riticuloendothelial system of the liver contains many immunologically active cells, acting as a 'sieve' for antigens carried to it via the portal system.
    - The liver produces albumin, the major osmolar component of blood serum.

Currently, there is no artificial organ or device capable of emulating all the functions of the liver. Some functions can be emulated by liver dialysis, an experimental treatment for liver failure. (Liver-Wikipedia, 2008).

2.4.3. FUNCTIONS OF BILE:

- It helps to emulsify and saponify fats in the small intestine by its alkalis, in this way the surface area is increased and the action of enzymes are increased.
- It stimulates peristalsis in the intestine.
- It is a channel for excretion of pigments and toxic substances from the bloodstream, such as alcohol and other drugs (Watson *et al*, 2000).

2.4.4. DISEASES OF THE LIVER

Many diseases of the liver are accompanied by jaundice caused by increased levels of bilirubin in the system. This bilirubin results from the breakup of the hemoglobin of dead red blood cells; normally, the liver removes bilirubin from the blood and excretes it through bile.

- Hepatitis, inflammation of the liver, caused mainly by various viruses but also by toxic poisons (e.g. alcohol), autoimmunity (autoimmune hepatitis) or hereditary conditions.
- Non-alcoholic fatty liver disease, a spectrum in disease, associated with obesity and characterized as an abundance of fat in the liver; may lead to hepatitis, i.e. steatohepatitis and/or cirrhosis.

- Cirrhosis is the formation of fibrous tissue in the liver, replacing dead liver cells. The death of the liver cells can for example be caused by viral hepatitis, alcoholism or contact with other liver-toxic chemicals.
- Cancer of the liver (primary hepatocellular carcinoma or cholangiocarcinoma and metastatic cancers, usually from other parts of the gastrointestinal tract).
- Wilson's disease, a hereditary disease which causes the body to retain copper.
- Primary sclerosing cholangitis, an inflammatory disease of the bile duct, likely autoimmune in nature.
- Primary biliary cirrhosis, autoimmune disease of small bile ducts
- Budd-Chiari syndrome, obstruction of the hepatic vein.
- Gilbert's syndrome, a genetic disorder of bilirubin metabolism, found in about 5% of the population.
- Glycogen storage disease type II, the build-up of glycogen causes progressive muscle weakness (myopathy) throughout the body and affects various body tissues, particularly in the heart, skeletal muscles, liver and nervous system (Sherlock and Dooley, 2002).
- Chronic Bile Duct Blockage: This condition can occur at birth (biliary atresia) or develop later in life (primary biliary cirrhosis). The cause of the latter remains unknown. When the bile ducts outside the liver become narrowed and blocked, the condition is called primary sclerosing cholangitis and is often associated with chronic ulceration of the colon (colitis) (Steele *et al*., 2007).
- Haemochromatosis: Hemochromatosis is the most common genetic liver disorder. It involves excess iron storage and is usually diagnosed in adults. (Gathenage *et al*., 2004.)

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2.4.5. LIVER FUNCTION TESTS (LFTs)

Liver function tests are series of enzyme tests used to ascertain the extent of hepatic (liver) damage either by injury or diseases. These Liver function tests consist of enzymes present in the liver and they are of great clinical significance. The test sample is either a plasma sample or serum sample collected by phlebotomy. There are different types of liver function tests, examples are the transaminases (e.g. alanine transaminase, aspartate transaminase), alkaline phosphatase, gamma-glutamyl transferase, 5'-nucleotidase, lactate dehydrogenase among others (Knight, 2005).

2.4.5.1. ALANINE TRANSAMINASE (ALT)

Alanine aminotransferase can also be called Serum Glutamic Pyruvate Transaminase (SGPT). Alanine aminotransferase (ALT or SGPT) catalyses the reversible transfer of amino group from alanine to α-ketoglutarate leading to the formation of pyruvate and glutamate. It is present in higher concentrations in the liver; lesser quantities are found in the kidney, heart and skeletal muscles. Injury or diseases affecting the Liver causes the release of ALT into the bloodstream thus elevating serum ALT levels.

Most ALT elevations generally are caused by liver dysfunction. This enzyme is specific and sensitive for hepatocellular diseases. Increased levels of ALT can signify Hepatic necrosis, hepatic ischemia, cirrhosis, hepatitis, hepatic tumour, cholestatis, hepatotoxic drugs, obstructive jaundice, severe burns, myocardial infarction, pancreatitis e.t.c. (Woman, 1999). In figure 2.18, ALT catalyzes the transfer of an α-amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate. (Darling *et al.*, 2000).

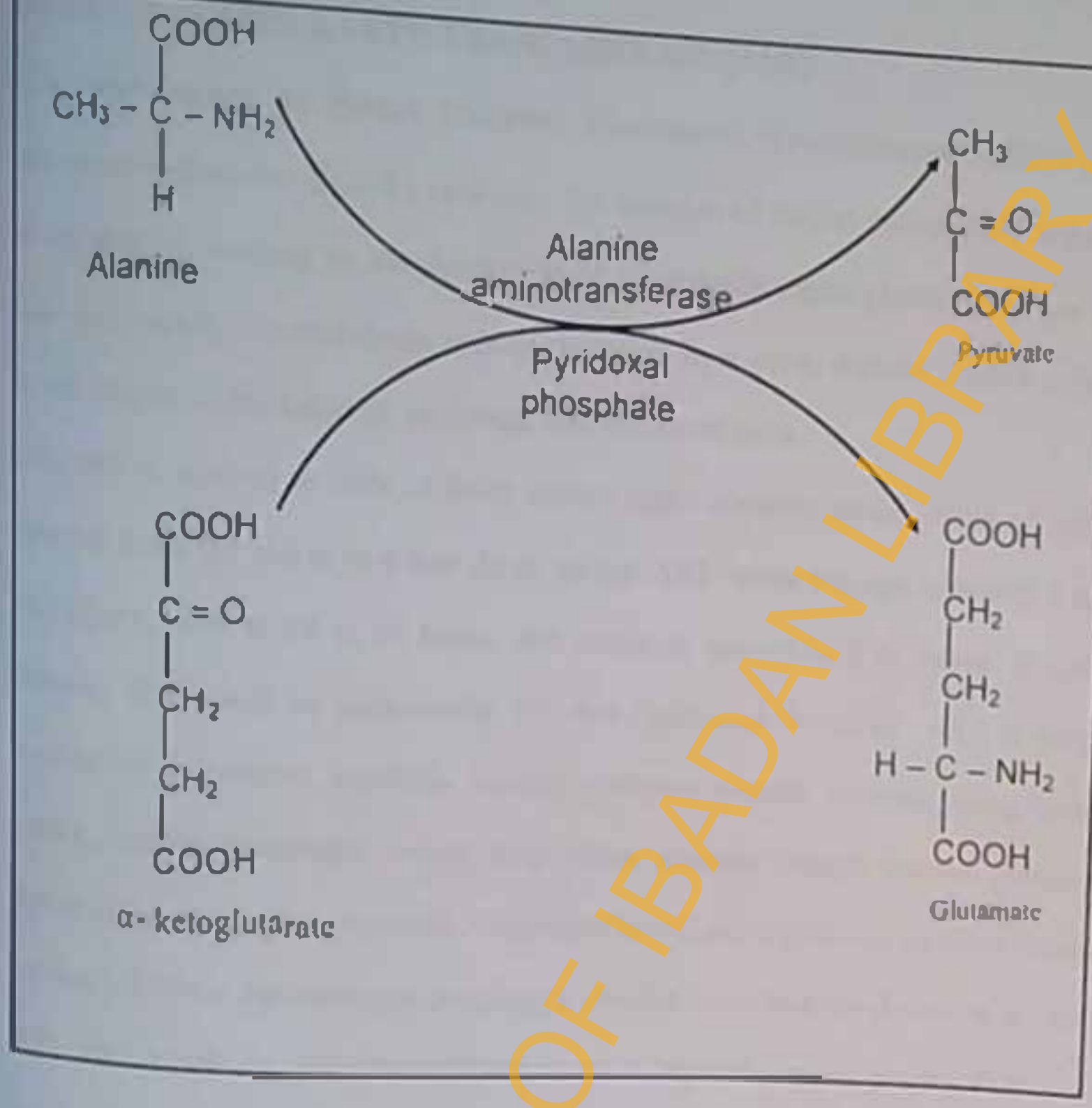

Fig. 2.18: Reaction of Alanine Aminotransferase

2.4.5.2. **ASPARTATE TRANSAMINASE (AST)**

It is also known as Serum Glutamic Oxaloacetic Transammase (SGOT). Aspartate aminotransferase (or SGOT) catalyses the transfer of amino group from aspartate to α-ketoglutarate, leading to the formation of oxaloacetate and glutamate. This enzyme is found in higher concentrations in heart muscles, liver cells, skeletal muscle cells, and to a lesser degree in the kidneys, pancreas, and red blood cells.

Diseases or injuries to cells of these tissues cause elevated serum levels of AST. AST is cleaved from the blood in a few days. Serum AST levels become elevated 8 hours after cell injury, peak at 24 to 36 hours, and return to normal in 3 to 7days. If cell injury is chronic, levels will be persistently elevated. Increased levels of AST is diagnostic of myocardial infarction, hepatitis, hepatic cirrhosis, hepatic necrosis, drug induced liver injury, hepatic metastatis, severe deep burns, skeletal muscle trauma, primary muscle diseases(e.g.myopathy, myositis). Decreased levels are significant of acute renal diseases, beriberi, diabetic ketoacidosis, pregnancy, chronis renal dialysis (Limdi et al, 2003).

AST also assists in early recognition of toxic hepatitis which results from exposure to drugs like acetaminophen and cholesterol lowering medications. Other disorders or diseases in which the AST determination can be valuable include acute pancreatitis, muscle disease, trauma, severe burn, and infectious mononucleosis. The AST test may be done at the same time as a test for ALT. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage and whether the liver or another organ has been damaged. Both ALT and AST levels can test for liver damage. (Nyblom *et al.*, 2004, Nyblom *et al.*, 2006).

2.4.5.2.1. **ISOENZYMES.**

There are two isoenzymes of Aspartate transaminase.

GOT 1, the cytosolic (soluble) isoenzyme which derives mainly from red blood cells and heart.

GOT 2, the mitochondrial isozyme which is predominantly present in liver. The plasma concentration of mitochondrial AST (mAST) is small (normally<4U/L) despite its considerable activity in heart and liver tissues (Rej, 1978). AST catalyses the transfer of an α-amimo group from Aspartate to α-ketoglutarate, producing oxaloacetate and glutamate (Fig. 2.19) (Darling, *et al.*, 2000).

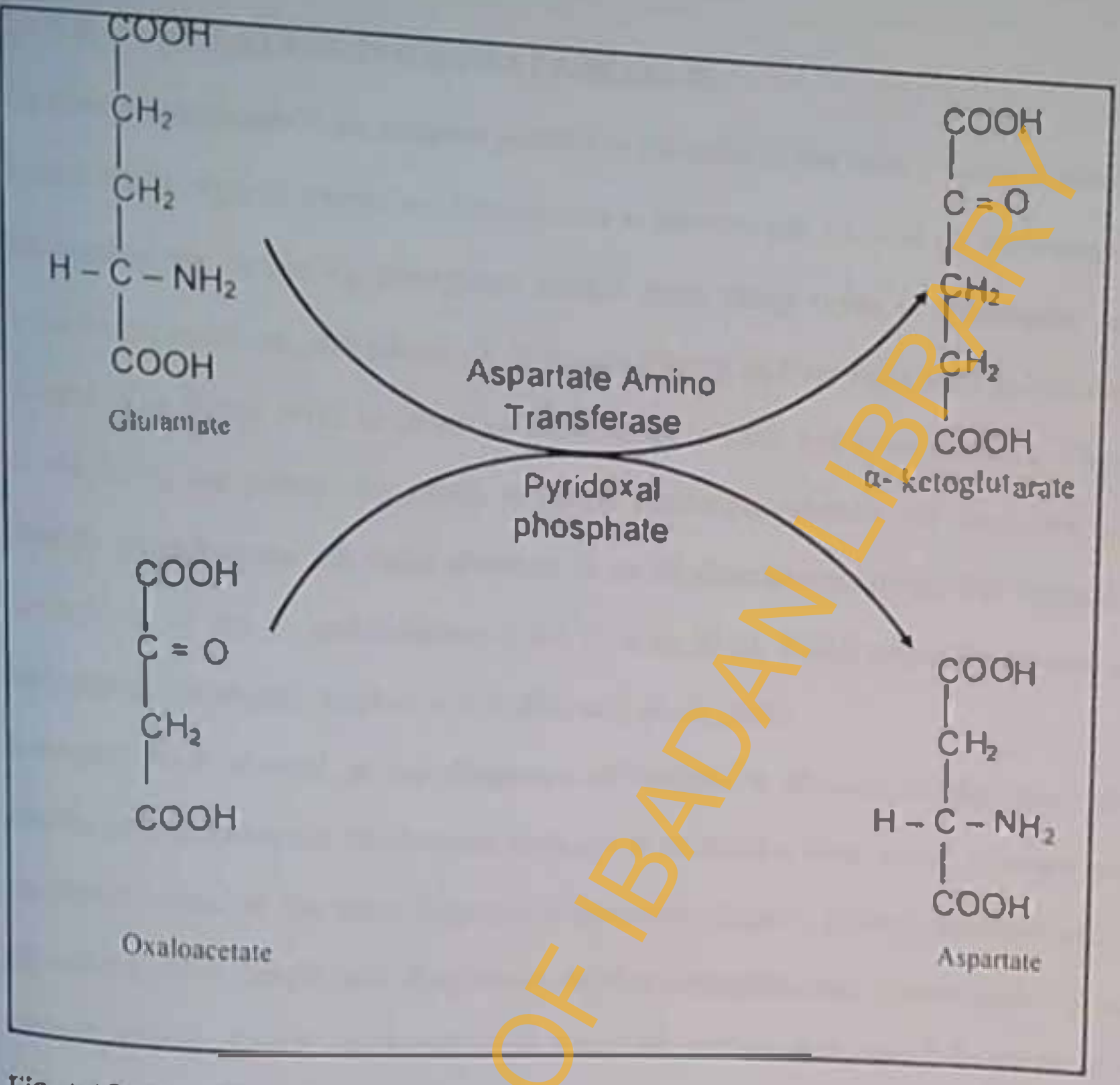

Fig. 2.19: Reaction of Aspartate Aminotransferase

2.4.5.3. ALKALINE PHOSPHATASE (ALP)

Alkaline phosphatase is an enzyme present in the cells of the biliary ducts. It catalyses the hydrolysis of organic phosphate monoesters at alkaline pH. i.e. it is an hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. It occurs mainly in liver cells next to bile ducts, and in bone. The blood level is raised in some types of liver and bone diseases. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. The optimal pH for the activity of the E. coli enzyme is 8.0 (Garen, *et al*, 1960) while the bovine enzyme optimum pH is slightly higher at 8.5. (Harada, *et al*, 1986).

Increased ALP is used in the diagnosis of infiltrative diseases of the liver, bile duct obstruction, intrahepatic cholestasis, primary or metastatic liver tumor, primary cirrhosis, metastatic tumor of the bone, hyperparathyroidism, Paget's disease, rheumatoid arthritis. Decreased ALP levels are diagnostic of hypophosphatemia, malnutrition, pernicious anemia, scurvy. The 5'-nucleotidase enzyme can aid the differential diagnosis of causes of ALP elevation (Palmer, 2004).

2.4.5.4. GAMMA-GLUTAMYL TRANSFERASE (GGT)

Gamma-glutamyl transpeptidase (GGT) catalyses the transfer of amino acids and peptides across the cellular membrane and possibly participates in glutathione metabolism. The highest concentration of this enzyme are found in the kidney, spleen, heart, intestine, brain, and prostate gland. This test is used to detect liver cell dysfunction. It is the most sensitive enzyme for detecting biliary obstruction. Increased levels is indicative of liver diseases (e.g. hepatitis, cirrhosis, hepatic, necrosis, hepatic tumour, metastasis, hepatotoxic drugs, cholestasis, jaundice), myocardial infarction, alcohol ingestion, pancreatic diseases (Dufour et al. 2000). GGT catalyses the reaction between gamma-glutamyl-p-nitro anilide and glycylglycine to produce p-nitroanilide and gamma-

glutamyl glycylglycine. The increase in absorption at 405nm is followed in a zero order reaction and is directly proportional to the enzyme activity.

L-□-glutamyl-p-nitroanilide + glycylglycine $\xrightarrow{\text{GGT}}$ L-□-glutamylglycylglycine +p-nitroaniline (Szasz, 1969).

GGT is involved in glutathione metabolism by transferring the glutamyl moiety to a variety of acceptor molecules including water, certain L-amino acids and peptides, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress, (Schulman *et al.*, 1975; Yokoyama.,2007).

GGT is involved in leukotriene metabolism (Rauff *et al.*,1985). GGT is found on the cell surface on all cells, with particularly high concentrations in the liver, bile ducts, and kidney. The enzyme is also present in other tissues, such as the epididymis. The GGT present in serum appears to originate primarily from the hepatobilary system, and its activity is elevated in all forms of liver diseases. GGT is more sensitive than ALP in detecting obstructive jaundice, cholangitis, and cholecystisis. High levels of GGT are also seen in patients with either primary or secondary liver cancer. Increased levels are also seen in sera of heavy drinkers or patients with alcohol cirrhosis. In patients receiving anticonvulsant drugs such as phenytoin and Phenobarbital, increased levels of the enzyme in serum may reflect induction of a new enzyme activity and the toxic effects of alcohol and other drugs on microsomal structures in liver cells.

In summary, GGT is the most sensitive enzymic indicator of hepatobiliary disease available at present; normal values are rarely found in the presence of liver disease. However, GGT is of little value in attempting to discriminate between different kinds of liver diseases. 'Normal' levels of the enzyme are seen in cases of skeletal disease, in children older than one year of age and in healthy pregnant women (conditions in which

ALP is elevated). Thus, measurement of GGT levels in serum can be used to ascertain whether observed elevations of ALP are due to skeletal disease or reflect the presence of hepatobiliary disease (Teitz, 1987).

2.5 BLOOD

Blood is a specialized bodily fluid that delivers necessary substances such as nutrients and oxygen to the body's cells and transports waste products away from these same cells (Williams *et al*., 1989). In vertebrates, it is composed of blood cells suspended in a liquid called blood plasma which comprises 55% of blood fluid and is mostly water (90% by volume). Plasma contains dissolved proteins, glucose, mineral ions, hormones, carbon dioxide (plasma being the main medium for excretory products' transportation), platelets and blood cells themselves (Ganong, 2003). The blood cells present in blood are mainly red blood cells (also called RBCs or erythrocytes) and white blood cells, including leukocytes and platelets. The most abundant cells in vertebrate blood are red blood cells. These contain hemoglobin, an iron-containing protein, which facilitates transportation of oxygen by reversibly binding to this respiratory gas and greatly increasing its solubility in blood. In contrast, carbon dioxide is almost entirely transported extracellularly dissolved in plasma as bicarbonate ion (Alberts, 2005).

Blood is circulated around the body through blood vessels by the pumping action of the heart. In animals having lungs, arterial blood carries oxygen from inhaled air to the tissues of the body, and venous blood carries carbon dioxide, a waste product of metabolism produced by cells, from the tissues to the lungs to be exhaled. Medical terms related to blood often begin with *hemo-* or *hemato-* (also spelled *haemo-* and *haemato-*) from the Ancient Greek word αἷμα (*haima*) for "blood". In terms of anatomy and histology, blood is considered a specialized form of connective tissue, given its origin in the bones and the presence of potential molecular fibers in the form of fibrinogen (Shmukler, 2004).

2.5.1. FUNCTIONS OF BLOOD

Blood supplies oxygen, nutrients (glucose, amino acids, and fatty acids) to tissues and removes waste (carbon dioxide, urea, and lactic acid) from the same. It has immunological functions (including circulation of white blood cells and detection of foreign material by antibodies), coagulation functions (which is one part of the body's self-repair mechanism), messenger functions (including the transport of hormones and the signaling of tissue damage), regulatory function on body pH (the normal pH of blood is in the range of 7.35 - 7.45 covering only 0.1 pH unit) and core body temperature (Shmukler, 2004).

2.5.2 CONSTITUENTS OF BLOOD

Blood accounts for 7% of the human body weight (Alberts, 2005), with an average density of approximately 1060 kg/m3, very close to pure water's density of 1000 kg/m3. The average adult has a blood volume of roughly 5 liters (1.3 gal), composed of plasma and several kinds of cells (occasionally called *corpuscles*); these formed elements of the blood are erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). By volume, the red blood cells constitute about 45% of whole blood, the plasma about 54.3%, and white cells about 0.7% (Shmukler, 2004).

2.5.2.1. RED BLOOD CELLS OR ERYTHROCYTES

The red blood cell or erythrocyte count ranges from 4.7 to 6.1 million in male and from 4.2 to 5.4 million in female. In mammals, mature red blood cells lack a nucleus and organelles. They contain the blood's hemoglobin and distribute oxygen. The red blood cells (together with endothelial vessel cells and other cells) are also marked by glycoproteins that define the different blood types. The proportion of blood occupied by red blood cells is referred to as the hematocrit, and is normally about 45%. The combined surface area of all red blood cells of the human body would be roughly 2,000 times as great as the body's exterior surface (Robert, *et al.*, 2006).

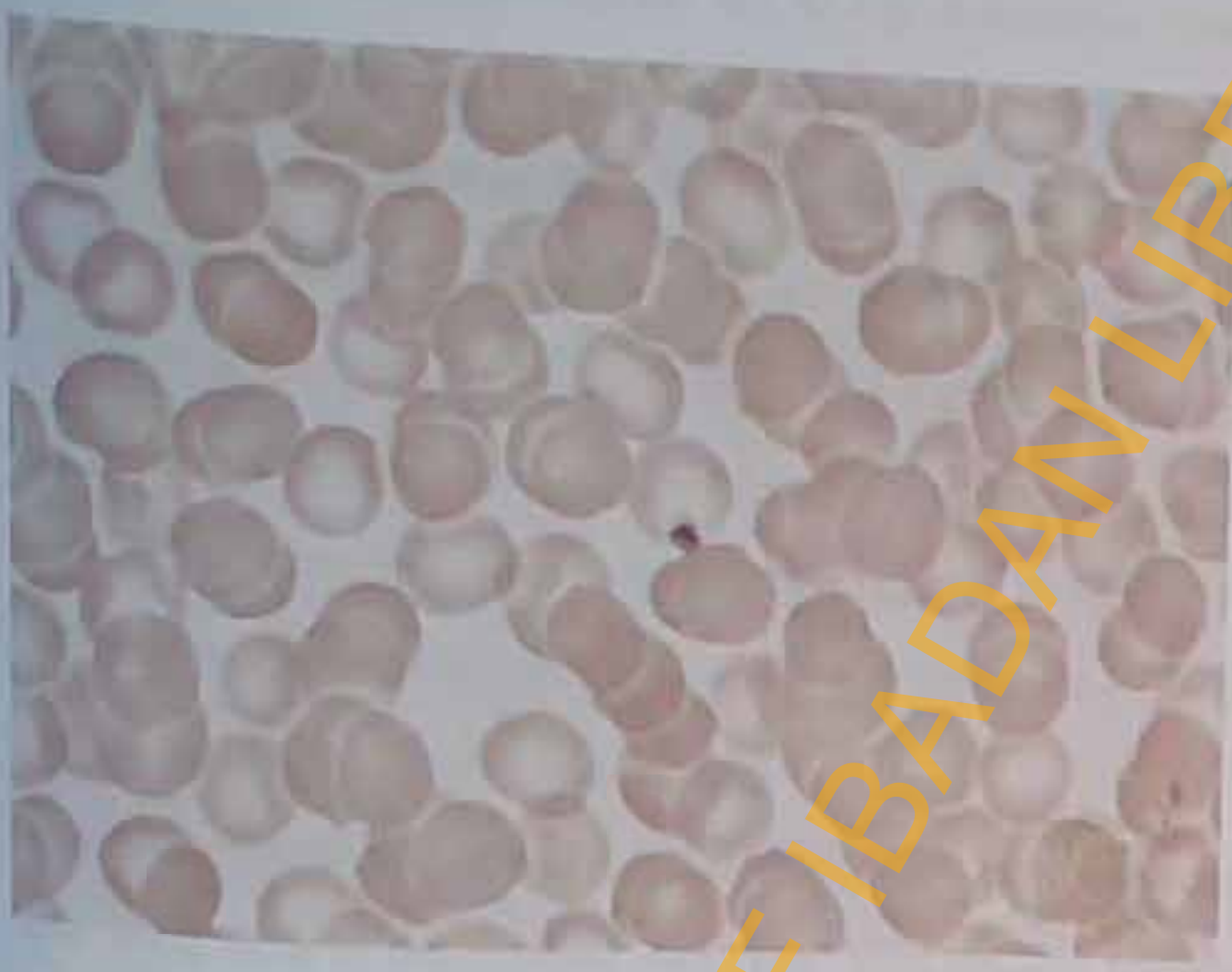

Fig. 2.20: Histology of the Red Blood Cells (Erythrocytes) (Ganong, 2003).

2.5.2.2 WHITE BLOOD CELLS OR LEUKOCYTES

There are about 4,000–11,000 leukocytes. White blood cells are part of the immune system; they destroy and remove old or aberrant cells and cellular debris, as well as attack infectious agents (pathogens) and foreign substances. The cancer of leukocytes is called leukemia (Ganong, 2003). The five types of white blood cells produced by the marrow are shown below in (Fig. 2.21) and they include;

- **Neutrophils:** These are the most numerous white cells, comprising over 62%. They are the first to attack an infection, and thus some doctors will count the nuetrophil level as well as the total white count. Neutrophils tend to gather at the site of infection which may appear red or feel warm, such as with a fever (Costanzo, 2007).
- **Eosinophils:** These cells react to allergies and hurt the foreign bodies (antigens) before they can hurt us (Mortensen *et al*., 2005).
- **Basophills:** These are the rarest white cells. They release histimine, which attracts the other white cells to the infected area. They also produce heparin which dissolves clots (and is used in ports to prevent new clots) (Shuster *et al*., 2004).
- **Lymphocytes:** These do not only fight infections but also provide immunity to certain diseases. These cells produce the antibodies that are so important to life (Costanzo, 2007).
- **Monocytes:** This last type goes into an area after the infection has been killed and cleans up the debris and damage left behind (Mortensen *et al*., 2005).

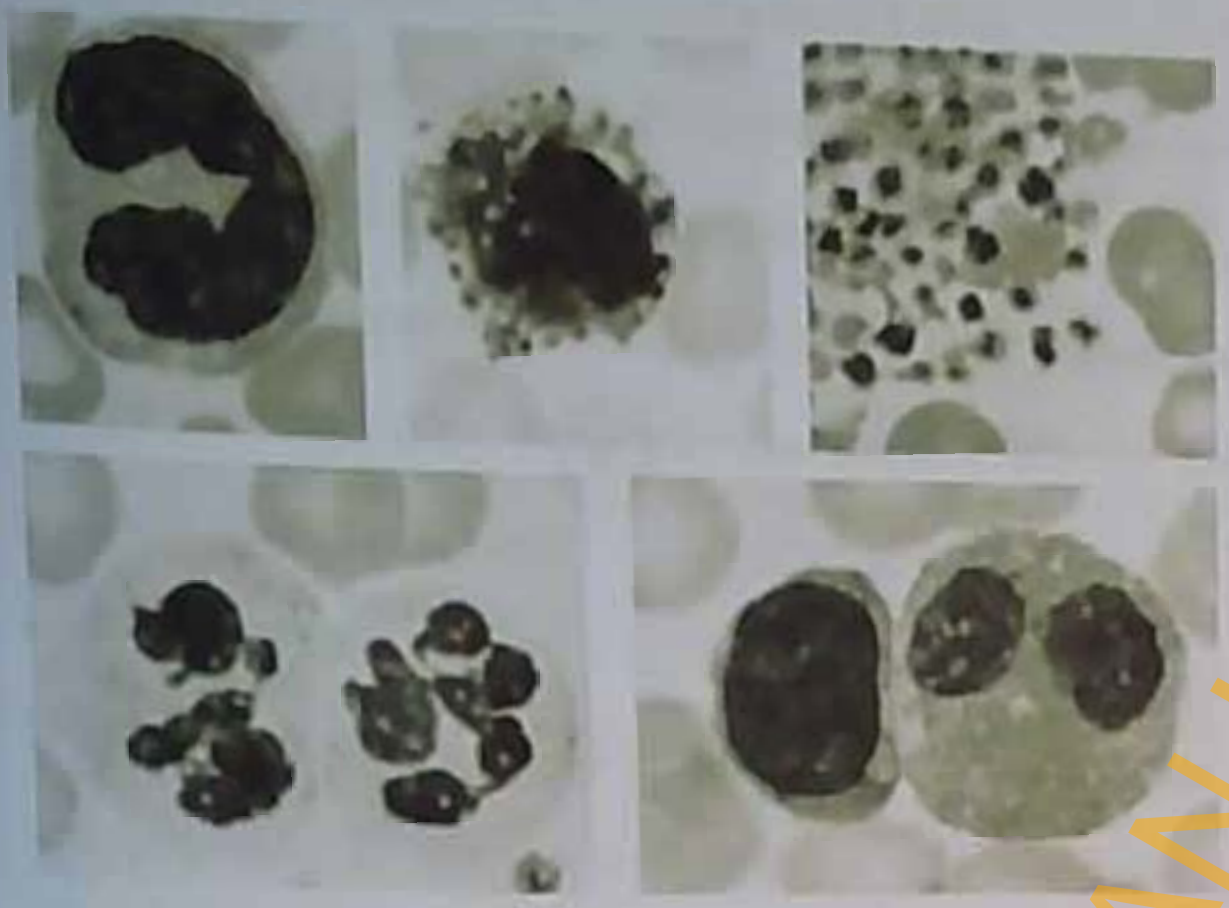

Fig. 2.21: White blood cells in a field of red cells.

(Top left) Monocyte, (top centre) basophil, (top right) platelets, (bottom left) two neutrophils, (bottom right) lymphocyte and eosinophil, respectively (Ganong, 2003).

2.5.2.3 PLATELETS OR THROMBOCYTES

There are about 200,000–500,000 thrombocytes. Thrombocytes, also called platelets, are responsible for blood clotting (coagulation). They change fibrinogen into fibrin. This fibrin creates a mesh onto which red blood cells collect and clot, which then stops more blood from leaving the body and also helps to prevent bacteria from entering the body (Ganong, 2003).

2.5.2.4 PLASMA

About 55% of whole blood is blood plasma, a fluid that is the blood's liquid medium, which by itself is straw-yellow in color. The blood plasma volume totals of 2.7 – 3.0 litres (2.8-3.2 quarts) in an average human. It is essentially an aqueous solution containing 92% water, 8% blood plasma proteins, and trace amounts of other materials. Plasma circulates dissolved nutrients, such as glucose, amino acids, and fatty acids (dissolved in the blood or bound to plasma proteins), and removes waste products, such as carbon dioxide, urea, and lactic acid. Other important components include serum albumin, blood-clotting factors (to facilitate coagulation), immunoglobulins (antibodies), lipoprotein particles, various other proteins, and various electrolytes (mainly sodium and chloride) (Williams *et al.*, 1989).

2.5.3. HEMATOLOGICAL DISORDERS

2.5.3.1. ANEMIA

Insufficient red cell mass (anemia) can be the result of bleeding, blood disorders like thalassemia, or nutritional deficiencies; and may require blood transfusion. Several countries have blood banks to fill the demand for transfusable blood. A person receiving a blood transfusion must have a blood type compatible with that of the donor (Austin and Perkins, 2006).

2.5.3.2. DISORDERS OF CELL PROLIFERATION

Leukemia is a group of cancers of the blood-forming tissues. Noncancerous overproduction of red cells (polycythemia vera) or platelets (essential thrombocytosis) may be premalignant. Myelodysplastic syndromes involve ineffective production of one or more cell lines (Shuster *et al.*, 2004).

2.5.3.3. DISORDERS OF COAGULATION

Hemophilia is a genetic illness that causes dysfunction in one of the blood's clotting mechanisms. This can allow otherwise inconsequential wounds to be life-threatening, but more commonly results in hemarthrosis, or bleeding into joint spaces, which can be crippling. Ineffective or insufficient platelets can also result in coagulopathy (bleeding disorders). Hypercoagulable state (thrombophilia) results from defects in regulation of Platelet or clotting factor function, and can cause thrombosis (Williams *et al.*, 1989).

2.5.3.4. INFECTIOUS DISORDERS OF BLOOD

Blood is an important vector of infection. HIV, the virus, which causes AIDS, is transmitted through contact with blood, semen or other body secretions of an infected person. Hepatitis B and C are transmitted primarily through blood contact. Owing to blood-borne infections, bloodstained objects are treated as biohazards. Bacterial infection of the blood is bacteremia or sepsis. Viral infection is viremia. Malaria and trypanosomiasis are blood-borne parasitic infections (Dominguez *et al.*, 1981).

2.5.4. HEMATOLOGICAL PARAMETERS

2.5.4.1. WHITE BLOOD CELL COUNT

It is the number of white blood cell count in a cubic millimeter of whole blood. A decrease in WBC count shows low level of infection in experimental rats or may relate to suppression of the production of the white blood cell resulting from toxic reactions to substances, and vice versa for an increase in white blood cell count (Schalm *et al.*, 1975).

- **LEUKOCYTOSIS**

Abnormally high number of white blood cells (leukocytes) in the blood circulation, defined as more than 10,000 leukocytes per cubic millimetre of blood. Leukocytosis is most commonly the result of infection. It may also occur after strenuous exercise, convulsions (e.g., epilepsy), emotional stress, anesthesia, the administration of epinephrine, pregnancy and labour, and lack of oxygen (as in the early phases of adaptation to high altitude). Leukocytosis is also observed in certain parasitic infestations, intoxications (metabolic or chemical), chronic diseases (e.g., leukemia), and allergic reactions (Cheesbrough, 2006).

- **LEUKOPENIA**

Leukopenia is characterized by leukocyte counts that are abnormally low (below 4,000 per cubic millimetre). Like leukocytosis, which is usually due to an increase of neutrophils (neutrophilia), leukopenia usually is due to a reduction in the number of neutrophils (neutropenia). Of itself, neutropenia causes no symptoms, but persons with neutropenia of any cause may have frequent and severe bacterial infections. Agranulocytosis is an acute disorder characterized by severe sore throat, fever, and marked fatigue associated with extreme reduction in the number of neutrophilic granulocytes or even their complete disappearance from the blood (Cheesbrough, 2006).

2.5.4.2. PACKED CELL VOLUME (PCV)

This is the fraction of whole blood that consists of red blood cells. PCV measures the percentage by volume of packed red blood cell in whole blood sample after centrifugation. An increase in packed cell volume indicates that the test animals are not anemic; it may rather be an indication of polycythaemia (Wyme and Edwards, 2003).

2.5.4.3. HAEMOGLOBIN TEST

It measures the amount of haemoglobin in grams in one deciliter of white blood and provides an estimate of oxygen carrying capacity of red blood cells. Hemoglobin is

measured by using a visual comparative technique. Hemoglobin values are expressed in grams per litre (g/l) or grams per deciliter (g/dl).

2.5.4.4. PLATELET COUNT

Platelet is involved in blood clotting. An increase in platelet count should lead to increase in bleeding and decrease in clotting time (Cheesbrough, 2006).

2.5.4.5. RED BLOOD CELL INDICES

Red cell indices most frequently used in the investigation of anemia are;

- **MEAN CELL HAEMOGLOBIN CONCENTRATION (MCHC)**

The MCHC gives the concentration of hemoglobin in g/l in 1 litre of packed red cells. It is calculated from the hemoglobin and packed cell volume as follows;

$$MCHC\ (g/l) = \frac{Hb\ (g/l)}{PCV\ (l/l)}$$

- **MEAN CELL HEMOGLOBIN (MCH)**

The MCH gives the amount of hemoglobin in picograms (Pg) and is calculated thus;

$$MCH\ (pg) = \frac{Hb\ (g/l)}{RBC \times 10^{12}/L}$$

- **MEAN CELL VOLUME (MCV)**

The mean red cell volume (MCV) provides information on red cell size. It is measured in femtolitre (fl) and is determined from the PCV and RBC counts as follows;

$$MCV\ (fl) = \frac{PCV\ (l/l)}{RBC \times 10^{12}/L}$$ (Baker *et al.*, 2000).

2.6. HISTOPATHOLOGY

Histopathology (compound of three Greek words: ἱστός histos "tissue", πάθος pathos "disease-suffering", and -λογία -logia) refers to the microscopic examination of tissue in order to study the manifestations of disease. (Histopathology-Wikipedia, 2010).

2.6.1. COLLECTION OF TISSUES

Histopathological examination of tissues starts with surgery, biopsy, or autopsy. The tissue is removed from the body or plant, and then placed in a fixative which stabilizes the tissues to prevent decay. The most common fixative is formalin (10% formaldehyde in water) (Histopathology-Wikipedia, 2010).

2.6.2. PREPARATION FOR HISTOLOGY

The collected tissues are then prepared for viewing under a microscope using either chemical fixation or frozen section. Chemical fixation involves transforming the samples to a cassette (a container designed to allow reagents to freely act on the tissue inside). This cassette is immersed in multiple baths of progressively more concentrated ethanol, to dehydrate the tissue, followed by toluene or xylene, and finally extremely hot liquid (usually paraffin).

During the 12 to 16 hour process, paraffin will replace the water in the tissue, turning soft, moist tissues into a sample miscible with paraffin, a type of wax. This process is known as tissue processing. The processed tissues are taken out of the cassette and set in a mold. Through this process of embedding, additional paraffin is added to create a paraffin block which is attached to the outside of the cassette. The process of embedding then allows the sectioning of tissues into very thin (2 - 7 micrometer) sections using a microtome. The microtome slices the tissue ready for microscopic examination. The slices are thinner than the average cell, and are layered on a glass slide for staining (Mitchell *et al*., 2007).

2.6.3. STAINING OF THE PROCESSED HISTOLOGY SLIDES

Staining can be done to slides processed by the chemical fixation or frozen section slides. To see the tissue under a microscope, the sections are stained with one or more pigments. The aim of staining is to reveal cellular components; counterstains are used to provide contrast. The most commonly used stain in histopathology is a combination of hematoxylin and eosin (often abbreviated H&E). Hematoxylin is used to stain nuclei blue, while eosin stains cytoplasm and the extracellular connective tissue matrix pink. There are hundreds of various other techniques which have been used to selectively stain cells. Other compounds used to color tissue sections include safranin, Oil Red O, congo red, silver salts and artificial dyes.(http://www.scribd.com/doc/4448747/Perl).

2.6.4. INTERPRETATION

The histological slides are examined under a microscope by a pathologist, a medically qualified specialist. This medical diagnosis is formulated as a pathology report describing the histological findings and the opinion of the pathologist. In the case of cancer, this represents the tissue diagnosis required for most treatment protocols (Histopathology-Wikipedia, 2010).

2.7. HERBS

The history of the use of herbs dates back to the time of the early man who had the crudest tools as his implements and used stones to start his fire. The fear of illness and death as well as the necessity to feed and of health protection has led man of all times and under all skies to resort to anything that nature can offer them (Koumare, 1985).

The art of using herbs to enhance his health must have come to the early man in the most unscientific way. Herbs are groups of plants to which the early man resorted for preserving his health against diseases. The early man was distinct from other animal neighbours because of his ability to use rational thought rather than rely on instinct as a basis of his action. Thus, he deliberately selected specific plants and materials for the treatment of his

ailment. This selection was not certainly based on a prior knowledge of the plants' constituents but on certain other factors like seasonal or astronomical, mystical or religious factors or signatures of nature e.t.c. which he accepted as influencing his life (Shelland, 1979).

This selection procedure was often a trial and error method, which at times becomes very dangerous even costing valuable lives, but once the attributes of a plant, beneficial or harmful become known to him, he would not normally forget it and could recognize the plant any time he comes across it. He would then use it as being beneficial or discard it as being dangerous. In this way, early man acquired sufficient knowledge about medicinal plants and their various therapeutic uses gradually increased in volume as civilization progressed (Ghani 1985).

The early man used herbs in their raw form and cooked form to keep fit and since that time, the use of herbs has been known and accepted by all nations on the surface of the earth. Herb is therefore, defined to mean any plant or part of plant, which is valuable for medical treatment, nutrition, food seasoning, dying and colouring of other materials. The most important uses of herbs are the medicinal uses. (Kafaru, 1994)

Despite modern development in the treatment of the human body, herbal remedies have been continuous and universal. Modern medicine in the beginning depended on herbal remedies, for plants were the fundamental source of therapeutic products for professional healers of the earliest times. Traditional healers have claimed success in the treatment of several diseases including, breast cancer, skin disorders, sickle cell anemia, venerealdiseases, diabetes and some forms of mental illnesses and as a result of this, herbal medicine has become a basis for research.

The flowers, leaves, barks and roots of various herbs have been used to prepare infusion, decoction, as well as dried forms made into powder for treating various illnesses (Kafaru,

1994). In Asia, Latin America, Africa and India the extensive use of natural plants as primary remedies, due to their pharmacological properties, is quite common (Conco, 1991).

The herbs commonly used for medicinal purposes include Mistletoe, Garlic, Onion, Almond, Ginger, *Momordica charantia* e.t.c.

2.7.1. ***Momordica charantia* (Bitter Melon).**

Family: Cucurbitaceae

Genus: *Momordica*

Species: *Charantia*

Synonyms: *Momordica Chinensis, M. elegans, M. indica, M. operculata, M. Sinensis, Sicyos Fauriei.*

Common Names: Bitter melon, papailla, bitter gourd, balsam pear, karela, ejinrin e.t.c.

The first written information of the vines appeared in 1813 when Ainsile (Ainsile, 1813) mentioned its use as a pot-vegetable. In 1826, the same author (Ainsile, 1826) mentioned the medicinal uses of the vine in the treatment of bleeding piles, in certain bowel affections and in curing wounds. In Jamaica, Ainslie quoted, "The natives used boiled leaves as well as a decoction of the plant itself, equally to promote the lochiae."

In 1891 Dymock (Dymock and Hooper, 1891) gave a complete description of the plant and its uses, stating it to be a kind of panacea for use in scabies, leprosy and other cutaneous diseases. Fruit and leaves have also been used as anthelmintic, emetic, purgative, in bilious affection, in night blindness, tonic stomachic, anti-rheumatic, in gout and in diseases of the spleen and liver.

In 1894 Bailey (Bailey, 1894) mentioned the use of the rind in medicine. In Batvia, (1898) Peckolt (Peckolt, 1898) discovered an alkaloid in the vine and described the use of the vine as an anthelmintic, purgative and emetic, and in 1904, he (Peckolt, 1904) verified the discovery of the alkaloid and called it momordicin.

To the Yorubas, bitter melon is known by the common name ejinrin weve.

The common name for *Momordica charantia* is Bitter Melon and it is from the family, cucurbitaceae. Although its country of origin is uncertain, the plant is commonly cultivated for its fruit in tropical regions of India, China, East Africa and Central and South America. It is herbaceous, climbing or having prostate vines with simple or forked tendrils (Garau *et al*., 2003; Rivera, 1941). The plant is described as having lobed leaves, yellow flowers and edible and bitter-tasting orange - yellow fruit. The unripe fruit is green and is cucumber -shaped with a bumpy looking surface. The parts of the plant used medicinally include the fruit, leaves, seeds, whole plant and seed oil. (Arion, 1999; Cunnick and Takenioto, 1993).

The Latin name Momordica means "to bite", referring to the jagged edges of the leaves, which appear as if they have been bitten. The stem of the plant are branched and puberulous, the leaf blade about 5 - 12cm long and as broad, the leaf is prominently nerved, having 5-7 lobes. The tendrils of the plant with which it supports itself is simple, slender and pubescent. The plant is monoecious. The fruit looks like a warty gourd, usually oblong, resembling a small cucumber about 5-7cm long.

The young fruit is emerald green, turning to yellow when ripe. At maturity, the fruit splits into three irregular valves that curl backwards and release numerous reddish - brown or white seeds cased in scarlet arils. The flower of the plant has five sepals, five petals and five male stamens on the end of a warty ovary. All parts of plant, including the fruit, taste very bitter. However, the fruit is wholesome and esteemed as vegetables when young, it may be sliced and preserved after drying for use in off-season. Parboiling with a dash of salt may remove some of the bitter taste of the plant (Tropical plant database, 2007; Raw materials, 1962).

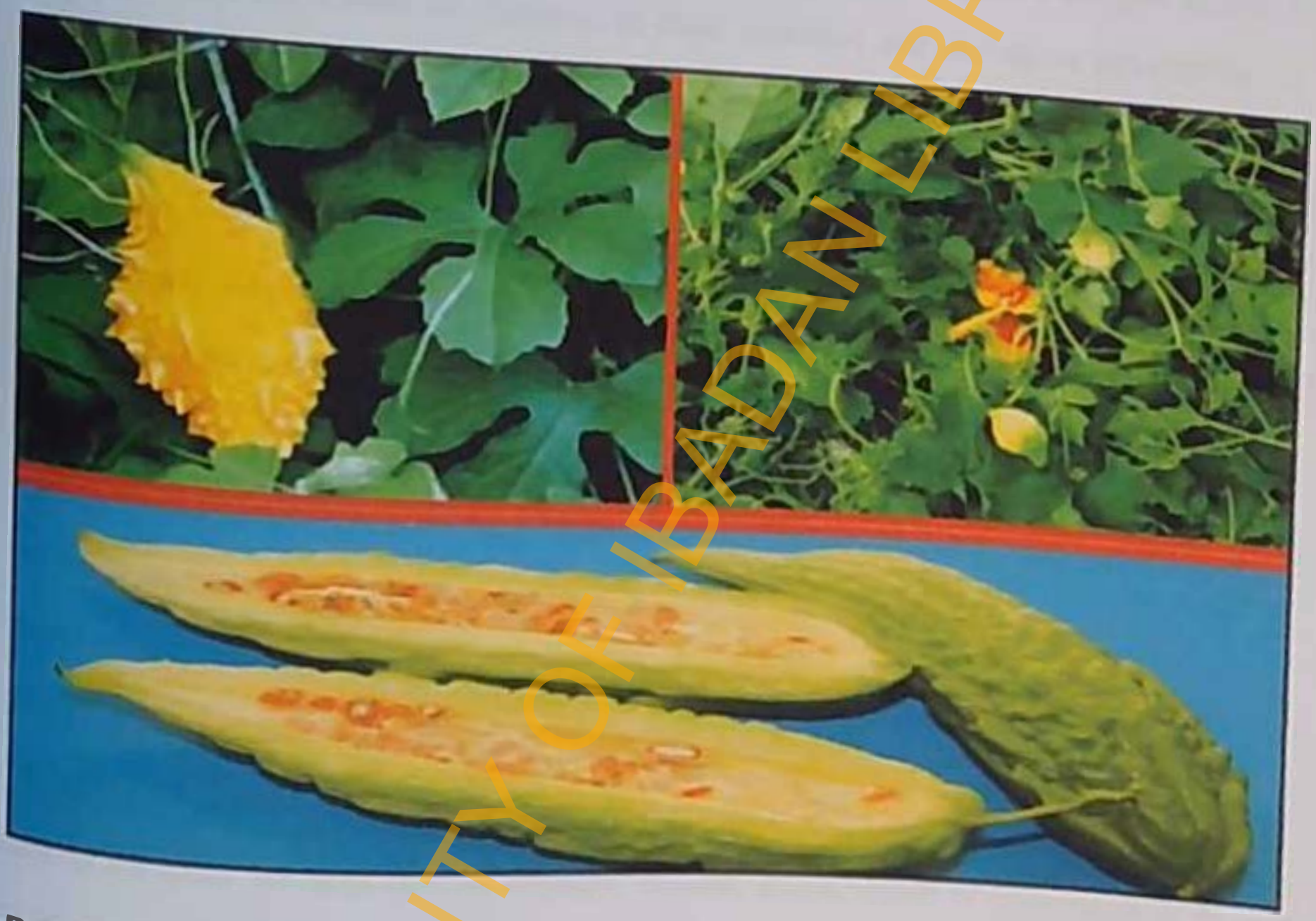

Fig. 222: *Momordica charantia*. Leaves, fruits and seeds

Bitter melon is cultivated in Asia, Africa, South America, and India and is used mostly as a traditional medicine in china, India and Africa (Anon. 1999).

2.7.1.1. TRIBAL AND HERBAL USES

In the Amazon, local people and indigenous tribes grow bitter melon in their gardens for food and medicine. They add the fruit and/or leaves to beans and soup for a bitter or sour flavour. Medicinally, the plant has a long history of use by the indigenous peoples of the Amazon. A leaf tea is used for diabetes, to expel intestinal gas, and as an antiviral for measles, hepatitis and feverish conditions. It is used topically for sores, wounds, and infections and internally and externally for worms and parasites. Bitter melon has also been used as both food and medicine through out Asia, as a therapeutic remedy in a variety of illnesses such as leukemia, diabetes, asthma, insect bites, menstrual cycle problems, stomach problems, as well as many other maladies.

In Brazilian herbal medicine, bitter melon is used for tumors, wounds, rheumatism, malaria, vaginal discharge, inflammation, menstrual problems, diabetes, colic, fevers, and worms. It is also used to induce abortions and as an aphrodisiac. It is prepared into a topical remedy for the skin to treat vaginitis, hemorrhoids, scabies, itchy rashes, eczema, leprosy and other skin problems. In Mexico, the entire plant is used for diabetes and dysentery, the root is a reputed aphrodisiac. In Peruvian herbal medicine, the leaf or aerial parts of the plant are used to treat measles, malaria, and all types of inflammation. In Nicaragua, the leaf is commonly used for stomach pain, diabetes, fevers, colds, coughs, headaches, malaria, skin complaints, menstrual disorders, aches and pains, hypertension, infections, and as an aid in childbirth (Tropical plant database, 2007). In Nigeria, the juice of the leaves and fruits is employed in folk medicine for the treatment and management of dysentery, piles, diarrhoea, malaria fever and skin diseases (Sofowora, 1984).

2.7.1.2. PHYTOCHEMICALS (ACTIVE CONSTITUENTS OF *MOMORDICA CHARANTIA*).

Bitter melon contains an array of biologically active plant chemicals including triterpenes, proteins and steroids. In numerous studies, various investigators have reported the presence of tannins, saponins, vitamins, peptides, amino acids, flavonoids and alkaloids in aqueous extracts of *M.charantia* (Barbieri *et al.*, 1980; Dalziel, 1959; Sofowora., 1982). At least three different groups of constituents found in all parts of bitter melon have clinically demonstrated hypoglycemic (blood sugar lowering) properties or other actions of potential benefit against diabetes mellitus. These chemicals that lower blood sugar include a mixture of steroidal saponins known as charantins, insulin-like peptides, and alkaloids. The hypoglycemic effect is more pronounced in the fruit of bitter melon where these chemicals are found in greater abundance (Tropical plant database 2007).

One chemical has clinically demonstrated the ability to inhibit the enzyme guanylate cyclase that is thought to be linked to the cause of psoriasis and also necessary for the growth of leukemia and cancer cells. (Takemoto, 1980; Takemoto, 1983; Claflin , 1978; Vesely , 1977). In addition, a protein found in bitter melon, momordin, has clinically demonstrated anticancerous activity against Hodgkin's lymphoma in animals (Terenzi, 1996). Other proteins in the plant, alpha- and beta-momorcharin and cucurbitacin B, have been tested for possible anticancerous effects. A chemical analog of these bitter melon proteins has been developed, patented, and named "MAP-30"; its developers reported that it was able to inhibit prostate tumor growth (Lee-Huang, 1996). In another study, it was reported that a hot water extract of the entire plant inhibited the development of mammary tumors in mice. (Nagasawa, 2002).

Numerous in vitro studies have also demonstrated the anti-cancerous and anti-leukemic activityof bitter melon against numerous cell lines including liver cancer, human

leukemia, melanoma and solid sarcomas. (Takemoto, 1982; Takemoto, 1983; West, 1971; Zhu, 1990). Bitter melon (and several of its isolated phytochemicals) also has been documented with invitro antiviral activity against numerous viruses including Epstein-Barr, herpes, and HIV viruses (Frame, 1998).

In an in vivo study, a leaf extract demonstrated the ability to increase resistance to viral infections as well as to provide an immunostimulant effect in humans and animals (increasing interferon production and natural killer cell activity) (Huang, 1990). Two of these proteins-alpha- and beta-momorcharin-have also been reported to inhibit HIV virus in in-vitro studies (Lee-Huang, 1990; Lee-Huang, 1995). In one study, HIV-infected cells treated with alpha- and beta-momorcharin showed a nearly complete loss of viral antigen while healthy cells were largely unaffected (Lee-Huang, 1990).

In 1996 the inventors of the chemical protein analog MAP-30 filed a U.S. patent, stating it "was useful for treating tumors and HIV infections. In treating HIV infections, the protein is administered alone or in conjunction with conventional AIDS therapies" (Lifson, 1989). Another clinical study showed that MAP-30's antiviral activity was also relative to the herpes virus in-vitro Bourinbaiar *et al.*, 1996). This plant has also been reported to have anti- leukemia and antiviral activities (Ng et al., 1994). The array of plant chemicals and their biological activities in Biuer melon include the following:

(1) **5 - Hydroxytryptamine (5HT)**: This is a saponin found mostly in the fruit; it is allergenic (Mitchell, and Rook, 1923); Cancer - preventive (Stitt, 1990); Cerebrophilic and insecticidal (Harborne and Baxter, 1983) and a Pesticide (Duke, 1992).

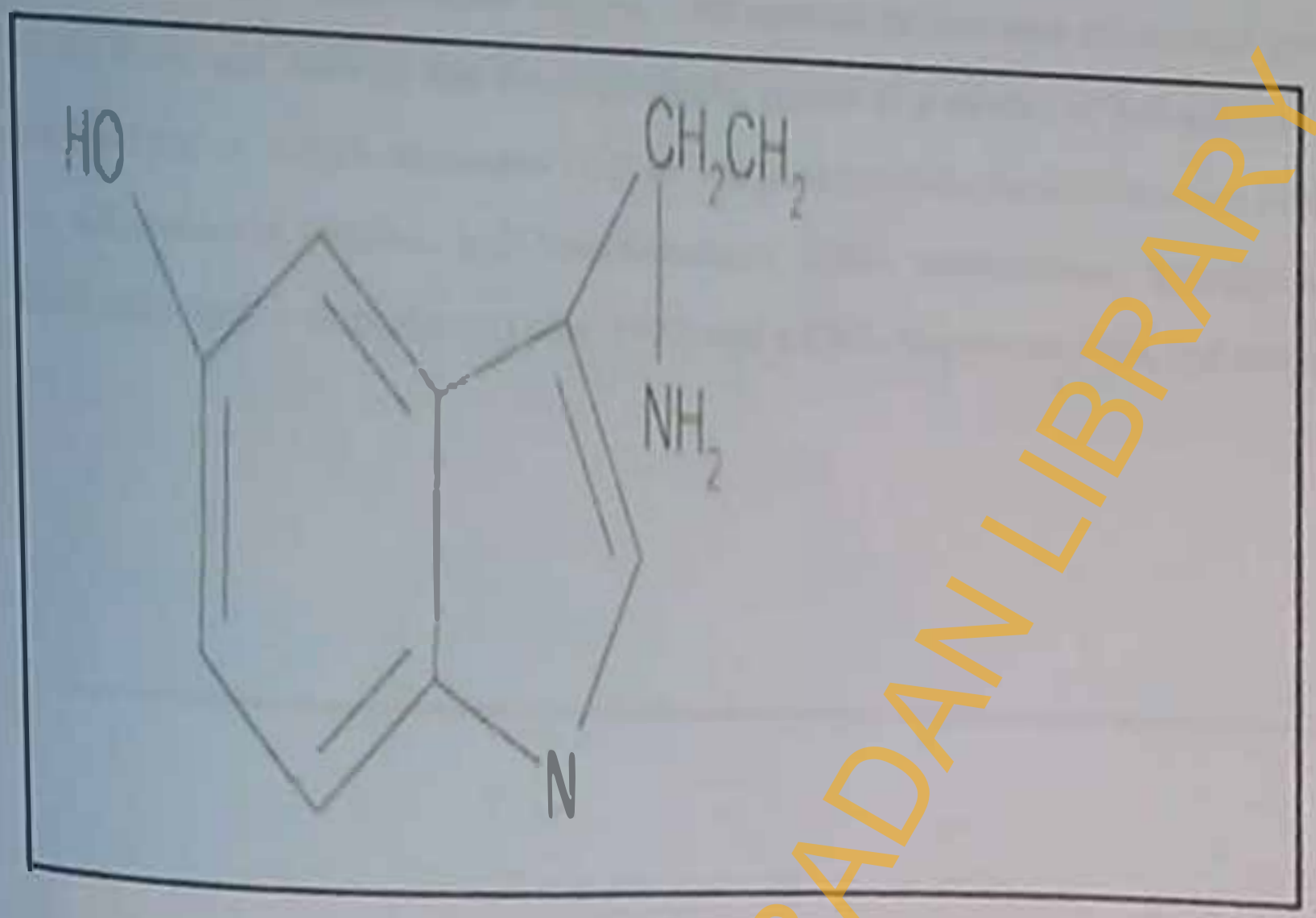

Fig. 2.23: Structure of 5-hydroxytryptamine (5-HT) (Fiche especc, 1989).

(2) **Beta-Sitosterol-D-Glucoside:** Sucrow, 1965 reported the presence of steroidal glucoside in the fruits and showed that these glucosides consist of a mixture of β-D-glucoside of β - sitosterol and of Δ-5,25 - stigmadiest -3- β- ol. The β-sitosterol-D-glucoside has been reported to be antispasmodic (Malini, and Vanithakumari, 1989), antitumorous, hypoglycemic, a CNS-Stimulant, a convulsant (Duke, 1992) and a CNS- Depressant (Rizk, and Al-Nowaili).

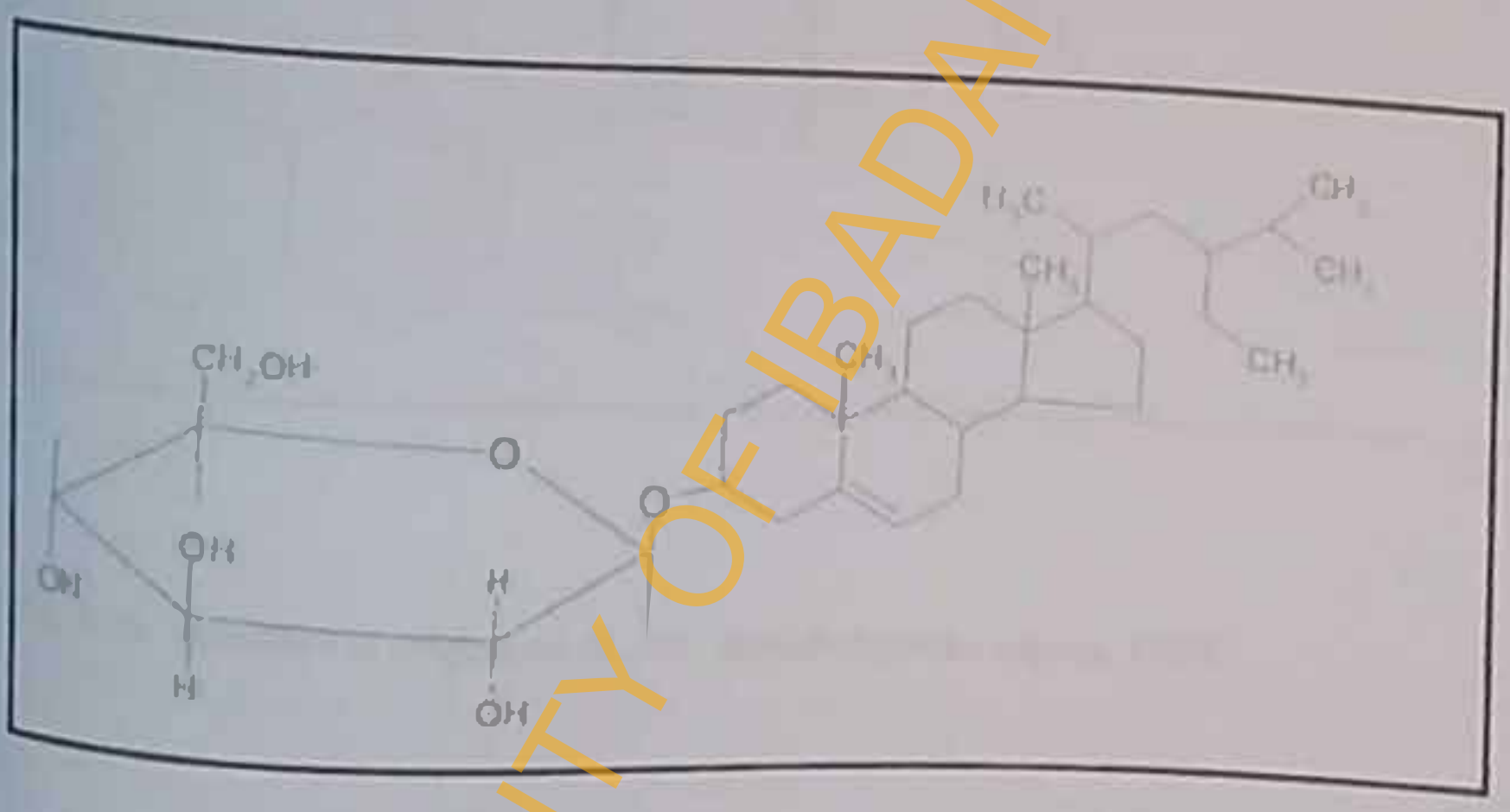

Fig 2.24: Structure of (β- D-glucoside of β- sitosterol) (Fiche espece, 1989).

(3) Stigmasta - 5, 25-dien-3- β -ol: this is another sterol found in the fruit of momordica cherantia.

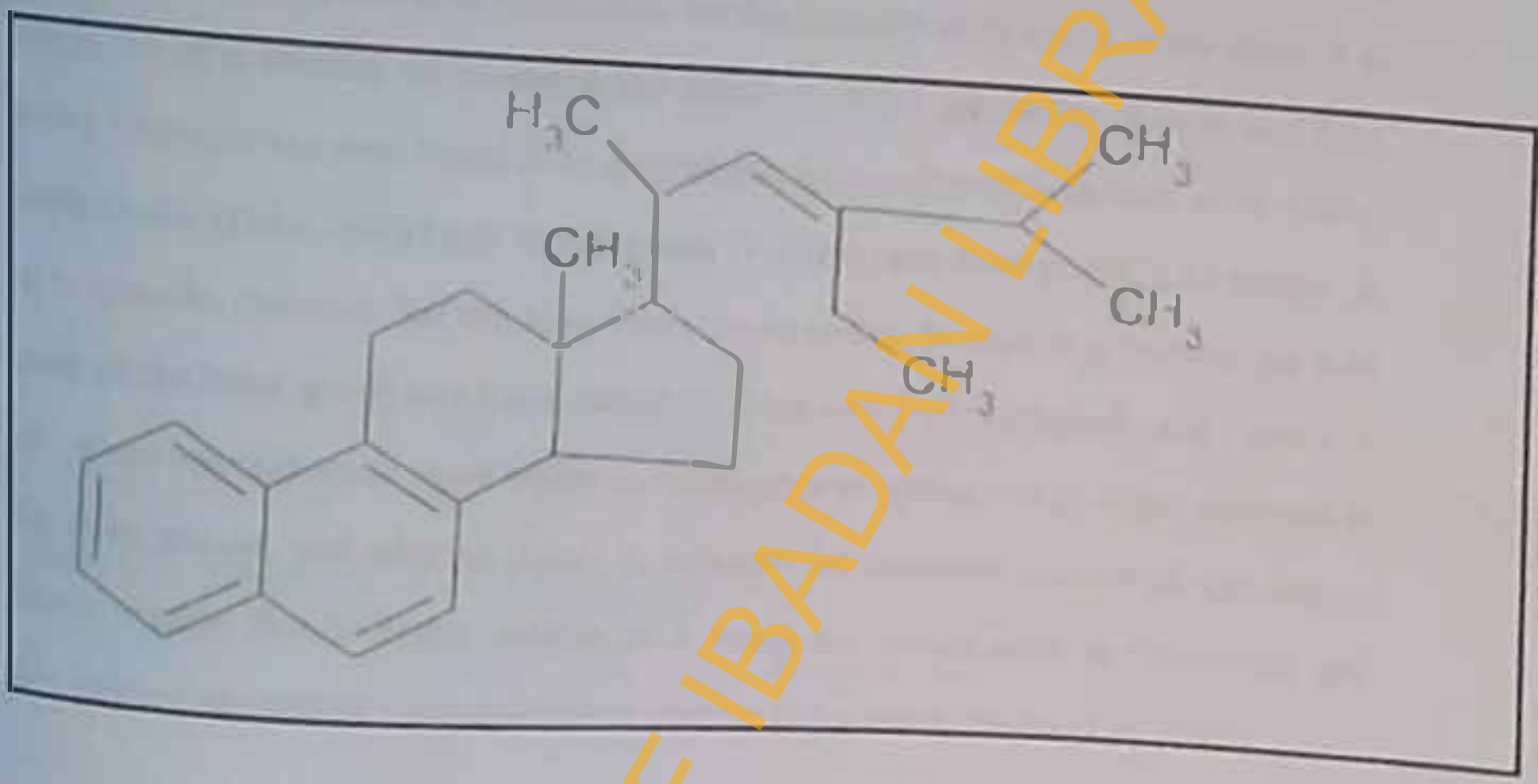

Fig. 2.25: Structure of Stigmasta -5, 25- dien-3-ol (Fiche espece, 1989).

(4) **Charantin:** This is a non-nitrogenous neutral principle found in every part of momordica charantia (leaf, fruit, seed, and vine). It gave a positive colour test for phytosterolins, and on hydrolysis yielded glucose and a sterol (Vasistha *et al*.,1960-61). It is one of the active principles responsible for the hypoglycemic action of the plant. It is composed of a mixture of sitosterol and stigmastadienol glucosides (Raman and Lau, 1996). Charantin has been found to be antidiabetic, an abortificient (Grenand, *et al*, 1987), antitesticular (Duke, 1992) and hypoglycemic (Pizzomo, and Murray, 1985). Charantin is an insulin-like chemical that can lower blood sugar and cholesterol. It is found in the fruit seeds of the bitter gourd and has a molecular mass of 9.7 kDa (Parkash et al., 2002). It serves as a hypoglycemic agent which increases glucose uptake and glycogen synthesis in the liver, muscle, and adipose tissue. A molecule of charantin consists of aglycone, a steroid portion that is highly soluble in a non-polar solvent such as chloroform and dichloromethane and on hydrolysis gives glucose and a sterol (Jesada et al., 2007).

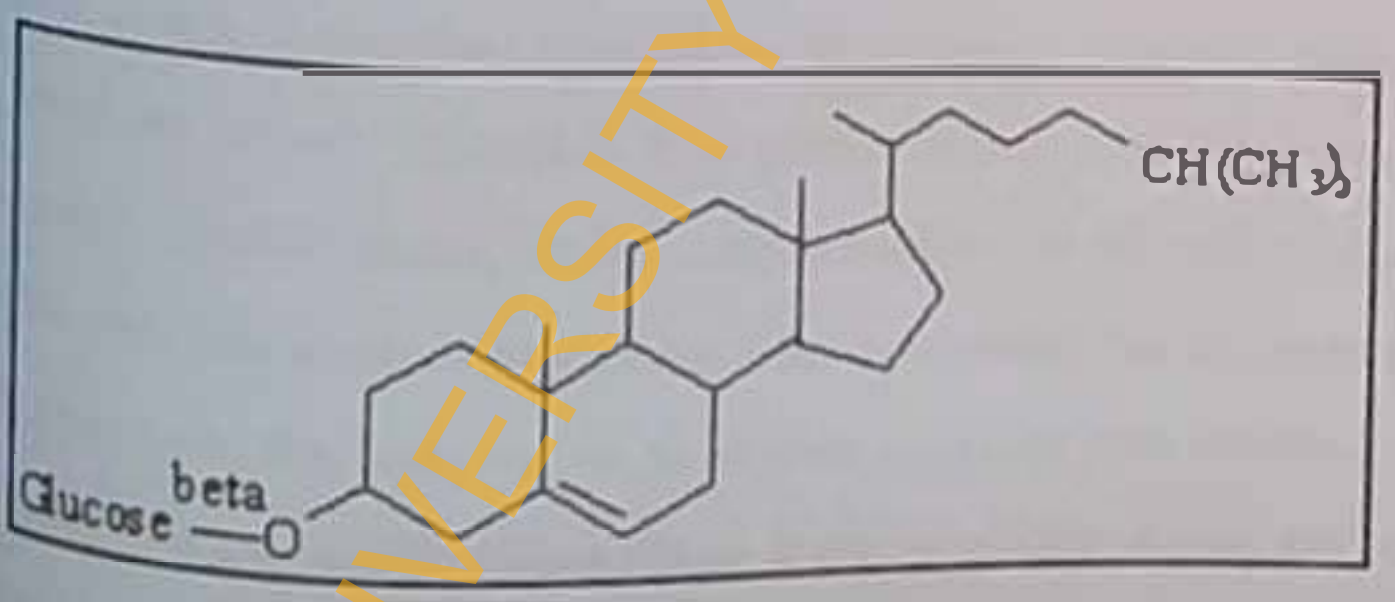

Fig. 2.26: Structure of Charantin (Sharma, *et al*., 1960).

(5) Momordicine

Momordicine is an alkaloid and is also an important part of the chemical make-up of the bitter gourd because this is the chemical responsible for the bitterness of the vegetable and has a stomachic effect (Huang *et al.*, 1990). It is a novel cucurbitane glucoside with the chemical formula; 7-*O* -d -glucopyranosyl-3, 23-dihydroxycucurbita-5, 24-dien-19-al. It comes in different forms; ranging from Momordicine I- IV. Momordicine II and IV deterred oviposition by *L. trifolii* significantly when bioassays were carried out on kidney bean leaves treated at 75.6 and 20.3μ g/cm2 Leaf surface, respectively. There was no synergistic effect on oviposition deterrent when the two compounds were combined in their natural abundance (Raintree Nutrition, Inc., 2007).

Other chemical components of *Momordica charantia* include 5-α- stigmasta -7,25-dien -3-β-ol, α-elaeosteric, ascorbic acid, ascorbigen (a bound form of ascorbic acid), Ash, β-carotene, β- sitosterol, calcium, carbohydrates, cholesterol, citrulline, copper, cryloxanthin, diosgenin, elasterol, fat, fiber, flavochrome, fluoride, fluorine, GABA, Galacturonic acid, iodine, iron, lanosterol, lead, linoleic acid, lutein, lycopene, magnesium, manganese, momordicosides (A-L), mutachrome, niacin, nickel, nitrogen, oleic acid, oxalate, oxalic acid, pectin, peroxidase, phosphorus, phytofluence, pipecolic acid, polypeptide - p, potassium, protein, Riboflavin, Rubixanthin, sodium, stearic acid, stigimasterol, sugars, thiamin, titanium, urease, vicine, water, zeaxanthin, zeinoxanthin (Duke, 2004).

The fruits and leaves of Momordica Charantia contain two alkaloids, one of them being momordicin. The seeds contain an alkaloid (m.p 236°) and anthelmintic principle in the germ, they also contain urease (Airan & Gihatge, 1930; Rivera, 1941; Airan & Gihatge, 1950; Nath & Ullah, 1956; Rehm *et al* 1957; Rehm & Wessels, 1957).

2.7.1.3. BIOCHEMICAL/MEDICINAL USES OF *MOMORDICA CHARANTIA*

Most commonly, Momordica charantia is used for its antidiabetic properties. Other common uses have included antimicrobial and antifertility. Traditionally, bitter melon has been used

as a folk remedy for tumors, asthma, skin infections, gastrointestinal disorders, and hypertension and for many other ailments (Anon 1999; Duke *et al.* 2002). Complementary and alternative medicine indications include Diabetes mellitus, appetite stimulant (in traditional Chinese medicine), cancer, HIV infection and gastrointestinal infections (Zhang, 1992; Raman and Lau, 1996). An Ayurvedic herbal preparation of bitter melon has been used for the treatment of increased intraocular pressure. (Mistry and Patel, 1991). A less common use of bitter melon has been in as an insecticide in tropical countries (Cunnick and Takemoto, 1993).

2.7.1.4. PLANT SUMMARY (LEAF/STEM)

In this study, the leaf decoction of *Momordica charantia* was used.

The Leaves' main actions include: Anticancerous, antiviral, antibacterial, digestive stimulant and hypoglycemic. It is used for treatments of cancer, viral infections (HIV, herpes, Epstein Barr, hepatitis, influenza, and measles), bacterial infections (staphylococcus, streptococcus, and salmonella), as a bitter digestive aid (for dyspepsia and sluggish digestion) for diabetes, as antifungal, anti-inflammatory, antimalaria, antiparasitic, antiseptic, bitter, carminative (expels gas), digestive stimulant, febrifuge (reduces fever), hypotensive, lactagogue (promotes milk flow), menstrual stimulator, purgative, vermifuge (expels worms) and a wound healer (Tropical plant database, 2007).

2.7.1.5. MECHANISM OF ACTION OF M. *CHARANTIA*.

Bitter Melon contains four very promising bioactive compounds; which are charantin, momordicin, momordin and v-insulin. These compounds activate a protein called AMPK, which is well known for regulating fuel metabolism and enabling glucose uptake, processes which are impaired in diabetics. Bitter melon contains a lectin that has insulin-like activity. The insulin-like bioactivity of this lectin is due to its linking together two insulin receptors. This lectin lowers blood glucose concentrations by acting on peripheral tissues and, similar to insulin's effects in the brain, suppressing appetite. This lectin is

likely a major contributor to the hypoglycemic effect that develops after eating bitter melon and why it may be a way of managing adult-onset diabetes. Lectin binding is nonprotein specific, and this is likely why bitter melon has been credited with immunostimulatory activity - by linking receptors that modulate the immune system, thereby stimulating said receptors (Bakhru, 1997).

A novel phytochemical in bitter melon has clinically demonstrated the ability to inhibit an enzyme named *guanylate cyclase*. This enzyme is thought to be linked to the pathogenesis and replication of psoriasis, and implicated in leukemia and other cancers (Manabe et al., 2003). *Momordica charantia* extracts have broad-spectrum antimicrobial activity, having been shown to prevent infection by numerous viruses, bacteria, parasite organisms, and fungi. Although mechanism have not been determined for all organisms, in the case of viral infection it is thought that certain bitter melon constituents prevents viral penetration of the cell wall (Cunnick and Takemoto, 1993). The immune-stimulating properties of *M.charantia* extracts may also contribute to decreased rates of microbial infection observed in animal studies. Animal studies demonstrate *M. charantia* extracts, particularly the saponin fraction, have lipid lowering effects resulting into inhibition of pancreatic lipase activity and subsequent decreased lipid absorption (Oishi *et al.*, 2007). Another study demonstrates *M. charantia* juice has an inhibitory effect on membrane lipid peroxidation (Ahmed, 2001).

2.7.1.6. **MEDICINAL USES OF LEAF DECOCTION OF *M. CILIRANTIA***

It has been observed that medicinal and fruit bearing plants (including *M.charantia*) are widely used mainly as decoctions and juice preparations (Schunourlo et al., 2005).

The leaves of Momordica charantia may be boiled to make a decoction. Enough leaves should be used to give decoction a strong bitter taste and colour. This decoction is drunk as preventative treatment for many problems, such as stomachache, fever, infectious diseases, arthritis, diabetes, hypertension, even cancer. The decoction may also be used as

a skin wash, or added to the bath water. To make the taste of the decoction more tolerable, the leaves may be boiled with mint, or the decoction may be tempered with sugar, honey, or milk. The decoction relieves skin rashes and heat rash. A decoction of the leaves may be taken at the onset of infectious diseases, and the course of the disease will be mitigated. The decoction, taken regularly, has been used to regulate blood sugar and control diabetes. Also, a decoction of the chopped fresh bitter gourd (four ounces) could be boiled gently in a pint of water until half the liquid has boiled off. The decoction should be taken once a day (Pinkney *et al*, 1998).

2.7.1.7. **THE PROPERTIES/ ACTIONS DOCUMENTED BY RESEARCH.**

Popularity of M.charantia in various systems of traditional medicine for several ailments (antidiabetic abortifacient, anthelmintic, contraceptive, dysmenorrheal, eczema antimalaria, gout, jaundice, abdominal pain, leprosy, kidney (stone), piles, laxative, purgative, rheumatism, fever and scabies) focused the investigator's attention on this plant. Over 100 studies using modern techniques have authenticated its use in diabetes and its complications (neuropathy, cataract, insulin resistance), as antibacterial, as well as antiviral agent (including HIV infection), as anthelmintic and abortifacient. Most importantly, the studies have shown its efficacy in various cancers (lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of tongue and larynx, human bladder carcinomas and Hodgkin's disease) (Grover and Yadav, 2004).

Ng *et al.* (1986), have reported the *in vitro* antifertility effect of alpha- and beta-momorcharin in male rats. Also, Chan and colleagues in 1984 reported the termination of early pregnancy in the mouse by beta- momorcharin (Chan *et al*., 1984). In vitro studies indicate bitter melon fruit and seed extracts inhibit the growth of several cancer cell lines, including prostate adenocarcinoma, (Chilin *et al*.1978), human colon cancer (Caco-2 cells), (Yasui *et al*., 2005), and the highly metastatic breast cancer cell line MDA- MB

231 (Lee-Huang *et al.*, 2000). Various aqueous, ethanol, and methanol extracts of the leaves have demonstrated *in-vitro* antibacterial activities against *E. coli*, *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Streptobacillus and Streptococcus* (George, 1949; Hussain, 1991; Omoregbe, 1996 and Khan, 1998) an extract of the entire plant was shown to have antiprotozoal activity against Entamoeba histolytica (Bhakuni,1988).

The fruit and fruit juice have demonstrated the same type of antibacterial properties and, in another study, a fruit extract has demonstrated activity against the stomach ulcer-causing bacteria Helicobacter pylori (Yesilada, 1999). Other properties/actions documented by traditional use include anthelmintic (power to destroy worms), anti-bacterial, antibiotic, antidiabetic, anti-inflammatory, anti-leukemic, antimicrobial, anti-mutagenic, anti-mycobacterial, anti-oxidant, anti-tumor, anti-ulcer, anti-viral, aperitive, aphrodisiac, astringent (arresting secretion), carminative, cytostatic, cytotoxic, depurative, hormonal, hypo- holesterolemic, hypotensive, hypotriglyceridemic, hypoglycemic (reducing sugar in the blood), immunostimulant, insecticidal, lactagogue (increase the secretion or flow of milk), laxative, purgative, refrigerant, stomachic (promoting digestion and improve appetite), styptic, tonic, vermifuge (Zhu *et al.*, 1990).

2.7.1.8. TOXICITY OF *MOMORDICA CHARANTIA*

In animals, the principal toxicity is to the liver and reproductive system. These effects have not yet been reported in humans. Chronic administration of an alcohol extract of bitter melon fruit was associated with testicular lesions and a state of infertility in dogs. In addition, the red arils of the seeds are reported to be toxic to children, and the fruit is contraindicated during pregnancy (Bakhru, 1997). Oral ingestion of bitter melon fruit is safe as demonstrated by long-term consumption of the fruit in Asian cultures. Subcutaneous injection of p-insulin extracted from *M charantia* appears to be safe; however, intravenous injection of *M. charantia* extracts is significantly more toxic and not recommended. (Tropical Plant Database, 2007).

Reported adverse effects of bitter melon include hypoglycaemic coma and convulsions in children, increases (gamma)-glutamyl transferase and alkaline phosphatase levels in animals, and headaches. (Basch *et al.*, 2003). It has been reported that gastric administration of juice from the leaves of Momordica species decreased fertility in female mice from 90% (controls) to 20%. The effect of the drug was reversible, with treated mice reverting to the control fertility rate after an estrous cycle. No pathological changes were seen in maternal organs of treated mice. (Stepka, *et al.*, 1974). However, chronic administration of an alcoholic extract of bitter melon fruit was associated with testicular lesions and a state of infertility in dogs. Animals received 1.75grams daily orally for 60 days, the seminiferous tubules were completely devoid of spermatozoa, and tubular diameters were minimal. Spermatozoa were also absent in the lumen of the epididymis and vas-deferens. (Dixit, *et al.* 1978).

CHAPTER THREE

MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Wistar strain albino rats (male), aged between 12 and 14 weeks and weighing between 180g and 250g were purchased from both pathogen free colonies in the Veterinary Anatomy animal house University of Ibadan, Nigeria and the Physiology Animal House Ladoke Akintola University of Technology, Ogbomoso, Nigeria. They were housed in netted wooden cages under controlled conditions of light (12h light/12h dark cycle). The animals were fed with pelleted feeds and water ad libitum and allowed to acclimatize over a period of two weeks. The animals were then grouped into five (i.e. Control, Groups A, B,C and D), with the control receiving feed and water only through the 30-day experimental period and the different groups receiving differing dosages of the leaf decoction of M.charantia ranging from 35mg/100g, 45mg/100g, 55mg/100g and 65mg/100g body weight. Body weights (bw) were taken at Day 1(D1), Day 6 (D6), Day 11 (D11), Day16 (D16), Day 21 (D21), Day 26 (D26) and Day 31 (D31).

3.2. MEDICINAL PLANT

The medicinal plant M.charantia was purchased mainly from the Elewe onio section of the Oje market in Ibadan and authenticated at the herbarium, department of Botany, University of Ibadan Nigeria. Fresh leaves of the plant were made into a decoction.

3.2.1. PREPARATION OF THE LEAF DECOCTION OF M.CHARANTIA

Decoction was made according to a modification of the method of Cunnick and Takemoto, 1993. In brief, the plants were leafed and the leaves were rinsed with distilled water, drained, chopped and then weighed. 100g of the chopped leaves were boiled in 200ml of distilled water until the volume came to half (100ml). Thus, in the end 100g of leaves were extracted in 100ml of distilled water.

3.3. PROTEIN ESTIMATION

Protein was estimated by the procedure of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as standard.

3.3.1. PRINCIPLE

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue-purple colour complex, with maximum absorption in the region of 750nM wave length, when reacted with Folin-Ciocalteau reagent, which consists of Sodium tungstate molybdate and Phosphate. Thus, the intensity of colour depends on the amount of these aromatic amino-acids present and will thus vary for different proteins.

Most protein estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein because of its low cost, high purity and ready availability. The method is sensitive down to about 10µg/ml and is probably the most widely used protein assay despite it being only a relative method subject to interference from tris buffer, EDTA, non-ionic and cation detergents, carbohydrates, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

REAGENTS

Reagent A.

2% Na_2CO_3 in 0.1M NaOH solution; 2.0g of Na_2CO_3 and 0.4g of NaOH pellets were dissolved in about 50mls of distilled water and then made up to the mark of the 100mls standard volumetric flask with distilled water. The reagent was stored at room temperature.

Reagent B.

2% Na-K Tartarate solution

2.0g of Sodium-potassium tartarate ($Na\text{-}K.C_4O_6$) was dissolved in 50mls of distilled water and then made up to the 100mls mark of the volumetric flask with distilled water. Reagent was kept at room temperature.

- **Reagent C.**

1% Copper Sulphate Solution.

1g of hydrated Copper Sulphate ($CuSO_4.5H_2O$) was dissolved in 50mls distilled water and made up 100mls in a standard volumetric flask.

- **Reagent D.**

Alkaline Copper Solution

This was prepared fresh before use by mixing 50ml of reagent A with 0.5ml of reagent B and 0.5ml of reagent C. The tartarate solution was added first to prevent the solution from becoming cloudy.

- **Reagent E.**

Folin Ciocalteau Reagent Solution:

This is the colour reagent. This reagent contains Phosphomolybdictungstic complex, and bromine water. The reagent is commercially available in 2N, it is diluted with distilled water to 1N just before use. The reagent is kept in a black container because it is photolytic.

Standard Protein Solution: 1mg/ml BSA solution was prepared by dissolving 5mg of BSA in 5mls of distilled water.

3.3.2. PROCEDURE

Varying volumes of (100µl to 500µl) of the prepared standard BSA solution were used as shown in the protocol. Each test tube was made up to 1ml with distilled water and 3mls of reagent D was added to the protein samples, mixed and then allowed to stand at room temperature for 10mins. 0.3mls of reagent E was added very quickly and mixture vigorously shaken immediately. After 30mins of standing at room temperature, the

absorbances were read at 750nM using a Beckman UV Spectrophotometer. The resulting values were the data used to plot the standard protein curve.

For mitochondrial protein concentration determination, 10µl of mitochondria was diluted with 990µl of distilled water and this volume was used in place of BSA solutions. Absorbances were taken at 750nM. Readings were taken twice and the average used in the calculation for mitochondrial protein estimation.

TABLE 3.1 : PROTOCOL FOR PROTEIN ESTIMATION (Lowry et al., 1951).

Test tubes	1	2	3	4	5	6
Standard BSA (µl)	–	100	200	300	400	500
Distilled water (µl)	1,000	900	800	700	600	500
Reagent D (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Reagent E (ml)	0.3	0.3	0.3	0.3	0.3	0.3

Experiments were run in duplicates.

3.4. ASSAY FOR MITOCHONDRIAL SWELLING.

300μM $CaCl_2$/mg mitochondrial protein was used to induce MMPTP opening according to a modification of the procedure of Lapidus and Sokolove (1993).

3.4.1. PROCEDURE

The low ionic strength mitochondria were isolated using a method described by Johnson and Lardy (1967). On the 31st day, overnight fasted animals were sacrificed by "cervical dislocation" and dissected quickly. The liver was rapidly excised, trimmed to remove excess tissue and washed in buffer A. Thereafter the liver was weighed, chopped and suspended in buffer A to make a 10% suspension of tissue in buffer. Immediately the liver suspension was homogenized on ice using a glass-Teflon potter homogenizer. The homogenate was sedimented twice at 2500 rpm for 5mins to remove the nuclear fraction and cellular debris. Supernatant obtained was centrifuged at 13000 rpm for 10 mins and the mitochondrial fraction obtained was washed three times at 12000 rpm for 10 mins with buffer B. An MSE cold centrifuged was used. The mitochondria were immediately dispensed into 1 ml Eppendorf tubes as aliquots and used fresh.

3.4.2. PREPARATION OF BUFFERS

Buffer A (Isolation buffer).

210 mM Mannitol, 70 mM Sucrose, 5mM Hepes, 1M KOH and 1mM EGTA (pH= 7.4)

Preparation: 0.6g of Hepes, 19.15g of Mannitol, 12g of Sucrose and 0.19g of EGTA were dissolved in 480 ml of distilled water, standardized with 1M KOH (pH=7.4) and then made up to 500 ml.

Buffer B (Washing buffer).

210 mM Mannitol, 70 mM Sucrose, 5mM Hepes, 1M KOH and 0.5% BSA (pH= 7.4).

Preparation: 0.6g of Hepes, 19.15g of Mannitol, 12g of Sucrose and 2.5mg of BSA were dissolved in 480 ml of distilled water, standardized with 1M KOH (pH=7.4) and then made up to 500 ml.

- **Buffer C (Swelling buffer).**

210 mM Mannitol, 70 mM Sucrose, 5mM Hepes, 1M KOH (pH= 7.4).

Preparation: 0.6g of Hepes, 19.15g of Mannitol, 12g of Sucrose were dissolved in 480 ml of distilled water, standardized with 1M KOH (pH=7.4) and then made up to 500 ml.

All buffers were stored at 4°C. Reagents were purchased from Sigma and Co, USA.

3.5. DETERMINATION OF MITOCHONDRIAL SWELLING

3.5.1. PRINCIPLE

The principle behind this method is that when the mitochondria swell their refractive index changes and thus less light passes through the cuvette, resulting in a decrease in the light absorbance measured with a spectrometer.

Changes in volume of liver mitochondria were measured quantitatively at 540nm in a Beckman UV spectrophotometer based on the procedure of Lapidus and Sokolove, 1993.

PREPARATION OF REAGENTS NEEDED FOR STOCK SOLUTIONS.

- **4mM Spermine.**

0.01393g of Spermine was dissolved in a little distilled water and then made up to 10mls in a 10ml-standard volumetric flask.

- **80μM Rotenone.**

0.000316g of Rotenone was first dissolved in a little quantity of 95% Ethanol and then made up to 10mls with Ethanol in a 10ml-volumetric flask.

- **250mM Sodium succinate.**

0.6753g of Sodium succinate was dissolved first in a little quantity of distilled water and then made up to 10mls mark in a 10ml-volumetric flask.

- **12mM $CaCl_2$**

0.01332g of $CaCl_2$ was dissolved first in a little quantity of distilled water and then made up to 10mls in a 10ml-volumetric flask

- Swelling buffer (Buffer C)

Preparation as described under preparation of buffer C

3.5.2. PROCEDURE

Three assays were carried out for each group member.

I Swelling without triggering agent

II Swelling with triggering agent.

III Spermine Inhibition.

Mitochondria (0.4mg of protein/ml) were pre-incubated in the presence of rotenone in swelling buffer for about 3 minutes at 30^{0}C prior to the addition of $CaCl_2$ (triggering agent). 30 seconds later 250mM sodium succinate was added and swelling rate was quantified at 540nm based on the procedure described by Lapidus and Sokolove (1993). For assay without triggering agent, addition of $CaCl_2$ was omitted; spermine was added immediately after the addition of rotenone, just before the addition of mitochondria and the mixture was incubated for 3 minutes in a water bath at 30^{0}C with thorough mixing for spermine inhibition determination. Liver samples from untreated animals were used for the in-vitro induction of Membrane Permeability transition pore by 300μM $CaCl_2$/mg mitochondrial protein. The procedure above (with triggering agent) was used.

TABLE 3.2: PROTOCOL FOR MITOCHONDRIAL SWELLING ASSAY

Sample	Swelling buffer(µl)	Rotenone (µl)	Spermine (µl)	Mitochondria (µl)	$CaCl_2$ (µl)	Sodium Succinate(µl)
Blank	2,500	M	S	H	Buffer	
Without Triggering agent	2,385	25	–	40	–	50
With Triggering agent	2,360	25	–	40	25	50
Spermine Inhibition	2,297.5	25	62.5	40	25	50

Data reported are representative of multiple (2) experiments per animal.

3.6. SEMEN EVALUATION

The rats were sacrificed, placed on dorsal recumbency and their testicles were removed through a lower abdominal incision. The right and left epididymis were trimmed off the body of the testes and semen samples were collected using a Pasteur pipette, from the tail of the epididymis with a scapel blade. Testes as well as epididymes were harvested and preserved in 10% (v/v) formo-saline for histopathology.

Semen samples obtained were promptly analyzed for the following: motility, percentage viability, sperm concentration, and motphology using conventional methods described by Zemjanis, 1977. Colour and consistency were determined by visual assessment and volume of ejaculate read from a graduated collecting tube.

3.6.1. Volume

This was done by volume displacement; semen was collected in to 5ml of normal saline and then the volume determined.

3.6.2. Mass Activity

It was done according to the method described by Oyeyemi *et al.* (1996). Procedure: A rather thick drop of semen was pipetted unto a warmed glass slide and viewed at magnification of x40.

3.6.3. Motility

This is the percentage of sperm cells in a unidirectional progressive motion over a field of microscope.

3.6.3.1. Procedure

Motility was determined by putting a drop of the collected semen on a clean warmed glass slide with a few drops of 2.9% sodium citrate solution. This was gently rocked and covered with a cover slip (the use of the cover slip provides a uniform film, restricts the floating of sperm cells and delays drying of the smear) then viewed at magnification of x40 (Zemjanis, 1977). The mobility test is considered to provide the most significant

information about the quality of semen though it is subjective to factors which if not properly managed could alter its sensitivity.

3.6.4. Determination of percentage viability

Semen smears were made and stained with Eosin.Nigrosine stain for determination of percentage viability. The Eosin-Nigrosine stain differentiates dead from live cells and stains the background satisfactorily. The staining mixture contains 1% Eosin B and 5% of the background stain, Nigrosine in 3% sodium citrate dehydrate solution. The staining mixture can be kept for a long period of time if refrigerated.

The live-dead staining principle is based on the observation that certain stains, in this case eosin B, penetrate and stain the dead sperm cells, whereas, the viable cells repel this stain. Staining should be done without delay.

3.6.4.1. Procedure

Dilution was done by the use of appropriate amounts of the staining mixture. A relatively small drop of semen was placed on a warm slide. A 5 to 10 times larger drop of warm staining mixture was added or placed next to the semen. The semen and staining mixture were gently mixed together with an applicator stick. Not more than 3 to 5 secs were allowed for mixing. A smear was then made and quickly air-dried. The slide was examined at magnification of x100 and at least 100 stained and unstained cells were counted and the percentage of each group is estimated. The live-dead count supplements rather than replaces the motility tests, (Laing, 1988; Zemjanis, 1977).

3.6.5. Examination of Cells' Morphology

The purpose of this evaluation is to determine the presence and incidence of abnormal forms. The method was according to that of Zemjanis, 1977.

3.6.5.1. Procedure

A drop of semen was placed on a clean warm glass slide with two drops of Wells and Awa stain. These were gently mixed together and a smear was made on another clean

wann slide (to avoid cold shock), then air-dried. The slide was observed under a light microscope (x 100 magnification) for the presence of abnormal cells out of at least 400 sperm cells from several fields of the slides. The number of sperm cells and percentage of abnormal sperm cells were noted and recorded.

3.6.6. Evaluation of Sperm Concentration

The improved Neubaur haemocytometer method as described by Zemjanis, 1977, was used for this evaluation.

3.6.6.1. Procedure

Semen was pipetted to 0.5 mark on the pipette (using the red blood cell pipette) and this was made up to 1.10 mark on the pipette with normal saline which serves to dilute and fix the spermatozoa present. The pipette is then introduced into a pipette shaker and allowed to mix. About 2 to 3 drops of the diluent were discarded from the pipette before it was introduced into the counting chamber of the haemocymeter for counting. Data obtained were analyzed using the student t-test at a significant ($p<0.05$) level.

3.7. LIVER FUNCTION TESTS

3.7.1. BLOOD SAMPLE COLLECTION AND PREPARATION

On the 31st day, treated rats (groups which received between 35mg/100g bw and 65mg/g bw of the leaf decoction of *M.chloranta*) and control (the group which received only feed and water *ad libitum*) were sacrificed by cervical dislocation, after having been starved overnight and blood samples were collected by cardiac puncture into sterile universal sample tubes. The blood samples were allowed to stand for about 30mins in order to clot. The clotted samples were then centrifuged at 4,000 r.p.m for 10mins and the supernatant which was the serum was separated (pipetted) into new and sterile sample containers then stored at 4°C. Randox Diagnostic Kits were used for all the Liver function tests.

3.7.2. ASSAY OF SERUM ALANINE TRANSAMINASE (ALT)

The assay was performed using the method of Reitman and Frankel, 1957;

3.7.2.1. PRINCIPLE

Glutamic pyruvic Transaminase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine.

α-Ketoglutarate + L-Alanine $\rightleftharpoons$ L-Glutanate + Pyruvate.

REAGENTS

Randox Assay Kit was used and the composition is as follows;

Solution 1.

This is the buffer containing Phosphate buffer (100mM/L, pH7.4), L-Alanine (200mM/L) and α-Ketoglutarate (2.0mM/L).

Solution 2.

This is the colour developing reagent. It is 2,4-dinitrophenylhydrazine (2.0mM/L). the reagents were stored at 4°C.

3.7.2.2. PROCEDURE

The blank was prepared by incubating 0.2mls of solution 1 for exactly 30mins at 37°C, then pipetting 0.2mls of solution 2 and then 0.04mls of sample in a 3ml spectrophotometric cuvette. The solution was mixed and allowed to stand for exactly 20mins at 20 to 25°C, after which 2.0ml of 0.4Mol/L sodium hydroxide was added. The solution was again mixed and used as sample blank after 5mins. Absorbance was taken at 546nm. This procedure guides against stimulation of Transaminases in some sera by high concentration of aldehydes, ketones or oxoacids.

For the sample, 0.04ml of sample is mixed with 0.2ml of solution 1 and incubated for exactly 30mins at 37°C. after which 0.2ml of solution 2 is added and the solution is mixed and allowed to stand for exactly 20 mins. at 25°C. 2ml of 0.4M NaOH was added and after brief shaking, the absorbance was taken at 546nm after 5mins.

TABLE 3.3: PROTOCOL FOR THE DETERMINATION OF SERUM ALANINE TRANSAMINASE (ALT) ACTIVITY (Reitman and Frankel, 1957).

	Sample Blank	Sample
Sample		
Solution I	-	0.04ml
Solution was mixed and incubated for exactly 30min at 37°C.	0.2ml	0.2ml
Solution 2		
Sample	0.2ml	0.2ml
Solution was mixed and allowed to stand for exactly 20min at 25°C	0.04ml	-
Sodium Hydroxide	2.0ml	2.0ml

Spectrophotometric measurements were done against a sample blank.

3.7.3. ASSAY OF SERUM ASPARTATE TRANSAMINASE (AST)

The assay was performed using the method of Reitman and Frankel, 1957;

3.7.3.1. PRINCIPLE

Glutamic oxaloacetic Transaminase is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine

$$\alpha\text{-Keloglutarate} + \text{L-Aspartate} \xrightleftharpoons{AST} \text{L-Glutamate} + \text{oxaloacetate.}$$

REAGENTS

Randox Assay Kit was used and the composition is as follows;

Solution 1.

This is the buffer containing Phosphate buffer (100mM/L, pH 7.4), L-Aspartate (200mM/L) and α-Ketoglutarate (2.0mM/L).

Solution 2.

This is the colour developing reagent. It is 2,4-dinitrophenylhydrazine (2.0mM/L). The reagents were stored at 4°C

3.7.3.2. PROCEDURE

The blank was prepared by incubating 0.2mls of solution 1 for exactly 30mins at 37°C, then pipetting 0.2mls of solution 2 and then 0.04mls of sample in a 3ml spectrophotometric cuvette. The mixture was mixed and allowed to stand for exactly 20mins at 20 to 25°C, after which 2.0ml of 0.4Mol/L sodium hydroxide was added. The mixture was again mixed and used as sample blank after 5mins. Absorbance was taken at 546nm. This procedure guides against stimulation of Transaminases in some sera by high concentration of aldehydes, ketones or oxoacids.

For the sample, 0.04 of sample is mixed with 0.2ml of solution 1 and incubated for exactly 30mins at 37°C, after which 0.2ml of solution 2 was added and the mixture is mixed and allowed to stand for exactly 20 mins. at 25°C. 2ml of 0.4M NaOH was added and after a brief shaking, the absorbance was taken at 546nm after 5mins.

TABLE 3.4: PROTOCOL FOR THE DETERMINATION OF SERUM ASPARTATE TRANSAMINASE (AST) ACTIVITY (Reitman and Frankel, 1957).

	Sample Blank	Sample
Sample	-	0.04ml
Solution 1	0.2ml	0.2ml
Solution was mixed and incubated for exactly 30min at 37°C.		
Solution 2	0.2ml	0.2ml
Sample	0.04ml	-
Solution was mixed and allowed to stand for exactly 20min at 25°C		
Sodium Hydroxide	2.0ml	2.0ml

Spectrophotometric measurements were done against a sample blank.

3.7.4. **ASSAY OF SERUM ALKALINE PHOSPHATE (ALP)**

The ALP activity was determined using an optimized standard method described by Englehardt, *et al.*, (1970).

3.7.4.1. **PRINCIPLE**

P-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ Phosphate + p-nitrophenol (Deutsche Gesellcschaft fir Klinische chemie, 1972).

ALP catalzes the hydrolysis of the phosphate group on p-mitrophenyl phosphate to yield p-nitrophenol. The amount of p-nitrophenol produced is proportional to the ALP activity determined spectrophotometrically.

REAGENTS

Solution 1. (R1 a).

Buffer containing 1mol/L (pH 9.8) of Diethanolamine buffer and 0.5mMol/L of $MgCl_2$.

Solution 2. (R1b).

Substrate containing 10mMol/L of nitrophenylphosphate. Reagents were stored at 4^0C.

3.7.4.2. **PROCEDURE**

The catalog number of the kit used was AP 501(20mls for 5x20mls). 20mls of buffer R1a was mixed with 1 vial (20mls) of substrate (R1b) and the reconstituted reagent served as the working reagent.(used fresh). 0.50mls of the working reagent was added to 0.01ml of the test sample and the resulting solution was mixed. The initial absorbance was read at 405nm and the timing started simultaneously. Absorbances were read again after 1,2 and 3 minutes.Changes in enzyme activity were determined using the formular for micro analysis; U/L = 2760x ΔA at 405nm/min.

TABLE 3.5: PROTOCOL FOR THE DETERMINATION OF SERUM ALKALINE PHOSPHATE (ALP) ACTIVITY (Englehardt, *et al.*, 1970).

Formulae	Macro	Semi-Macro	Micro
Sample	0.05ml	0.02ml	0.01ml
Reagent (25°C, 30°C, 37°C.).	3.00ml	1.00ml	0.50ml

Spectrophotometric measurements were done at room (25°C) and against air.

Experiments were duplicated for each animal.

3.7.5. ASSAY OF SERUM GAMMA-GLUTAMYL TRANSFERASE (GGT).

The assay method used was done according to the method of Ladenson (1980).

3.7.5.1. PRINCIPLE

L-γ- glutamyl-p-nitroanilide+ glycylglycine $\xrightarrow{GGT}$ L-γ-glutamylglyglycine + p-nitroaniline (Szasz, 1969).

L-γ-glutamyl-p-nitroaniline and glycylglycine are donor and acceptor of the glutamyl group respectively. γ-glutamyl transferase transfers the γ-glutamyl group of L-γ-glutamyl-p-nitroaniline to glycylglycine. The amount of p-nitroanilide liberated is proportional to the GGT's activity (determined spectrophotometrically).

REAGENT

The substrate contains Tris buffer (71.5mM/L, pH 8.25), Glycylglycine (126mM/L), L-γ-glutamyl-p-nitroanilide (4mMol/L) and surfactants.

3.7.5.2. PROCEDURE

The catalog number of the kit used was GT 1065 (20x3.0ml). One vial of substrate was reconstituted with 3.0ml of deionized water and the reconstituted reagent served as the working reagent (used fresh). 1.0ml of the working reagent was added to 0.10ml of sample in a 1ml cuvette at 25°C and the resulting solution was mixed. The initial absorbance was read at 405nm and the timing started simultaneously. Absorbances were read again after 1,2 and 3 minutes.

Changes in enzyme activity was determined using the formular below:

$$\frac{\Delta A/\text{min} \times \text{total assay vol (ml)} \times 1000}{E \times \text{path (cm)} \times \text{light (cm)} \times \text{sample vol. (ml)}} = \text{U/L GGT}$$

$\Delta A/\text{min}$ = change in Absorbance per minute

1000 = factor for converting ml to litre

E = molar absorpontivity of p-nitroaniline

$9.9 cm^2$/ mol at 405nm

Factor = 2.2ml x1000/ 9.9 x 1cm x 0.2ml =1111

U/L = 1111 x A 405nm.

TABLE 3.6: PROTOCOL FOR THE DETERMINATION OF SERUM GAMMA-GLUTAMYL TRANSFERASE (GGT) ACTIVITY (Ladenson, 1980).

Fonnulae	Macro	Semi-Macro
Sample	0.20ml	0.10ml
Reagent (25°C, 30°C, 37°C,).	2.00ml	1.00ml

Spectrophotometric measurements were done at room (25°C) and against air. Experiments were duplicated for each animal.

3.8. HEAMATOLOGICAL STUDIES

Blood samples were collected intraocularly into Na^+EDTA bottles on the 31st day, after the animals have been fasted overnight. The heamatological studies were performed within as short time as possible in the Veterinary Medicine Laboratory, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. Red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV), heamoglobin concentration and the RBC indices (MCH (mean corpuscular heamoglobin), MCV (mean corpuscular volume) and MCHC (mean corpuscular heamoglobin concentration)) were all estimated. RBC indices were calculated from the RBC count, HB concentration and PCV estimations. All data were expressed as Mean ± SD and statistically analyzed with the student's t-test and One-way ANOVA. $P < 0.05$ was considered statistically significant.

3.9. HISTOPATHOLOGY

Samples of liver were obtained from sacrificed animals and fixed in 10% (v/v) formo-saline for histopathological studies. Same was done with the testes and epididymes.

3.9.1. PROCEDURE

The tissues collected (livers, testes and epididymis) were removed from the fixative after two (2) days, dehydrated through ascending grades of alcohol (70%, 80%, 90% and absolute) cleared in xylene, infiltrated, embedded in paraffin wax and cut into 5 micron a piece on Reichert ultra microtome for light microscope studies. They were then mounted on slides and stained with haematoxyline and eosin (H and E) according to routine procedure for light microscope. Tissues prepared were examined for qualitative differences in comparison to the normal untreated rats, which served as control. The slides were examined at magnification of X100.

3.10. DATA ANALYSIS

The difference (Mean ±SD) between the control groups and the experimental groups were examined using the one-way Analysis of Variance (ANOVA) and Student's t-test. p-values less than 0.05 were considered as significant. All data were analyzed using Excel and Origin 7.0. Computer Software Packages.

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CHAPTER FOUR

4.0. EXPERIMENTS AND RESULTS

EXPERIMENT 1: EFFECTS OF THE LEAF DECOCTION OF *MOMORDICA CHARANTIA* ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION (MMPT) PORE.

INTRODUCTION

Mitochondrial Permeability Transition (MPT) has been found to be involved in the regulation of apoptosis, as the mitochondrial proapoptotic factors such as cyt. c, AIF and Smac/Diablo, which are normally confined to the mitochondrial matrix are released through it into the cytosol. Once released, cyt. c binds with Apaf-1 which prompts the activation of caspases in the presence of ATP/dATP (Petronilli *et al.*, 2001). The realization that apoptosis is a gene-directed program has had profound implications for the understanding of developmental biology and tissue homeostasis, for it implies that cell numbers can be regulated by factors that influence cell survival as well as those that control proliferation and differentiation.

Moreover, the genetic basis for apoptosis implies that cell death, like any other metabolic or developmental program, can be disrupted by mutation. In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy (Thompson, 1995).

Kerr *et al.* (1972), have raised the possibility that a large percentage of cell loss from tumors was due to apoptosis and this hypothesis has been confirmed by subsequent studies which revealed a high frequency of apoptosis in spontaneously regressing tumors and in tumors treated with cytotoxic anticancer agents (Kerr *et al.*, 1994). Taken together, these observations suggested that apoptosis contributed to a high rate of cell loss in malignant tumors and, moreover, could promote tumor regression (Lowe and Lin, 2000).

It is now well established that anticancer agents induce apoptosis, and that distruption of apoptotic programs can reduce treatment sensitivity (Schmitt and Lowe, 1999). Sun, *et al.* (2004), identified representatives from various classes of chemopreventive agents from in-vitro studies with sufficient evidence to provide a detailed account of their apoptotic mechanisms. Most of these compounds can activate caspases through intrinsic effector mechanisms that are regulated by Bcl-2 family members (e.g inhibition of Bcl-2 expression or induction of Bax expression) or the mitochondrial permeability transition (e.g dissipation of mitochondrial inner transmembrane potential) (Sun, *et al.*, 2004).

The popularity of the plant *M.charantia* in various systems of traditional medicine for several ailments suggests that the plant contains bioactive agents that will be potentially useful in drug development. Over 100 studies using modern techniques have authenticated its use in diabetes and its complications. Most importantly, some of these studies have shown its efficacy in various cancers including breast cancer, skin tumor, prostatic cancer, and Hodgkin's disease (Grover and Yadav, 2004). The aim of this study was therefore to elucidate the effect of the decoction of *M.charantia* on MMPT pore as this may have a direct link to its anticancer properties.

PROCEDURE

Mitochondria were isolated from the livers of animals as stated in Chapter 3, Materials and Methods.

Three assays were carried out for animals in each group.

Swelling without triggering agent

Swelling with triggering agent.

Spermine inhibition.

Mitochondria (0.4mg of protein/ml) were pre-incubated in the presence of rotenone in swelling buffer for about 3 minutes at 30°C prior to the addition of $CaCl_2$ (triggering agent). Thirty seconds later 250mM sodium succinate was added and swelling rate was

quantified at 540nm in a Beckman UV spectrophotometer based on the procedure of Lapidus and Sokolove (1993).

For assay without triggering agent, addition of 300μM $CaCl_2$ was omitted; spermine was added immediately after the addition of rotenone, just before the addition of mitochondria and the mixture was incubated for 3 minutes in a water bath at 30°C with thorough mixing, for the determination of spermine inhibition.

Liver samples from untreated animals were used for the in-vitro induction of Membrane Permeability transition pore by 300μM $CaCl_2$ /mg mitochondrial protein.

RESULTS

Fig.1 shows that there were no significant changes in the volumes of intact mitochondria respiring on succinate in the absence of calcium, while the ion induced the opening of mitochondrial permeability transition pore up to about 12 folds in the presence of succinate and rotenone. In Fig.2, spermine, a standard inhibitor of calcium-induced opening of mitochondrial membrane permeability transition (MMPT) pore reversed the opening of the pore induced by calcium, by about 65%. Fig.3 shows the effect of *Momordica charantia* (*M. charantia*) at 35mg/100g bw on MMPT pore. Here, about a 4-fold increase in permeability transition was observed. Accordingly, swelling was almost completely reversed in the presence of 4mM spermine. Fig. 4 shows that at 45mg/100g bw, about 8-fold increase in permeability transition was obtained. This decoction-induced pore opening was also reversed by 4mM spermine.

In Fig 5, *M. charantia* at 55mg/100g bw was shown to increase MMPT by about 11folds. This increase was also reversed by spermine. Fig.6 shows the effect of 65mg/100g bw of the decoction of *M. charantia* on MMPT pore opening. A 9-fold increase in MMPTP opening was observed. This increase is lower than that observed at 55mg/100g bw, but much more than what was obtained without the triggering agent (Ca^{2+}).

Thus, the degree of opening of the pore was dose-dependent, with a minimal effect of 4-fold increase at 35mg/100g bw and a maximal effect of 11-fold increase was obtained at 55mg/100g bw. The use of ANOVA and students'-test also supports these results, showing a significant increase ($P<0.05$) in swelling between and within groups in a somewhat dose-dependent manner.

The extents of opening of the pore with Ca^{2+} in the presence of the decoction were affected in a dose-related manner. At 35mg/100g bw, there was still a significant increase in the decoction-induced opening of the MMPT pore in the presence of calcium to the tune of about 9 folds. At 45mg/100g bw the increase became less pronounced (9 folds). and at 55mg/100g bw which gave the highest induction in the presence of the decoction only, there was no significant additional induction caused by calcium in the presence of the decoction (11 folds).

The extent of opening of the pore at 65mg/100g bw by calcium in the presence of the decoction is however not as much as that seen with 55mg/100g bw, because the extent of induction of opening of the pore at 65mg/100g bw was only 9 folds as opposed to the 10-fold increase obtained in the presence of extra mitochondrial calcium. The changes in absorbance representing the effects of the leaf decoction of *M.charantia* on MMPTP in the absence and presence of calcium ion are summarized in Table 4.1 below. Data is reported as Mean ± SD.

CONCLUSION

The leaf decoction of *M.charantia* induced MMPTP opening in a dose-dependent manner. However, further opening observed in the presence of Ca^{2+} was only significant for group A. This may mean that the decoction induced almost complete openings in the other groups

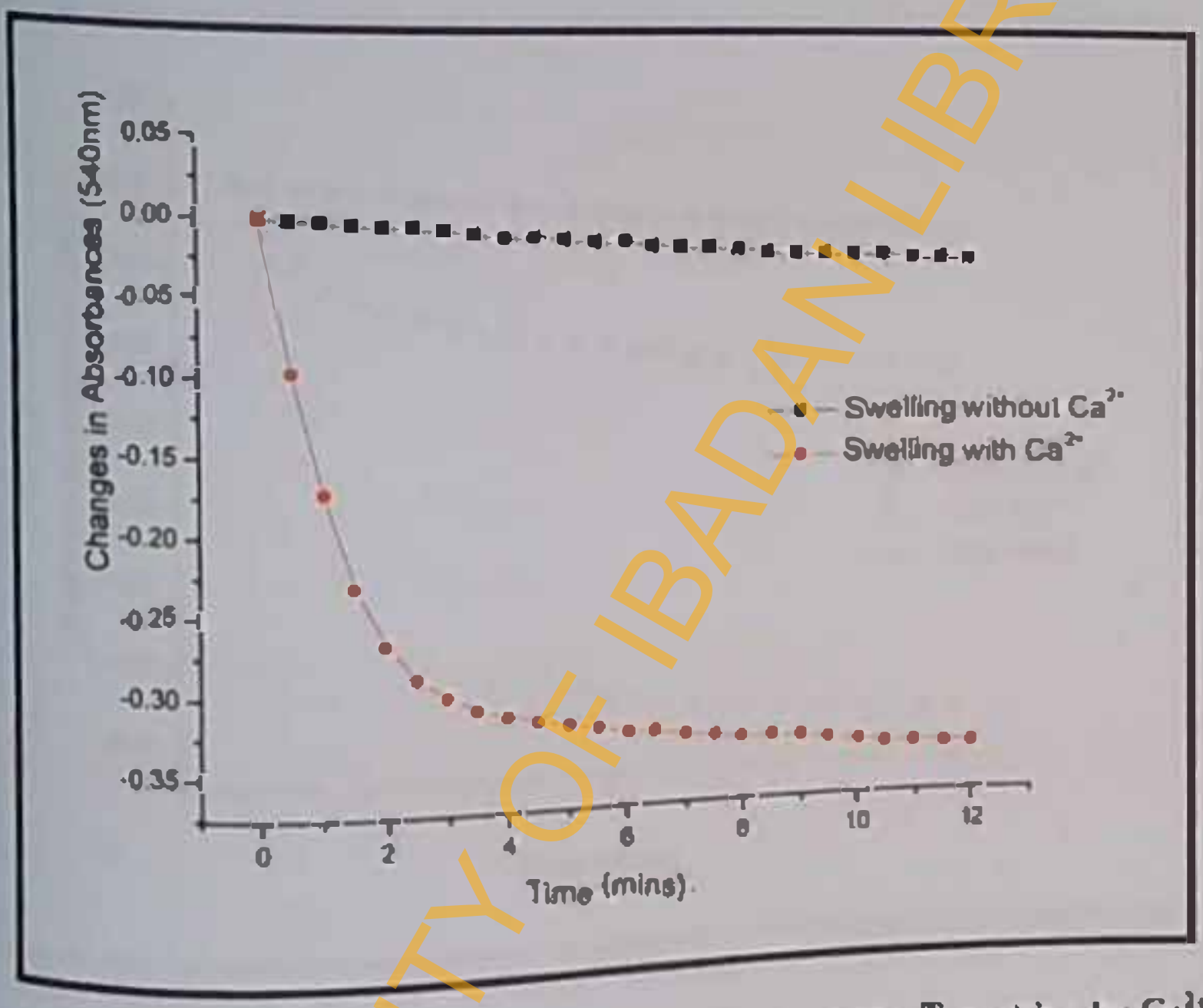


Fig 4.1a: Induction of Mitochondrial Membrane Permeability Transition by Ca^{2+} in normal (control) rats.

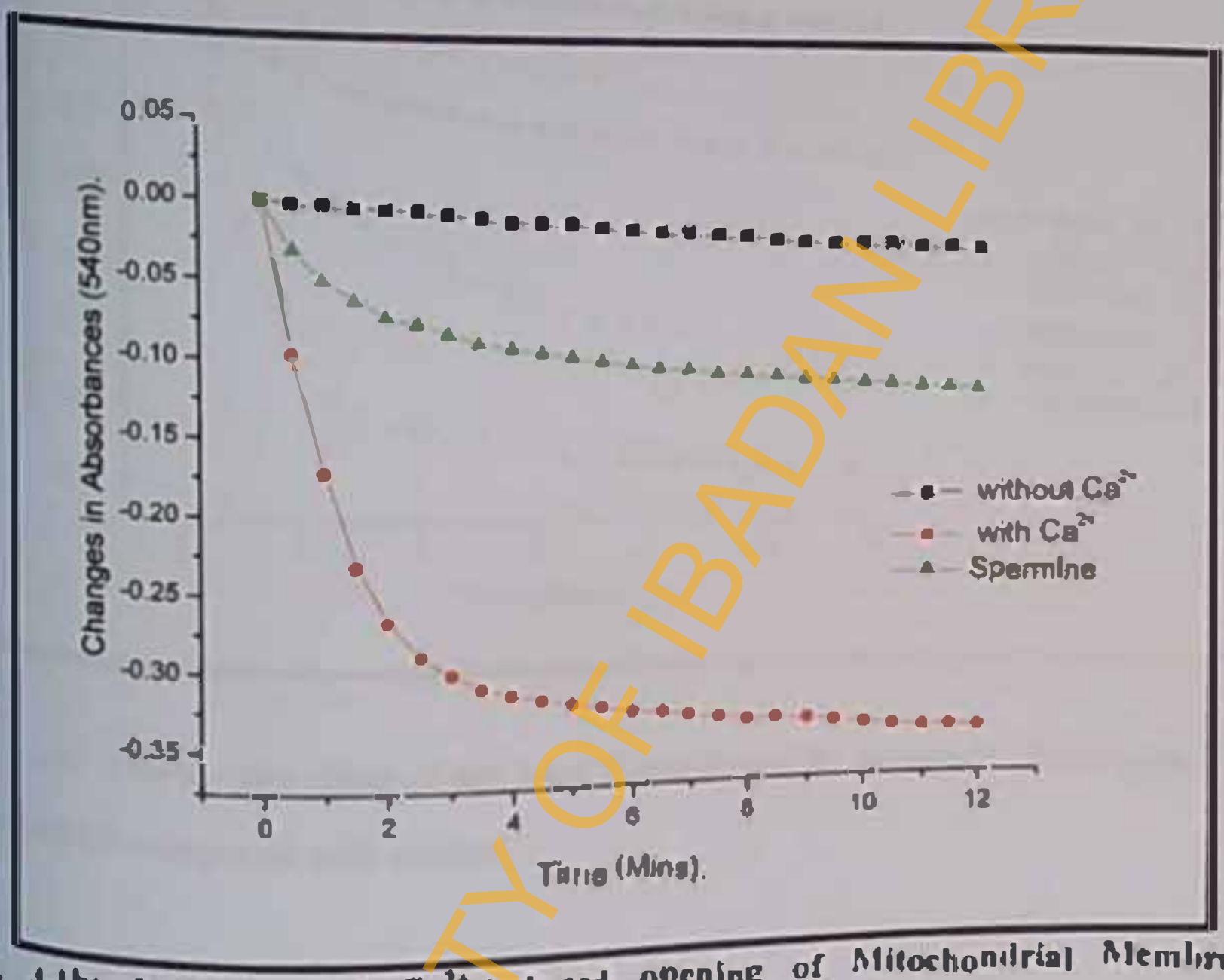


Fig 4.1b: Inhibition of Ca^{2+}-induced opening of Mitochondrial Membrane Permeability Transition Pore by spermine, a standard inhibitor.

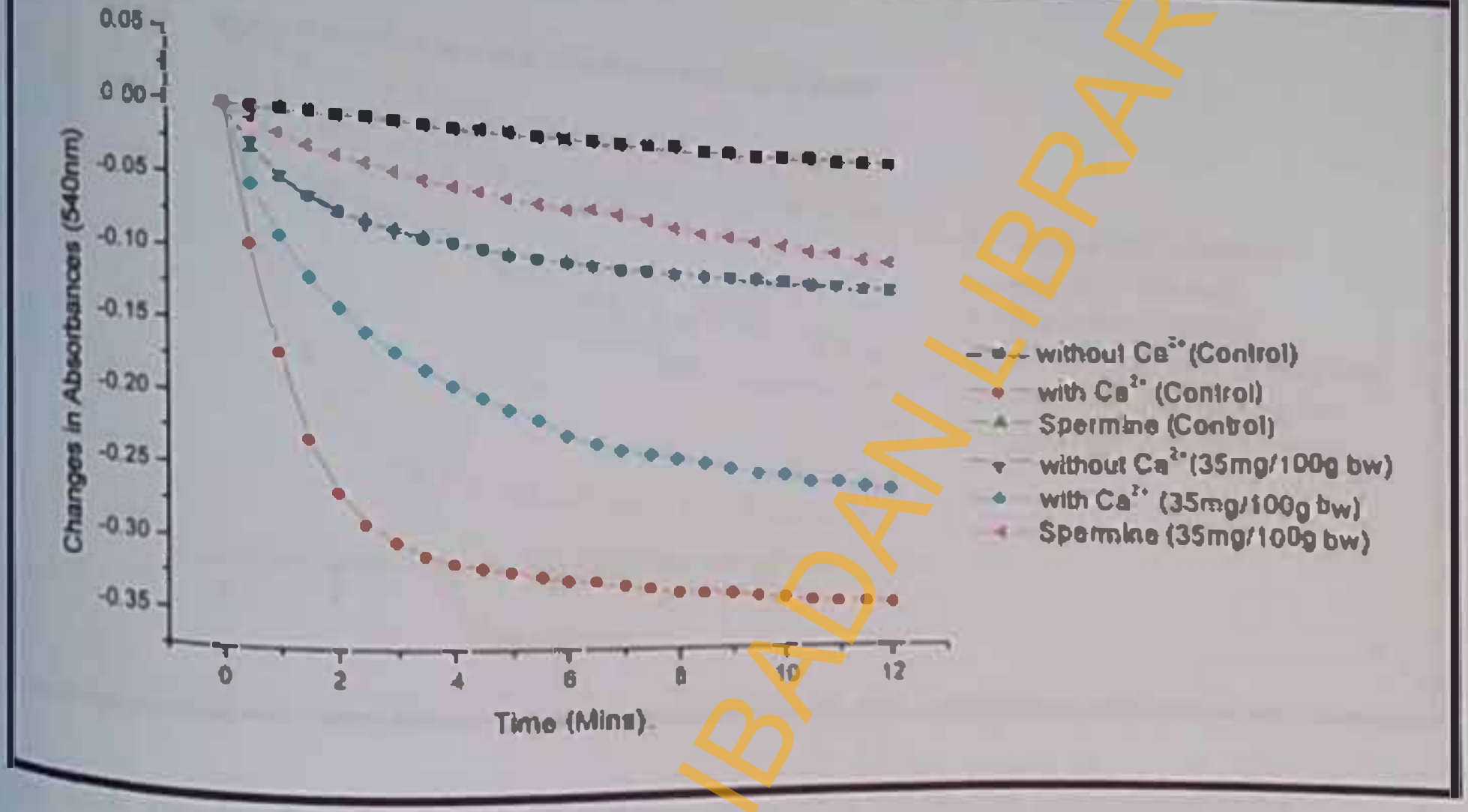


Fig. 4.1c: The in-vivo effect of the Leaf decoction of *M. charantia* (35mg/100g BW) on MMPTP compared with control.

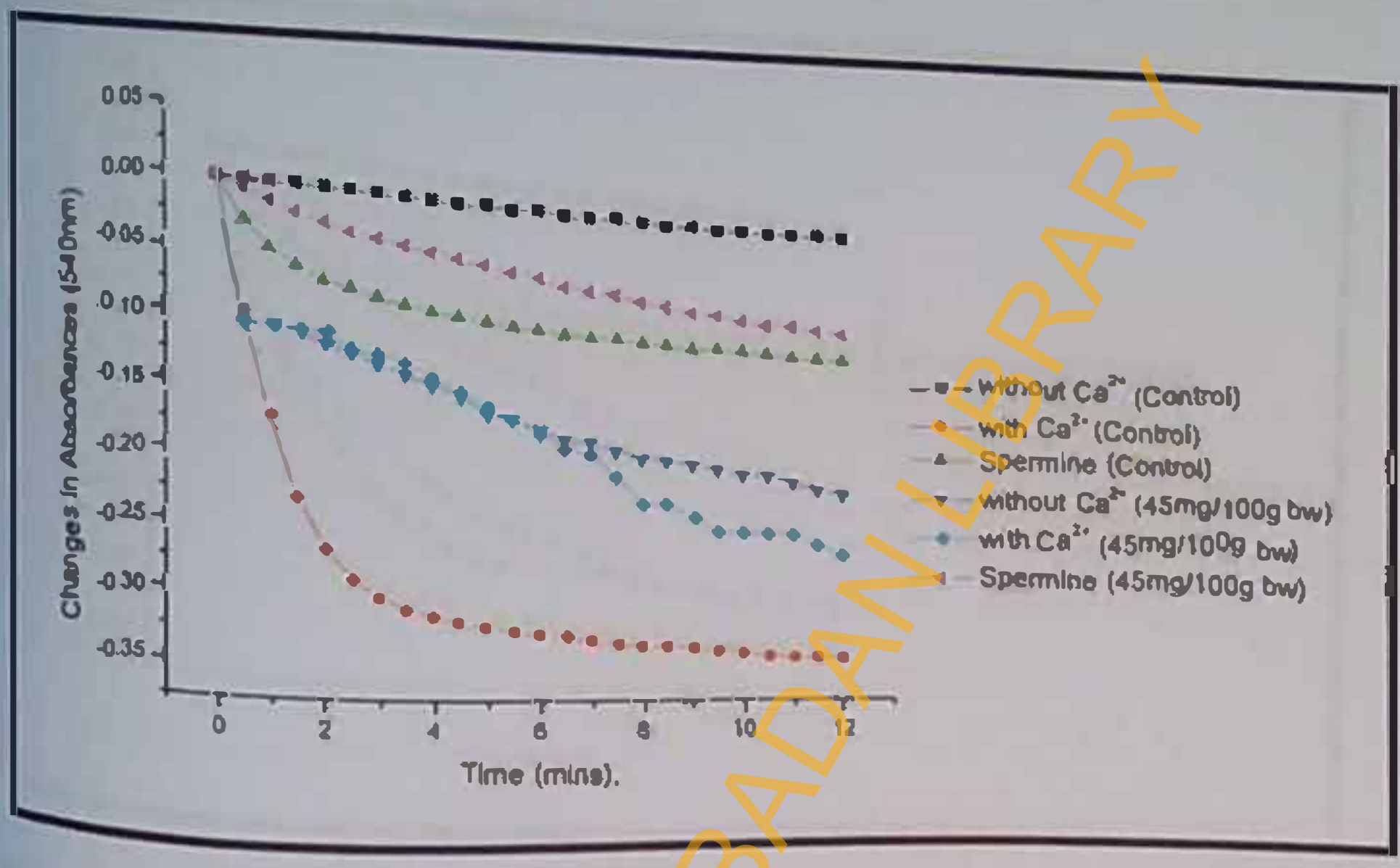


Fig 4.1d: The in-vivo effect of the Leaf decoction of *M. charantia* (45mg/100g BW) on MMPTP compared with control.

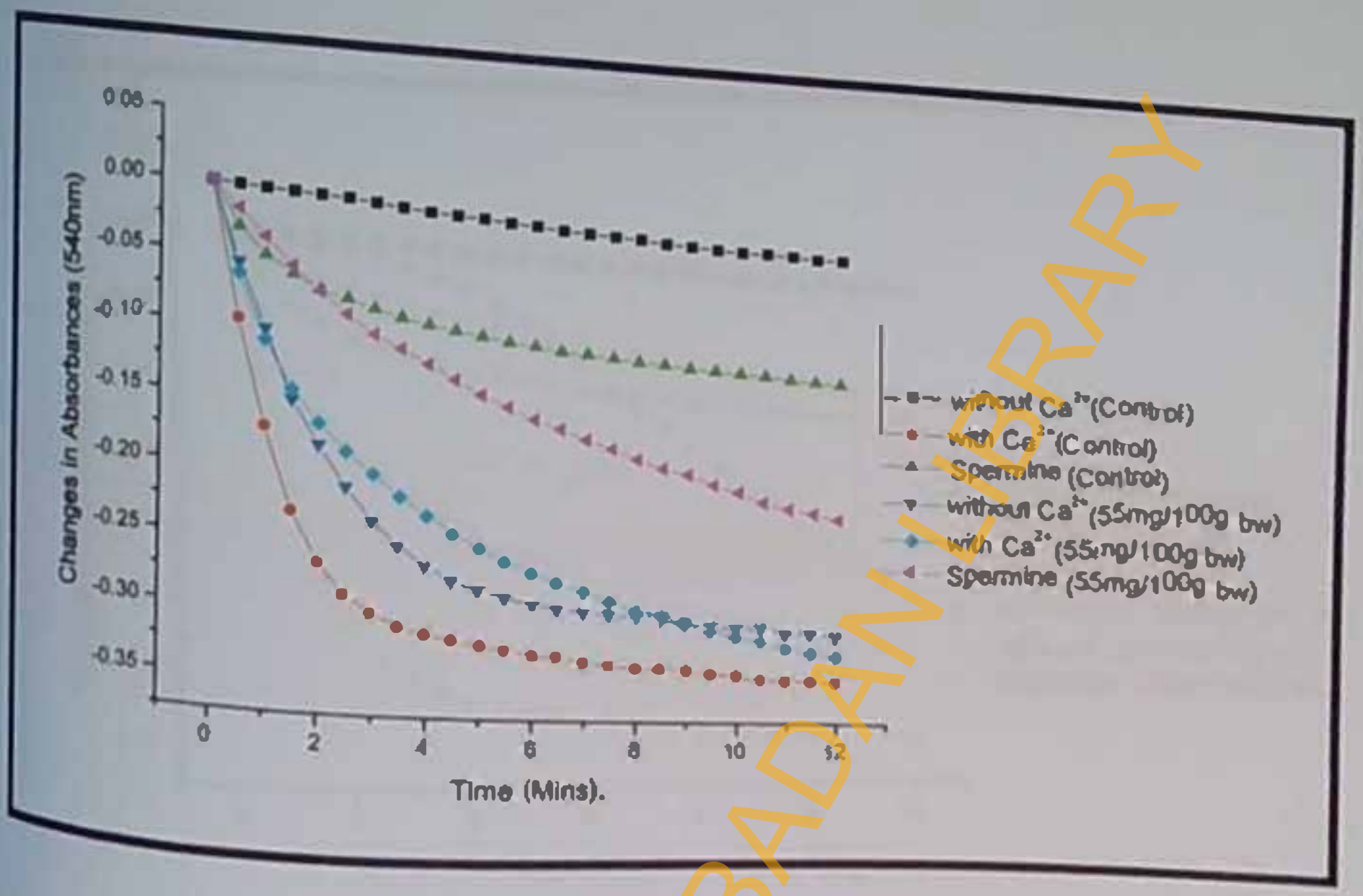


Fig. 4.1e: The in-vivo effect of the Leaf decoction of *M. charantia* (55mg/100g BW) on MMPTP compared with control.

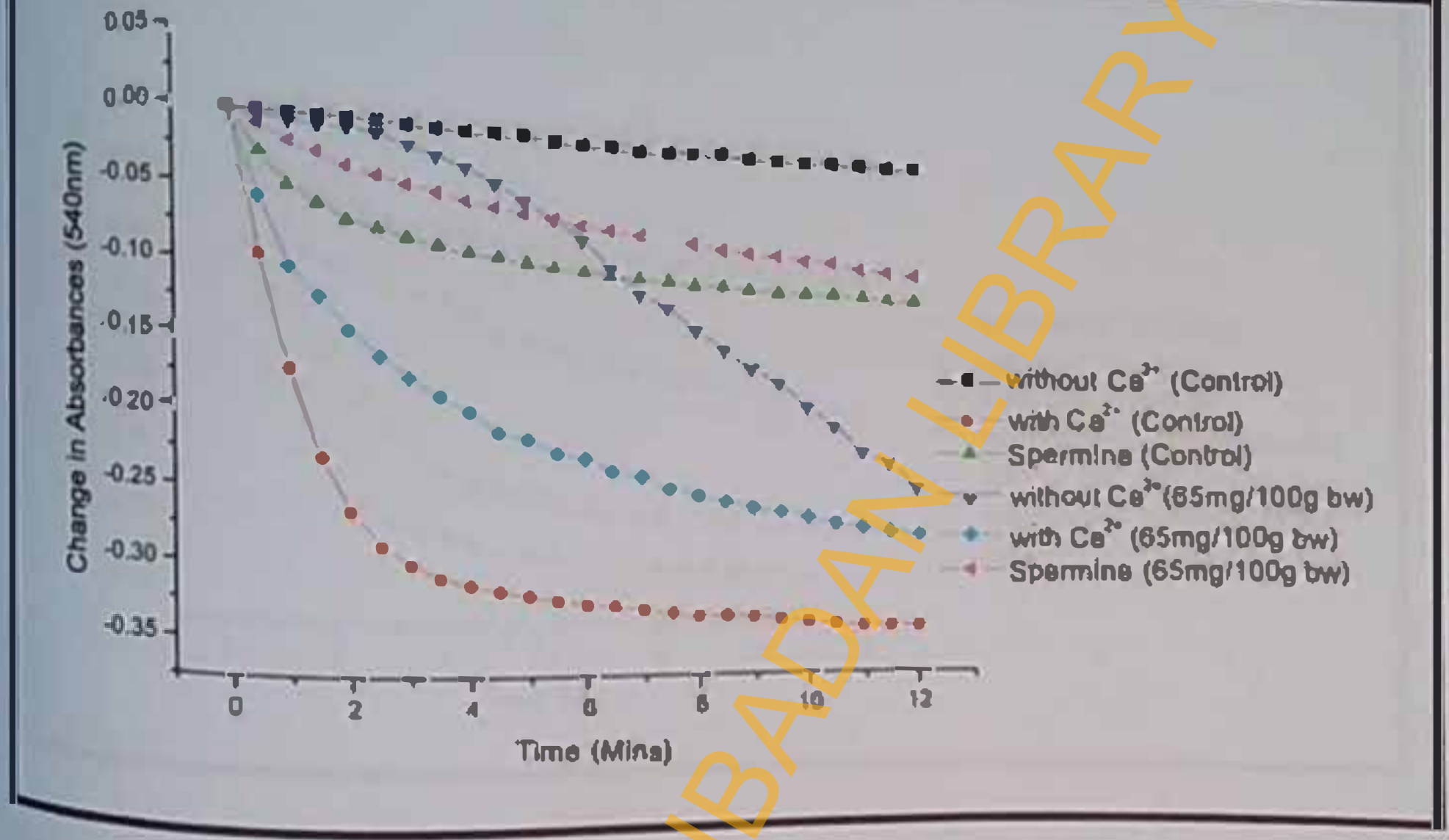


Fig. 4.1f: The in-vivo effect of the Leaf decoction of *M. charantia* (65mg/100g BW) on MAPTP compared with control.

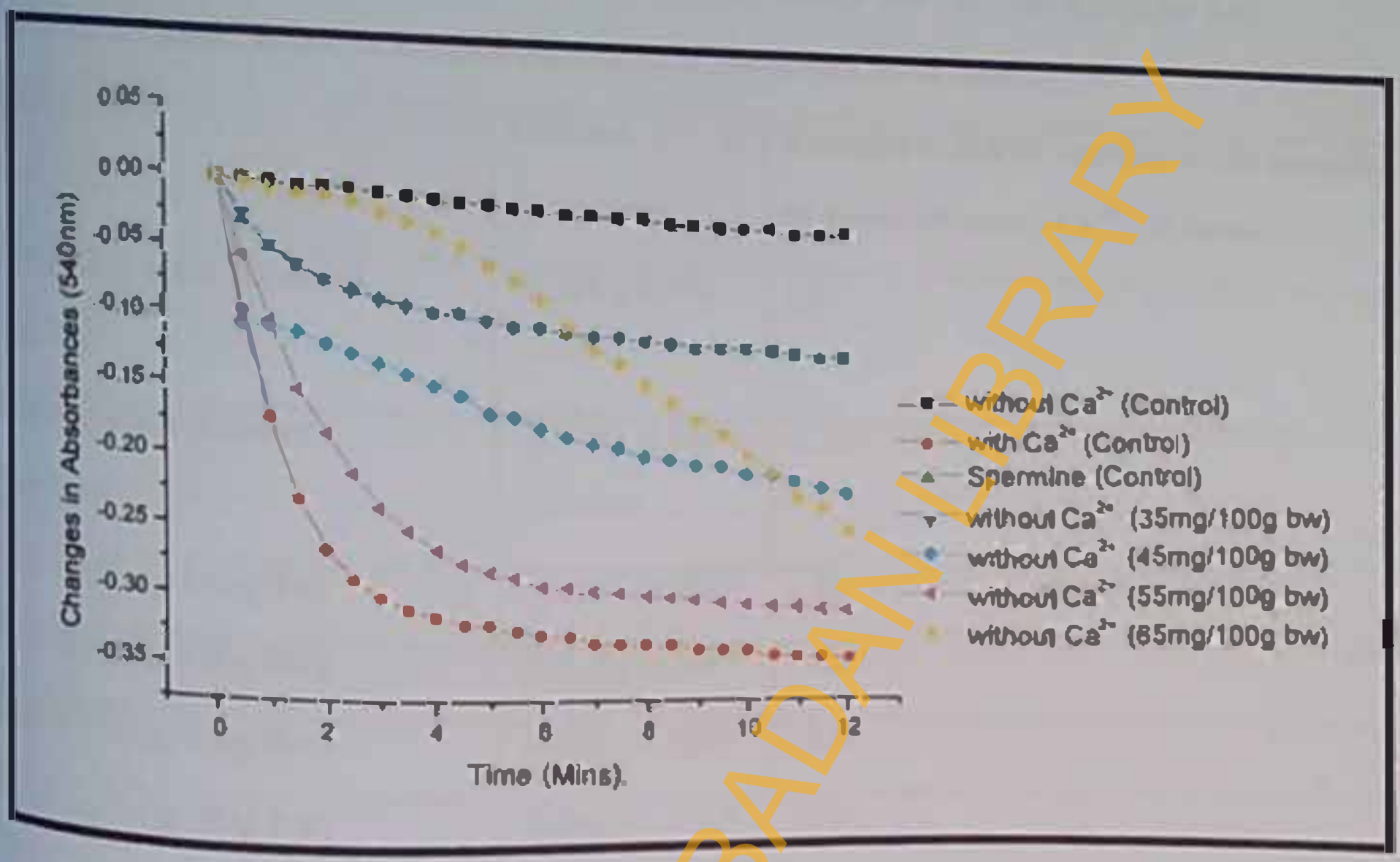


Fig. 4.1g: Comparism of the effects of the different dosages of *M. charantia* on MMPT Pore between groups and with control.

TABLE 4.1: EFFECTS OF THE LEAF DECOCTION OF *M.CHARANTIA* ON MMPTP IN THE ABSENCE AND PRESENCE OF CALCIUM ION.

Groups (n=4)	Changes in Absorbance	Increases in MPTP opening in the presence of decoction and/or Ca^{2+} (in Folds)
No triggering agent (Control) (-Calcium).	-0.029 ± 0.004	1.0
Triggering agent (Control) (+Calcium).	-0.344 ± 0.045*	11.86
Group A (35mg/100g Bw)	-0.119 ± 0.035*^{a}	4.10
Group B (45mg/100g Bw)	-0.222 ± 0.045*	7.66
Group C (55mg/100g Bw)	-0.309 ± 0.026*	10.66
Group D (65mg/100g Bw)	-0.249 ± 0.043*	8.59
Group A (35mg/100g Bw) + Calcium.	-0.263 ± 0.074*^{a}	9.07
Group B (45mg/100g Bw) + Calcium.	-0.267 ± 0.087*	9.21
Group C (55mg/100g Bw) + Calcium	-0.325 ± 0.039*	11.21
Group D (65mg/100g Bw) + Calcium.	-0.281 ± 0.025*	9.69

* Values which significantly ($P<0.05$) decreased when compared with control (No triggering agent).

^{a} values which significantly ($P<0.05$) differ when compared to each other.

EXPERIMENT 2: DETERMINATION OF THE EFFECTS OF THE LEAF DECOCTION OF *M. CHARANTIA* ON SPERMIOGRAM AND MORPHOLOGICAL CHARACTERISTICS OF SPERMATOZOA IN MALE WISTAR ALBINO RATS.

INTRODUCTION

Reproductive ability in the male comprises the production of semen containing normal spermatozoa (quality) in the number (quantity), together with the desire and ability to mate (Setchell, 1977). The testes (male glands) in all mammals are paired encapsulated, oval organs consisting of seminiferous tubules separated by interstitial tissue. Their sizes vary according to the species. In rodents and ungulates, they can be as much as one percent or more of the body weight. In human and some apes, they are considerably smaller (Harcourt *et al*, 1981). The epididymis is an extremely large convoluted structure, which is closely attached to the dorsal part of the lateral surface of the testicle (Setchell, 1977; Oyeyemi *et al*., 2000).

The functions of the epididymis include storage, maturation and absorption of sperm cells. Changes occurring in sperm during epididymal migration have been correlated with the function and integrity of the testis and epididymis (Rao, 1971). This has led to the classification of sperm defects into primary and secondary or major and minor sperm defects (Bloom, 1973) although Moss *et al*., (1988) classified these defects into primary, secondary and tertiary or miscellaneous.

Abnormal spermatozoan morphologies may be caused by testicular abnormalities that are present at birth (congenital), enlargement of veins within the scrotum (varicole veins), high fever, illicit drug use and infections (www.healthcaremagic.com, 2009).

Over 100 studies using modern techniques have authenticated the use of *M. charantia* in diabetes and its complications (neuropathy, cataract, insulin resistance), as antibacterial,

as well as antiviral agent (including HIV infection), as anthelmintic and abortifacient. Most importantly, the studies have shown its efficacy in various cancers (Grover and Yadav, 2004). In animals, the principal toxicity is to the liver and reproductive system. These effects have not yet been reported in humans. Chronic administration of an alcohol extract of bitter melon fruit was associated with testicular lesions and a state of infertility in dogs and the fruit is contraindicated during pregnancy (Bakhru, 1997). Also, Ng *et al.* have reported the in vitro antifertility effect of alpha- and beta- momorcharin in male rats (Ng *et al.*, 1986). Chan and colleagues in 1984 also reported the termination of early pregnancy in the mouse by beta- momorcharin (Chan *et al.*, 1984).

This study seeks to determine the effects of the leaf decoction of *M.charantia* on spermiogram and morphological characteristics of spermatozoa in male wistar albino rats.

PROCEDURE

The rats were sacrificed, placed on dorsal recumbency and their testicles were removed through a lower abdominal incision. The right and left epididymis were trimmed off the body of the testes and semen samples were collected using a Pasteur pipette, from the tail of the epididymis with a scapel blade. Semen samples obtained were promptly analyzed for the following; motility, percentage viability, sperm concentration, and morphology using conventional methods described by Zemjanis, (1977) at the Veterinary Surgery and Reproductive Laboratory, University of Ibadan, Nigeria. Colour and consistency were determined by visual assessment and volume of ejaculate read from a graduated collecting tube. Each group (except group C, where the demise of an animal was recorded), was made up of four animals.

RESULTS

Significant ($P<0.05$) reductions in sperm motility (77.5±50% at 35mg/100g bw, 72.5±50% at 45mg/100g bw, 73.33±5.77% at 55mg/100g bw and 75±5.77% at

65mg/100g bw compared with control (92±2.87%)) and sperm cell concentrations (i.e 64.25±8.26 (65mg/100g bw) < 66±7.21 (55mg/100g bw) < 57.75±4.79 (45mg/100g bw) < 70.25±1.26 (35mg/100g bw) < 82±2.45x10^6 cells/ml (control) were observed for all animals which were orally exposed to the decoction. Likewise, significant reduction ($P<0.05$) i.e 86.25±4.79%, 91.67±2.89% and 88.75±2.50% respectively compared to the control (96.5±1.73%) in percentage viability were seen for animals that received 45mg/100g bw and above. Morphological abnormalities of sperm cells above the proposed percentage (Control = 7.69%, 35mg/100g bw = 9.60%, 45mg/100g bw =12.94%, 55mg/100g bw =13.84% and 65mg/100g bw = 13.02%).) range of 10% allowed for breeding (Reece, 1997; Rozeboom, 2000) animals were also observed in animals that received 45mg/100g bw and above. Tables 4.2 and 4.3 summarize the effects of the leaf decoction of *M. charantia* on the spermiogram and morphological characteristics of spermatozoa in male wistar albino rats. Data is reported as Mean ± SD.

CONCLUSION

M. charantia elicited toxic effects of sub-acute (30-day) oral administration of its leaf decoction in albino rats with respect to male fertility.

TABLE 4.2: EFFECTS OF DIFFERENT DOSAGES OF *M.CHIARANTIA* ON THE SPERMIOGRAM OF EXPERIMENTAL RATS.

Groups / Parameter	Control 0mg/100g BW(n=4)	Group A 35mg/100gBW (n=4)	Group B 45mg/100gBW (n=4)	Group C 55mg/100gBW (n=3)	Group D 65mg/100gBW (n=4)
Motility (%)	92.50±2.89	77.50±5.00[b]	72.50±5.00[b]	73.33±5.77[b]	75.00±5.77[b]
Percentage (%) Live. dead ratio	96.50±1.73	93.25±3.95	86.25±4.79[b]	91.67±2.89[b]	88.75±2.50[b]
Cell count (X10⁶ cells/ml)	82.00±2.45	70.25±1.26[b]	57.75±4.79[b]	66.00±7.21[b]	64.25±8.26[b]
Volume (ml)	0.15±0.06	0.15±0.06	0.13±0.05	0.13±0.06	0.18±0.05

b = values which decreased significant ($P<0.05$) when compared with control

TABLE 4.3: EFFECTS OF *M.CHARANTIA* ON THE MORPHOLOGICAL CHARACTERISTICS OF SPERMATOZOA IN EXPERIMENTAL RATS.

Groups / Parameter	Control 0mg/100gBW (n=4)	Group A 35mg/100gBW (n=4)	Group B 45mg/100gBW (n=4)	Group C 55mg/100gBW (n=3)	Group D 65mg/100gBW (n=4)
NHWT	15 (0.94%)	17 (1.02%)	21 (1.30%)	17 (1.41%)	19 (1.18%)
NTWH	16 (1.00%)	21 (1.23%)	26 (1.63%)	16 (1.32%)	20 (1.24%)
BT	4.0 (0.25%)	23 (1.41%)	28 (1.74%)	26 (2.16%)	34 (2.08%)
RT	19 (1.19%)	23 (1.41%)	36 (2.24%)	32 (2.66%)	36 (2.21%)
CT	21 (1.31%)	23 (1.41%)	33 (2.07%)	23 (1.90%)	33 (2.02%)
CMP	25 (1.56%)	24 (1.47%)	31 (1.95%)	25 (2.07%)	34 (2.09%)
BMP	23 (1.44%)	25 (1.60%)	27 (1.71%)	22 (1.82%)	31 (1.90%)
LT	-	-	5.0 (0.30%)	6.0 (0.50%)	5.0 (0.30%)
TSC	1600 (100%)	1640 (100%)	1605 (100%)	1205 (100%)	1625 (100%)
TNC	1477	1483	1398	1038	1413
TAC	123	157	207	167	212
%AC	7.69%	9.60%	12.94%	13.84%	13.02%

NHWT = Normal head without tail

RT = Rudimentary tail

CT = Curved tail

BMP = Bent mid-piece

TSC = total sperm cells

TAC = Total abnormal cells

NTWH = Normal tail without head

BT = Bent tail

CMP = Curved mid- piece

LT = Looped tail

TNC = Total normal cells

%AC = Percentage abnormal cells.

EXPERIMENT 3: DETERMINATION OF THE LEAF DECOCTION OF *MOMORDICA CHARANTIA* ON LIVER FUNCTION OF NORMAL ALBINO RATS.

INTRODUCTION

The liver is the largest gland of the body. It normally weighs about 1.5kg. It is an organ in the upper abdomen and fitting under the diaphragm. It has two main lobes, the right lobe being much larger than the left (Chopra, 2002). The various functions of the liver, including the production of bile for emulsification, Gluconeogenesis, Glycogenolysis, Glycogenesis, the breakdown of insulin and other hormones, protein metabolism, lipid metabolism, cholesterol synthesis, production of triglycerides (fats), production of coagulation factors, breakdown of haemoglobin for the production of metabolites that are added to bile as pigment, breakdown of toxic substances and most medicinal products in a process called drug metabolism, convertion of ammonia to urea, production of red blood cells in the first trimester fetus, and the production of albumin are carried out by the liver cells or hepatocytes. (Liver-Wikipedia, 2008).

Many diseases of the liver are accompanied by jaundice caused by increased levels of bilirubin in the system (bilirubin results from the breakup of the hemoglobin of dead red blood cells; normally, the liver removes bilurubin from the blood and excretes it through bile this, a dysfunctional liver can not do, thus, jaundice results). Common examples of liver diseases are: Hepatitis (inflammation of the liver, caused mainly by various viruses but also by some poisons (e.g. alcohol), autoimmunity (autoimunune hepatitis) or hereditary conditions), non-alcoholic fatty liver disease (a spectrum in disease, associated with obesity and characterized as an abundance of fat in the liver; may lead to a hepatitis, ie: steatohepatitis and/or cirrhosis), cirrhosis (the formation of fibrous tissue in the liver, replacing dead liver cells which could be caused by viral hepatitis, alcoholism or contact

with other liver-toxic chemicals), cancer of the liver (primary hepatocellular carcinoma or cholangiocarcinoma and metastatic cancers, usually from other parts of the gastrointestinal tract) (Sherlock and Dooley, 2002), chronic bile duct blockage (a condition which may occur at birth (biliary atresia) or develop later in life (primary biliary cirrhosis) and haemochromatosis (the most common genetic liver disorder which involves excess iron storage and is usually diagnosed in adults) (Gallenage *et al*., 2004).

Liver function tests are series of enzyme tests used to ascertain the extent of hepatic (liver) damage either by injury or diseases. These Liver function tests consist of enzymes present in the liver and they are of great clinical significance. The test sample is either o plasma sample or serum sample collected by phlebotomy. There are different types of liver function tests, examples are the transaminases (e.g. alanine transaminase, aspartate transaminase), alkaline phosphatase, gamma-glutamyl transferase, 5'-nucleotidase, lactate dehydrogenase among others (Knight, 2006).

Momordica charantia is used for its antidiabetic properties. Other common uses have included antimicrobial and antifertility. Traditionally, bitter melon has been used as a folk remedy for tumors, asthma, skin infections, gastrointestinal disorders, and hypertension and for many other ailments (Allon 1999; Duke *et al*. 2002). Complementary and alternative medicine indications include diabetes mellitus, appetite stimulant (in traditional Chinese medicine), cancer, HIV infection and gastrointestinal infections (Zhang, 1992; Raman and Lau, 1996). An Ayurvedic herbal preparation of bitter melon has been used for the treatment of increased intraocular pressure (Mistry and Patel, 1991).

A less common use of bitter melon has been as an insecticide in tropical countries (Cunnick and Takemoto, 1993). Possible toxic effects of *Momordica charantia* on key hepatic enzymes in animals have been documented (Tennekoon *et al*., 1994). These include increase in hepatic enzymes such as serum γ-glutamyl transferase and serum alkaline phosphatase (Bakhru,

1997). This current experiment thus aims at determining the effects of the leaf decoction of *M charantia* on liver function of normal albino rats.

PROCEDURE

On the 31st day, treated rats (groups which received between 35mg/100g bw and 65mg/g bw of the leaf decoction of *M charantia*) and control (the group which received only feed and water *ad libitum*) were sacrificed by cervical dislocation, after having been starved overnight and blood samples were collected by cardiac puncture into sterile universal sample tubes. The blood samples were allowed to stand for about 30mins in order to clot. The clotted samples were then centrifuged at 4,000 r.p.m for 10mins and the supernatant which was the serum was separated (pipetted) into new and sterile sample containers then stored at 4°C. Randox Diagnostic Kits were used for all the liver function tests. The assays for alanine transaminase (ALT) and aspartate transaminase (AST) were performed using the method of Reitman and Frankel (1957), gamma-glutamyl transferase(γGT) was assayed according to the method of Ladenson (1980), while alkaline phosphatase (ALP) levels were assayed based on the optimized standard method described by Englehardt, *et al*, (1970). Each group (except group C, where the demise of an animal was recorded), was made up of four animals.

RESULTS

The results obtained show no significant ($P<0.05$) differences for ALT and AST when compared with control however, there were significant increases ($P<0.05$) in a somewhat dose-dependent pattern [181.65±69.80 at 35mg/100g bw, 211.10±31.45 at 45mg/100g bw, 213.20±39.42 at 55mg/100g bw and 218.90±34.25 at 65mg/100g bw compared with control (92.55 ± 7.76)] for ALP and [42.33 ±7.56 at 35mg/100g bw, 50.77 ± 4.48 at 45mg/100g bw, 54.35 ± 5.31 at 55mg/100g bw and 51.34 ± 3.22 at 65mg/100g bw relative to control (20.86 ± 1.07)] for γGT. Figures 4.2.1 and 4.2.2 are graphical representations of the ALT and AST activities and the ALP and γGT activities

respectively, in the control and decoction- treated rats. Table 4.2 summarizes the effects of the leaf decoction of *Momordica charantia* on liver function of normal albino rats. Data is reported as Mean ± SD.

CONCLUSION

The leaf decoction of *M. charantia* caused somewhat dose-dependent significant increases in the ALP and γGT levels of normal (control) rats. The leaf decoction may be hepatotoxic on prolonged use.

Fig. 4.3a: Effects of different dosages of the decoction of *M. charantia* on serum ALT and AST activities.

There were no significant differences observed between the control groups and the different groups.

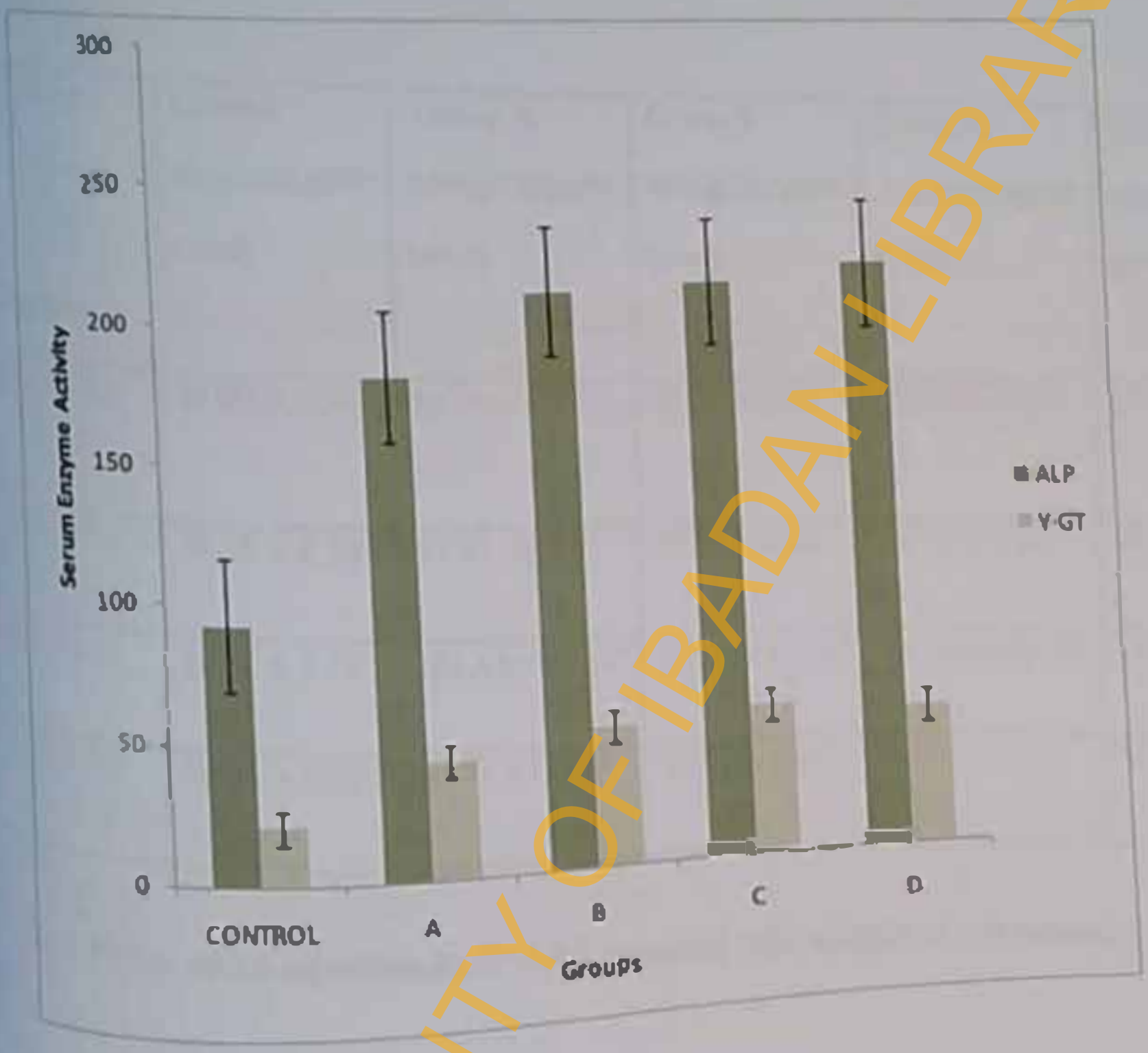


Fig. 4.3b: Effects of different dosages of *M. charantia* on Serum ALP and γ-GT levels.

Note: There were significant ($P < 0.05$) increases in ALP and γGT for Groups A, B, C and D compared with control.

TABLE 4.4: EFFECTS OF THE LEAF DECOCTION OF *ALCHARNEA* ON LIVER FUNCTION OF NORMAL ALBINO RATS.

Groups / Parameter	Control 0mg/100gBW (n=4)	Group A 35mg/100gBW (n=4)	Group B 45mg/100gBW (n=4)	Group C 55mg/100gBW (n=3)	Group D 65mg/100gBW (n=4)
ALT (U/L)	25.00 ± 7.70	24.25 ± 7.37	22.50 ± 4.20	29.67 ± 4.51	27.5 ± 7.05
AST (U/L)	54.85 ± 8.30	55.13 ±8.05	55.55 ± 4.06	5943 ± 1.10	57.08 ± 8.66
ALP (U/L)	92.55 ± 7.76	181.65± 69.80[a]	211.10±31.45[a]	213.20±39.42[a]	218.90±34.25[a]
GGT (U/L)	20.86 ± 1.07	42.33 ±7.56[a]	50.77 ± 4.48[a]	54.35 ± 5.31[a]	51.34 ± 3.22[a]

a = Values which significantly ($P<0.05$) increased when compared with control.

EXPERIMENT 4: IN-VIVO EFFECTS OF THE LEAF DECOCTION OF *M.CHARANTIA* ON HEAMATOLOGICAL PROFILE OF EXPERIMENTAL RATS.

INTRODUCTION

Irrespective of the target organs, the decoction is conveyed to it sites of action through the blood stream. Blood accounts for 7% of the human body weight (Alberts, 2005), with an average density of approximately 1060 kg/m3, very close to pure water's density of 1000 kg/m3. The average adult has a blood volume of roughly 5 liters (1.3 gal), composed of plasma and several kinds of cells (occasionally called *corpuscles*); these formed elements of the blood are erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). By volume, the red blood cells constitute about 45% of whole blood, the plasma about 54.3%, and white cells about 0.7%. Blood supplies oxygen, nutrients (glucose, amino acids, and fatty acids) to tissues and removes waste (carbon dioxide, urea, and lactic acid) from the same. It has immunological functions (including circulation of white blood cells and detection of foreign material by antibodies), coagulation functions (which is one part of the body's self-repair mechanism), messenger functions (including the transport of hormones and the signaling of tissue damage), regulatory function on body pH (the normal pH of blood is in the range of 7.35 - 7.45 covering only 0.1 pH unit) and core body temperature (Shmukler, 2004).

Blood could be diseased as do other tissues and the diseases of blood include anemia (insufficient red cell mass) which can result from bleeding, blood disorders like thalassemia, or nutritional deficiencies (Austin and Perkins, 2006), leukemia (a group of cancers of the blood-forming tissues) (Shuster *et al.*, 2004), Hemophilia (a genetic illness that causes dysfunction in one of the blood's clotting mechanisms and can allow otherwise inconsequential wounds to become life-threatening) (Williams *et al.*, 1989) and

infectious disorders of blood (blood is an important vector of infections) including HIV, Hepatitis B and C, bacterial infection of the blood (bacteremia or sepsis), viral infection (viremia) and blood-borne parasitic infections such as malaria and trypanosomiasis (Dominguez *et al*., 1981).

Momordica charantia extracts have broad-spectrum antimicrobial activity, having been shown to prevent infection by numerous viruses, bacteria, parasitic organisms, and fungi. Although the mechanisms have not been determined for all organisms, in the case of viral infection, it is thought that certain bitter melon constituents prevents viral penetration of the cell wall (Cunnick and Takenjoto, 1993). An in-vitro study using human plasma demonstrated the prolongation of activated partial thromboplastin time by *Momordica charantia* trypsin inhibitor-II. No actual human cases of coagulation disorders have been reported (Hayashi *et al*., 1994). Although several studies have been carried out on the different pharmacological activities of *Momordica charantia*, not much has been done on its effects on haematological profiles in either experimental animals or man thus, this study aims at determining the in-vivo effects of the leaf decoction of *M.charantia* on haematological profile of experimental rats.

PROCEDURE

Blood samples were collected intraoccularly into Na$^+$EDTA bottles on the 31st day, after the animals have been fasted overnight. The heamatological studies were performed within as short time as possible in the Veterinary Medicine Laboratory, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. Red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV), heamoglobin concentration and the RBC indices (MCH (mean corpuscular heamoglobin), MCV (mean corpuscular volume) and MCHC (mean corpuscular hearnoglobin concentration)) were all estimated. RBC indicies were calculated from the RBC count, HB concentration and PCV estimations.

All data were expressed as mean ± SD and statistically analyzed with the student's t-test and One-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

An assessment of the blood parameters of animals orally exposed to the leaf decoction of *M charantia* showed no significant differences in the Red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV), heamoglobin concentration and the RBC indices (MCH, MCV and MCHC). Tables 4.5 and 4.6 show the erythrocyte and leucocyte values (Mean ± SD) in control and rats treated with the leaf decoction of *M charantia*.

CONCLUSION

The Leaf decoction of *M.charantia* had no significant effects on the heamatological profile of experimental rats which were exposed to it over a period of 30 days.

TABLE 4.5: EFFECTS OF DIFFERENT DOSAGES OF *M.CHARANTIA* ON THE ERYTHROCYTE VALUES OF EXPERIMENTAL RATS.

Groups / Parameter	Control 0mg/100gBW (n=4)	Group A 35mg/100gBW (n=4)	Group B 45mg/100gBW (n=4)	Group C 55mg/100gBW (n=3)	Group D 65mg/100gBW (n=4)
RBC ($X10^{12}$/L)	13.74±0.74	12.48±1.48	12.09±0.64	13.03±0.61	14.40±0.25
PCV (%)	38±1.83	38±0.82	36±2.16	37±1.00	41.0±0.82
Hb (g/dl)	12.6±0.49	12.48±0.41	11.9±0.66	12±0.00	13.0±0.00
MCV (fl)	27.67±0.46	29.95±3.68	29.74±0.23	28.41±0.55	28.47±0.33
MCH (Pg)	9.18±0.23	10.09±0.99	9.84±0.16	9.22±0.43	9.03±0.15
MCHC (g/dl)	33.17±0.45	32.82±0.39	33.06±0.43	32.45±0.88	31.72±0.63

There were no significant ($P<0.05$) differences in the treated animals when compared with Control.

TABLE 4.6: EFFECTS OF DIFFERENT DOSAGES OF *M.CILIRANTIA* ON THE LEUCOCYTE VALUES OF EXPERIMENTAL RATS.

Groups / Parameter	Control 0mg/100gBW (n=4)	Group A 35mg/100g BW (n=4)	GroupB 45mg/100gBW (n=4)	GroupC 55mg/100gBW (n=3)	Group D 65mg/100gB (n=4)
Total WBC (x10³/L)	21.68±6.46	23.45±6.04	20.80±3.86	21.33±0.90	25.93±0.41
Lymphocytes (%)	71.25±4.50	69.50±6.14	64.00±4.32	59.67±0.58	66.75±5.74
Neutrophils (%)	28.75.9±4.50	30.50±6.14	38.50±7.55	40.33±0.58	33.25±5.74
Absolute Lymphocytes (x10³/L)	15.42±4.78	16.32±4.45	13.28±2.25	12.73±0.65	17.32±1.76
Absolute Neutrophils (x10³/L)	6.26±2.03	7.13±2.10	7.53±1.97	8.60±0.26	8.60±1.36

There were no significant ($P<0.05$) differences in the treated animals when compared with Control.

TABLE 4.6: EFFECTS OF DIFFERENT DOSAGES OF *M.CHARANTIA* ON THE LEUCOCYTE VALUES OF EXPERIMENTAL RATS.

Groups / Parameter	Control 0mg/100gBW (n=4)	Group A 35mg/100g BW (n=4)	GroupB 45mg/100gBW (n=4)	GroupC 55mg/100gBW (n=3)	Group D 65mg/100gBW (n=4)
Total WBC (10^9/L)	21.68±6.46	23.45±6.04	20.80±3.86	21.33±0.90	25.93±0.41
Lymphocytes (%)	71.25±4.50	69.50±6.14	64.00±4.32	59.67±0.58	66.75±5.74
Neutrophils (%)	28.75.9±4.50	30.50±6.14	38.50±7.55	40.33±0.58	33.25±5.74
Absolute Lymphocytes (10^9/L)	15.42±4.78	16.32±4.[illegible]	13.28±2.25	12.73±0.65	17.32±1.706
Absolute Neutrophils (10^9/L)	6.26±2.03	7.13±2.10	7.53±1.97	8.60±0.26	8.60±1.36

There were no significant ($P<0.05$) differences in the treated animals when compared with Control.

EXPERIMENT 5.0: HISTOPATHOLOGICAL STUDIES

INTRODUCTION

Histopathology (compound of three Greek words: Ιστός histos "tissue", πάθος pathos "disease-suffering", and -λογία -logia) refers to the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides (Histopathology-Wikipedia, 2010).

PROCEDURE

Liver, testis and epididymal samples were obtained from sacrificed animals and fixed in 10% (v/v) formo-saline for histopathological studies. The collected tissues (livers, testes and epididymis) were removed from the fixative after two (2) days, dehydrated through ascending grades of alcohol (70%, 80%, 90% and absolute) cleared in xylene, infiltrated, embedded in paraffin wax and cut into 5 micron a piece on Reichert ultra microtome for light microscope studies. They were then mounted on slides and stained with haematoxyline and eosin (H and E) according to routine procedure for light microscope. Tissues prepared were examined for qualitative differences in comparison to the normal untreated rats, which served as control. Magnification was set at X 100.

RESULTS

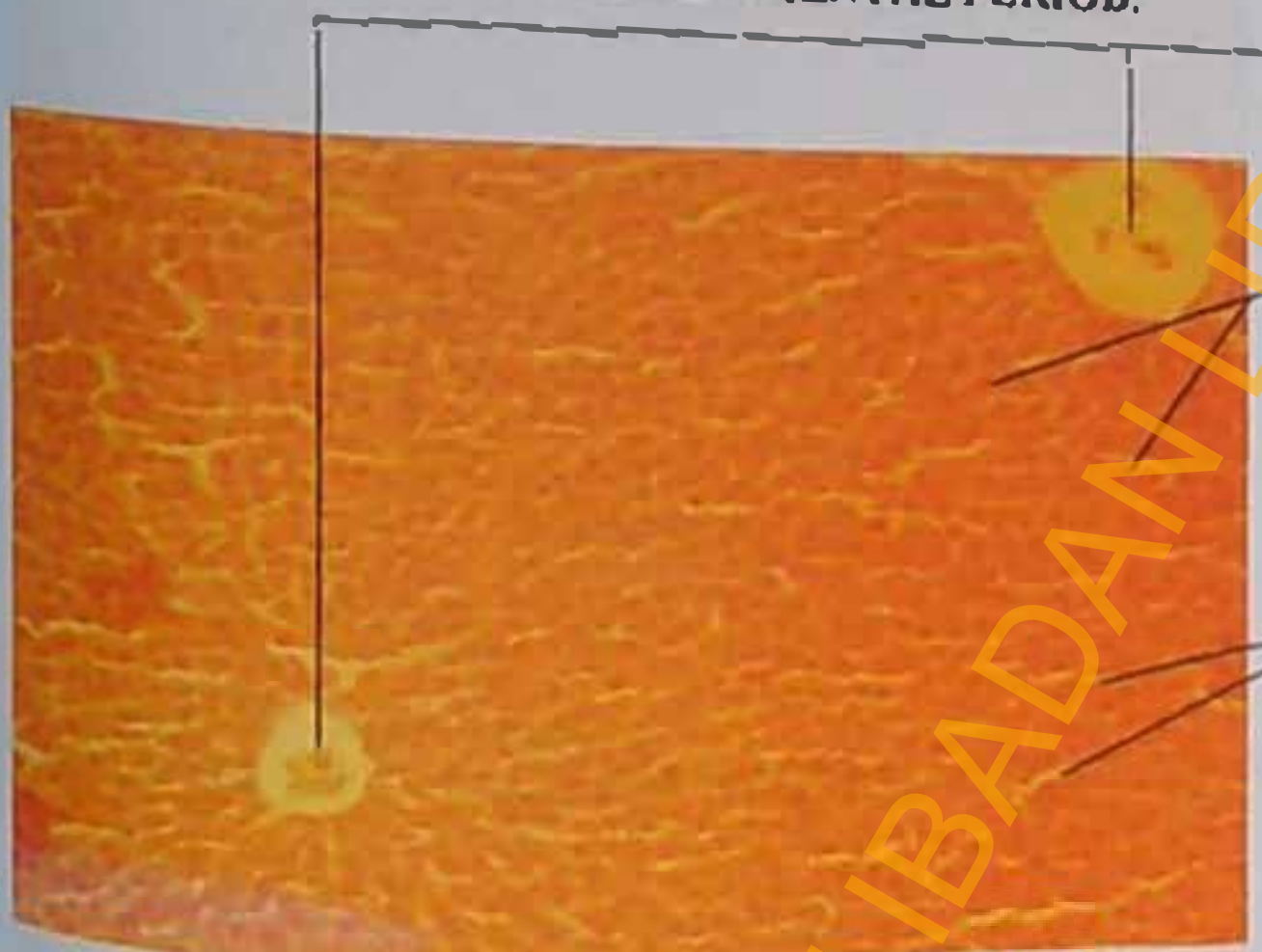
For liver histopathology, the control group showed normal tissue conditions. Group A presented with normal tissue conditions, group B showed moderate vascular hemorrhage. Group C showed multifoci fatty degeneration and severe vascular hemorrhage and Group D showed moderate vascular degeneration, distruption of the sinusoids and severe degeneration. Degenerations ranging from mild to severe were observed in the histopathologies of testes and epididymis of each treated group as the dosages increased.

In group A, mild basal membrane and cellular degenerations were observed in the testes while the epididymis was normal. In group B, severe basal membrane degeneration, cellular heinorrhage and tubular vacuolations were observed in the testes while a mild tubular degeneration as well as an increase in spermatids was seen in the epididymis. In group C, there was a moderate tubular degeneration in the testes while the epididymis showed a mild tubular degeneration with an increase in spermatids. In group D, severe basal membrane and tubular degenerations were observed in the testes while the epididymis presented with a mild tubular degeneration.

CONCLUSION

Oral administration of the leaf decoction of *M. charantia* over a 30-day experimental period had a dose-dependent hepatotoxic and anti-spermatozoal effects on Normal Wistar Albino rats.

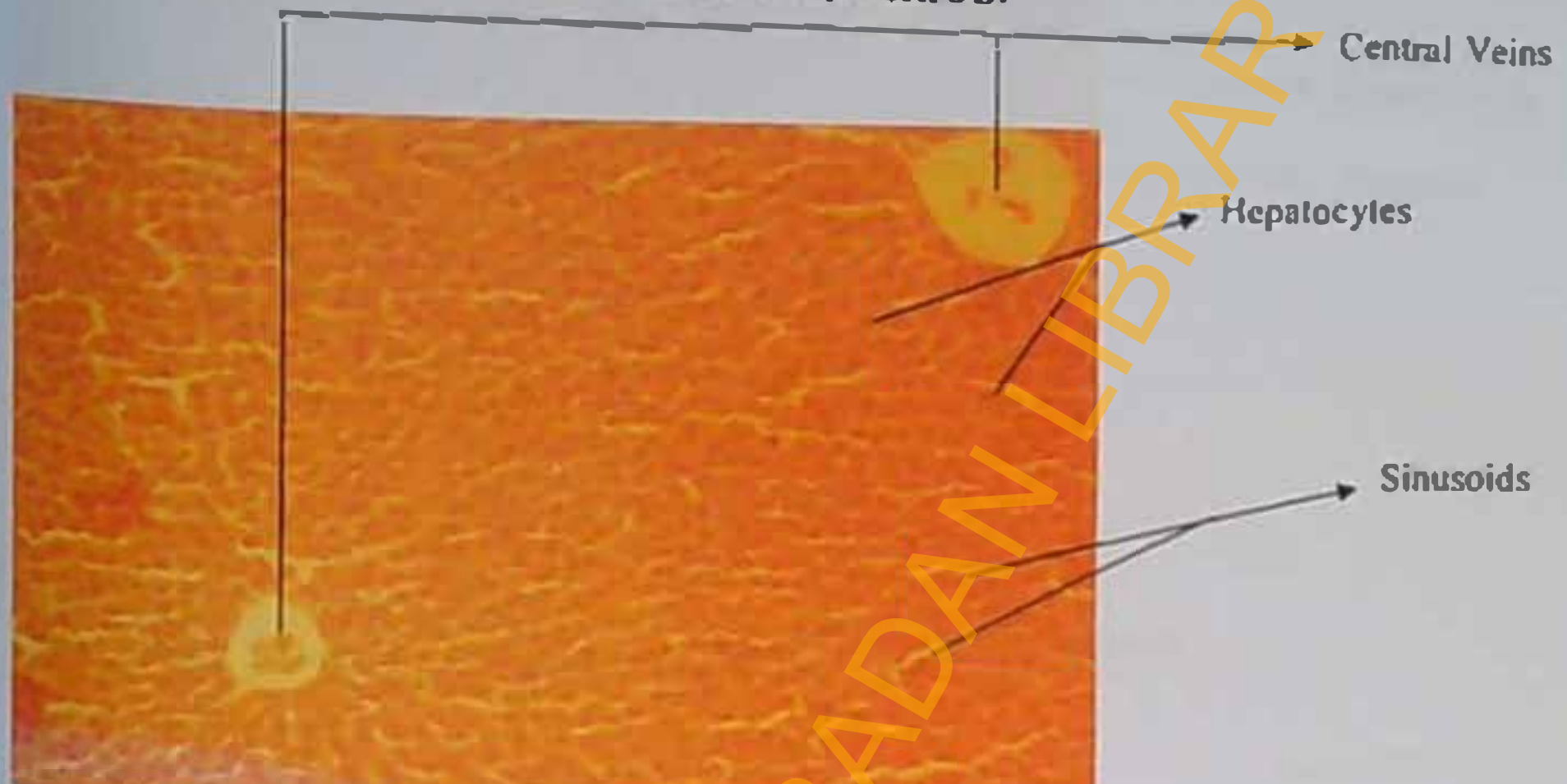
HISTOPATHOLOGY OF THE LIVER SHOWING THE EFFECTS OF THE LEAF DECOCTION OF *MORMORDICA CHARANTIA* AT DIFFERENT DOSAGES OVER A 30-DAY EXPERIMENTAL PERIOD.



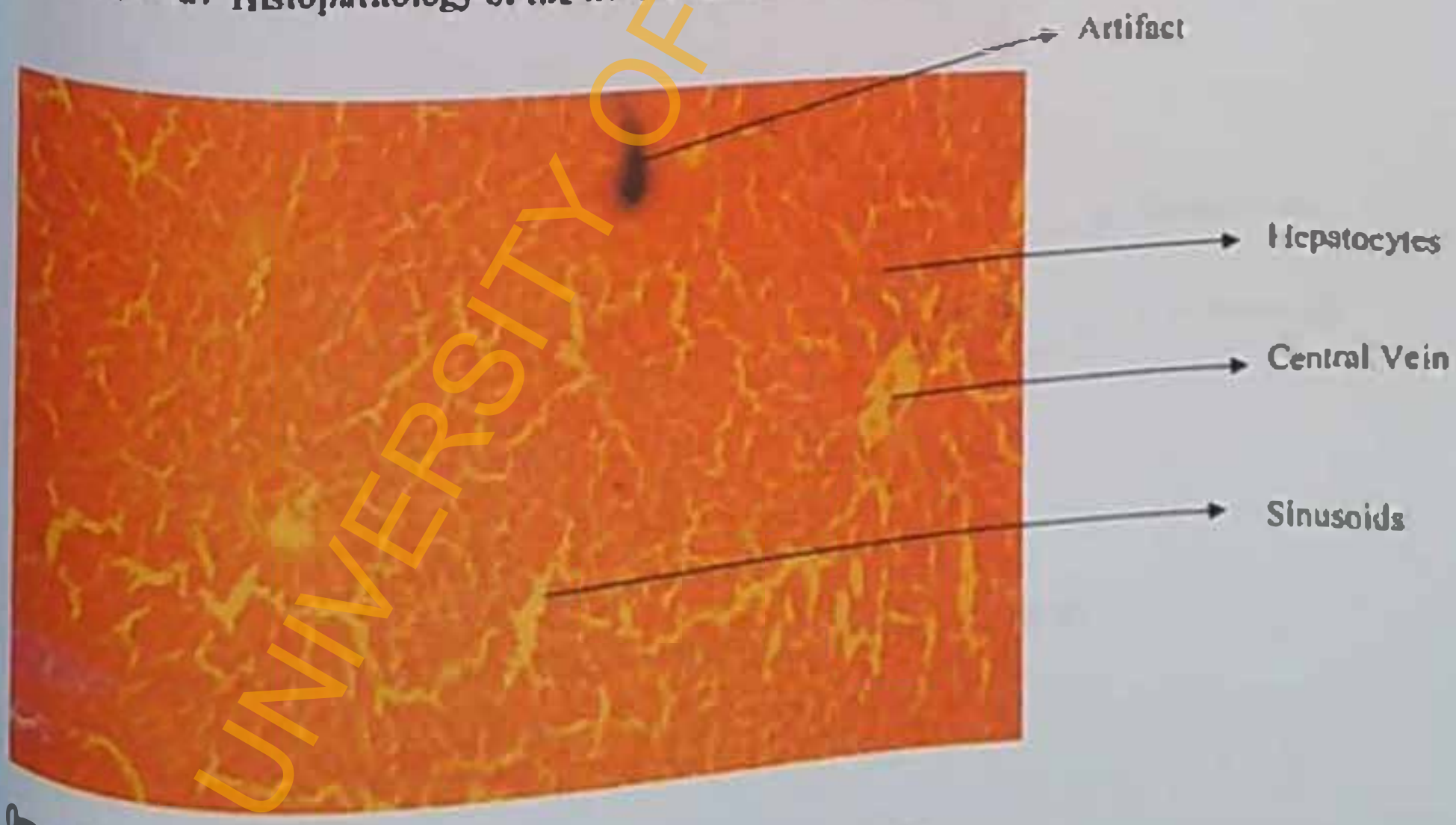
0mg/100g body weight (Control).
Figure 4.5.1a: Histopathology of the liver in normal animals.

0mg/100g body weight (Control)
Figure 4.5.1b: Histopathology of the liver in normal animals.

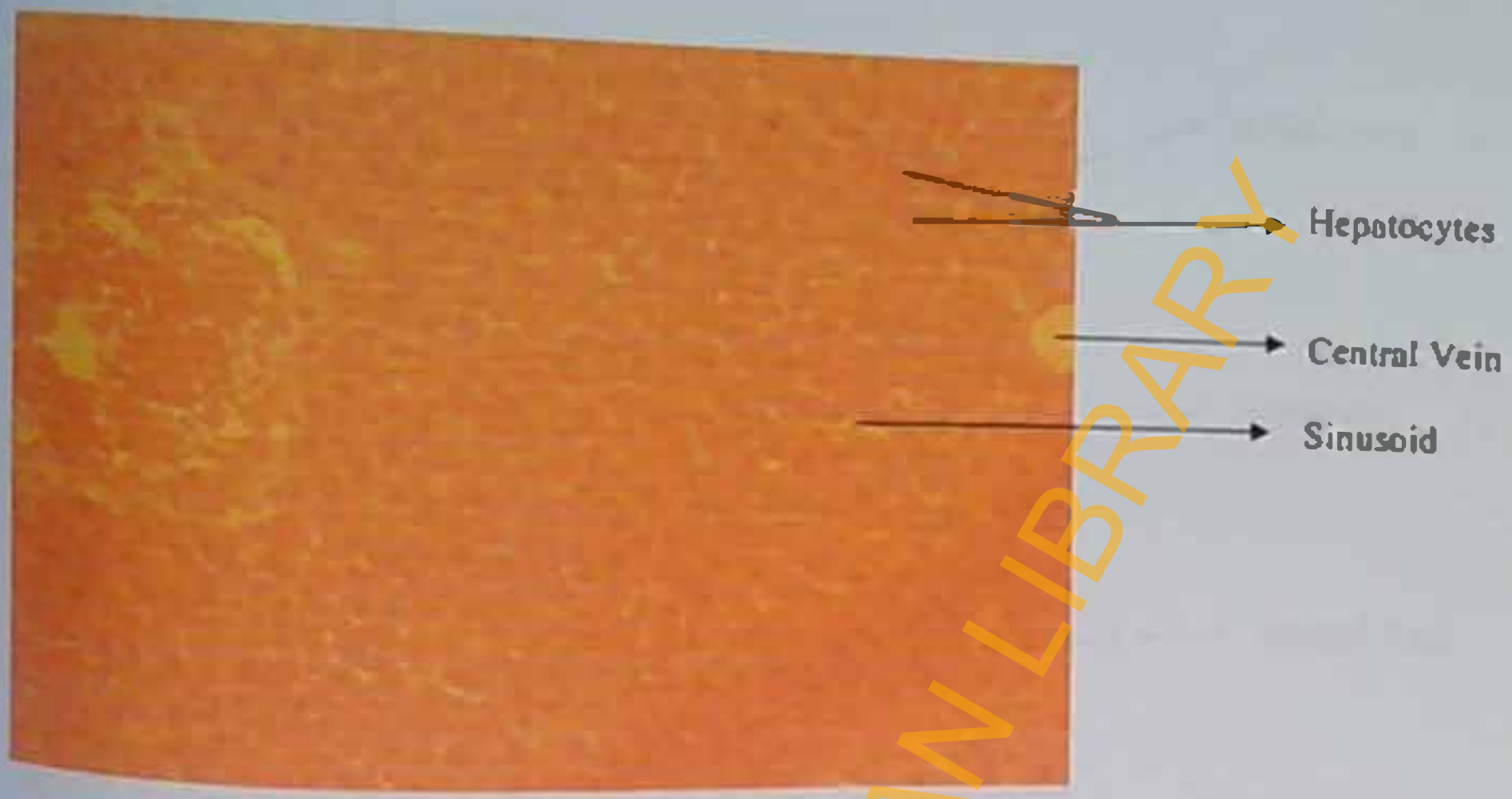
HISTOPATHOLOGY OF THE LIVER SHOWING THE EFFECTS OF THE LEAF DECOCTION OF *MORMORDICA CHARANTIA* AT DIFFERENT DOSAGES OVER A 30-DAY EXPERIMENTAL PERIOD.



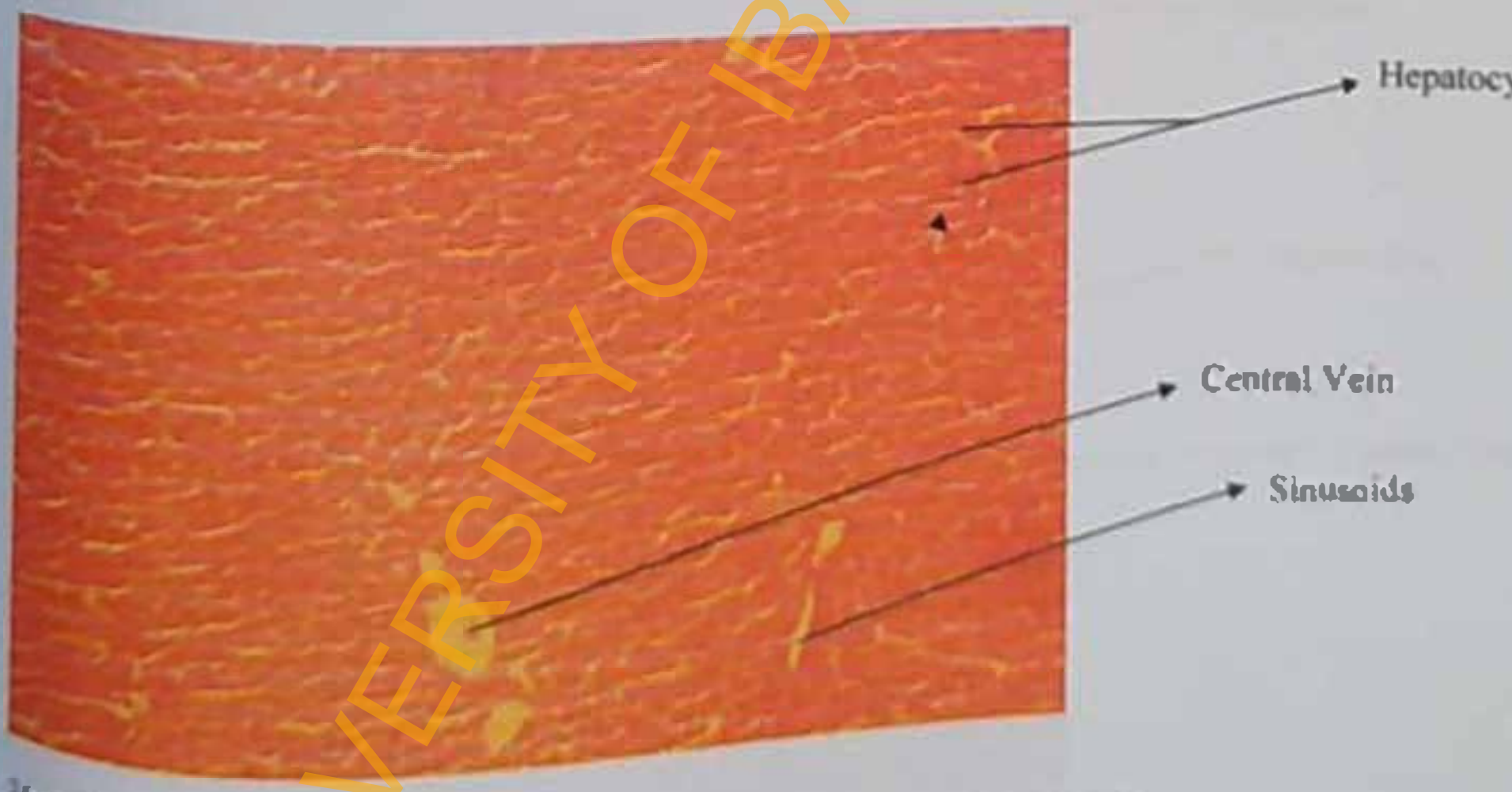
0mg/100g body weight (Control).
Figure 4.5.1a: Histopathology of the liver in normal animals.



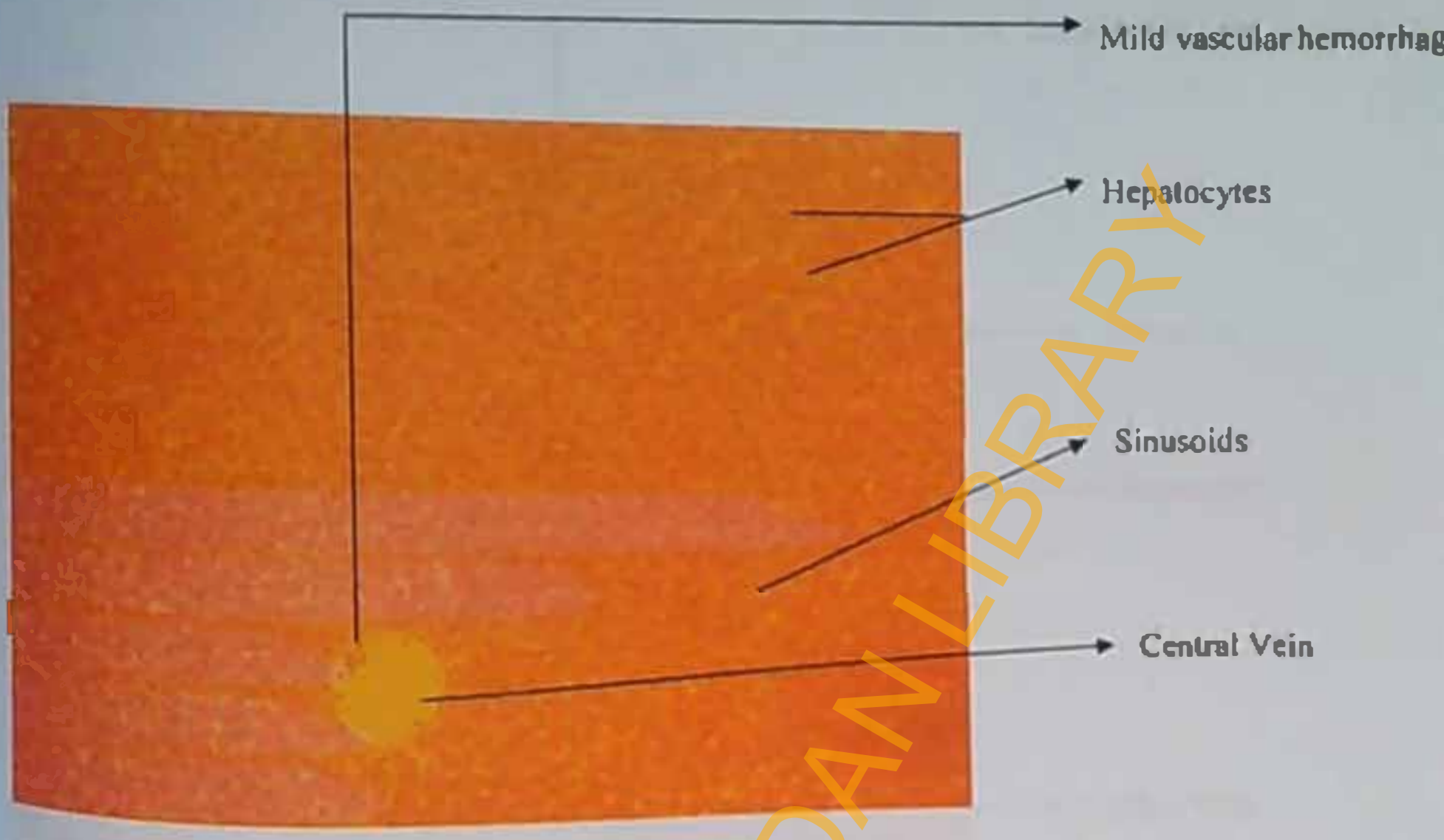
0mg/100g body weight (Control).
Figure 4.5.1b: Histopathology of the liver in normal animals.



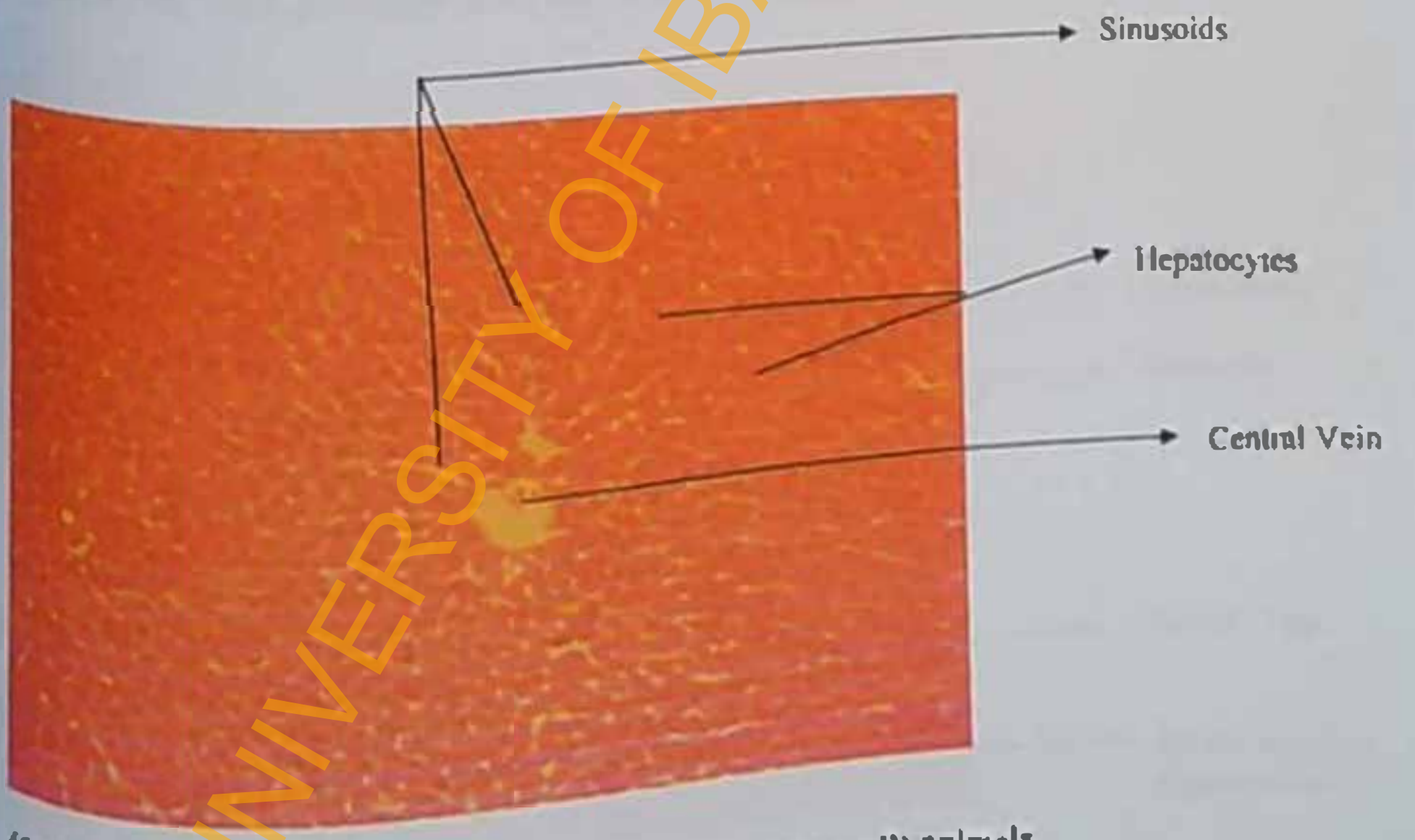
35mg/100g body weight (Normal)
Figure 4.5.2a: Histopathology of the liver in tested (Group A) animals



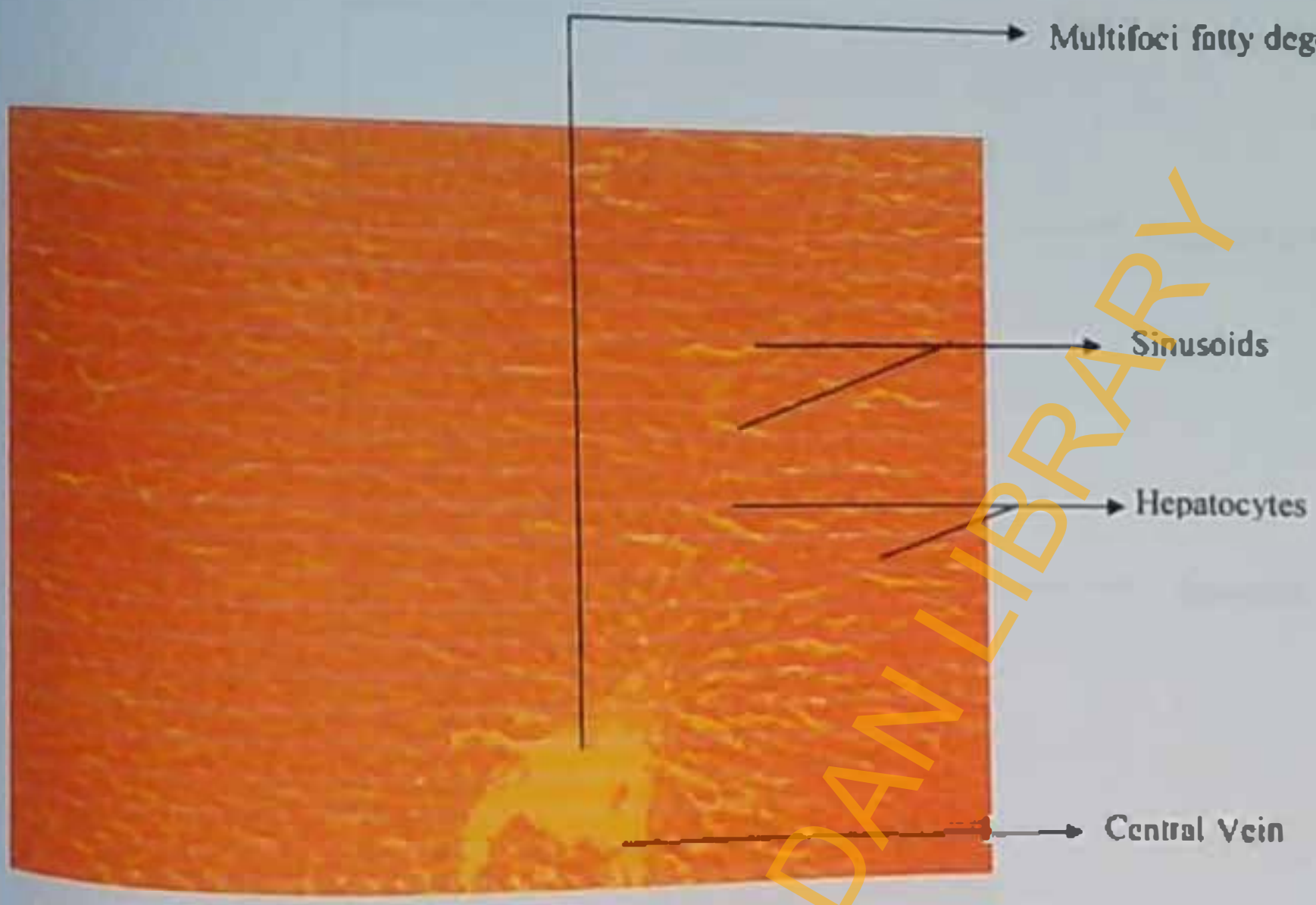
35mg/100g body weight (Normal)
Figure 4.5.2b: Histopathology of the liver in tested (Group A) animals



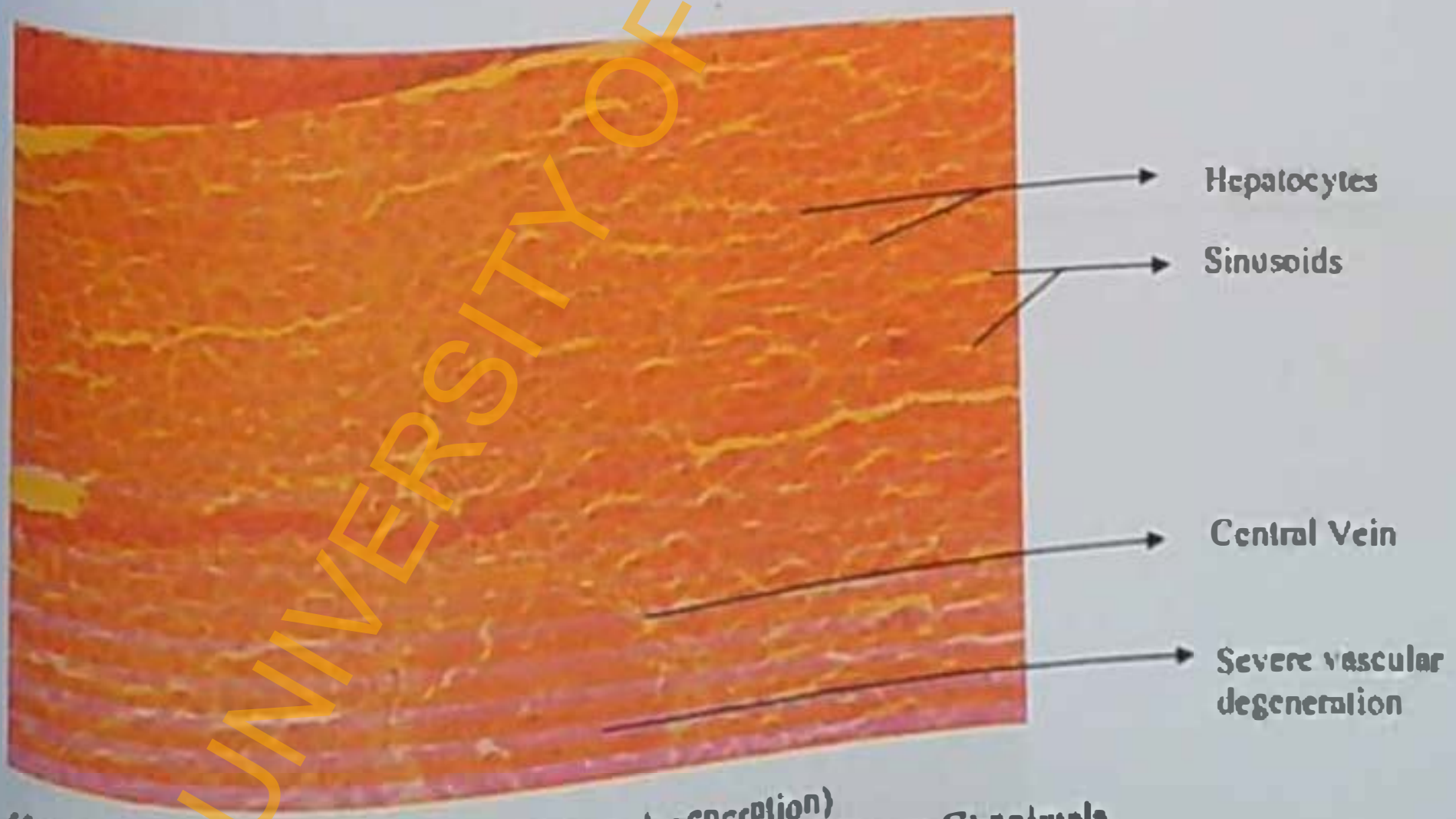
45mg/100g body weight (Moderate vascular hemorrhage)
Figure 4.5.3a: Histopathology of the liver in tested (Group B) animals



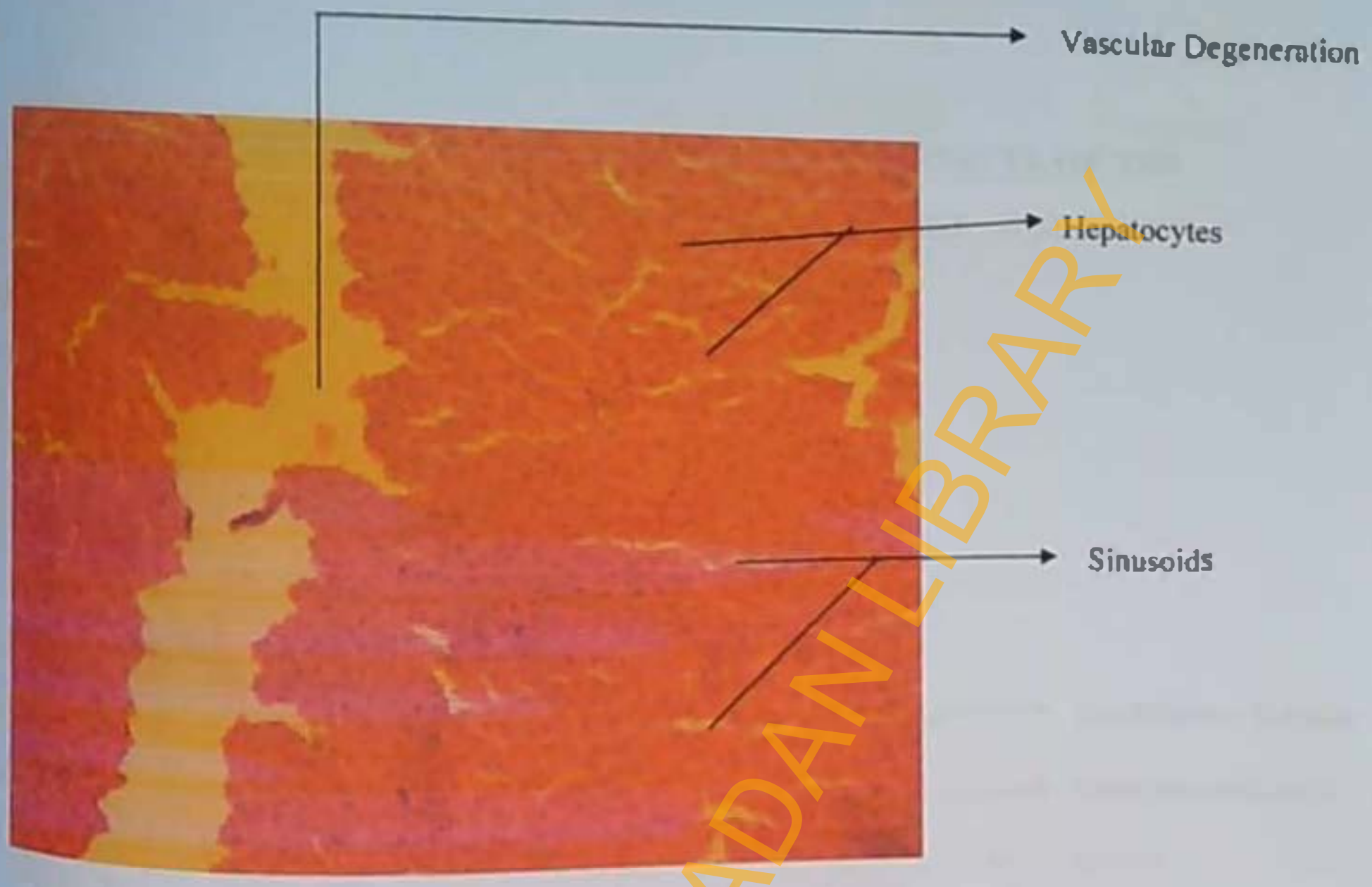
45mg/100g body weight (Normal)
Figure 4.5.3b: Histopathology of the liver in tested (Group B) animals



55mg/100g body weight (Multifoci fatty degeneration)
Figure 4.5.4a: Histopathology of the liver in tested ((Group C) animals

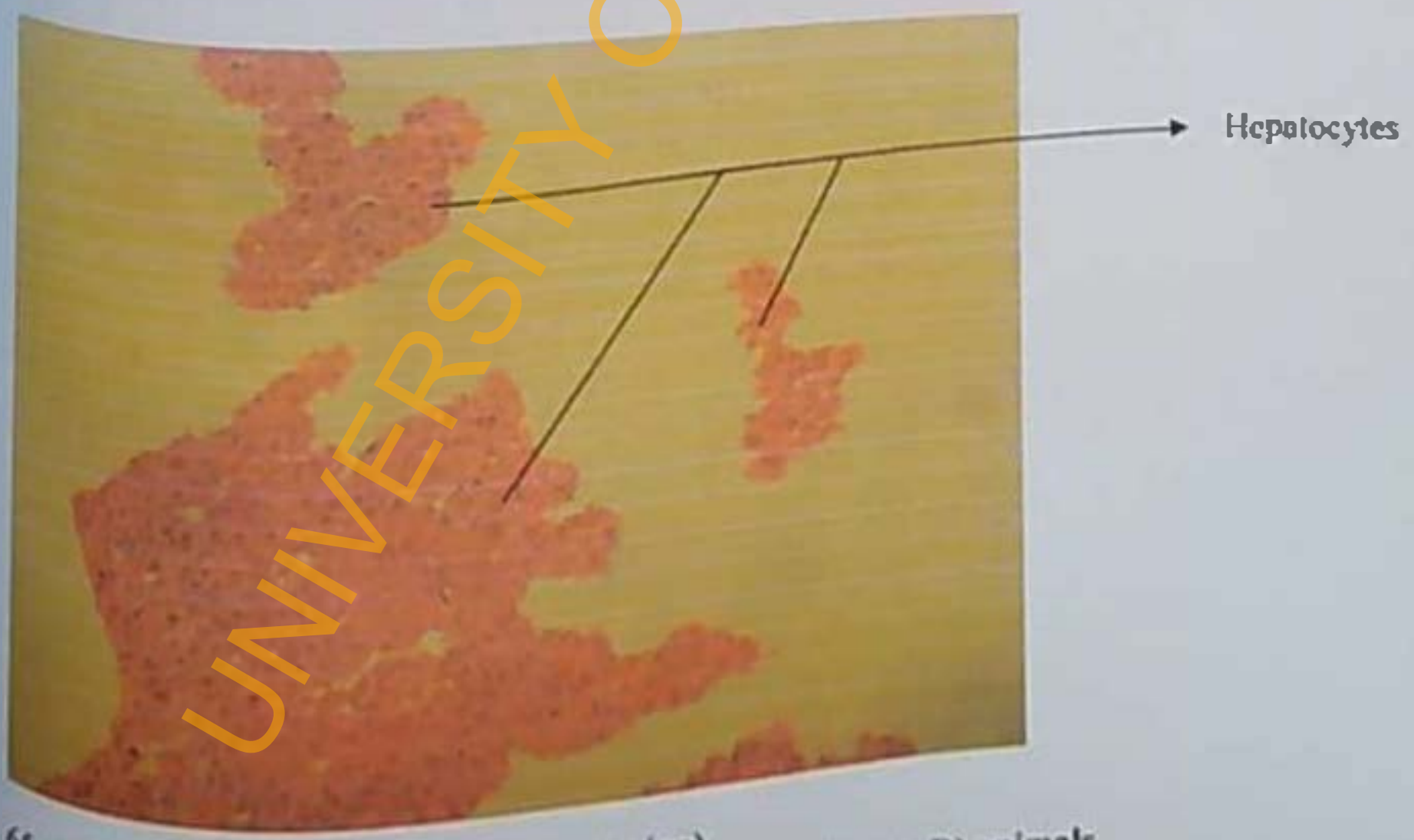


55mg/100g body weight (Severe vascular degeneration)
Figure 4.5.4b: Histopathology of the liver in tested ((Group C) animals



65mg/100g body weight (Moderate vascular degeneration and disruption of the sinusoids)

Figure 4.5.5a: Histopathology of the liver in tested (Group D) animals



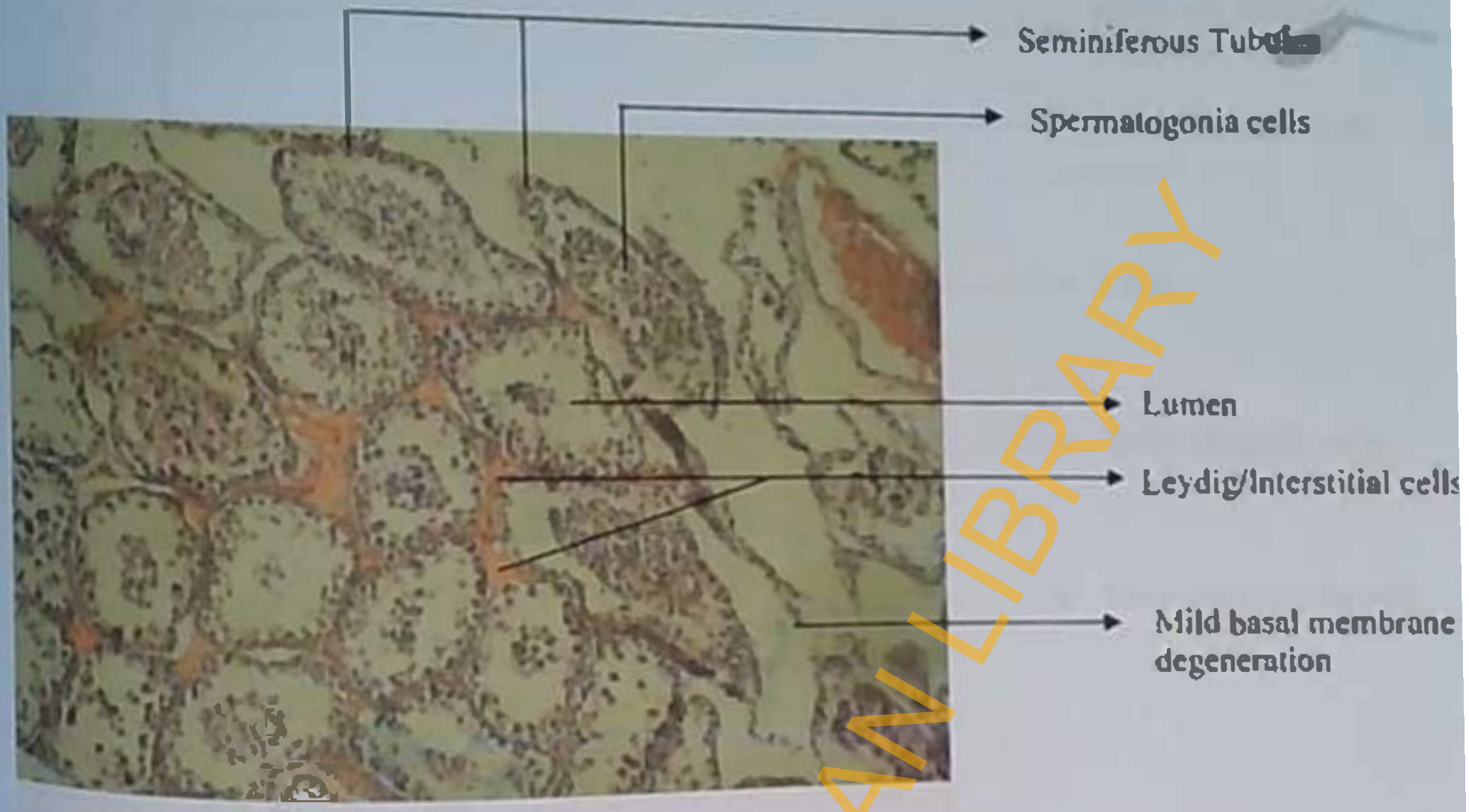
65mg/100g body weight (Severe degeneration)

Figure 4.5.5b: Histopathology of the liver in tested (Group D) animals

HISTOPATHOLOGY OF THE TESTES SHOWING THE EFFECTS OF THE LEAF DECOCTION OF *MORMORDICA CHARANTIA* AT DIFFERENT DOSAGES OVER A 30-DAY EXPERIMENTAL PERIOD.

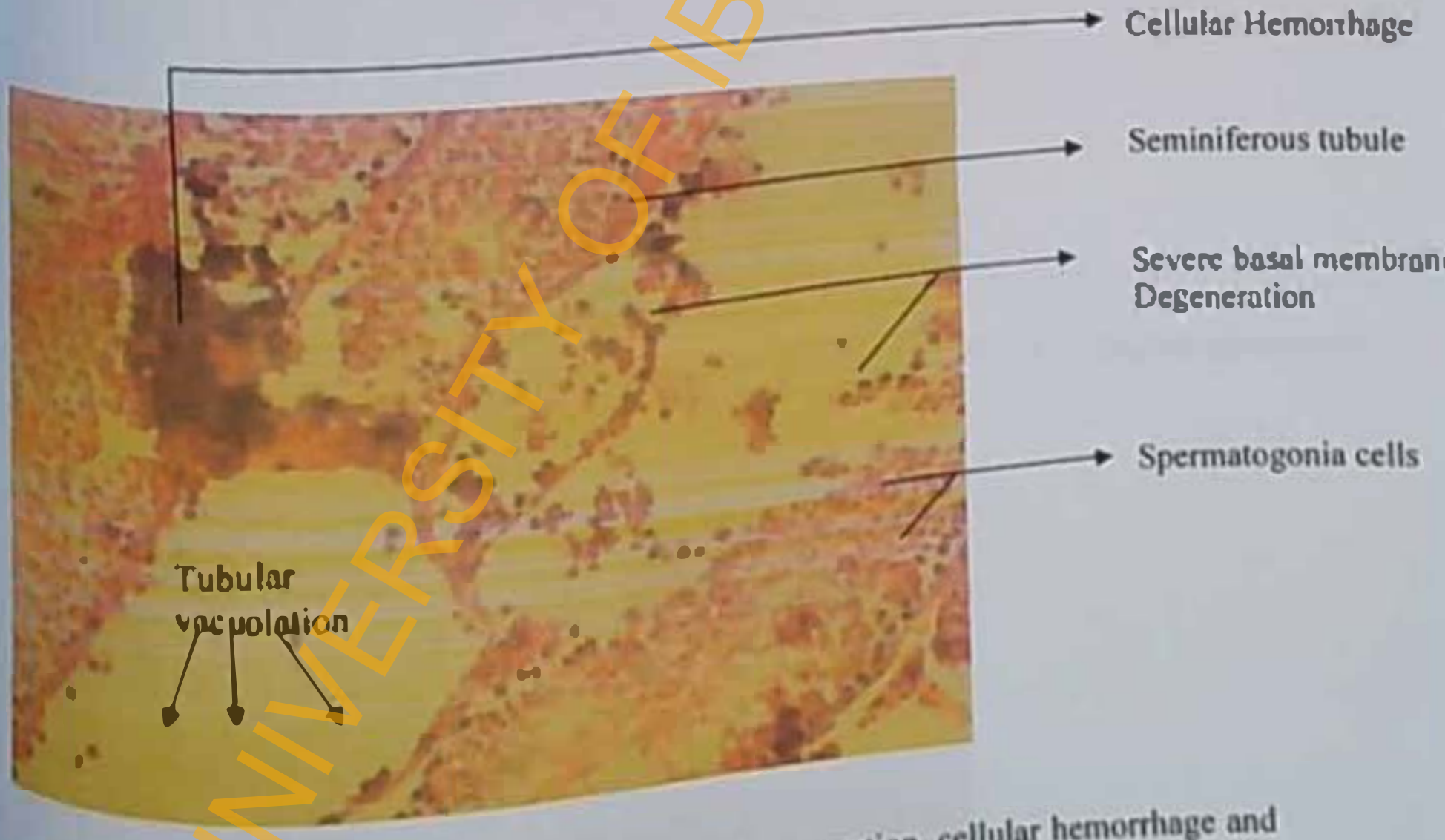
0mg/100g body weight (Normal Testis Histology).

Figure 4.5.6: Histopathology of testis in normal (control) animals



35mg/100g body weight (Mild basal membrane and cellular degeneration).

Figure 4.5.7: Histopathology of testis in tested animals.



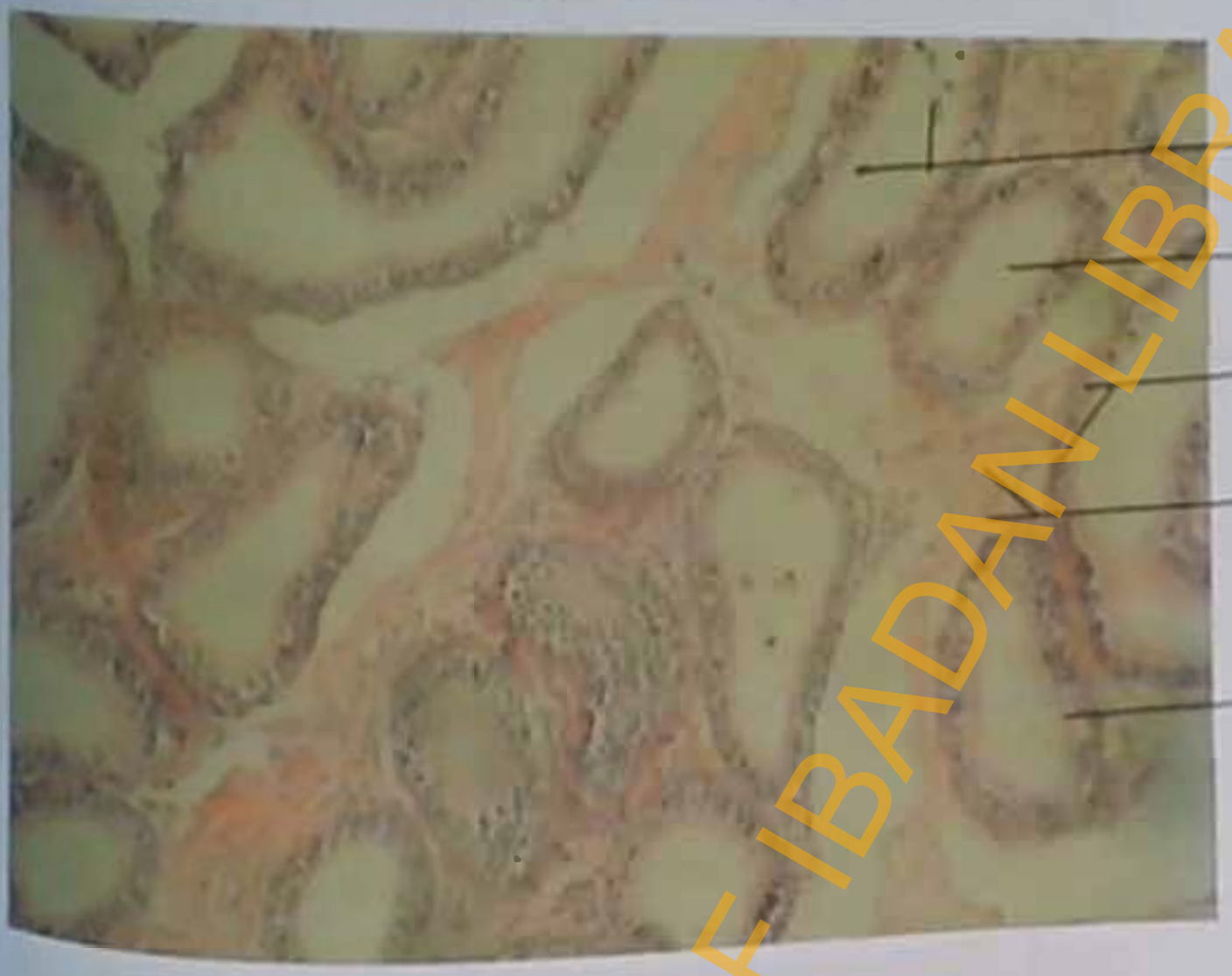
45mg/100g body weight (Severe basal membrane degeneration, cellular hemorrhage and tubular vacuolation).

Figure 4.5.8: Histopathology of testis in tested animals.

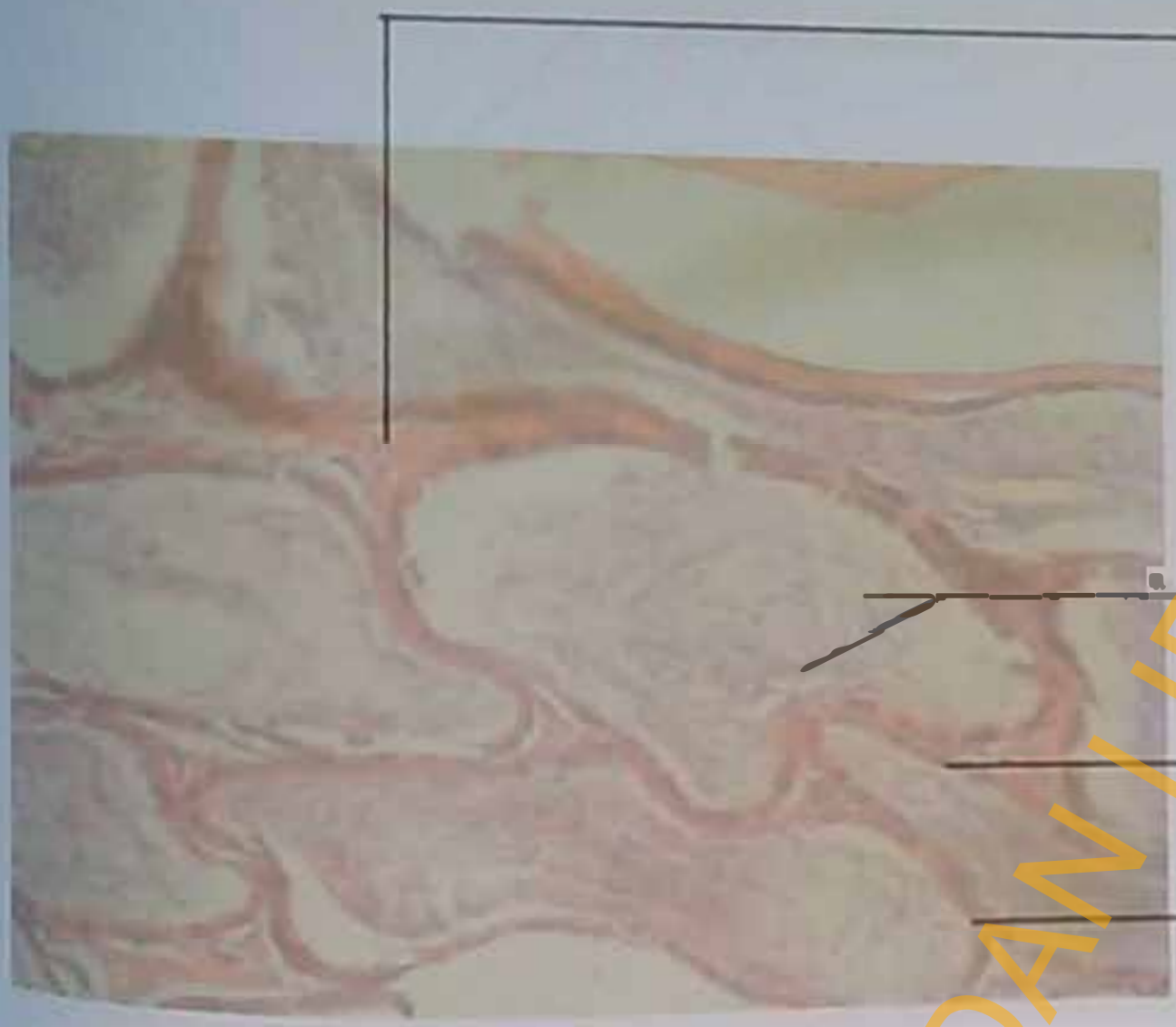
55mg/100g body weight (Moderate tubular degeneration).
Figure 4.5.9: Histopathology of testis in tested animals.

95mg/100g body weight (Severe basal membrane and tubular degeneration).
Figure 4.5.10: Histopathology of testis in tested animals.

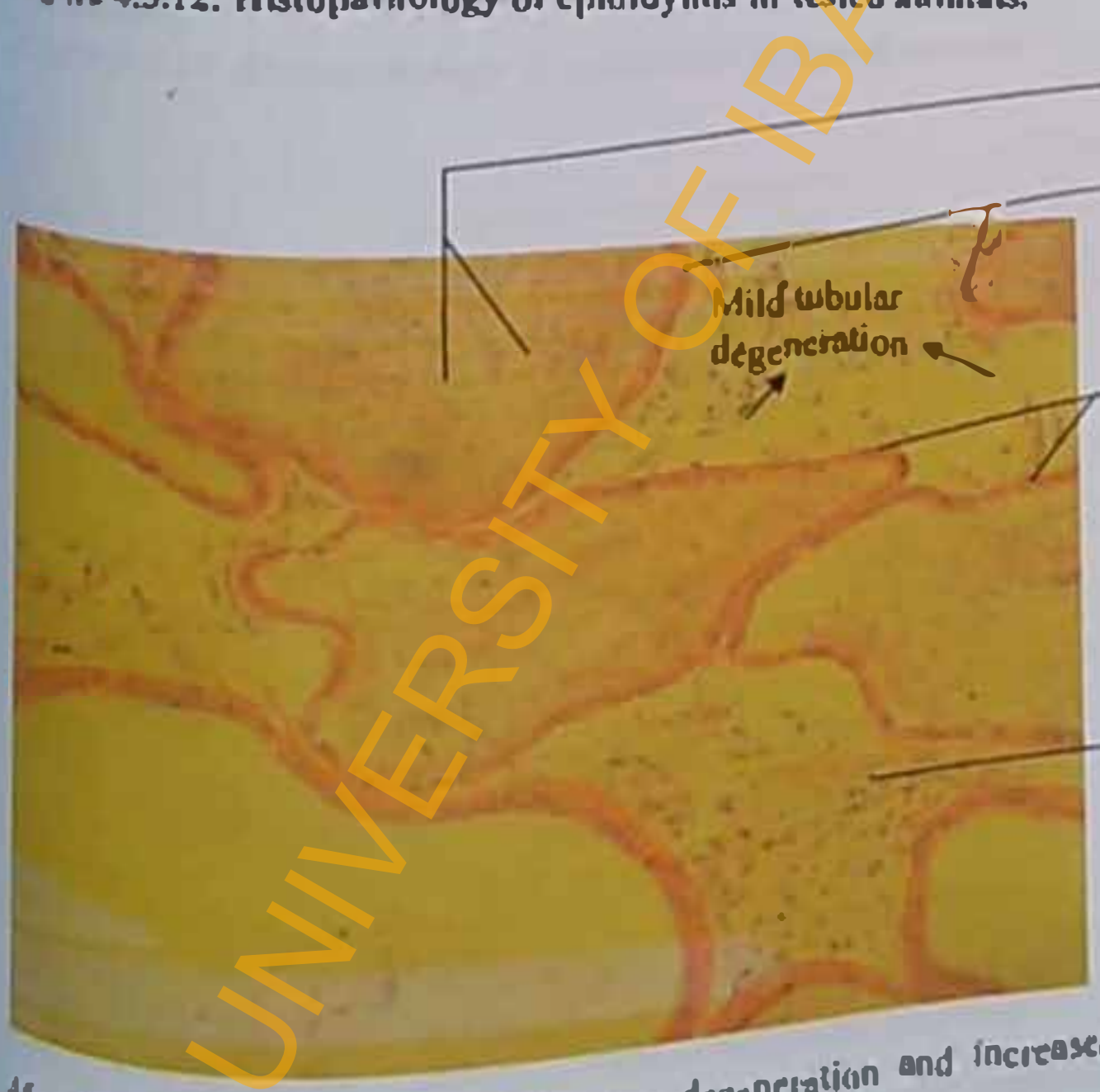
HISTOPATHOLOGY OF THE EPIDIDYMIS SHOWING THE EFFECTS OF THE LEAF DECOCTION OF *MORMORDICA CHARANTIA* AT DIFFERENT DOSAGES OVER A 30-DAY EXPERIMENTAL PERIOD.



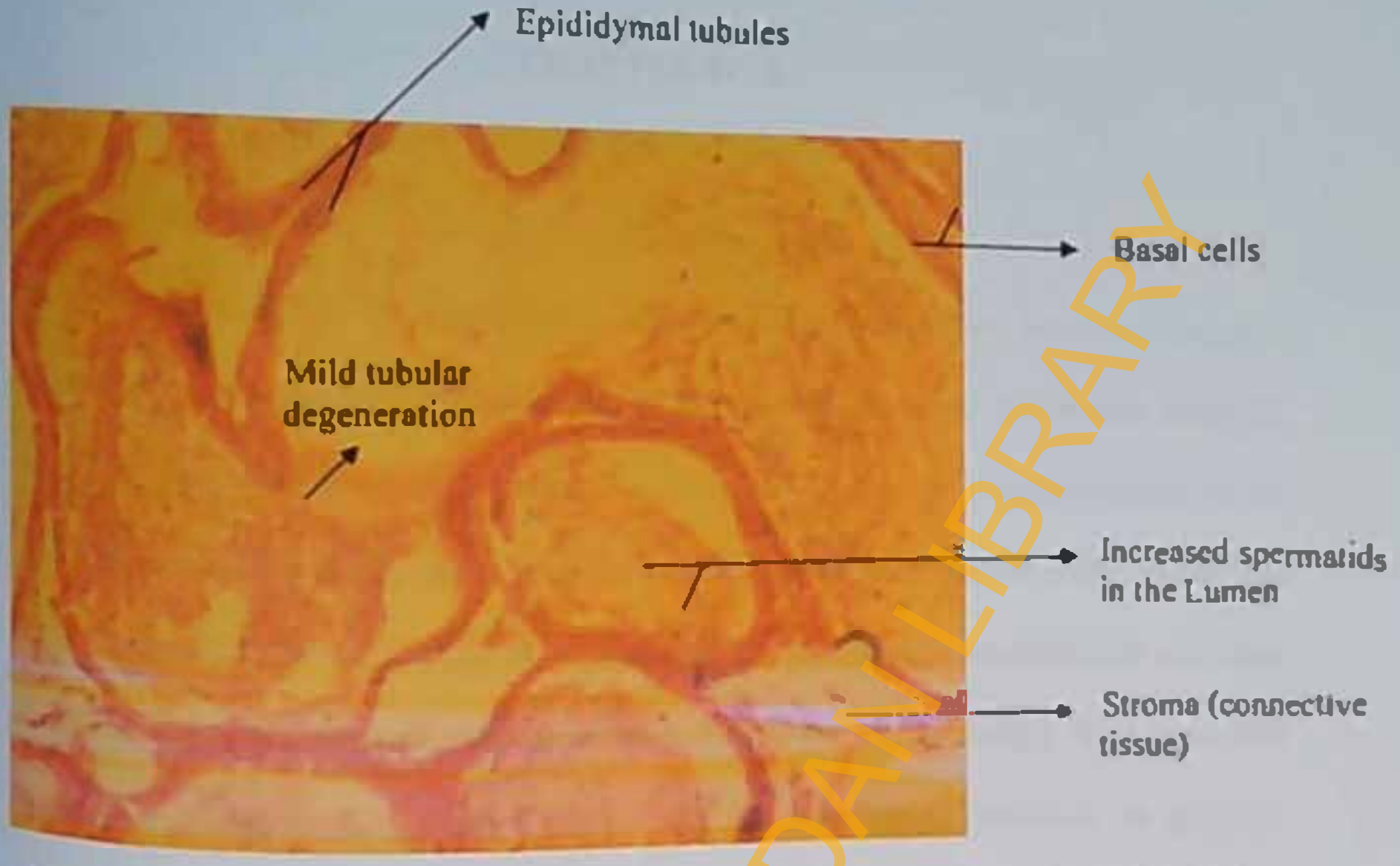
0mg/100g body weight (Normal histology of epididymis)
Figure 4.5.11: Histopathology of epididymis in normal (control) animals



35mg/100g body weight (Normal epididymis with increased spermatids).
Figure 4.5.12: Histopathology of epididymis in tested animals.

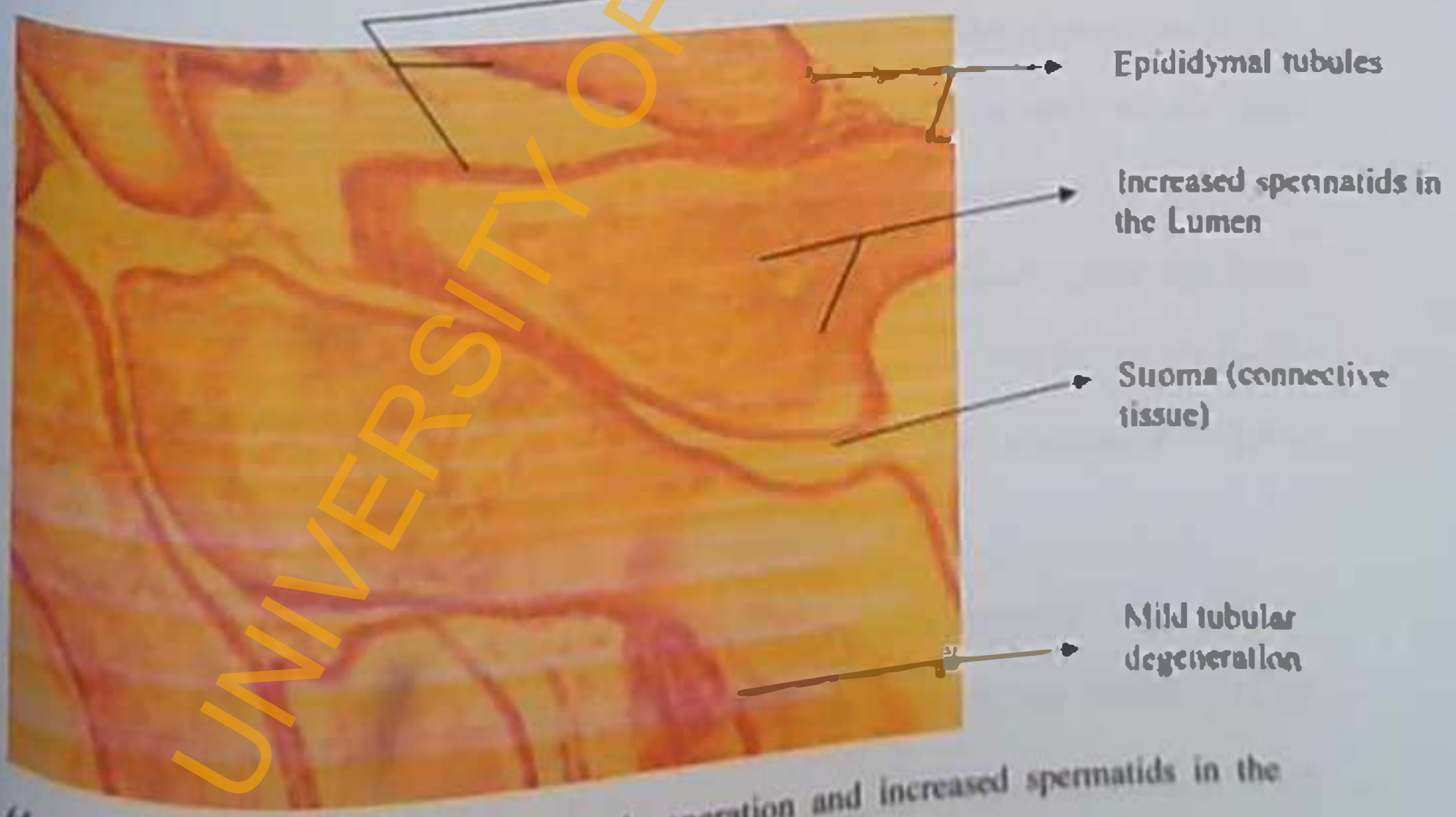


45mg/100g body weight (Mild tubular degeneration and increased spermatids in the epididymis).
Figure 4.5.13: Histopathology of epididymis in tested animals.



55mg/100g body weight (Mild tubular degeneration and increased spermatids in the epididymis).

Figure 4.5.14: Histopathology of epididymis in tested animals.



65mg/100g body weight (Mild tubular degeneration and increased spermatids in the epididymis).

Figure 4.5.15: Histopathology of epididymis in tested animals.

CHAPTER FIVE

5.1. DISCUSSION

Apoptosis is one of the most potent cellular defenses against cancer, because it destroys potentially deleterious mutated cells (Reed, 1999). A current and efficacious approach that is in the limelight is nutritional modulation via an array of phytochemicals which cause the induction of apoptosis in damaged cells such as in cancer cells. In fact, an efficient apoptotic process in normal tissues prevents malignant transformation and helps to correct age-related tissue damage (Zhang and Herman, 2002a&b). It is now well established that anticancer agents induce apoptosis, and that disruption of apoptotic programs can reduce treatment sensitivity (Halestrap and Davidson, 1990).

A greater understanding of the pivotal events associated with carcinogenesis will facilitate the use of natural products/medicinal plants as a key strategy to prevent cancer and initiate apoptosis. Indeed, experimental evidence indicates that phytochemicals can modulate the complex multistage process of carcinogenesis at each of the three recognized stages of initiation, promotion and malignant progression (Chen and King, 1995). A critical stage of apoptosis is the opening of the mitochondrial membrane permeability transition pore because the release of cytochrome C into the cytosol finally commits the cell to self destruction (Duckworth and Johnson, 1993; Jacobson *et al*., 1994; Newmeyer *et al*., 1994).

The results obtained in this study revealed that there were dose-dependent and significant increases in the induction of the opening of the MMPT pore by the decoction of *M. balsamina*. Specifically, maximal induction of about 11 folds observed at 55mg (10%)g bw

suggests that the ingestion of extracts of the plant could result in the opening of pore in cancer cell lines. This view is supported by previous observation that bioactive agents that alter mitochondrial membrane function and/or dissipate the mitochondrial potential can induce apoptosis. For example, epigallocatechin gallate (EGCG) in green tea, depolarizes mitochondria in numerous human cell lines including prostate and lung cells, leading to apoptosis (Galati and Brien, 2000).

The vanilloid curcumin, found in tumeric, and capsaicin, found in chili peppers, can open the MPTP and collapse mitochondrial potential, leading to induction of apoptosis (Galati and Brien, 2000). The flavonoid baicilin induces apoptosis in T lymphocytes by inducing cytochrome c release and distrupting MMP before activation of caspase 3 (Ueda *et al.*, 2001). Curcumin, a polyphenol, induces mitochondrial swelling and collapses the MMP, resulting in apoptosis in numerous cell types (Morin *et al.*, 2001; Kim *et al.*, 2003). Beta carotene, a carotenoid found in carrot, can induce release of cytochrome c from mitochondria and alter mitochondrial membrane potential in different tumor cell lines derived from leukemia, colon adenocarcinoma, and melanoma cells (Palozza *et al.*, 2003). Lycopene, a non-provitamin A carotenoid found in tomatoes, can depolarize the mitochondria of human prostate cancer cells, induce cytochrome c release and ultimately induce apoptosis (Hantz *et al.*, 2005).

This suggestion is further underscored by the finding that *M. charantia* has anti- leukemia and antiviral activities (Ng *et al.*, 1994), inhibits the growth of several cancer cell lines, including prostate adenocarcinoma, (Clafin *et al.*, 1978), human colon cancer (Caco-2 cells) (Yasui *et al.*, 2005), the highly metastatic breast cancer cell lines MDA 231 (Lee-Huang *et al.*, 2000) and also causes mitochondrial swelling as observed in this study. Thus,

the decoction of *M. charantia* certainly contains bioactive agents that will alter mitochondrial membrane function and/or dissipate the MMP therefore ultimately inducing apoptosis. The finding that the effect of *M.charantia* is dose-dependent suggests that the active components of the plant may be interacting with specific components of the pore such as adenine nucleotide translocase (ANT). Although it is yet to be determined which of the active components exerts the effect, there is incontrovertible evidence that exposure to *M.charantia* will elicit opening of the pore and subsequently the release of cytochrome c and activation of the execution caspases.

Further work will certainly involve the use of purified components of *M.charantia* on the apoptotic process in cancer cell lines. Consequent to the trend of observations made on the effects of the leaf decoction of *M.charantia* on MMPTP, Fertility screening in terms of semen evaluation and examination of sperm cells' morphologies were carried out in order to verify if the apoptotic process evident in the liver cells of experimental rats treated with the leaf decoction were localized or extended to other body organs. Interestingly, there were significant reductions ($P<0.05$) in sperm motility and sperm cell concentrations for all animals which were orally exposed to the decoction compared to the control group and a significant reduction ($P<0.05$) in percentage viability in animals that received 45mg/100g bw and above.

These observation agree with the findings of Naseem *et al.* (Naseem *et al.*, 1998), Dixit *et al* (Dixit *et al.*, 1978) and Basch *et al.* (Basch *et al.*, 2003) on the effects of *M.charantia* on male fertility and also suggests that there was probably an elevated rate of apoptosis in the sperm cells thereby causing death or deformation of the cells. Morphological abnormalities of sperm cells above the proposed percentage range of 10% (Reece, 1997;

Rozeboom, 2000) allowed for breeding animals were also observed in animals that received 45mg/100g bw and above, signifying a possible negative effect of the decoction on sperm morphology, also possibly arising from enhanced apoptosis.

The decoction of *M.charantia* is drunk for prevention or treatment of several health problems, such as stomach ache, fever, infectious diseases, arthritis, diabetes, hypertension, cancer and to regulate blood sugar and control diabetes (Pinkney, 2007). Since this decoction is conveyed to it sites of action through the blood stream, irrespective of their target organ, we deemed it fit to adduce the effects of the decoction on blood parameters. It was observed that there were no significant differences in the Red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV), haemoglobin concentration and the RBC indices (MCH, MCV and MCHC) when compared to the untreated (control) animals. This observation is in consonance with the work of Platel, *et al.*, (1993) and suggests that orally ingested leaf decoction of *M. charantia* at dose levels 35mg, 45mg, 55mg and 65mg/100g bw is safe on the blood parameters in treated albino rats.

The results obtained when the liver function tests were carried out on the experimental animals orally treated with the varying dosages (35mg-65mg/100g bw) of the leaf decoction followed a somewhat dose-dependent pattern in which the ALP and γGT levels significantly ($P<0.05$) increased for all groups relative to the control, though no significant differences were observed for ALT and AST levels.

Tennekoon *et al.*, (1994), has reported similar results albeit, with no consistent histopathological defects. The group concluded that their results may be due to enzyme induction or mechanisms not obvious at the histological level (Tennekoon *et al.*, 1994).

This is not the case here as histopathology for group B showed moderate vascular hemorrhage; Group C, multifoci fatty degeneration and severe vascular hemorrhage and Group D, moderate vascular degeneration, distruption of the sinusoids and severe degeneration. The hemorrhage may be as a result of damage caused to the cells of the liver while the fatty degeneration may be attributed to the hypocholesterolemic and hypotriglyceridemic effects of *M. charantia* (Ahmed *et al*,. 2001; Jaysooriya *et al*,2000). These observations imply that the decoction may be hepatotoxic if ingested chronically at relatively high doses.

In conclusion *M. charantia* induced MMPT pore, explaining its potency in cancer therapy in traditional medicine. The leaf decoction of *M. charantia* has hepatotoxic properties as shown in the increased GGT and ALP levels as well as in the consistent dose-dependent histopathological defects. Also, possible toxic effects of sub-acute (30-day) oral administration of leaf decoction of *M. charantia* in albino rats were observed, suggesting that this popular anti-cancer agent could pose some danger to humans especially, in regard to male fertility in individuals who rely on oral administration of the decoction in treating various ailments. However, the leaf decoction seems to be safe on haematological profile as there were no significant differences between values of the haematological parameters of treated rats when compared to the control group.

5.1 SUMMARY OF RESULTS

From this study we conclude that orally administered leaf decoction of *M. charantia* induced liver Mitochondrial Membrane Permeability Transition Pore (MMPTP) in normal albino rats.

The leaf decoction of *M. charantia* caused increases in the percentage of abnormal spermatozoa in male albino rats.

Orally administered leaf decoction of *M. charantia* caused significant reduction in sperm motility and concentration of normal male albino rats.

The percentage viability in male albino rats treated with the leaf decoction of *M. charantia* significantly reduced.

In general the leaf decotion of *M. charantia* had a negative effect on fertility in male albino rats as has been observed by Naseem *et al.*, (1998) and Basch *et al.*,(2003).

The leaf decoction of *M. charantia* significantly increased the ALP and GGT levels in treated rats. This is in consonance with the work of Tennekoon *et al.* (1994).

On the other hand, the ALT and AST levels were not signicantly different from control in animals upon treatment with the leaf decoction of *M. charantia*.

Histopathological studies of the liver showed negative effect of the leaf decoction of *M. charantia*.

Histopathological studies of the epididymes and testes also showed negative effects of the leaf decoction of *M. charantia* on fertility in male albino rats.

CONTRIBUTIONS TO KNOWLEDGE

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

1. The leaf decoction of *Momordica charantia* is a potent inducer of the Rat Liver Mitochondrial Membrane Permeability Transition (MMPT) pore and this may be the link between it and cancer chemotherapy.
2. The leaf decoction of *Momordica charantia* has antifertility effects as has been seen on its effects on experimental rats' spermiogram and spermatozoa morphologies.
3. The leaf decoction of *Momordica charantia* has hepatotoxic effects in experimental rats.
4. The leaf decoction of *Momordica charantia* seems to be safe on haematological profile as there were no significant differences between values of the haematological parameters of treated rats when compared to the control group.

In all, we conclude that the leaf decoction of *Momordica charantia* has a dose-dependent antifertility and hepatotoxic effects on experimental animals which were orally exposed to it over a period of 30 days. Also, the leaf decoction is able to open the mitochondrial membrane permeability transition pore in a somewhat dose-dependent manner in orally treated experimental rats. Treatment being over a period of 30 days.

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APPENDIX

1.0 DETERMINATION OF REAGENTS VOLUMES NEEDED FOR SWELLING ASSAY MEDIUM.

CONSTITUENTS CONCENTRATION ARE AS FOLLOW:

- 0.8µm Rotenone
- 5mM Sodium succinate
- 300µM Ca^{2+} per mg mitochondrial protein
- 0.1mM Spermine
- Final volume of the swelling assay = 2,500µL

1.1 PREPARATION OF ROTENONE

Molar mass of Rotenone = 394.40

1M = 394.4g in 1000ml

1M = 3.944g in 10ml

1µM = 3.944g x 10^{-6} in 10ml

80µM = 3.944g x 10^{-6} x 80g in 10ml

= 0.00031552g

= 0.000316g in 10ml

Therefore M_1 = 80 µM, M_2 = 0.8 µM

V_1 = ? and V_2 = 2,500 µL

From the mole ratio, $M_1V_1 = M_2V_2$

$$V_1 = \frac{2,500 \times 0.8}{80} = 25\ \mu L$$

1.2 PREPARATION OF SPERMINE

Molar mass = 348.19g

1M = 348.19g in 1000ml

1mM = 3.4819g X 10^{-3} in 10ml

4mm = 3.4819g X 10^{-3} X 4g in 10ml

= 0.0139278g in 10ml

= 0.01393g in 10ml

Therefore M_1 = 4mM, M_2 = 0.1mM

V_1 = ? and V_2 = 2,500 µL

From the mole ratio, $M_1V_1 = M_2V_2$

$$V_1 = \frac{2,500 \times 0.1}{4} = 62.5\ \mu L$$

1.3 PREPARATION OF $CaCl_2$

Molar mass = 111g

1M = 111g in 1000ml

1µM = 1.11g X 10^{-6} in 10ml

12,000µM = 1.11 X 10^{-6} X 12,000g in 10ml

= 0.01332g in 10g

Therefore , M_1 = 12,000 µM, M_2 = 120µM(300 µM x 0.4)

V_1 = ? and V_2 = 2,500 µL

From the mole ratio, $M_1V_1 = M_2V_2$

$$V_1 = \frac{2,500 \times 120}{12000} = 25\ \mu L$$

1.4 Preparation of sodium succinate

Molar mass = 270.1

1M = 270.1g in 1000ml

1mM = 2.710g x 10^{-3} in 10ml

250mM = 2.710g x 10^{-3} x 250 in 10ml

= 0.675g

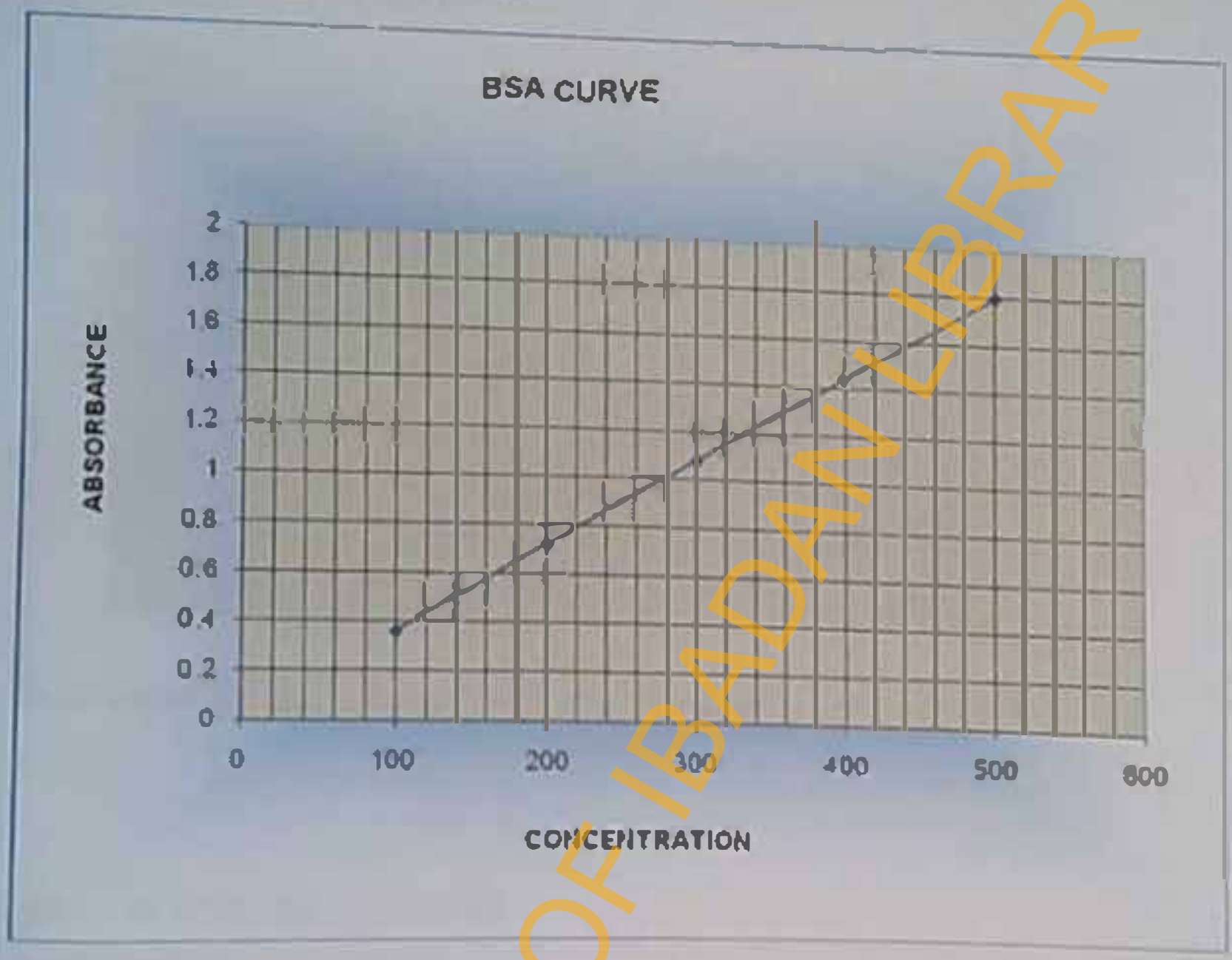
Therefore, M_1 = 250mM, M_2 = 5mM

V_1 = ? and V_2 = 2,500 µL

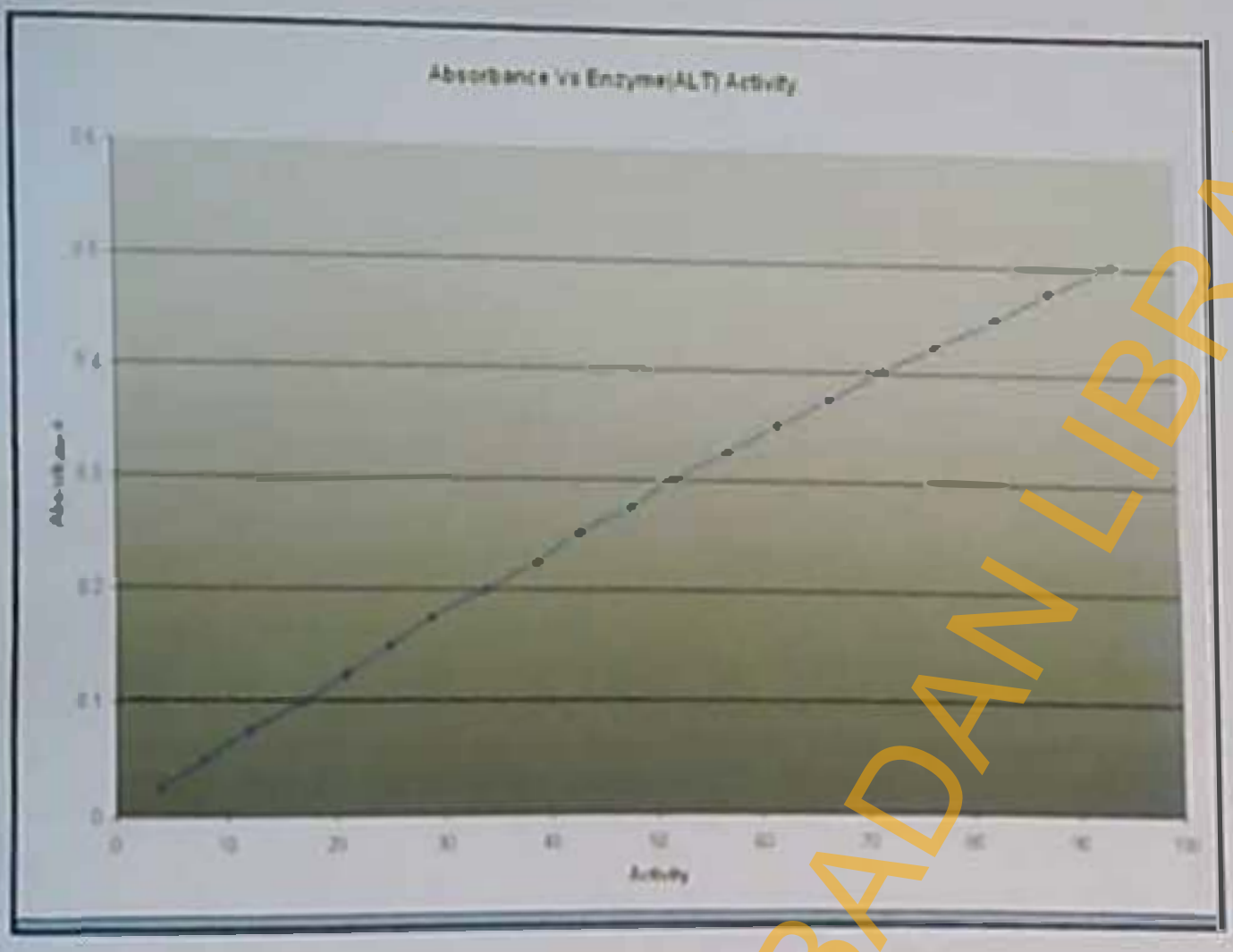
From the mole ratio, $M_1V_1 = M_2V_2$

$$V_1 = \frac{2,500 \times 5}{250} = 50\ \mu L$$

2.0 BSA STANDARD CURVE



3.0 STANDARD ALT CURVE.



4.0 STANDARD AST CURVE.