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A Biochemical Study of Gossypol and Lactate Dehydrogenase X Binary Interactions

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A BIOCHEMICAL STUDY OF GOSSYPOL AND LACTATE DEHYDROGENASE X BINARY INTERACTIONS

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ABSTRACT

A BIOCHEMICAL STUDY OF GOSSYPOL AND LACTATE DEHYDROGENASE-X BINARY INTERACTION

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The goal of this project was to study the mechanism of action of gossypol through its (a) binary interaction with native and trypsin digested lactate dehydrogenase-X (LD-X), a sperm-specific isozyme, and (b) its binding in vitro in primary cultures of spermatogenic cells. Mouse LD-X was cleaved with trypsin before and after treatment with gossypol. This was followed by high performance chromatography (HPLC) separation of the LD-X tryptic peptide fragments to observed alterations in separation patterns as indicators of intramolecular disturbance in the enzyme molecule. Definite alterations in peptide fragment peaks were observed in the presence of gossypol and suggest conformational or intramolecular modifications in the enzyme structure attributable to direct binding with gossypol or its degradation products. Whether there are gossypol interactions unique for LD-X was not determined. The binding of ^{14}C-gossypol to cytosolic and membrane fractions of spermatogenic cell suspensions revealed that most of the gossypol was located in the membrane fractions. However, the relative amount of LD-X bound gossypol was greater in the cytosol than the membrane fractions. A model of gossypol binding in spermatogenic cells is proposed.

DEDICATION

This project is dedicated in loving memory of my guardian, Mrs. Mae Maxwell Yates, an endearing lady who took a young, neglected girl in whom she saw potential and then provided all the tangible and intangible nurturing that made this venture possible. It is further dedicated to my devoted husband, Fred, and my son, Tim, who supported me and often tolerated my neglect of home and family in this pursuit.

ACKNOWLEDGEMENT

I thank Dr. James H. Yuan for his encouragement and direction throughout this project and for planting the seed that led to my application for the MARC fellowship that funded my studies. I am also grateful to each member of my research committee, namely Dr. Keith Carson, Dr. Laura Moen and Dr. Nancy Alexander for their recommendations and insights throughout this project. Dr. Moen was generous in sharing her knowledge and expertise in protein chemistry and the use of her laboratory for the SDS-PAGE. Dr. Carson was also kind enough to assist me in my attempts to isolate hepatocytes, and his calm demeanor often renewed my faith in the "system". I am particularly grateful to Dr. Alexander who continued to serve on my research committee even after having assumed a position elsewhere.

This project could not have been possible without the ^{14}C-gossypol provided by Dr. Martin Sonenberg of Sloan Kettering Memorial Cancer Hospital, New York . I am most indebted to this research scientist and his coworkers.

My neighbors and dear friends Mr. Robert Heard and Dr. Gladys Heard were helpful in the preparation of this manuscript. Bob's knowledge and guidance in computer literacy, as well as the use of his computer hardware, were most valuable in the completion of the manuscript. Gladys' encouragement and proof-reading were also greatly appreciated. I will forever be grateful to Mr. David Hankins for his friendship, his initial assistance with the HPLC system and his expertise in computer literacy. Finally, I thank all those individuals, faculty, staff and fellow students, who have assisted me or contributed in anyway to the fulfillment of this project.

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INTRODUCTION

A. Background

The potential male contraceptive properties of gossypol were well-documented during the late 1970's and the 1980's. Primary among its characteristics is its antispermatogenic effect. The mechanism by which this compound exerts its sperm-specific effect has not been clearly delineated, although several hypotheses have been advanced. Being a hydrophobic polyphenolic compound with a highly reactive carbonyl moiety, gossypol has been shown to bind to plasma proteins with high affinity and to traverse membranes by unmediated diffusion [1-3]. Within the cell this compound may interact with membrane components, cytosolic components and mitochondrial enzymes or metabolites [1-9].

The goal of this project was to probe the biochemical interactions of gossypol with mouse lactate dehydrogenase-X (C_4). This sperm-specfic isozyme is inhibited by gossypol and has been shown [10-14] to have a favored affinity for gossypol when compared to other lactate dehydrogenase isozymes that are found in the testis and in other somatic organs. These studies would allow a better understanding of how the gossypol affects spermatogenesis.

To appreciate this study a background discussion on male reproduction and spermatogenesis, gossypol and lactate dehydrogenase-X follows.

1. Male reproduction and spermatogenesis

The male reproductive system is housed mainly within the scrotum. It consists of the testes, an elaborate transport tubule called the epididymis and vas deferens, and

accessory glands that provide secretions comprising 95% of the seminal ejaculate (Figure 1). The testes are enclosed by a testicular capsule composed of three distinct layers. The outermost layer is composed of the visceral and parietal portions of the tunica vaginalis. The inner parietal portion lines the scrotum and forms a cavity with the outer serous membrane viceral portion of the tunica vaginalis [15, 16]. The middle layer of the testicular capsule is the tunica albuginea, a prominent fibrous structure in which smooth muscle fibers have been observed. The third, innermost capsule layer is the tunica vasculosa, which is a very thin structure of loose areolar connective tissue with a rich microvasculature.

The testis itself is composed of two major anatomical and functional compartments, the seminiferous tubules and interstitial tissue. The interstitial tissue is composed of Leydig cells, blood vessels, an extensive lymphatic system, and numerous macrophages and myoid cells [15, 16]. The seminiferous tubules form convoluted loops that empty at both ends into the rete testis which connects with the epididymis by way of the ductuli efferentes. The seminiferous tubules contain developing spermatogenic cells and nurturing Sertoli cells. The germinal spermatogenic cells are arranged in the basal circumference and the developing spermatogenic cells progress within the Sertoli cells toward the lumen of the seminiferous tubule (Figure 2). This forms the seminiferous epitheilium and is the site of spermatogenesis.

The Leydig cell, which is regulated by luteinizing hormone (LH), is the site of steroidogenesis in the male reproductive system. Androgenic steroids are synthesized de novo from available acetate to form cholesterol, from which pregnenolone is produced through side chain cleavage. Androstenediol and testosterone are formed from pregnenolone through the 5-Δ or 4-Δ pathway which involves sequential reactions with an isomerase, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17-20 lyase and 17β-hydroxylsteroid dehydrogenase, respectively [16, 23].

Figure 1

The male reproductive system. *The scrotal sac houses the testes which are composed of two major anatomical and functional components. These are the seminiferous tubules where spermatogenesis occurs, and the interstitial tissue where the Leydig cells synthesize and secrete androgens. (1) Epididymis (2) Ductules efferentes (3) Vas deferens (4) Rete testis (5) Seminiferous tubule (6) Tunica albuginea (7) Interstitial tissue. [23B]*

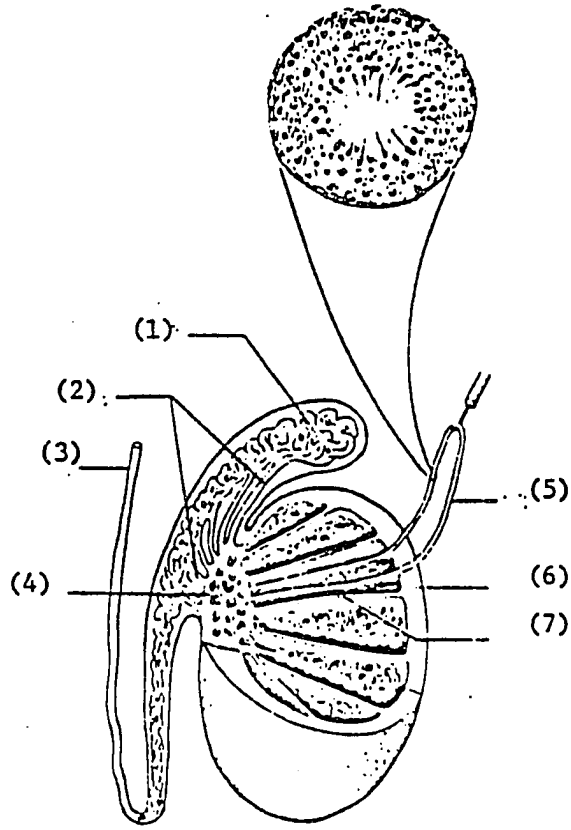
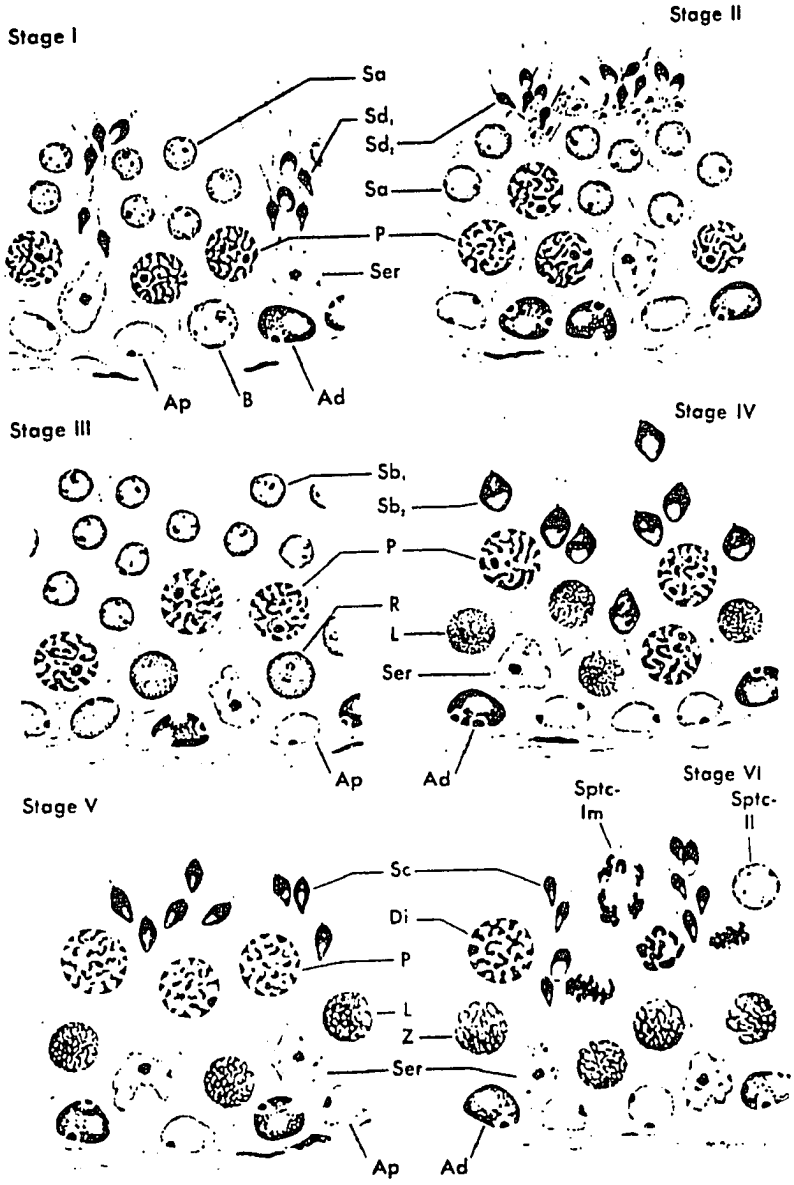


Figure 2

Spermatogenic cells. *Spermatogenic cells are arranged in synchronous associations in Sertoli cells to form the seminiferous epithelium with the most immature cells, spermatogonia, in the basal region progressing to the elongated spermatids and spermatozoa in the luminal region. Ser= Sertoli, S= Spermatogonia, Z= zygotene, L= Leptotene spermatocyte, P= Pachytene spermatocyte, T= Spermatid. [16]*



The Sertoli cell or sustentacular cell represents a channel of communication from the vascular supply outside the basement membrane to all developing spermatogenic cells surrounding it. This cell also has specific receptors to follicle stimulating hormone (FSH) which stimulates the maturation of spermatogenic cells and the production of an androgen binding protein [16, 19]. Transport of nutrients takes place centripetally through the Sertoli cell. Spermatogonia and spermatocytes lie juxtaposed to the Sertoli cell plasma membrane and large portions of the spermatids are embedded in its cytoplasm. Contiguous Sertoli cells form tight junctures which provide a selective blood-testis barrier for all spermatogenic cells, except the basal spermatogonia [23, 23B]. Substances from blood capillaries and lymph vessels must traverse the cytoplasm of the Sertoli cell to reach these spermatogenic cells. Cyclic changes also occur within the Sertoli cell itself [20]. As immature spermatogenic cells ascend toward the lumen, the nucleus of the Sertoli cell rises; the cell itself remains anchored to the basement membrane. As spermatozoa are released into the lumen, the nucleus of the Sertoli cell returns to the basal portion of the cell and the cycle begins again with the next spermatic generation. The decrease in the fat and carbohydrate content of the Sertoli cell prior to the release of spermatozoa into the lumen is evidence of its sustaining ability for developing spermatogenic cells [19].

The process of sperm development can be divided into spermatocytogenesis, meiosis and spermiogenesis [23]. During spermatocytogenesis the immature germinal cells, spermatogonia, divide by mitosis to form the premeiotic cells; while during meiosis the primary and secondary spermatocytes progress by the process of reduction division to form immature haploid spermatids [15]. Spermiogenesis begins with the spermatid stages wherein the oval spermatids mature and differentiate to flagellated spermatozoa in as many as 19 steps. The spermatids develop into mature spermatozoa through a metamorphosis that

involves cytoplasmic and nuclear modifications, formation of new organelles, and the acquisition of flagella which endow independent directional motility to the sperm.

There are synchronous spermatogenic cellular changes and associations within the seminiferous epithelium which succeed one another cyclically in any given area of seminiferous tubules (Figure 2). Each complete sequence of changes in these cellular associations represents a spermatogenic cycle [16, 19, 20]. The formation of specific cellular associations and the sequence of their appearance in a given area of the seminiferous tubule epithelium are highly synchronized. The composition of each of these cellular associations is defined by the step of development of spermatids present in that cellular group. These cellular associations succeed one another in time in any given area of the seminiferous tubules and this succession of cell groups comprises a cycle. Each cycle consists of several stages, and several cycles lead to the complete maturation of spermatozoa [16, 20].

As shown in Figure 2, the least mature germ cell that enters the spermatogenic cycle (spermatogonia type A) is located closest to the basement membrane. This cell can be traced through the mitotic divisions (spermatogonia type B and intermediate), formation of meiotic cells (preleptotene, leptotene, pachytene spermatocytes), reduction division (secondary spermatocytes) and formation of spermatids. Spermatids are located close to the tubular lumen and develop into spermatozoa which are released into the lumen. The immotile luminal spermatozoon is then transferred to the epididymis for storage until ejaculation or autolysis. The spermatozoa continues its maturation in the epididymis where additional morphological and biochemical changes occur that are essential for acrosomal activity and sperm motility. Figure 3 illustrates the mature spermatozoa.

Spermatogenesis is a well-defined orderly process, albeit unlike that of the monthly cyclic release of one ovum in the female gonad, the ovary. Once begun at puberty it

is an average forty-two (mouse) to seventy-two day event (man) that continues throughout the life of the male, and results in the daily production of millions of mature spermatozoa. Spermatogenesis in the rat takes approximately sixty-four days and entails four and one half cycles with fourteen stages in each complete cycle (Figure 4), while in the mouse the process takes approximately forty-two days entailing four cycles of twelve stages each [16]. In man, the cellular associations appear to be less well-defined in the spermatogenic process, wherein only six cell associations are observed in the seminiferous epithelium (Figure 2). Spermatogenesis in man requires 74 $\pm$ 5 days that include 4.5 cycles with approximately 15 days per cycle and six stages per cycle [16, 23, 23B].

The major metabolic reactions occuring in the spermatozoa involve pathways for the utilization of simple sugars to provide chemical energy necessary for motility and to maintain cellular osmotic balance. Very little, if any, sugar is converted to glycogen. Hexose catabolism to pyruvate or lactate by the Embden-Meyerhof pathway and oxidation of either pyruvate or lactate or both in the mitochondria seem to be the major mechanisms for energy production. Energy requirements of spermatocytes and spermatids are supported by lactate and pyruvate, but not by glucose [21]. Lactate also appears to maintain intracellular ATP and stimulate protein synthesis [22] in these cells. Glycolysis proceeds at almost the same rate both anaerobically and aerobically [23]. The high rate of glycolysis in spermatozoa is similar to that of many tumor cells which reflects, in part, an absence of significant feedback on the glycolytic pathway by mitochondrial metabolites. A possible significant portion of the energy generated by the mitochondrial electron transport system is converted into energy needed to carry out non-ATP dependent ion transport, which may be very active in motile cells [22].

Figure 3

Mature spermatozoa. *Through the process of spermatogenesis the spherical non-differentiated spermatogonia develops into this flagellated cell with a prominent nucleus and mitochondrion that make up the head and middle piece, respectively. [23]*

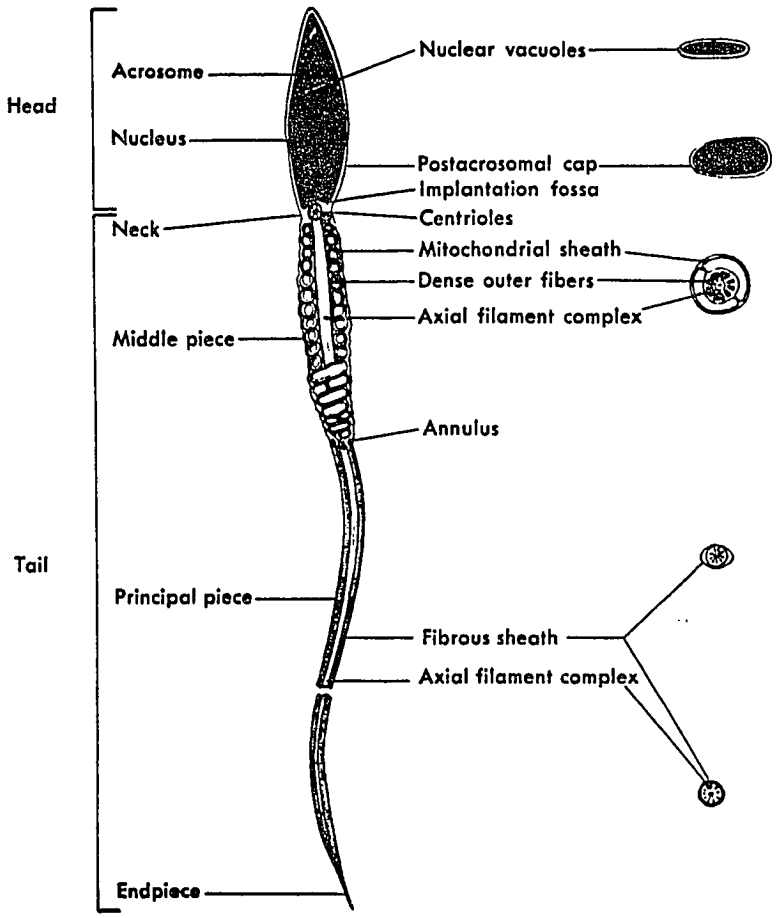
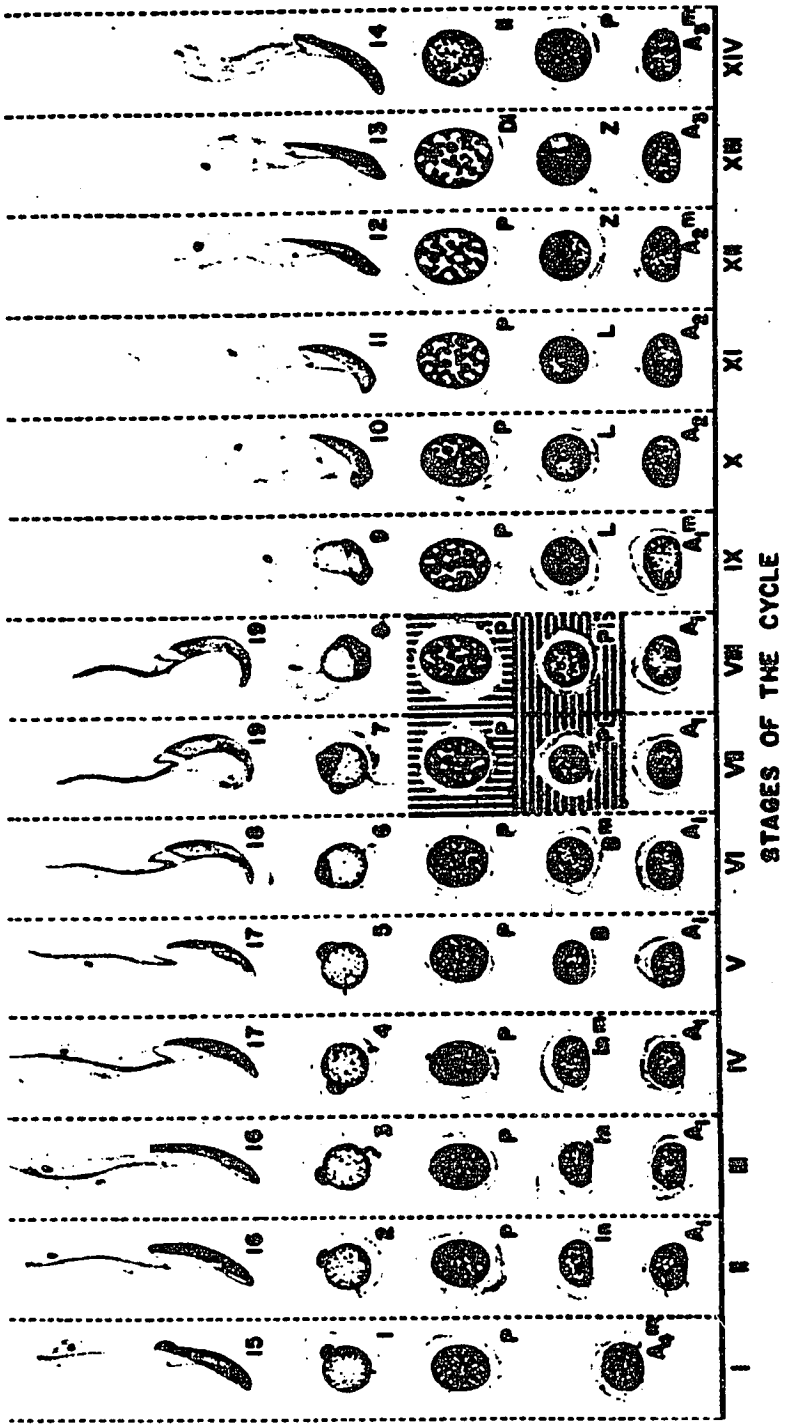


Figure 4

Spermatogenesis in the rat. *During spermatogenesis in the rat there are 14 stages in a complete cycle and 4.5 cycles . (A) Type A spermatogonia, (In) Inter-mediate spermatogonia, (B) Type B spermatogonia, (L) Leptotene spermatocyte, (Z) Zygotene spermatocyte (P) Pachytene spermatocyte, (Di) Dikinesis, (1-19) Spermatids. [16]*



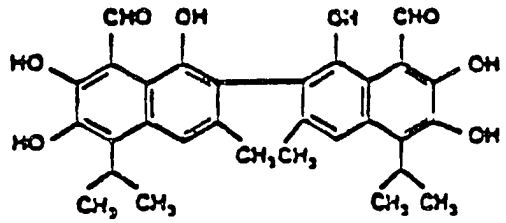
Certain stages in the spermatogenic process appear to be more sensitive to heat, toxins and radiation, and are referred to as lethal stages [15]. Cells in the lethal stage are observed to die upon exposure to such agents more readily than other spermatogenic cells. In the rat under normal conditions there are four stages at which spontaneous degeneration takes place, and these are considered to be vulnerable steps in spermatogenesis. These include spermatogonia type A in stage XII; primary spermatocytes at stages XII-XIV; spermatids in steps 9, 10 and 11 of the acrosome phase of spermatid developmemt; and spermatids in steps 17 through 19, the last half of spermatid maturation [15, 16]. Usually it is within one of these stages that the spermatogenic cells are damaged by lethal agents; however critical metabolic properties and phases of nucleic synthesis at each stage determine how these cell are damaged. At low doses of ionizing radioactivity spermatogonia A and B are damaged significantly, while spermatocytes and spermatids are fairly resistant, with damage occurring at high doses of radioactivity [15].

2. *Gossypol*

Gossypol is a yellow polyhydroxylated binaphthalene compound isolated from the pigment glands of cotton plants. It was first isolated by J.J. Longmore in 1886 and was crystallized as its acetic acid complex, gossypol acetic acid, by Marchelewski in 1899 [1, 24]. In 1958 J.D. Edwards elucidated and confirmed its structure by total synthesis [25]. The chemical name for gossypol is 1,1', 6,6',7,7'- hexahydroxy 3,3'-dimethyl-5,5'-diisopropyl-2,2'-binaphthyl- 8,8'-dialdehyde, and its structure (Figure 5) shows an asymmetrical polyphenolic dinaphthyl dialdehyde with a molecular weight of 519 daltons. It is a highly lipophilic compound that is heat sensitive, relatively insoluble in alkaline aqueous solvents and very soluble in ninety-five percent ethanol. It also has highly reactive carbonyl and phenolic groups. The carbonyl groups can react with acids, bases, oxygen, amine and other functional

Figure 5

Tautomeric forms of gossypol. *(1) dialdehyde (2) dilactol or hemiacetal and (3) monoaldehyde-monolactol. [1].*



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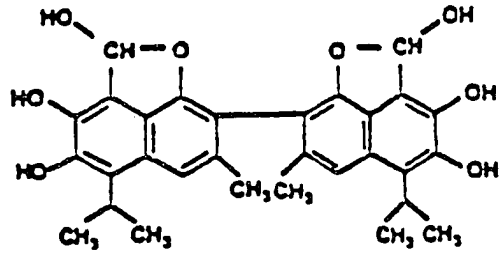

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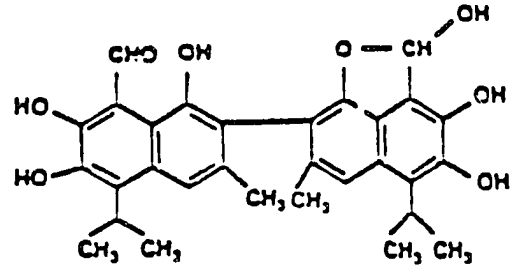


3

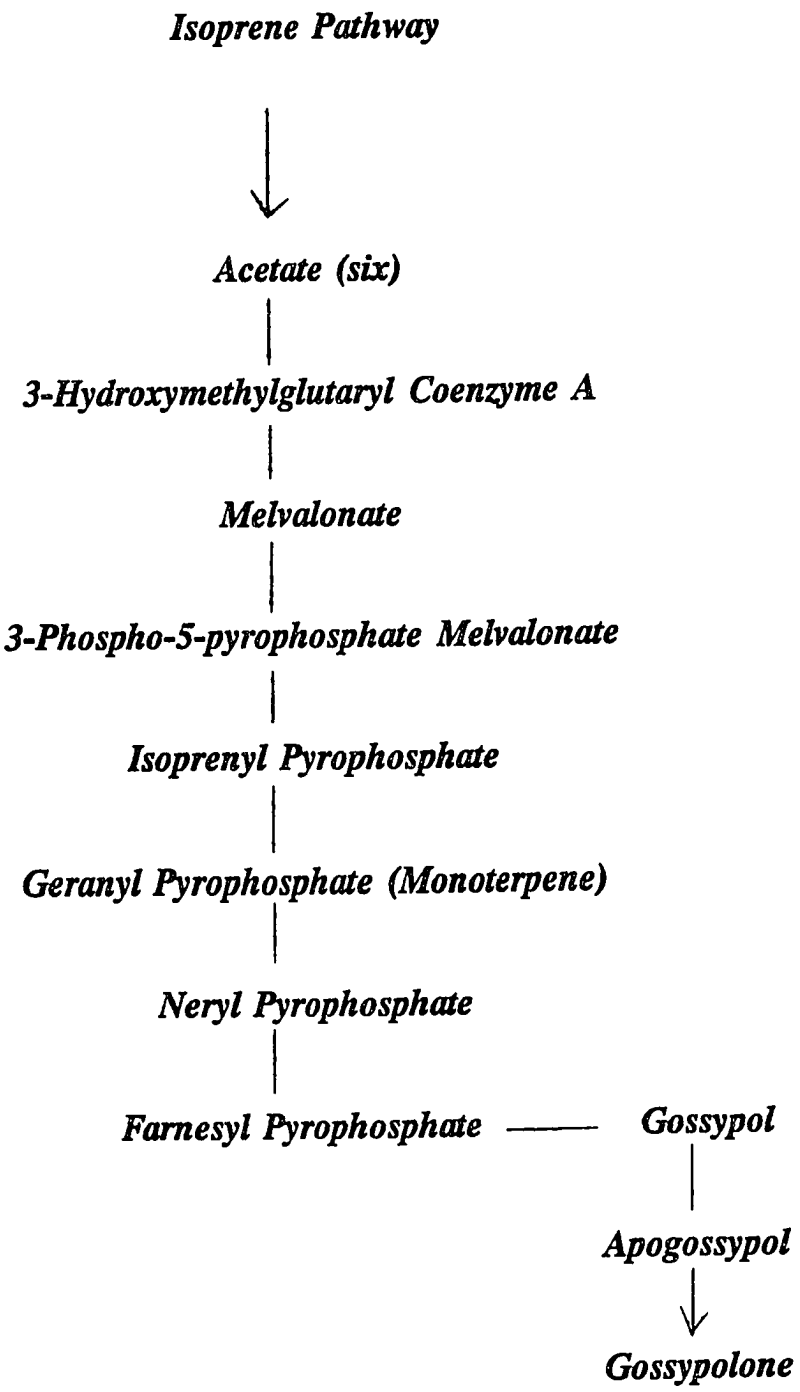
groups in biochemical reactions; while the phenolic groups may readily form esters [25]. Gossypol is also a reducing agent and, as such, is extremely susceptible to oxidation. The rotation about the C-C bond linking the two naphthyl rings is restricted and results in (+) and (-) enantiomeric forms [4]. It occurs mainly in three tautomeric forms as an aldehyde, a hemiacetal or a phenolic quinoid (Figure 5).

Gossypol is synthesized ***in situ*** *by the isoprenoid pathway [26] requiring an initial six acetate groups much like that of cholesterol biosynthesis (Figure 6). Two of its major metabolites are apogossypol, resulting from the removal of the two aldehyde groups, and gossypolone with keto groups at the 1,1' and 4,4' positions. Lyman et al. [27] used ^{14}C-formyl gossypol to determine its biosynthetic pathway and to study gossypol decomposition and deposition in various tissues and the major excretion route for gossypol in chickens. Eighty-nine to ninety-five percent of orally ingested ^{14}C-gossypol was excreted in the bowel with relatively smaller amounts retained by various tissues [27]. The retained labeled gossypol or gossypol metabolite was distributed throughout the body. More than half of this amount was concentrated in the liver followed in decreasing amounts in the kidney, blood, muscles, spleen, lung, heart, testicles and brain [27, 28]. A water soluble metabolite, possibly protein bound or oxidized, as well as an insoluble, nonpolar metabolite were also recovered.*

Gossypol may bind with plasma proteins or remain unbound. Increasing levels of protein seem to decrease the toxicity of gossypol [1, 2, 4, 25, 29] and suggest that such complexes decrease the release of lipid-soluble gossypol, which otherwise readily permeates the tissues. Like most lipophilic drugs, gossypol is likely to be metabolized mainly in the liver by mixed-function oxidation to render a more polar compound which may be conjugated for excretion through the biliary duct. The carbonyl and hydroxyl groups would be the most probable sites for these reactions. Haspel and Corin [29] suggested a model for gossypol

Figure 6

Biosynthesis of gossypol. *Isoprene is synthesized from six molecules of acetate. In an initial pathway similar to that of cholesterol synthesis, 3-hydroxymethyl glutaryl Coenzyme A is formed and the pathway continues to form gossypol.*



uptake by unmediated diffusion (Figure 7) after observing ^{14}C-gossypol uptake and efflux by murine erythroleukemia cells in the presence and absence of bovine serum albumin (BSA). In protein-free media, gossypol reached steady state within one minute, while the addition of 0.1% BSA decreased gossypol uptake, and the steady state was reached between 10 and 20 minutes. When ^{14}C-gossypol distribution between particulate and soluble fractions of cells treated with subsaturable (5 μM) and saturable (30 μM) levels of ^{14}C-gossypol was determined, 95% of the gossypol was found in the membrane fraction and only around 5% gossypol was found in the soluble or cytosolic fraction; suggesting that gossypol was mainly membrane associated [29]. However, the addition of NaCN at concentrations that completely inhibited the uptake of oxygen and ATP synthesis did not appreciably inhibit the uptake of ^{14}C-gossypol, further suggesting that ^{14}C-gossypol uptake does not appear to require ATP.

Because of the economic importance of cottonseed products, such as cottonseed oil and cottonseed meal, the pharmacologic and toxicologic properties of gossypol were studied and determined very early and well into the early 1960's and 1970's, long before its contraceptive potential was realized. It has also been shown to exhibit antibacterial [4, 30] and antiviral [31] properties, as well as anti-tumorgenic and cytocidal activity [25, 31] against erythroleukemia, human melanoma and colon carcinoma cell lines.

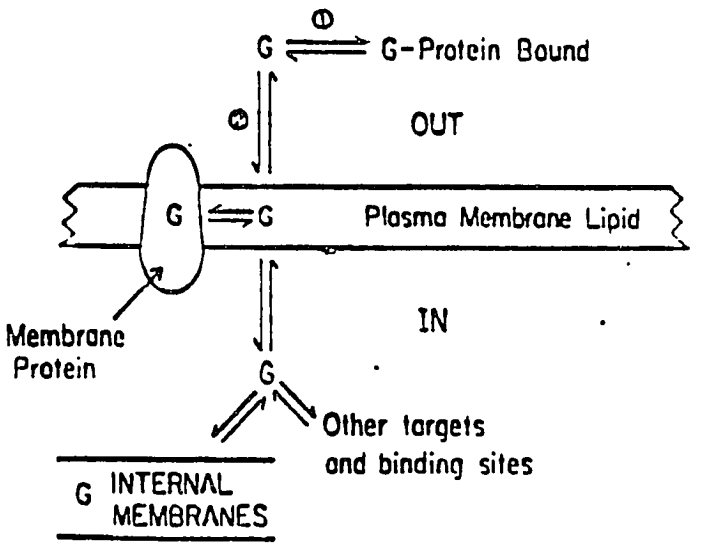
The role of gossypol as an effective male antifertility agent was discovered in China in the early sixties in a rural area where crude cotton seed oil was used in cooking [1]. Subsequent investigations in which the three enamtomers and the racemic mixture of gossypol were compared, have confirmed conclusively that the (-)-gossypol are the more effective antifertility forms of the compound [4, 11, 24, 32-35] .

Pharmacokinetic and toxicological studies of this compound have been performed in animals [36-45]. Gossypol is cytotoxic to mature caudal epididymal spermato-

Figure 7

Corin-Haspel model for gossypol uptake by unmediated diffusion in non-testicular cells *Only free gossypol traverses the lipid bilayer of the plasma membrane by unmediated diffusion. The extracellular protein-bound gossypol is not a substrate for such unmediated diffusion within the plasma membrane. Once dissolved in this lipid phase the gossypol may interact with membrane components, enter the cytosolic compartment and interact with intracellular targets [1].*

Entry of Gossypol into Cells :
A Model Based on Unmediated Diffussion



zoa [24, 41, 46-48] and certain spermatogenic cells in animal testes, particularly spermatocytes and spermatids [24, 49]. Sertoli cells, Leydigs cells and spermatogonia are relatively unaffected [1, 35, 37, 50, 51] at antifertility concentrations, though a recent study shows gossypol binding to peptides isolated from Sertoli cells [52, 53]. Serum lutenizing hormone (LH) and follicle stimulating hormone (FSH) levels and the response of the pituitary gland to gonadotrophin releasing hormone (GnRH) are also not significantly influenced [50].

Clinically, gossypol has been shown to be an effective male antifertility agent [46, 50, 54] with an efficacy of 99.89% and some incidences of well-defined adverse side effects [24, 49, 54]. Although the compound has been used as a male contraceptive agent, these toxic effects make gossypol an undesirable contraceptive agent in much of the western world and China. The most significant deleterious physiological effect is hypokalemia that may not be completely ameliorated with potassium supplementation or potassium sparing agents [5, 38, 50, 60]. Other toxic effects include hepatotoxicity, nephrotoxicity, anemia, anorexia, epididymal thickening, and cardiomyogenic arrhythmia [1, 5, 40].

Yu [56] observed equipotency between (+)- and (-)-gossypol in causing toxic effects in some somatic tissues in rats, but noted the stereo-selectivity of (-)-gossypol to induce antispermatogenic effects. He reasoned that gossypol may actually exhibit two categories of cytotoxicity toward different organs and tissues, which suggests that one may be able to eliminate one effect while preserving the other. However, a unified theory of its biochemical mechanisms is still being developed. Others [25] used gossypol derivatives wherein the carbonyl, hydroxyl and/or aldehyde groups were removed or substituted. Results from erythrocyte anion transport and spermicidal assays, imply that the aldehyde groups were essential for antispermatic activity by gossypol. The carbonyl groups were needed for

inhibition of anion transport, while substitution of the hydroxyl groups of gossypol seemed to have little influence on either the anion transport or spermicidal activity.

There have been numerous studies ***in vivo*** *and* ***in vitro*** *to elucidate the molecular mechanism of gossypol effects and to determine if the mechanism of action attributed to its antifertility properties is distinct from its universal somatic effects. These investigations have revealed several biochemical and molecular actions of gossypol. These include (a) membrane perturbations affecting membrane permeability, transduction and transport mechanisms [3, 9, 54, 56, 57], (b) inactivation or inhibition of oxido-reductases involved in anaerobic and aerobic metabolism [13, 35, 57-58, 60], (c) inhibition of cyclic nucleotide metabolism [59, 61], (d) inhibition of protein and nucleic acid synthesis [62, 63], (e) inactivation or inhibition of Na/K ATPase and Ca/K ATPase [6, 57, 59], and (f) uncoupling of electron transport and oxidative phosphorylation [64, 65]. Still, there are many unanswered questions about the mechanism of action of gossypol at the molecular and/or enzyme levels, and there is no conclusive hypothesis on a unique antifertility mechanism.*

3. Lactate dehydrogenase-X (LD-X)

The lactate dehydrogenase (L-lactate-NAD$^+$ oxido reductase E.C. 1.1.1.27) isozymes are mainly cytosolic nucleotide-dependent enzymes that catalyze the oxidation of lactic acid to pyruvate with nicotinamide adenine dinucleotide (NAD) as coenzyme. These enzymes also catalyze the reverse reaction wherein pyruvate is reduced to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH). In both reactions the enzyme displays absolute stereospecificity [66, 67] in the transfer of the prochiral hydrogen of the fourth carbon on the nicotinamide ring of NAD(H), resulting in the donation or acceptance of the hydride ion . This is an essential reaction in the anaerobic and aerobic synthesis of ATP.

There are five well-documented somatic lactate dehydrogenase (LD) isozymes. These are forms of the enzyme located in tissues other than the gonads. They are tetramers of 3 the A (M) and B (H) subunits that are coded by the ld-a and ld-b genes [68-70]. Each subunit has a molecular weight of 35,000 . The somatic isozymes are LD-$B_4(H_4)$, LD-B_3A_1 (H_3M_1), LD-B_2A_2 (H_2M_2), LD-B_1A_3 (H_1M_3), and LD-$A_4(M_4)$, respectively. Clinically these are recognized as LD-1(LD-B_4) through LD-5(LD-A_4) in accordance with their electrophoretic mobility in barbital buffer at pH 8.6. Although these isozymes are present in most tissues, the LD-$B_4(H_4)$ and LD-$B_3A_1(H_3M_1)$ are mainly associated with the myocardium and erythrocytes; while the LD-$A_4(M_4)$ isozyme is associated with skeletal muscle.

The LD-X or LD-C_4 isozyme is a homotetramer of four C subunits derived from the ld-c gene. It is sperm-specific and represents as much as 80% of total testicular LD isozymes [68]. It is heat stable and, unlike the LD-A_4 and LD-B_4, favorably utilizes ketovalearate and ketobutyrate as substrate [71]. Antibodies against LD-X do not cross react with the A and B subunits, but rabbit anti-mouse LD-X reacts with LD-X from several other species [72].

LD-X first appears in the pachytene spermatocytes [10, 73] and is essential for the metabolic activities in cells in the later stages of spermatogenesis [73]. This enzyme can only be found in spermatocytes, spermatids and spermatozoa [1, 72, 73] with the highest amount in the spermatids. The synthesis of LD-X begins specifically at the midpachytene stage of spermatocyte development and continues throughout spermatozoan differentiation [72, 73], with insignificant LD-X activity in cells prior to the midpachytene stage. In adult male mice there is substantial LD-X in the mid and late pachytene spermatocytes, higher levels in round spermatids and maximum concentration in elongated spermatids. The enzyme is located

in the cytosol of most other spermatogenic cells but is present almost exclusively in the mitochondria of the mature spermatozoa [71, 72].

LD-X appears to play an essential role in mito-chondrial metabolism in spermatozoa. Numerous hypotheses have been proposed regarding the specific expression of LD-X and its functional significance in spermatogenic cells. According to a study by Lardy and his co-workers [75], LD-X plays a unique metabolic role in spermatogenic cells. It appears to compete effectively for reducing equivalents (NADH, $FADH_2$) with the electron transport chain enzymes and permits the direct oxidation of the lactic acid produced by glycolysis. As such, LD-X may participate in shuttling these reducing equivalents from the cytosol to the mitochondria, where the energy yields are higher [73]. In addition to serving as the last enzyme for anaerobic glycolysis, it also functions as a key enzyme to provide NADH for intramitochrondrial oxidative phosphorylation . LD-X could be critical in a mitochondrial/cytoplasmic shuttle system much like malate dehydrogenase in gluconeogenesis. The 2-keto acids produced by transamination of branched chain amino acids could be reduced by cytosolic NADH in a reaction catalyzed by LD-X. The 2-hydroxy acids formed might then cross the inner mitochondrial membrane and be oxidized by LD-X, transferring a hydride ion to mitochondrial NAD, and finally to the respiratory chain [71].

The sperm-specific LD-X and the LD-A and LD-B isozymes have been purified and sequenced [68, 69, 70, 76, 77, 78]. X-ray crystallography and computer model simulations suggest four functional domains and the ternary complex is much like that illustrated in Figure 8. LD-X has a molecular weight of 140,000 daltons with each subunit being 35,000 daltons and consisting of 329 to 333 amino acids [77,78,79]. Since over 60% of its amino acids are non-polar it is a hydrophobic protein. Figure 9 shows a comparison of the amino acid sequence of mouse and rat LD-X. These include (a) an NH_2 terminal (a.a. 1-18), (b) a

Figure 8

Orientation of lactate/pyruvate substrate and the NAD/NADH coenzyme in the lactate dehydrogenase substrate and coenzyme domains. *The orientation of the substrate in the substrate binding domain is the same for all lactate dehydrogenase isoenzymes [79].*

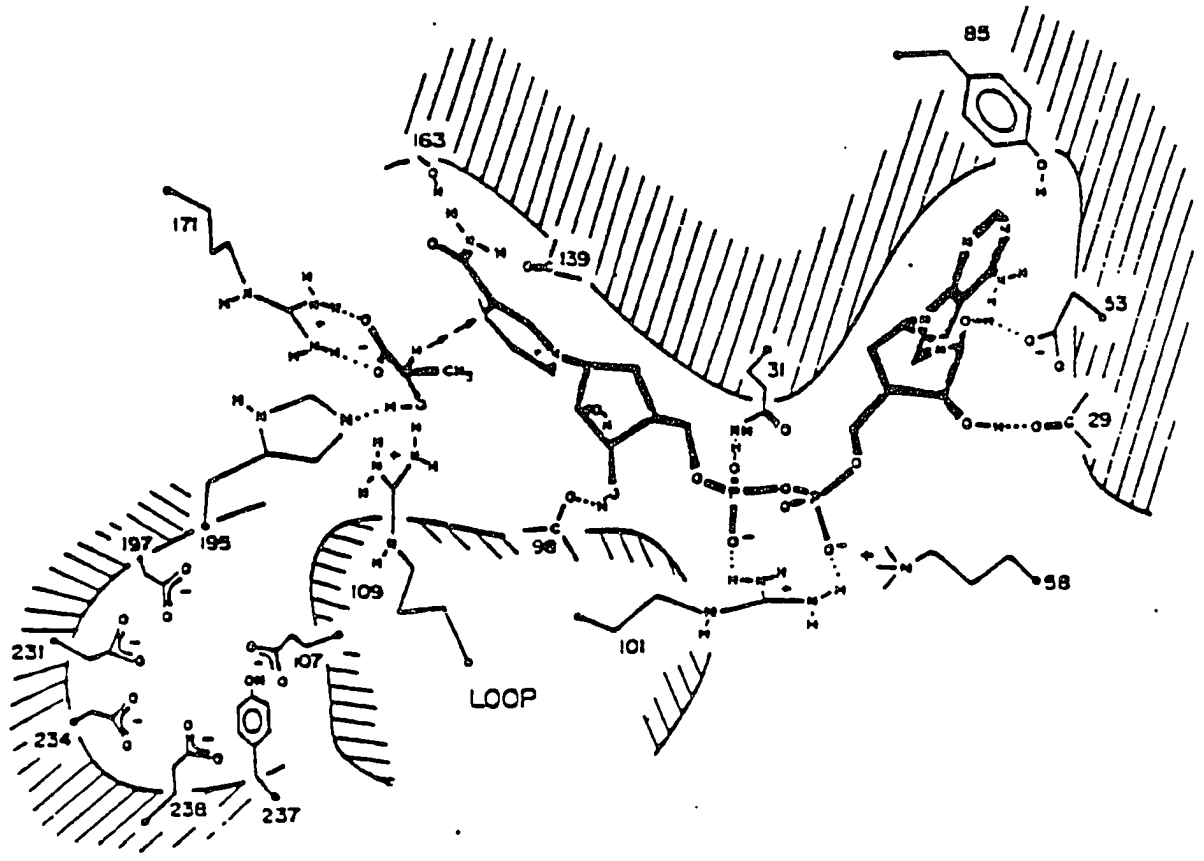
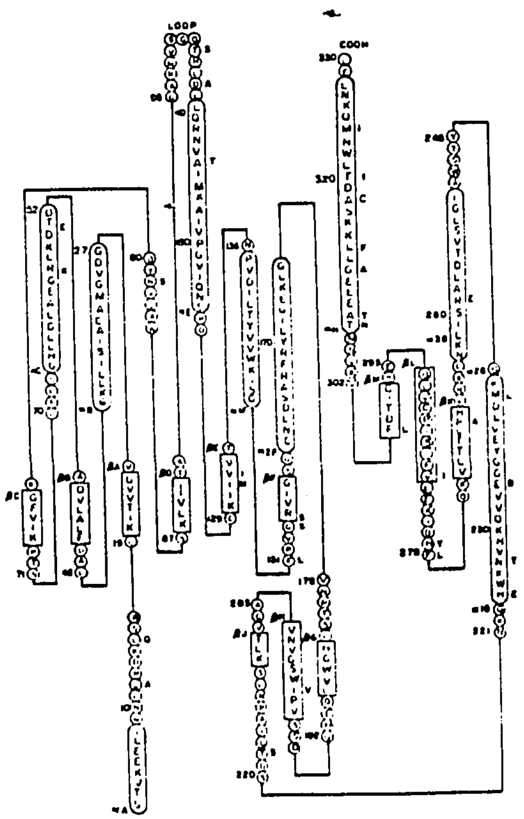


Figure 9

Amino acid sequence for mouse and rat LD-X. *(A) MC = mouse LD-X and RC = rat LD-X.* ***(B)*** *Tertiary structure the showing four functional domains [68,77].*

10 20
Ser-Thr-Val-Lys-Glu-Glu-Leu-Ile-Gln-Asn-Leu-Val-Pro-Asp-Lys-Leu-Ser-Arg-Cys-Lys-
Ala Gln
30 40
Ile-Thr-Val-Val-Gly-Val-Gly-Asp-Val-Gly-Met-Ala-Cys-Ala-Ile-Ser-Ile-Leu-Leu-Lys-
50 60
Gly-Leu-Ala-Asp-Glu-Leu-Ala-Leu-Val-Asp-Ala-Asp-Thr-Asp-Lys-Leu-Arg-Gly-Glu-Ala-
Glu Lys
70 80
Leu-Asp-Leu-Leu-His-Gly-Ser-Leu-Phe-Leu-Ser-Thr-Pro-Lys-Ile-Val-Phe-Gly-Lys-Asp-
90 100
Tyr-Asn-Val-Ser-Ala-Asn-Ser-Lys-Leu-Val-Ile-Ile-Thr-Ala-Gly-Ala-Arg-Met-Val-Ser-
Ser
110 120
Gly-Gln-Thr-Arg-Leu-Asp-Leu-Leu-Gln-Arg-Asn-Val-Ala-Ile-Met-Lys-Ala-Ile-Val-Pro-
Ser Ala Thr
130 140
Gly-Val-Ile-Gln-Asn-Ser-Pro-Asp-Cys-Lys-Ile-Ile-Val-Val-Thr-Asn-Pro-Val-Asp-Ile-
Met Ile
150 160
Leu-Thr-Tyr-Val-Val-Trp-Lys-Ile-Ser-Gly-Phe-Pro-Val-Gly-Arg-Val-Ile-Gly-Ser-Gly-
Leu Ser Ser
170 180
Cys-Asn-Leu-Asp-Ser-Ala-Arg-Phe-Arg-Tyr-Leu-Ile-Gly-Glu-Lys-Leu-Gly-Val-Asn-Pro-
190 200
Thr-Ser-Cys-His-Gly-Trp-Val-Leu-Gly-Glu-His-Gly-Asp-Ser-Ser-Val-Pro-Ile-Trp-Ser-
Val
210 220
Gly-Val-Asn-Val-Ala-Gly-Val-Thr-Leu-Lys-Ser-Leu-Asn-Pro-Ala-Ile-Gly-Thr-Asp-Ser-
Ser
230 240
Asn-Lys-Gln-His-Trp-Lys-Asn-Val-His-Lys-Gln-Val-Val-Glu-Gly-Gly-Tyr-Glu-Val-Leu-
Glu Thr Asp
250 260
Asp-Met-Lys-Gly-Tyr-Thr-Ser-Trp-Ala-Ile-Gly-Leu-Ser-Val-Thr-Asp-Leu-Ala-Arg-Ser-
Leu Glu
270 280
Ile-Leu-Lys-Asn-Leu-Lys-Arg-Val-His-Pro-Val-Thr-Thr-Leu-Val-Lys-Gly-Phe-His-Gly-
Ala Leu Tyr
290 300
Ile-Lys-Glu-Glu-Val-Phe-Leu-Ser-Ile-Pro-Cys-Val-Leu-Gly-Glu-Ser-Gly-Ile-Thr-Asp-
Ile
310 320
Phe-Val-Lys-Val-Asn-Met-Thr-Ala-Glu-Glu-Glu-Gly-Leu-Leu-Lys-Lys-Ser-Ala-Asp-Thr-
Leu Asn Thr Ala Phe Cys Ile
330
Leu-Trp-Asn-Met-Gln-Lys-Asn-Leu-Glu-Leu MC
Ile RC

A



B

coenzyme binding domain (a.a. 19-161), and (c) a substrate binding domain (a.a. 162-330). The substrate binding domain is highly conserved in all isozymes with three substrate binding amino acid residues (Arg-104, Arg-167, and His-191) that are conserved in all known LD isozymes including LD-X. The coenzyme binding domain includes a hydrophobic loop and alpha-D helix (a.a. 94-118) with the greatest variablity in the loop region and unconserved areas in a hydrophobic pocket in the LD-X isozymes of six different species [69, 76]. The relative hydrophobicity of the LD-X molecule and its loop region might facilitate complex formation between gossypol and the coenzyme binding site through hydrophobic interactions. This may explain why LD-X is the most sensitive of the three isozymes to gossypol inhibition. Since the sequence in the loop and alpha-D helix region of mouse and rat LDH-X isozymes is remarkably different from those of mammalian somatic isozymes, this loop region and its hydrophobicity suggest it is a possible site for gossypol binding. The Gln-98 involved in the hydrogen bonding of both apoenzyme and ternary complex is replaced by methionine in mouse and rat LD-X. The Glu-102 is also replaced by glutamine in LD-X. These differences may also account for the unique physical and chemical properties of the LD-X isozyme, since this region is of functional importance during catalysis.

The active sites of dehydrogenases must be easily accessible from solution to allow binding of both the coenzyme and the substrate [79]. Yet, when catalytic activity takes place there is a requirement for a hydrophobic environment in the active site. This is achieved through a combination of coenzyme and substrate binding, as well as conformational change of the enzyme which closes off the active site. The active site is then completely shielded from the solution. In the LD isozymes' holoenzyme structure this conformational change involves a loop, including the alpha-D helix, which covers the active site. Thus, the combined effect of conformational changes and the coenzyme /substrate binding is to shield off

the active site from solution [79]. A compulsory ordered mechanism in which the coenzyme binds to the enzyme before the hydroxy or keto acid substrate is suggested. Additionally, the proton uptake that occurs in the binding of NADH per unit of enzyme has been attributed to a NADH induced increase in the pK_a of histidine in the active site [76, 77, 79]. NAD is a flexible molecule which can adopt many configurations, such that adenine binds in the hydrophobic cavity, with the amino group oriented away from solution [79]. The fact that a large variety of compounds with aliphatic and aromatic groups are inhibitors of dehydrogenases is most probably due to the binding properties of this cavity.

If complete trypsin digestion of LD-X occurs and all peptide fragments are retrieved upon completion of digestion, the expected tryptic peptide fragments of LD-X using the Musick-Rossmann sequence map [68, 69] should yield thirty-three fragments (Table 1) beginning at the N-terminus. Yet with the probable loss of small fragments (one to three peptides) during the desalting step and the single amino acid residues, such as fragment 31, the empirical expected number of peptide fragments is approximately twenty-four fragments.

4. LD-X inhibition by gossypol

Although it is clear that the spermatogenic cells are adversely affected by gossypol, there is a lack of consensus as to its mechanism of action and, except in few instances [80], exactly which cells within the testicular compartment are directly affected. Is its effect an immediate interaction of gossypol with the cell, gossypol metabolites, or concomitant actions that adversely affect nursing or supporting cells?

Three possible antifertility mechanisms have been hypothesized [1, 81]. First, spermatogenesis could be hindered. This is an orchestrated event during which specific enzymes are often expressed to fulfill specific metabolic functions in developing cells [1]. If any of the sperm-specific or testis-specific enzymes are inhibited or inactivated by gossypol,

Table 1

Amino Acid Sequence of LD-X and Expected Tryptic Fragments

1. *Ala-Thr-Leu-Lys*
2. *Glu-Gln-Leu-Ile-Ala-His-Val-Ala-Glu-Gln-Ser-Glu-Thr-Ile-Ser-His-Gln-Lys*
3. *Ile-Thr-Val-Leu-Gly-Val-Arg*
4. *Gln-Val-Gly-Met-Ala-Cys-Gly-Ser-Ser-Ile-Leu-Met-Lys*
5. *Ser-Leu-Ala-Asp-Gln-Leu-Ala-Leu-Leu-Asp-Ala-Met-Glu-Asp-Lys*
6. *Met-Lys*
7. *Gly-Glu-Met-Met-Asp-Leu-Gln-His-Gly-Ser-Leu-Phe-Leu-Try-Thr-Pro-Lys*
8. *Leu-Ala-Val-Val-Thr-Ala-Gly-Val-Arg*
9. *Gln-Gln-Gly-Glu-Ser-Arg*
10. *Leu-Asn-Leu-Leu-Gln-Arg*
11. *Asn-Ala-Asn-Val-Phe-Ile-Ile-Leu-Pro-Arg*
12. *Ile-Val-Lys*
13. *Try-Ser-Pro-Asn-Cys-Leu-Ile-Leu-Val-Val-Ser-Asn-Pro-Gly-Asp-Val-Leu-Thr-Val-Ala-Trp-Lys*
14. *Ile-Ser-Gly-Phe-Pro-Val-Gly-Arg*
15. *Val-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asp-Ser-Ala-Arg*
16. *Leu-Arg*
17. *Asn-Val-Met-Ala-Ile-Lys*
18. *Ile-Val-Leu-Gly-Ser-Leu-Ser-Cys-His-Gly-Trp-Leu-Val-Gly-Arg*
19. *His-Gly-Asp-Ser-Gly-Val-Pro-Val-Trp-Leu-Gly-Met-Asn-Asn-Ala-Gly-Val-Leu-Gln-Asn-Leu-Asn-Gln-Gly-Met-Gly-Trp-Glu-Asn-Asp-Ser-Glu-Gly-Trp-Lys*
20. *Glu-val-His-Arg*
21. *Met-Val-Val-Glu-Ser-Ala-Try-Glu-Val-Ile-Lys*
22. *Leu-Lys*
23. *Gly-Tyr-Glu-Asn-Trp-Ile-Gly-Leu-Ser-Val-Ala-Glu-Ser-Ala-Glu-Thr-Val-Met-Lys*
24. *Asn-Leu-Try-Arg*
25. *Val-His-Pro-Val-Ser-Thr-Leu-Val-Lys*
26. *Glu-Leu-His-Glu-Ile-Lys*
27. *Glu-Glu-Val-Phe-Leu-Ser-Leu-Pro-Cys-Leu-Leu-Asn-Gln-Ser-Gly-Leu-Arg*
28. *Glu-Ile-Leu-Lys*
29. *Met-Leu-Leu-Lys*
30. *Pro-Glu-Glu-Val-Gly-Gln-Ser-Lys*
31. *Arg*
32. *Ser-Ala-Asp-Ile-Leu-Trp-Gly-Ile-Gln-Lys*
33. *Glu-Leu-Gln.*

its presence may lead to the metabolic destruction of certain spermatogenic cells, but not other cell types. LD-X is inhibited by gossypol, but so are several other oxido-reductases and membrane associated enzymes [1, 5]. Secondly, the spermatogenic cells may have specific "receptors" for gossypol. The specific binding of gossypol to certain spermatogenic cell receptors may be cytotoxic. Thirdly, since spermatogenic cells are rapidly dividing cells, gossypol might have a specific inhibitory effect on DNA or RNA synthesis during active mitosis and meiosis of spermatogenesis [81]. Although gossypol has been reported to compete with androgen binding proteins [18], to date there is little evidence to support the inhibition of spermatogenic membrane receptors by this compound. Some investigators report increased branch-chain breakage after exposure to gossypol [62] in rat liver cells. Others have observed no direct inhibition of protein or nucleic acid synthesis during the initial antifertility action of gossypol but a decrease in both as exposure to the compound continues [80].

To test the first of these hypotheses, studies were conducted to examine the effect of gossypol on the activity of several enzymes from mouse testis. Among the enzymes examined, lactate dehydrogenase (LD) isozymes and malate dehydrogenase (MDH) were shown to be significantly inhibited or inactivated by low concentrations of gossypol in solution [11, 71, 81]. The degree of enzyme inactivation is both gossypol and enzyme concentration dependent. Under the same experimental conditions, LD-X from mouse was inactivated faster than LD-B_4. NADH was shown to partially protect the enzyme against inactivation by gossypol. It was concluded that LD isozymes are inactivated by minor components of gossypol in solution and the male anti-fertility effect of gossypol may be related to the selective inactivation of sperm-specific LD-X. Of the three mouse LD isozymes, LD-X was shown to be selectively inhibited or inactivated by gossypol. Some postulate that sperm-sp-

ecific mitochondrial enzymes might be the binding or acting site of gossypol [11, 13, 33, 80], which results in impaired motility and interrupted spermatogenesis.

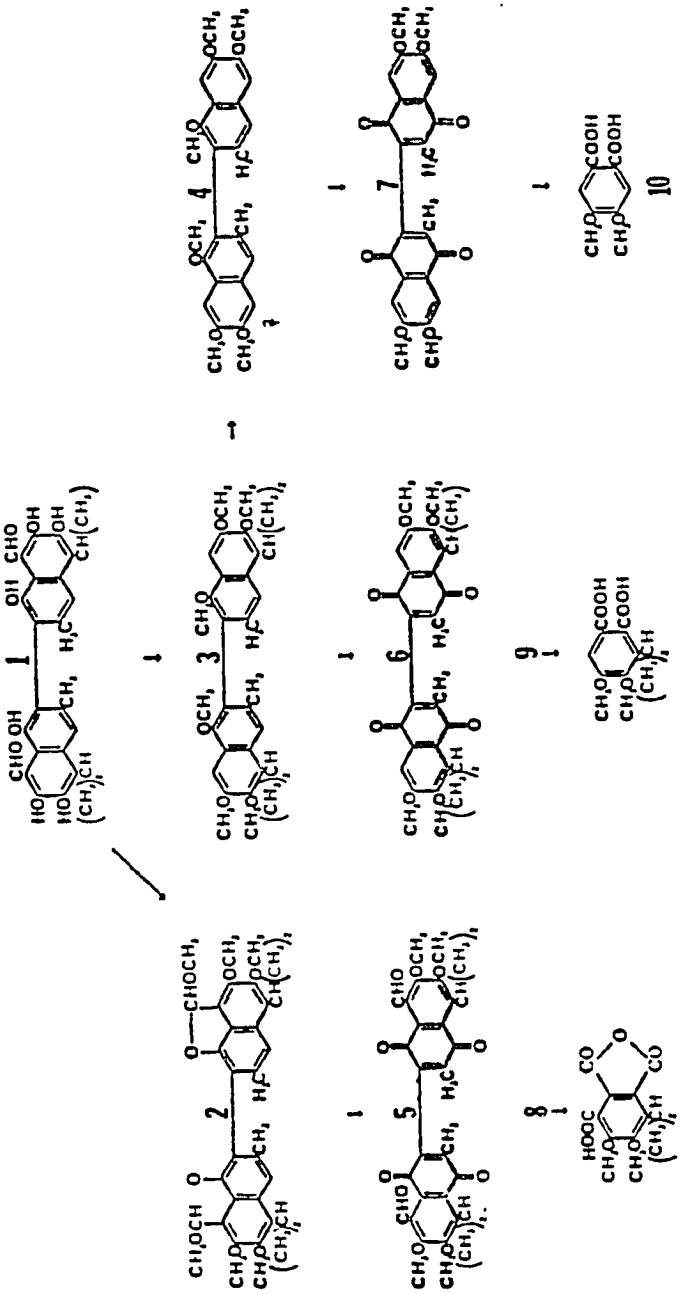
Additional studies [12, 33] also showed that enzyme inactivation by gossypol is both gossypol and enzyme concentration dependent, despite the fact that gossypol is always in great excess when compared to the amount of enzyme present [81]. This observation may imply that there are "active" minor component(s) in the gossypol sample that inhibit the enzymes. These minor components are either (a) the decomposition products or minor tautomeric forms of gossypol in neutral aqueous solution, or (b) impurities contaminated in the original gossypol sample. To distinquish between these two possibilities, gossypol samples were initially tested for their relative potency with respect to enzyme inactivation [34, 81]. To eliminate possible impurities in the original samples, attempts were made to purify gossypol samples by thin-layer chromatography using silica gel plates in different solvent systems. Gossypol from the single yellow UV-positive spot was recovered from the plates and redissolved in ethanol. During the course of these experiments, it was noticed that rechromatography of gossypol samples recovered from the first thin-layer chromatography showed the appearance of minor decomposition products on silica gel plates. In a separate experiment, the original sample in ethanol and solvents from any of the solvent systems used in earlier experiments were spotted together on silica gel plates without migration. Twenty minutes later, the gossypol recovered from the unmigrated spots on silica gel plates also showed a higher degree of potency in terms of its inactivation of LD-X. As a control, incubation of fresh silica gel powder with gossypol alone in ethanol did not significantly alter the potency of this compound on LD-X inactivation.

In an attempt to search for the "active" components of gossypol that lead to specific enzyme inactivation, the stability of gossypol was investigated mainly by thin-layer

chromatography under different experimental concentrations [11, 81]. Subsequently, it was found that gossypol has a half-life of less than 18 hours in neutral solution at room temperature. The stability of gossypol in neutral aqueous solution was also examined, when 0.5 mg (1 μM) gossypol was first dissolved in 0.5 ml of absolute ethanol followed by the addition of 1.5 ml of 50 mM sodium phosphate buffer, pH 7.0. The sample solution was not protected from light and stood at room temperature overnight. Within 24 hours seven distinct decomposition products were isolated by thin layer chromatography on cellulose plates with a solvent system of ethanol:acetic acid [34]. These were designated S-1, S-2, S-3, S-4, S-5, S-6 and S-7. When the inhibitory effect on mouse LD-X by each decomposition product was studied, S-1 and S-2 were the most potent inhibitors. Nuclear magnetic resonance, infrared and mass spectroscopic studies permitted chemical characterization of each decomposition product by determining the presence or absence of dienes, naphthalene, aliphatic aldehyde and carbonyl groups. The findings suggested that the two naphthalene ring systems separated first, followed by the splitting of the two rings of each napthyl group to form two components from each (Figure 10). One of these components was then assumed to further decompose. S-1 and S-2 were shown to be two of these later decomposition products. Compared to the original gossypol sample, the decomposition products also had a much lower absorbance ratio of 380 nm to 260 nm [34]. The minor active components of gossypol samples that may lead to enzyme inactivation have recently been identified [84]. Three major decomposition products were formed after incubation of gossypol in a phosphate buffer/ethanol solution at pH 7.55 for six hours as separated by a high pressure liquid chromatographic procedure. These decomposed products were isolated and subjected to mass spectroscopy, nuclear magnetic resonance, and fourier transform infrared spectroscopic studies. Results from these studies strongly indicate that the identity of the minor component of these decomposed products of

Figure 10

Gossypol derivatives and decomposition products. *(1) Gossypol* **(2)** *Gossypol hexamethyl ether* **(3)** *Apogossypol hexamethyl ether (4) Desapogossypol hexamethyl ether (5) Gossypolone tetramethyl ether (6) Apogossypolone tetramethyl ether (7) Desapogossypolone tetramethyl ether (8) Gossic acid (9) apogossypolic acid (10) m-Hemipinic acid [84]*



gossypol in neutral aqueous solution was either a lactol, a carboxylic acid, or a quinoid compound [84]; and that LD-X is mainly inactivated by minor components of gossypol preparations [34].

The lack of consistency in reported observations between LD-X inactivation studies ***in vivo*** *and* ***in vitro*** *could result from the combined effect of free gossypol along with gossypol metabolites derived through biotransformation in the liver* ***in vivo*** *systems, in contrast to the direct intracellular interactions of gossypol that occur in cell free systems. Although most observations suggest that the inactivation of LD-X was most probably responsible for the antifertility properties of gossypol, some recent studies refute this concept and propose that its antifertility effect may very well result from its general molecular action on nucleotide metabolizing enzymes [5, 80, 83], including LD-X. Others have reported [1, 5, 65,] that other testicular and non-testicular enzymes are also inhibited by gossypol. The basal actvity of a soluble form of adenylate cyclase associated with spermatocytes and spermatozoa was decreased by 70% in rats after treatment with 30 mg gossypol/Kg/day for seven weeks, while adenylate cyclase from other tissues was unchanged [5, 35].*

Morris [85] studied the inhibitory effect of isomers of gossypol upon testicular cytosolic LD-X ***in vitro****. Gossypol acetic acid increasingly inhibited LD-X prepared from testes in the mouse> rabbit > human > rat > hamster, but there was no relationship between this* ***in vitro*** *inhibition of LD-X activity and* ***in vivo*** *antifertility activity. In fact the antifertility activity as determined by fertilized ova was lowest in the mouse.*

Still the predominance of data have shown a high correlation between gossypol inhibition of LD-X and anti-fertility [36, 59, 81]. It should also be noted that variations in experimental protocol and substrates may also contribute to contradictory reports. Gossypol inhibition of LD-X with coenzymes NAD/NADH is competitive, while that with pyruvate/lactate

is non-competitive [13], and the order in which substrate or coenzyme is added in assay systems also affects enzyme activity [12, 34, 81].

Tanphachitr and Fitzgerald [53] reported preferential binding of ^{14}C-gossypol to a Sertoli cell line (TM-4) when compared to cell lines derived from kidney cells, fibroblasts and epithelial cells, and also characterized at least 15 proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography that conjugated with ^{14}C-gossypol. They suggested that conjugation of such proteins with gossypol may result in macromolecular dysfunction and abnormal structure. One class of proteins characterized in this report included peptides with apparent molecular weights of 35,000 and 37,000 daltons which, when based solely on molecular weight, could very well include gossypol/LD-X conjugates or conjugation products.

Since gossypol has been shown to be a highly effective non-hormonal antifertility agent, further probing of its biochemical mechanism of action could lead to the development of synthetic compounds or analogs exhibiting the desired antifertility effect without toxic side effects. It is extracted from an economical natural product; and its histochemical effects are reversible up to four years after cessation of oral ingestion at non-toxic antifertile concentrations [60]. However, the longer the compound is used, the greater the chance of permanent sterility. If developed into a practical contraceptive model for men, gossypol or its derivatives could perhaps have as great an impact on the control of world population as the female oral contraceptives. Potential gossypol-derived spermicidal contraceptive devices may still be envisioned, in spite of the pessimism with its toxicity and apparent limited specificity.

Inactivation of LD-X by gossypol and gossypol degradation studies suggest that interactions between the decomposed product(s) of gossypol or its metabolites and specific domains on the enzyme might be a plausible mechanism for its antifertility action. Experi-

ments designed to observe the characteristics of binding between the enzyme and gossypol, along with the isolation the peptide fragment(s) of LD-X that contain the linked inhibitor, might confirm this hypothesis.

B. Statement of the Problem

The control of population growth by contraception has become an increasingly important issue, especially in the under-developed countries. Thus far, there is no efffective noninvasive, reversible antifertility agent used by human males without some undesirable side effects; and, other than the condom and vasectomy, there is no readily available and acceptable male contraceptive agent. Sperm specific monoclonal antibodies and antispermatogenic chemical agents have been the modalities investigated as potential male contraceptives.

From preliminary studies in China, it appeared that the oral administration of gossypol in effective doses could result in severe oligospermia and azoospermia in several mammalian species including humans. Recent investigations have shown these and other pathognomonic properties to be almost 100% restored after cessation of gossypol ingestion in all species [35, 36, 83] except the human male, where only 92% reversibility of azospermia was observed [83]. Although eight percent irreversibility is significantly high from the perspective of the average male in modern western cultures, such may not be the case in certain third world cultures. Whether gossypol has the potential to become a useful male antifertility agent world-wide depends greatly on continous research probing the biochemical basis of its mechanism of action, as well as its toxicity and its antifertility reversibility.

The goal of the proposed research was to investigate interactions between gossypol and LD-X (LD-C_4) that might aid in defining a mechanism that could eventually lead to the development of synthetic compounds or a derivative of this economical and plentiful compound which would exhibit the desired antifertility effect without the unwanted toxic effects thus far

noted in human and non-human species. There is a consensus that there may be several targets for the action of gossypol in mammalian species. Whether LD-X is a primary target enzyme in sperm cells for gossypol remains to be fully resolved, yet its inhibition by this compound is unequivocal. The inactivation of LD-X by gossypol could lead to the severe impairment of both anaerobic and aerobic metabolism in spermatogenic cells, and it has been reported that the sugar uptake by human spermatozoa is diminished upon incubation with gossypol [33]. If LD-X is the key enzyme in glycolysis (fructolysis) within spermatozoa, its inactivation may indeed be responsible for the action of gossypol on the spermatozoan metabolism.

The research was designed to examine binding properties of gossypol with purified native LD-X, LD-X peptide fragments and cell bound LD-X in an attempt to determine whether the compound binds mainly to the enzyme through covalent binding or hydrophobic interactions. Most investigations that have been conducted by this laboratory and others to elucidate the biochemical properties of gossypol have focused on the characterization of LD-X/gossypol inhibition kinetics [1, 11-13, 81, 85] and gossypol degradation products [1, 34, 84]. This investigation focuses on the binding characteristics between the inhibitor (gossypol) and the enzyme (LD-X) without the presence of coenzyme or substrate. Thus, in this study, binary interactions are defined as interactions between the enzyme and gossypol.

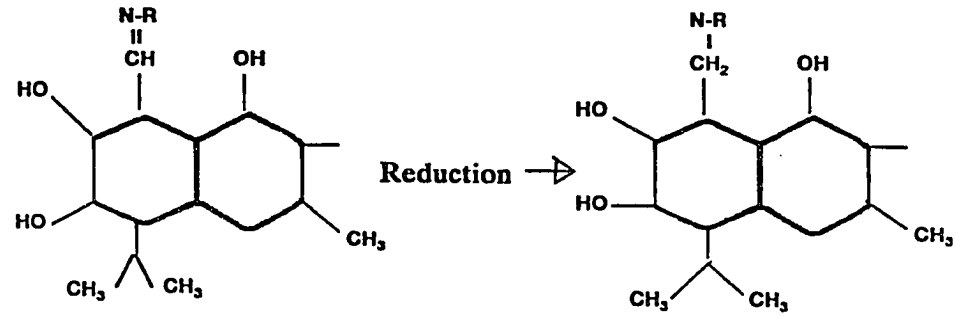
The questions to be answered are: 1. To what extent does native LD-X bind with gossypol? This will be determined by binding assays such as incubating increasing concentrations of gossypol with constant amounts of undigested enzyme to determine the binary affinity constant. 2. Are there alterations in the LD-X peptide fragments after treatment with gossypol? The answer to this question will be sought by observing high performance liquid chromotography (HPLC) separation patterns following cleavage of LD-X, in the presence and

absence of gossypol, with trypsin after partial reduction and carboxymethylation with dithiothreiotol and iodoacetamide, respectively. Amino acid analysis of altered peptide fragments would yield information relative to the domain(s) which would be most affected by gossypol binding. 3. To what extent does gossypol bind with testicular bound LD-X? This will be ascertained by comparing the total binding of ^{14}C-gossypol to cytosolic and membrane fractions of testicular cell suspensions to its binding to similar fractions that are complexed with anti-LD-X. The ^{14}-C binding in the anti-LD-X complex is assumed to be LD-X specific binding. 4. Does it appear that covalent gossypol LD-X binding contributes to its antifertility properties? Given the structure of gossypol and its chemical properties there are at least four ways by which it may interact with LD-X (Figure 11). These include (a) reduction of the quaternary molecule to cause conformation alterations through gossypol's role as a reducing agent, (b) covalent binding through the reactive carbonyl group at the C-8 and C-8' positions with the free NH_2 groups by Schiff condensation, (c) ester linkage at any of the six hydroxyl groups per molecule, and (d) hydrophobic interactions through its aliphatic side groups and its aromatic naphthyl rings. Binary binding studies may indicate if one or a combination of these mechanisms is associated exclusively with the unconserved portions of the LD-X molecule and suggest a mechanism of action of the gossypol male antifertility effect, or provide evidence on treatments to eliminate its toxic effects. Covalent binding would be suggested if the amino acid content of altered peptides reveals an abundance of amino acids with free amino side chains and these fragments show a relative higher binding with labelled gossypol under conditions more favorable to covalent binding. The protocol for this research is shown in Table 2.

Figure 11

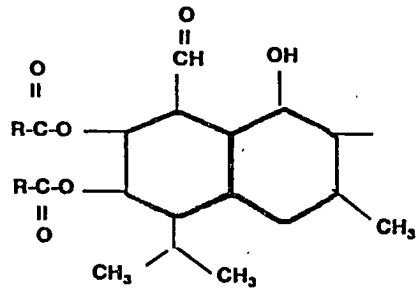
Possible types of LD-X interactions with gossypol. ***(a)*** *Covalent bonding through Schiff base linkage between carbonyl and amine groups.* ***(b)*** *Ester linkage at hydroxyl groups.* ***(c)*** *Interaction between alkyl side groups of amino acids and the methyl and propyl of gossypol.* ***(d)*** *Interaction between naphthyl ring or aromatic degradation products and aromatic amino acids.*

(a.)

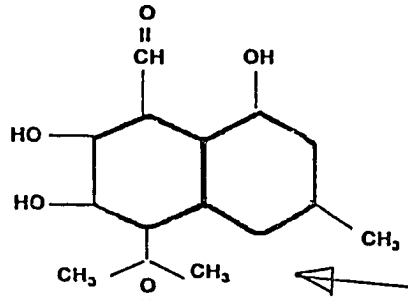


Schiff Base Linkage

(b.)

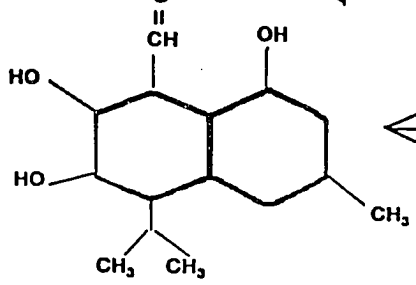


(c.)



Alkyl Side Chains

(d.)



Aromatic Amino Acid
(Histidine Phenylalanine)
Tyrosine
Tryptophan

Table 2

Experimental Protocol

1. *Extract and purify mouse LD-X.*
2. *Develop and isolate polyclonal mouse anti-LD-X gamma globulin.*
3. *Determine optimum conditions for immunoprecipitation of LD-X anti-LD-X complex.*
4. *Cleave enzyme by trypsin digestion.*
5. *Separate digested enzyme peptide fragments by HPLC.*
6. *Perform binding assays to observe ^{14}C-gossypol binding with undigested and digested LD-X .*
7. *Observe HPLC separation of LD-X before and after exposure to gossypol to determine alterations in tryptic peptide fragments.*
8. *Observe and compare the binding of ^{14}C-gossypol with testicular cell suspension bound LD-X in the cytosol and membrane components.*

Chapter II

EXPERIMENTAL

A. Materials

The ^{14}C-gossypol was graciously supplied by Dr. Martin Sonenberg, Memorial Sloan-Kettering Cancer Center, New York, N.Y. Mouse testicles and goat anti-rabbit IgG were purchased from Pel Freez Biological, Rogers, Arkansas. The HPLC peptide standards, lactate dehydrogenase isozyme M_4 and H_4, trifluoroacetic acid, triacetic acid, NADH, pyruvic acid, NAD, gossypol, gossypol acetic acid, dithiothrietol, iodoacetimide, urea, ammonium bicarbonate, urea-hydrogen peroxide, tetramethylbenzidine, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), Eagle's minimum essential media, collagenase, DNase, glutamine, cyanogen bromide, anti-rabbit IgG-horseradish peroxidase conjugate, DEAE-cellulose, TCPK trypsin, hexanediamine, Sepharose 4B, and streptomycin-penicllin were purchased from Sigma Chemical Co. St. Louis, MO. Glass distilled acetonitrile, HPLC grade, was obtained from EM Science. Nuncion Delta SI 24-well Multidish were purchased from Inter Med, Denmark. Aquasol II scintillation fluid was a product of DuPont Chemical Co. Heat-inactivated fetal calf serum was acquired from Gibco.

B. Equipment

All HPLC was performed using a dual pump system that consisted of two Varian 2510 pumps, a Varian 2584 static mixer, a Varian 2550 variable UV detector and a Varian 4290 integrator. A Partisil ODS-10 C_{18} and a Partisil ODS-5 C_{18} analytical columns were purchased from Alltech. A fast pressure liguid chromatography (FPLC) desalting Superfine

Sephadex C-25 column and a Frac-100 fraction collector were purchased from Pharmacia . An ISCO Model UA-5 absorbance fluorescence monitor coupled with a 5-mm light path flow cell was used to monitor effluents from the FPLC and the DEAE-cellulose columns. An Eppendorf Model 5414 and a Beckman Model J2-21 centrifuges were used for high speed centrifugation. A Beckman Model TJ-6 refrigerate centrifuge was used for low speed centrifugation. A Pharmacia Frac-100 fraction collector was used to collect chromatographic fractions. A Waters HPLC PicoTag System at Virginia Polytechnic Institute and University, Blacksburg, VA was used for amino acid analysis. It consisted of a Model 510 HPLC with a temperature control module, a Model 700 satellite WISP injector, a Model 484 tunable absorbance detector, system interface module and a Maxima 820 software. A Milton-Roy Spectoronic Model 1201 UV-visible spectrophotometer coupled with an IBM PS/2 Model 30 microcomputer and a Milton-Roy Spec-Scan software was used for all spectral and kinetic studies. A Corning Digital Model 110 pH meter was used for all pH measurements. A Labconco benchtop freezer-dryer was used for all lyophilysis work. A Beckman Model 5000CE liquid scintillation counter was used for measurement of ^{14}C-gossypol radioactivity.

C. Methods

1. 8-(6-Aminohexyl)-amino-adenosine 5'-monophosphate Sepharose-4B affinity chromatography

Cynaogen bromide activated Sepharose-4B suspended in 1.0 mM HCl was washed on a sintered glass filter with 1.0 mM HCl. This was followed by washing with 0.1 M sodium bicarbonate buffer containing 0.5 M NaCl, pH 8.4. Twenty milliliters 8-(6-aminohexyl)-amino-AMP was added to the sepharose and shaken overnight at 4°C. This was followed by a wash with 1.0 M NaCl and 10 mM potassium phosphate buffer, pH 6.5. This product was equilibrated with a pH 6.5 buffer for affinity chromotography or stored at 4°C.

2. Preparation of reduced NAD-pyruvate adduct

The reduced NAD-pyruvate adduct was prepared according to the procedure published previously [78]. Fifty milligrams NAD and 50 mg pyruvate were dissolved in 1.0 ml deionized water. The pH was adjusted to 11.5 by dropwise addition of 1.0 N NaOH. This mixture was allowed to react at room temperature in the dark for 20 minutes. The mixture was then diluted to 175 ml with 10 mM potassium phosphate buffer, pH 6.5, and 120 ml was employed for biospecific elution of LD-X from the affinity column being sure to protect the solution from light. The reduced NAD-pyruvate adduct is an inhibitor of LD-X and therefore, will compete with the affinity gel for LD-X binding, causing the LD-X to be eluted from the column.

3. Purification of LD-X from mouse testes

Twenty grams of frozen trimmed mouse testicles were suspended in 100 ml cold 0.15 M Tris-HCl buffer, pH 7.4, in a Waring blender. The testes were homogenized at the highest speed for 10-15 second intervals for one minute. The cold homogenized tissue was centrifuged at 15,000 x g for 25 minutes at 4°C. The supernatant was collected through cheese cloth, heated in a 65°C waterbath for 30 minutes, and centrifuged at 15,000 x g for 25 minutes. The supernatant was retained and adjusted to pH 6.5 with 50 mM KH_2PO_4. It was then loaded on the 8-(6-aminohexyl)-amino AMP-Sepharose affinity column equilibrated previously with 10 mM potassium phosphate buffer, pH 6.5. The column was then washed with additional 50 ml of the column buffer, followed by 150 ml of 50 mM potassium phosphate buffer, pH 6.5. The LD-X was then eluted from the column with 120 ml of the reduced NAD-pyruvate adduct. During the elution the column was enclosed within an aluminum foil jacket. The eluate containing LD-X was then concentrated to 10 ml in an Amicon ultrafiltration system equipped with a PM-10 membrane. This concentrate was then dialyzed against several

changes of the 10 mM potassium phosphate buffer pH 6.5 to remove excess adduct. The dialyzed concentrate was lyophilized and stored at -20°C.

4. Agarose gel electrophoresis

A Beckman Paragon electrophoresis system was used to assess the degree of purity of the purified LD-X preparations. Aliquots of sample or standard were applied to a 1% agarose gel and electrophoresis was carried out in a 1.4% 2-amino-2-methyl-1,3 propanediol glycine barbital aspartate buffer, pH 8.6, at 100 V for 20 minutes. The substrate consisted of 208 mM lithium L-lactate, 5.6 mM NAD, 2.4 mM nitroblue tetrazolium and 0.33 g phenylmethyl sulfate per liter. Gels were incubated in this substrate mixture at 45°C for 30 minutes. Bands were scanned with a Beckman densitometer.

5. SDS-PAGE electrophoresis

A Hoefer Scientific Instrument SE-600 Series vertical slab gel electrophoresis unit was used to perform sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on the purified LD-X fractions following a modified Laemmli [88,112] protocol. Samples were prepared by boiling 2-3 minutes after mixing one volume sample to four volumes of a sample buffer containing 1ml each of 10% SDS, 0.5 M Tris-HCl, pH 6.8, glycerol, 0.2 ml 2-mercaptoethanol and 0.2 ml 0.05% bromophenol blue. Samples were then applied to slab gel composed of 10% acrylamide separation gel and 3% acrylamide stacking gel, and electrophoresis was carried out in the Tris/glycine/SDS separation buffer at 20 mA/gel for 18 hours in a vertical cell with a cooling jacket. After fixation in 5% acetic acid, the gels were stained in 0.05% Commassie Blue, destained in 5% acetic acid and dried at 70°C under vacuum in a Bio Rad gel dryer. Band migrations were compared with molecular weight markers to determine approximate molecular weights.

6. Assay of LD-X activity

Lactate dehydrogenase activity was assayed by spectrophotometric assay [12] wherein the decrease in absorbance at 340 nm was monitored after the addition of coenzyme to a reaction mixture containing LD-X samples at room temperature. The reaction mixture contained 0.75 ml 0.2 M Tris-HCl, pH 8.0, 150 µl 10 mM pyruvate, 0.5 ml deionized water, and 50 µl diluted enzyme sample. The change in absorbance at 340 nm was recorded immediately after the addition of 50 µl 1.5 mM NADH using a Milton-Roy Spectronic 1200 in the kinetic mode.

7. Protein assay

The Lowry [88] method was used to measure protein concentration and a 1.0 mg/ml bovine serum albumin was utilized as a standard.

8. Preparation of rabbit anti-mouse LD-X antiserum

Two milligrams mouse LD-X was dissolved in 1.0 ml saline and suspended in 2.0 ml Freund's complete adjuvant. One milliliter was injected intramuscularly in the hind and front limbs at several sites in two New Zealand albino rabbits each. Two booster injections of 1.0 mg LD-X/ml saline suspended in 0.5 ml Freund's incomplete adjuvant were given at 2 and 4 weeks after the initial injection. Blood was collected from the marginal ear veins at 10 to 45 days after the last antigen injection. The serum was collected and stored at -70°C. An aliquot of each serum was titrated by an enzyme immunoassay (EIA) using microwells that had been coated with 1.0 ng LD-X per well. Two hundred microliter aliquots of serial dilutions of each antiserum were added to each antigen coated microwell and incubated at room temperature for one hour and then rinsed with phosphate buffered saline (PBS). This was followed by the addition of 200 µl of anti-rabbit IgG-horse radish peroxidase conjugate, incubation at room temperature for 45 minutes and rinsing again with PBS [100]. The assay

was continued by the addition of 100 µl of 0.057% (w/v) urea-hydrogen peroxide in a buffer containing 0.1 M citric acid and 0.1 M sodium phosphate, pH 5.0, and 100 µl of chromagen solution containing 0.02% (w/v) tetramethylbenzidine, 1% (v/v) DMSO, 10% (v/v) glycerol, and 40% (v/v) methanol. The assay was incubated at room temperature for 15 minutes and terminated with the addition of 100 µl 2.0 N sulfuric acid to each well.

9. Isolation of the gamma globulin fraction from rabbit anti-mouse LD-X antiserum

The gamma immunoglobulin (γ-Ig) fraction was isolated from the rabbit anti-mouse LD-X antisera by DEAE-cellulose column chromatography [89]. The resin was prepared by suspending 50 g of DEAE-cellulose in 1.0 L of 0.1 N HCl and stirring for 10 minutes at room temperature. It was then filtered in a glass fritted funnel and washed with approximately 1.0 L deionized water, 1.0 L of 0.1 N NaOH and another 1.0 L deionized water. The DEAE-cellulose resin was resuspended in 5 mM sodium phosphate buffer, pH 6.5, packed in a 0.5 x 20 cm column and equilibrated with this buffer.

The anti-mouse LD-X serum was diluted 10 fold in deionized water and dialyzed against the 5 mM sodium phosphate buffer, pH 6.5, until equilibrated with this buffer. The diluted antiserum was then applied onto the pre-equilibrated DEAE-cellulose column. The γ-Ig fraction was eluted with the 5 mM sodium phosphate buffer, pH 6.5, and the peak fraction collected by monitoring absorbance at 280 nm. The γ-Ig fraction was then concentrated to 1 ml with an Amicon ultrafiltration system equipped with a PM-10 membrane. To assess the purity, this fraction was subjected to electrophoresis on cellulose acetate membranes in 0.05 M barbital buffer, pH 8.6, using a Beckman microzone electro-phoresis system, at constant voltage of 250 volts for 20 minutes. The membrane was placed in a 0.2% Ponceau-S, 3% trichloroacetic acid and 3% sulfosalicylic acid fixative dye solution for 8 minutes; then rinsed

in 5% glacial acetic acid, dehydrated in absolute ethanol, cleared in 30% cyclo-hexanone/denatured alcohol for one minute and dried in an oven preheated to 100°C.

10. Tryptic digestion of proteins

Two methods were used to digest the proteins with trypsin, a traditional method [68, 69, 72] and the Basu and Modak method [92]. The traditional method involved digestion with trypsin after partial reduction and alkylation of the protein, and the addition of 2 M urea. Five to 10 mg protein (LD-X , LD-B_4, or BSA) was dissolved in 0.15 M Tris-HCl buffer pH 8.0, and was partially reduced by adding 0.01 M dithiothreitol and sitting at room temperature for two hours. Alkylation was accomplished by adding 0.04 M crystalline iodoacetamide and allowing the mixture to stand in the dark at 21°C for fifteen minutes. This mixture was dialyzed against deionized water at 4°C for 24 to 36 hours and then lyophilized or adjusted to pH 8.3 with 0.10 M ammonium bicarbonate buffer. The lyophilized alkylated protein was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.3 to yield 2 to 5 mg/ml. Urea was added to yield 2 M urea and the mixture was incubated at 37°C for 30 minutes. A volume of a 0.5 mg/ml TPCK trypsin (12,000 units/mg) in 0.01 N HCl was added to yield a 1/50 to 1/100 of trypsin to the alkylated protein, and the mixture was incubated at 37°C for one hour. This step was repeated three times over four hours span; after which, the mixture remained at 37°C for an additional 2 to 18 hours. The mixture was placed in a boiling bath for approximately one minute to stop the trypsin digestion, cooled to room temperature and centrifugated in an Eppendorf microfuge. Five hundred microliters of the digested peptide were applied to a Pharmacia Fast Desalting column (FPLC) HR 10/10 Sephadex G-25 that had been equilibrated with 0.1 M ammonium bicarbonate buffer, pH 8.3. The eluted volumes were monitored at 280 nm by an ISCO UV detector. Peak fractions were collected for UV scan, protein assay and HPLC analysis. These fractions were stored at -70°C until futher use.

In the Basu and Modak method of tryptic digestion 500 μg protein was dissolved in PBS. Two hundred micro-liters of cold 10% trichloroacetic acid (TCA) were added and the solution was mixed well and centrifuged in an Eppendorf microfuge for 10 minutes. The precipitate was then washed three times with cold 5% TCA and the TCA was removed by washing with ether three times. The remaining ether was removed by dessication under vacuum and the precipitate was resuspended in 0.1 M ammonium bicarbonate buffer, pH 8.3. TCPK trypsin was added (50 protein: 1 trypsin) and the mixture was incubated at 37°C for two hours. The same trypsin addition was repeated and incubation continued at 37°C overnight. Aliquots of this mixture were then used for UV scan, protein measurement or HPLC analysis.

11. Reverse phase high performance liquid chromatography

All reverse phase high performance liquid chromatography (RP-HPLC) analyses [72, 78, 90-94] were performed with a Varian system which was equipped with dual pumps, a mixer, a variable wavelength detector, an integrator, and a Valco column injector. A Whatman 4.6 mm x 250 mm Partisil 10 octyldodecyl silanyl (ODS-3) C_{18} analytical column for reverse phase HPLC served as the stationary phase. The mobile phase solvents utilized in most separations were Solvent A which contained 10% acetonitrile, 0.075% tri-flouroacetic acid (TFA) and 90% milli-Q water; while Solvent B contained 90% acetonitrile, 0.070% TFA and 10% milli-Q deionized water. The pH of both solvents was maintained at between 2.1 and 2.2. A peptide standard composed of 0.125 mg glycine-tyrosine and 0.5 mg each of valine-tyrosine-valine, methionine-enkaphlin, leucine-enkephalin and angiotensin II was applied to the column at least every forty-eight hours between sample runs and column equilibration. All samples and peptide standards were diluted or dissolved in Solvent A before being injected in 10 to 100 μl volumes onto the column. The HPLC peptide standard was reconsti-

tuted to yield 1 μg/ml and 10 μl was injected onto the column, but the yield of trypsin digested peptides required 50-100 μl injection volumes. After equilibrating the column with Solvent A, samples or standards were separated by linear gradient of Solvent B from 0 to 70%. HPLC separations were generally run at a flow rate of 0.5 ml/minute for 70 minutes or at a flow rate of 1.0 ml/minutes for 45 minutes. Peaks were monitored with a UV detector at 215 nm. Peak fractions were collected manually, concentrated by evaporation and/or frozen at -70°C.

12. Isolation of testicular cells

Adult (12-16 weeks) male mice of the CD-1 strain that were raised in the Old Dominion University Animal Laboratory and maintained on normal mouse chow and water ad libitum were used throughout this project. Three to four mice were euthanized by cervical dislocation and were thoroughly cleaned with 70% ethanol. After an incision into the peritoneal cavity, the testes were excised, placed in cold sterile PBS, defatted, and decapsulated. The decapsulated testes were placed in an 85 μm sieve mesh cup and with a sterile Pasteur pipette forcefully washed with Eagle's Minimal Essential Medium (MEM), pH 7.2, to dislodge interstitial cells [95, 96]. The supernatant was designated as fraction 1 (F1). The testes were then minced with fine scissors and again washed forcefully to dislodge spermatogenic cells [96, 97]. The filtrate, designated as fraction 2 (F2), was centrifuged at 500 rpm (200 x g) for 10 minutes and washed twice with MEM. The remaining tissue was treated with collagenase by adding 20 ml of collagenase, 1.0 mg/ml in MEM, at 32°C for 60 minutes with intermittent shaking [98, 99]. The tubular tissue was allowed to settle, the supernatant was collected and the remaining tissue was treated a second time with collagenase. Both supernatants collected after the collagenase treatment were pooled, centrifuged and washed three times with MEM. The cells retrieved were designated as fraction 3 (F3). The seminiferous cords remaining

after collagenase treatment were then treated with trypsin/DNase [96]. Twenty milliliters of MEM containing 1 mg/ml trypsin and 1 μg/ml DNase were added to the cords, and intermittently shaken at 32°C for 15 minutes. This suspension was then repeatedly and gently pipetted within approximately 3 minutes, centrifuged at 500 rpm (200 x g), and the supernatant decanted. The cells were resuspended in MEM, filtered through the 85 μm sieve mesh or a Nylon mesh, and washed thrice. This fraction was designated as fraction 4 (F4). Cell counts were performed on an aliquot of all cell fractions (F2, F3, F4) using the Neubauer hemocytometer, and viability was ascertained by trypan blue dye exclusion. The cells were then seeded 0.5 to 1 x 10^6 cells per 2 ml MEM with 2% fetal calf serum in 24-well dish multidish and cultivated at 32°C in 5% CO_2 for 24 to 36 hours.

13. Gossypol binding studies

a. Peptides

Native proteins, tryptic digested proteins and HPLC peak fractions were diluted in phosphate buffered saline, pH 7.4. Three protocols were employed for the binding assays. In one group of experiments varying concentrations (0 to 15 μM) of unlabeled gossypol and constant amounts of ^{14}C-gossypol were added to the protein or peptide solution. In a second group of experiments varying concentrations of ^{14}C-gossypol were added to the peptide or protein. In the third protocol constant amounts of unlabeled gossypol were mixed with the protein and increasing amounts of ^{14}C-gossypol were added. All mixtures were allowed to stand at room temperature for 0 to 60 minutes. After incubation these mixtures were stored at -70° C or aliquots were diluted in Solvent A and applied to an equilibrated HPLC column. Undiluted aliquots were used for protein determination, absorption scan (190 nm to 450 nm), and measurement of ^{14}C-radioactivity for direct binding and LD-X binding by immunoprecipitation when ^{14}C-gossypol was incubated with peptide/proteins. The bound radioactivity was

calculated by subtracting the ^{14}C activity on the filter or immunoprecipitate from the total ^{14}C-gossypol added. The K_D was calculated using the equation K_D = [protein] [total ^{14}C]/[^{14}C bound] with data obtained from experiments using the third protocol.

b. Testicular cells

Testicular cell suspensions F2, F3 and F4 were treated with ^{14}C-gossypol after a 24 hour incubation at 32°C in Eagle's MEM with 2% Fetal calf serum. Varying concentrations of ^{14}C-gossypol (0 to 150 nM) were added to the cell medium in 1 to 10 μl and incubated from 30 to 60 minutes. At the conclusion of incubation the cells were chilled 30 to 60 minutes at 4°C. The media was removed from the dish, and the cells scraped from the wells; 2 ml cold PBS was placed in each well and the media/cell suspension then centrifuged at 1200 rpm (500 x g) for 10-15 minutes. The cells were washed twice with 3.0 ml cold PBS. One milliliter distilled water was added to the remaining cell pellet, mixed on vortex mixer and allowed to stand at room temperature 30 minutes. This mixture was frozen at -70°C, followed by quick thawing and centrifuged at 3,000 rpm (1,000 g) for 25 minutes. The supernatant was collected as the lysate. The remaining membranous pellet was solubilized by adding 1 ml 1% triton X-100 in PBS, pH 7.4, thoroughly mixing, and sitting at room temperature for 30 minutes. Aliquots (100 to 200 μl) of the lysate and the solubilized pellet were placed in 6.0 ml scintillation fluid and counted for ^{14}C radioactivity to assess direct ^{14}C-gossypol binding to the lysate and pellet. A 200 μl aliquot of each was also retrieved for immunoprecipitation with rabbit anti-mouse LD-X γ-Ig to assess ^{14}C-gossypol binding to LD-X in the lysate and pellet.

14. LD-X immunoprecipitation assay

A 200 μl aliquot of the testicular cell lysate and solubilized membranous pellet or peptide solutions were placed in 4.0 ml plastic tubes. This was followed by the addition of

200 μl diluted (1/200) rabbit anti-mouse LD-X γ-Ig, vortex mixing and incubation at room temperature 45 to 60 minutes. Two hundred microliters of diluted (1/20) goat anti-rabbit γ-Ig were added. The mixture was thoroughly mixed and again incubated 45 to 60 minutes at room temperature. The precipitate was separated by centrifugation at 3000 rpm for 25 minutes. The supernatant was discarded and the ^{14}C radioactivity was counted only in the dissolved precipitate when testicular cell lysate and pellet were immunoprecipitated; while glass or nitrocellulose filters were used to collect the immunoprecipitate and the ^{14}C radioactivity was counted on the filters when peptide solutions were used.

15. Amino acid analysis

The quantitative amino acid analysis was performed in Dr. Bruce Anderson's laboratory at Virginia Polytechnical Institute and University. The Waters PicoTag amino acid analysis method [101] was used. This method requires precolumn derivatization with phenylisothiocyanide and the separation of phenylisothiocyanate amino acid derivatives by reverse phase HPLC (RP-HPLC). Peak peptide fractions of interest that had been collected from the HPLC separation of LD tryptic digest were transferred to Picotag sample tubes, dried under vacuum, and stored frozen if hydrolysis could not be continued. The concentrated samples were hydrolyzed by acid vapor phase hydrolysis. The samples were hydrolyzed in 6 N HCl/ phenol at 105°C in an oven for 24 hours, then allowed to cool. The cooled samples were derivatized by adding 10 μl of a redry solution and 20 μl of PITC derivatization solution and standing for 10 minutes. The dried derivatized samples were diluted to 100 μl with Picotag diluent, placed in sample vial on a sample tray. An automatic injector injected 4 μl sample aliquots onto a 3.9mm x 300 mm C_{18} column with 4 μm particle size and 3.0 ml void volume. The mobile phase consisted of Water's eluent A and eluent B. Eluent A contained 6% acetonitrile in 0.05% triethylamine (TEA) which contained a proprietary concentration of sodium

acetate trihydrate and 0.2 μg calcium disodium ethylene diamine tetraacetic acid (EDTA). Eluent B contained 60% acetonitrile in Milli-Q water with 0.2 μg EDTA. A linear gradient from 100% Eluent A to 54% within 21 minutes was run.

16. Statistical Analysis

Most experiments were repeated at least twice and each test sample was performed in duplicate. The standard error of the mean was calculated and data were analyzed for statistical significance by the student t test.

Chapter III

RESULTS

A. Purification of Mouse LD-X

Four preparations of mouse LD-X were purified. The average yield from 200 to 300 mouse testicles was 35 mg with a purity of approximately 95% as ascertained by SDS-PAGE. The range of specific activity was 49-70 U/mg protein and the purification averaged a 35% yield after heating the crude extract. Table 3 shows the specific activity and yield of one of these preparations. The agarose gel electrophoresis of each preparation showed a single band of LD activity at the expected mobility for the LD-X isozyme (Figure 11) and the SDS-PAGE showed one prominent protein band at the approximate molecular weight of 35,000 daltons, the molecular weight of the LD-X subunit (Figure 12). HPLC analysis of the purified mouse LD-X also showed one prominent peak for each preparation.

B. Rabbit Anti-mouse LD-X

The polyclonal rabbit anti-mouse LD-X antisera obtained from two rabbits after 3 injections of antigen had a titer of 8,000 from rabbit No.1 and a titer of 16,000 from rabbit No.2, when titrated using a solid phase enzyme immunoassay wherein microwells were each coated with 1.0 ng mouse LD-X. The DEAE-cellulose isolated γ-Ig from rabbit No. 2 was used in all subsequent immunoprecipitation assays in LD-X binding experiments. This fraction had a protein concentration of 13.6 to 15 mg/ml and a dilution of 1/200 proved to be suitable to obtain optimal precipitation with a 1/20 dilution of the Pel-Freez anti-rabbit IgG.

Table 3

Purification of Mouse LD-X

Sample	*Enzyme Activity U/ml*	*Protein Concentration mg/ml*	*Specific Activity U/mg*	*Fold Enrichment*	*Yield %*
Crude Extract	*288*	*44*	*6.54*	*1*	*100*
Heat treatment	*174*	*14.2*	*12.3*	*2*	*60*
Affinity chromatography	*98.7*	*1.39*	*74*	*11*	*34*

Mouse LD-X Preparation # 3

Figure 12

Agarose gel electrophoresis of mouse lactate dehydrogenase isozyme X. *Serum (A) samples were electrophoresed under the same conditions as the mouse LD-X (B). The LD-X isozyme is a cathodic fraction during electrophoresis in barbital buffer pH 8.6 and is located between isoenzyme LD-M_3H (4) and LD-M_4 when compared to the serum LD isoenzymes.*

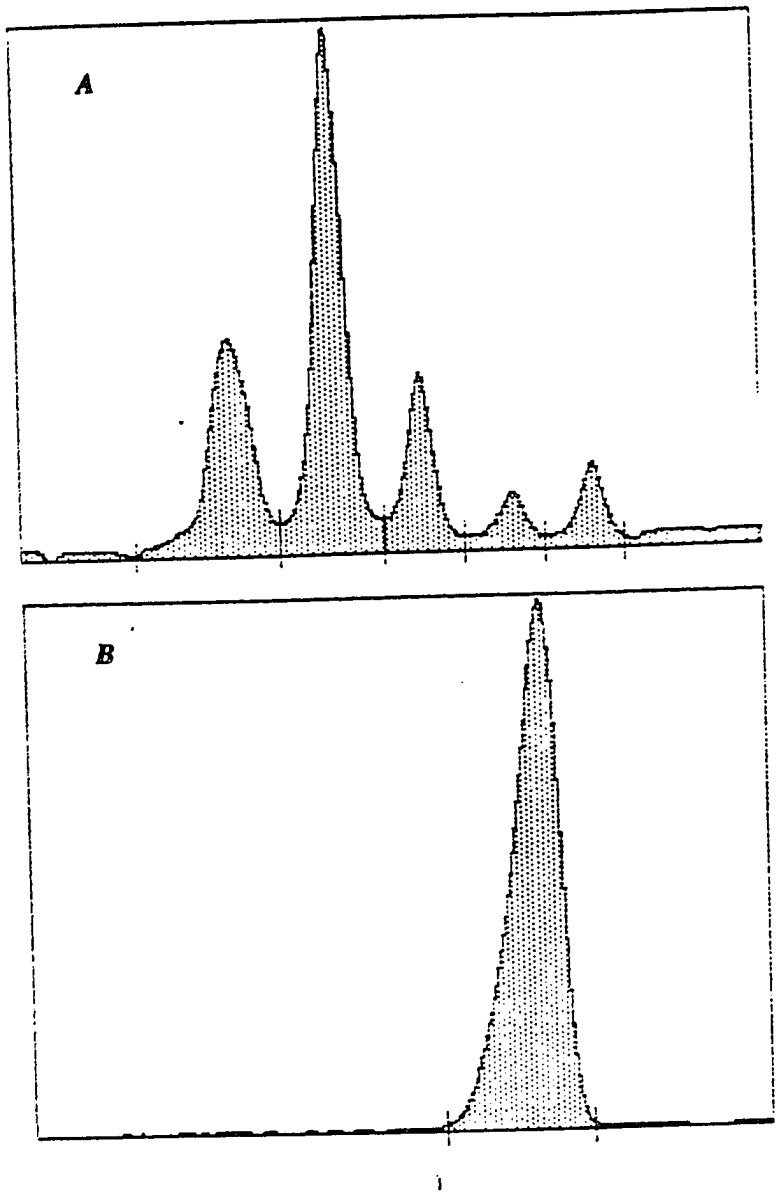
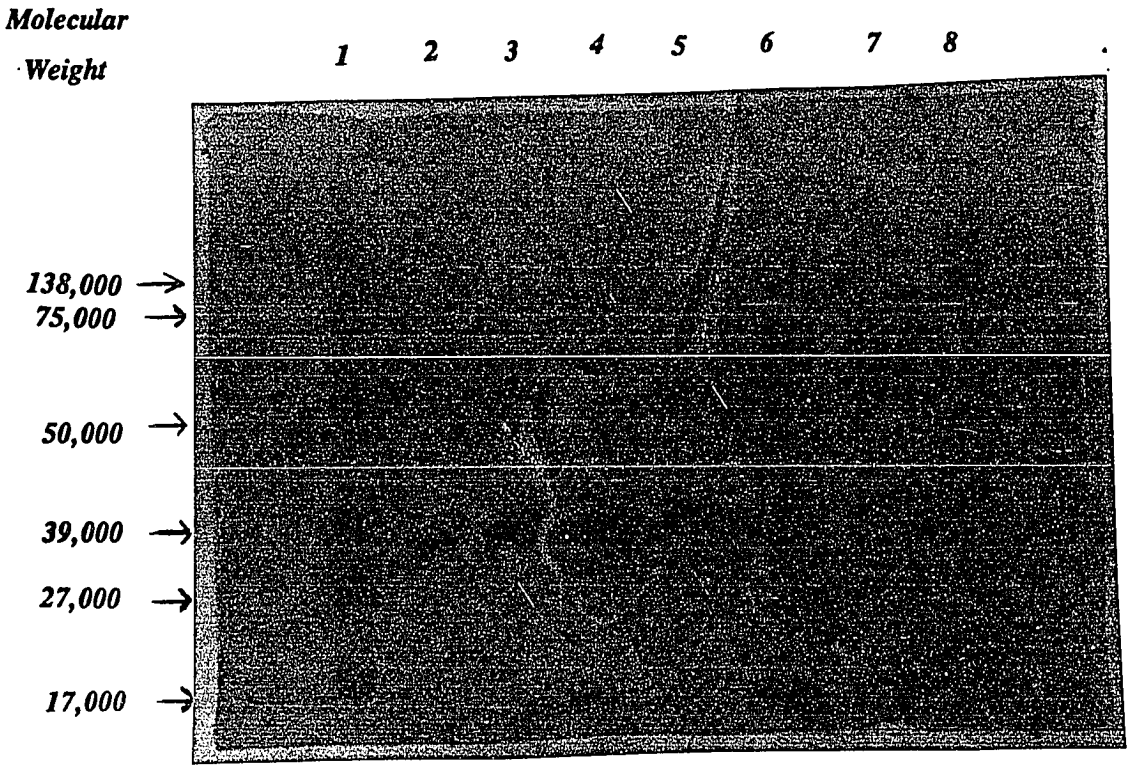


Figure 13

SDS-PAGE of purified mouse LD-X. *Lane 1 and 8 are molecular weight markers. Lane 2, 10 μg LD-X prep 4; lane 3, 10 μg LD-X prep 3; lane 4, 20 μg prep 4; lane 5, 20 μg prep 3; lane 56 μg crude extract before affinity column; lane 7, 44 μg crude extract after heating. LD-X prep 3 & 4 migrated at the approximate molecular weight of its subunit of 35,000 daltons. The separation gel contained 10% acrylamide.*

LD-X SDS-PAGE



C. Tryptic digestion of lactate dehydrogenase

Bovine serum albumin was first used to determine the extent of tryptic digestion by the traditional method [68, 69, 72] which required reduction and carboxymethylation and the Basu-Modak method [89] in which the washed TCA protein precipitate was digested with trypsin [89]. The traditional method proved to be the method of choice although it required more time even when using the FPLC column for desalting after trypsin digestion; because the Basu-Modak method, though less time-consuming, often resulted in the loss of the protein when removing the trichloroacetic acid by ether or showed difficulty in completely removing the ether itself. Peptide fractions were eluted from the Sephadex G-25 FPLC column in 0.1 M ammonium bicarbonate buffer, pH 8.3, and the absorbance of peptide fractions were monitored at 280 nm. Mouse LD-X or chicken LD-M_4, with and without incubation with unlabelled gossypol or ^{14}C-gossypol, were then digested. Figure 14 shows a spectroscopic UV-visible scan of trypsin digested LD-X peak fraction eluted from the FPLC desalting column. Similar elution patterns were also observed for LD-M_4 and BSA tryptic digested preparations. Since the Basu-Modak tryptic digest did not require desalting, only the spectroscopic scans were run on these fragmented peptides. The differences in the UV-vis spectral scan between 190 to 400 nm for LD-M_4 and LD-X with and without the addition gossypol were unremarkable. Both spectra showed increased absorbance in the 240 to 320 nm region when unlabeled gossypol (0.1 to 6 nM) was incubated with these proteins before trypsin digestion . This was due to the presence of gossypol and gossypol decomposition, because gossypol decomposition products show absorbance peaks at 254 nm [84] and the maximum peak absorbance for gossypol is at 272 nm wavelength. Figures 15 and Figure 16 show such scans of mouse LD-X before and after the addition of various amount of unlabeled gossypol.

Figure 14

A spectroscopic UV-vis scan of LD-X tryptic digest after desalting. *Eluate scanned between 190 nm to 400 nm wavelength.*

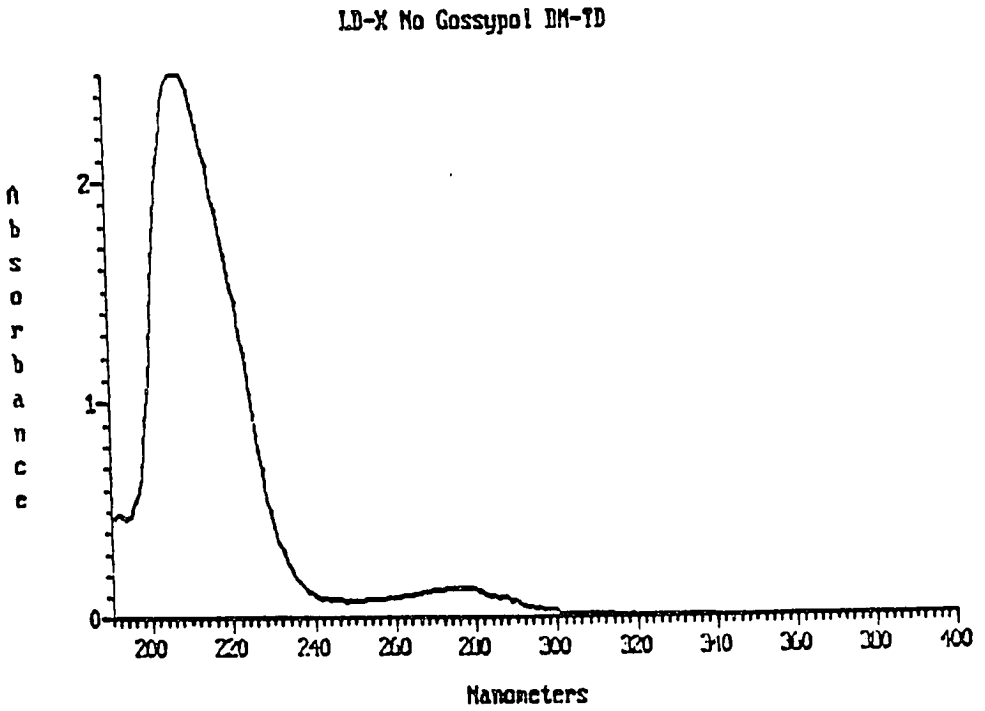


Figure 15

Spectroscopic scan of LD-X tryptic digest. *The eluates from the FPLC were scanned between 190 nm to 400 nm wavelengths before and after the addition of ^{14}C-gossypol or unlabelled gossypol. A second peak appears as the concentration of gossypol increases and represent peptide bond or unbound gossypol. A similar pattern was observed with LD-M_4 tryptic digest.*

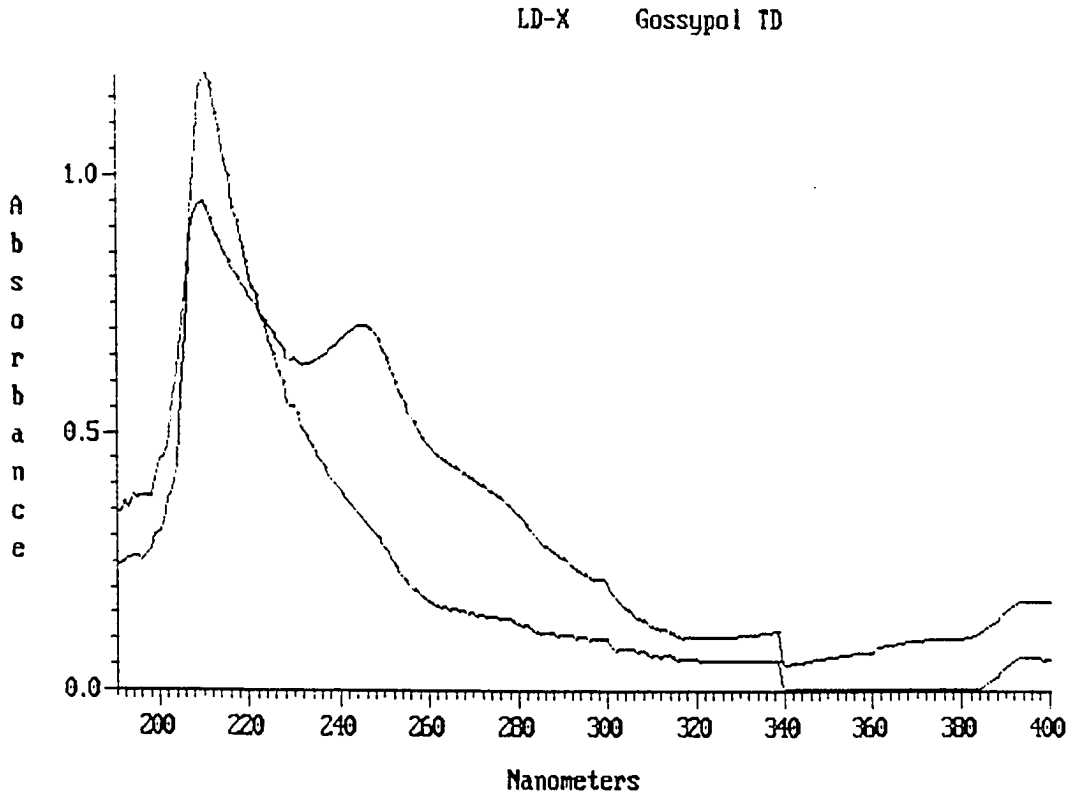
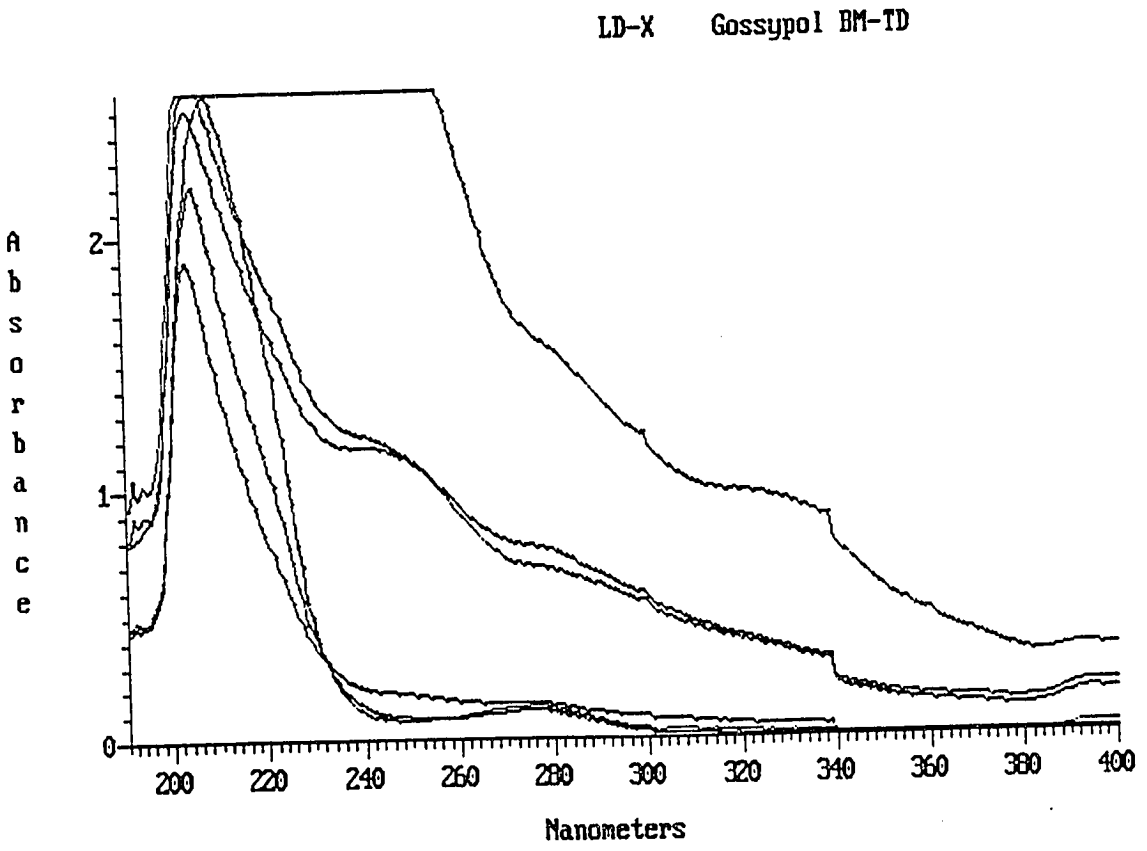


Figure 16

Spectroscopic scan of gossypol treated LD-X tryptic digest. *The isozyme was incubated with 0.1 to 6 nM unlabelled gossypol before trypsin digestion.*



HPLC analyses were performed using these trypsin digested peptides immediately following digestion or after being frozen and stored at 70°C. The binding of ^{14}C-gossypol to individual tryptic peptide fragments was run on one LD-X separation to determine if there was preferential binding to gossypol by any of the fragments.

D. HPLC analysis of LD-X and LD-M_4 tryptic digests

After extensive studies in search of a solvent system and the column conditions that would be most suitable for the analysis of peptides on HPLC, it was determined that a linear gradient elution with 5% to 65% solvent B on the C_{18} reverse phase column at a flow rate of 1.0 ml/min for 1 hour or 0.5 ml/min for 2 hours were the best. The injection of 10 uL of Sigma HPLC peptide standard (1.25 mg/2 ml Solvent A) gave a chromatogram with five peaks within 30 minutes of elution under these conditions at a flow rate of 1.0 ml/min. These represented the five peptides and the increasing retention times of angiotensin II, methionine enkephlin, leucine enkephlin, valine-tyrosine-valine and glycine-valine respectively (Figure 17). For calibration, this standard was routinely injected after equilibrating the column prior to HPLC separations of peptide samples.

Samples of trypsin digested peptides obtained from untreated BSA, LD-X or LD-M_4, and after incubation of LD-X or LD-M_4 with unlabelled gossypol or ^{14}C-gossypol were first dissolved in Solvent A and injected onto the previously equilibrated C_{18} column. These samples were then separated under the same conditions as the peptide standard, but were injected in volumes of 50 to 100 µl because of the extremely low concentration of peptide fragments obtained after trypsin digestion of 5 to 10 mg native proteins. Peak fractions were collected for ^{14}C-gossypol binding studies, UV/visible spectroscopic scan and/or amino acid analysis.

The HPLC chromatogram of the tryptic digest of native LD-X showed separations

Figure 17

HPLC chromatogram of peptide standards. *Sigma HPLC peptide standard contained angiotensin II, methionine enkephlin, leucine enkephlin, valine-tyrosine-valine and glycine-valine. Gradient 90 water:10 acetonitrile: 0.075 TFA to 40 water: 60 acetonitrile:0.075 over 60 minutes. 215nm wavelength. Attenuation = 32. Flow rate= 1ml/min. Chart speed = 0.25 cm/min.*

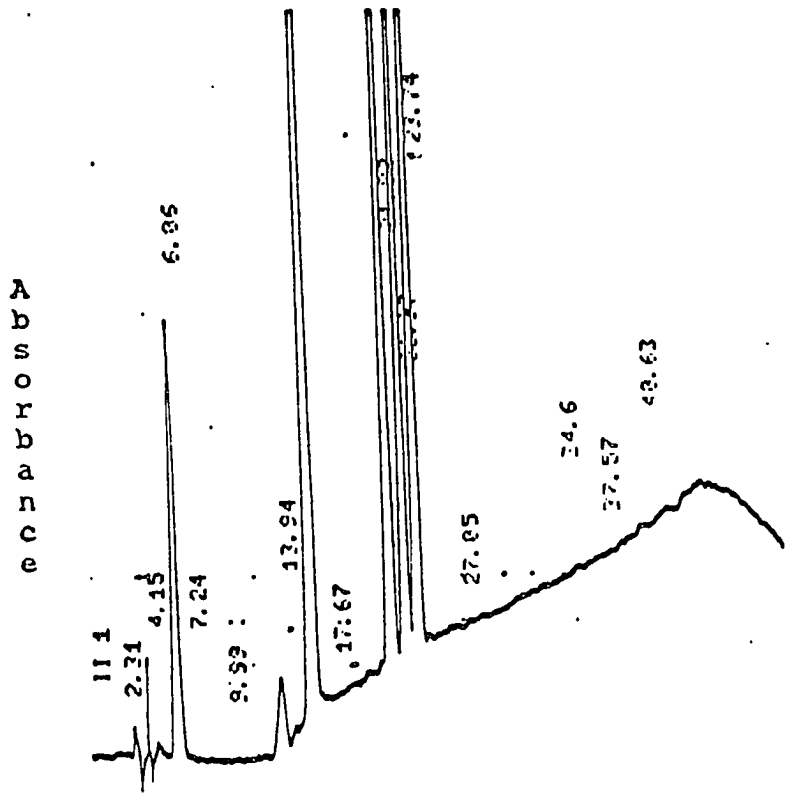
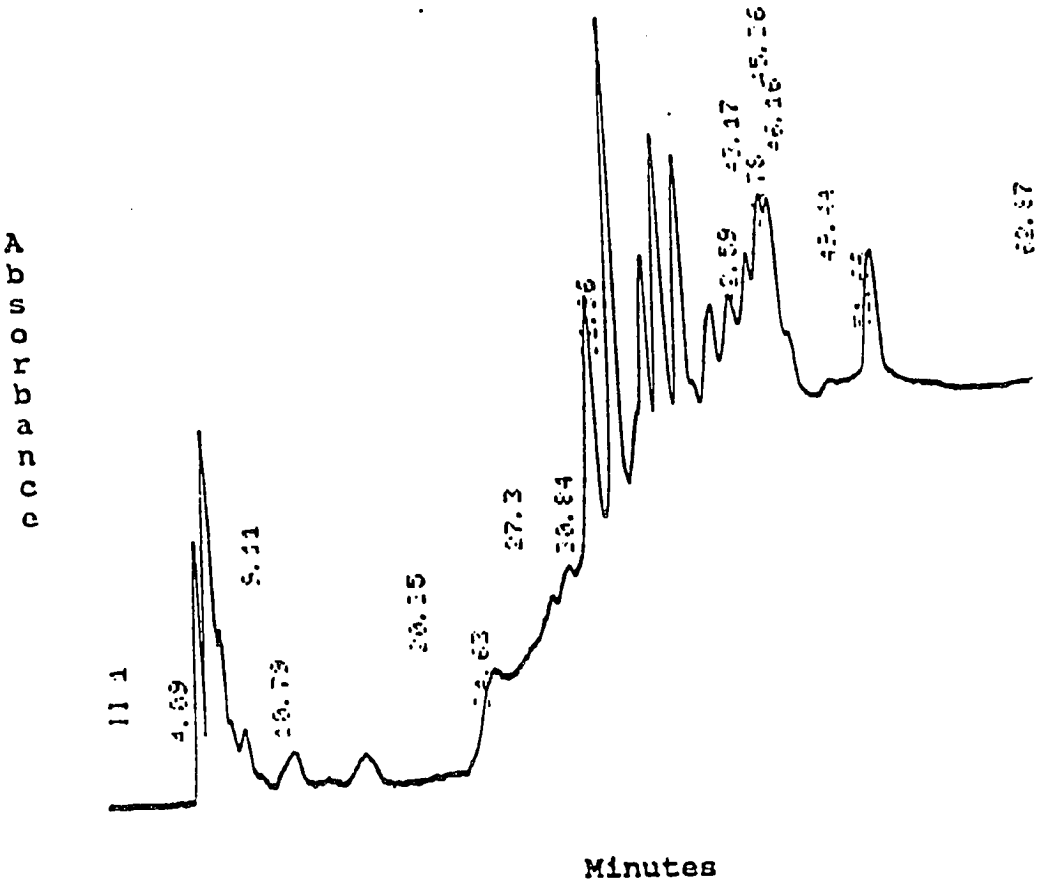


Figure 18

The HPLC chromatogram of tryptic digest of mouse LD-X without incubation with gossypol. *Gradient 90 water:10 acetonitrile: 0.075 TFA to 40 water: 60 acetonitrile: 0.075 over 60 minutes. 215nm wavelength. Attenuation = 32. Flow rate= 1ml/min. Chart speed = 0.25 cm/min.*



with 19 peaks out of an expected twenty-four peptide fragment peaks (Figure 18). One group of experiments involved the preincubation of LD-X and LD-M$_4$ with unlabeled gossypol prior to the tryptic digestion. LD-X was preincubated with 0.1 μM to 6μM gossypol and LD-M$_4$ was preincubated with 0.7 μM to 39 μM for 30 to 60 minutes at room temperature before trypsin digestion. The HPLC chromatograms obtained after digestion showed separation patterns that were markedly altered when compared to the digests of these enzymes without gossypol preincubation. Figure 19 shows HPLC chromatograms of LD-X peptide fragments retrieved with and without incubation with 0.01 μM (A) to 0.25 μM (B) unlabelled gossypol. When unlabelled gossypol was incubated with LD-X before trypsin digestion there was an increase in the number of peaks and a shift in other peaks. This was most apparent between peaks 8 and 18 (A & B) for LD-X. In the absence of gossypol the most prominent peaks were peaks 8 (Rt=38), 11 (Rt=42.74), 12 (Rt=44) and 19 (Rt=52). In the presence of gossypol additional peaks were observed and there were 9 prominent peaks. Peak heights for peaks 9, 10, and 11 were increased (B); and there were three additional peaks between peak 7 and 8, two peaks between peaks 12 and 13, and three peaks between peaks 6 and 7. As the unlabeled gossypol concentration increased from 0.01 μM to 0.25 μM the retention times increased only slightly. In one of these experiments where the urea step was inadvertently omitted 25 peaks were obtained at 0.01 μM with 19 major peaks. As the gossypol concentration increased to 0.1 μM there were still 25 peaks with at least 7 peaks that were altered and retentions time were only slightly increased. This demonstrates gossypol's capacity as a reducing agent and it's denaturing properties. Gossypol likely acts to unfold the enzyme by competing with bonds that are critical in maintaining the enzyme's tertiary and quaternary structure. Peak alterations, though dissimilar, were also observed with the gossypol treated

Figure 19

The HPLC chromatogram of tryptic digest of mouse LD-X after incubation with unlabelled gossypol. *The tryptic digest was treated with (A) 0.01μM and (B) 0.1 μM gossypol. Gradient 90 water:10 acetonitrile: 0.075 TFA to 40 water: 60 acetonitrile:0.075 over 60 minutes. 215nm wavelength. Attenuation = 32. Flow rate= 1ml/min. Chart speed = 0.25 cm/min.*

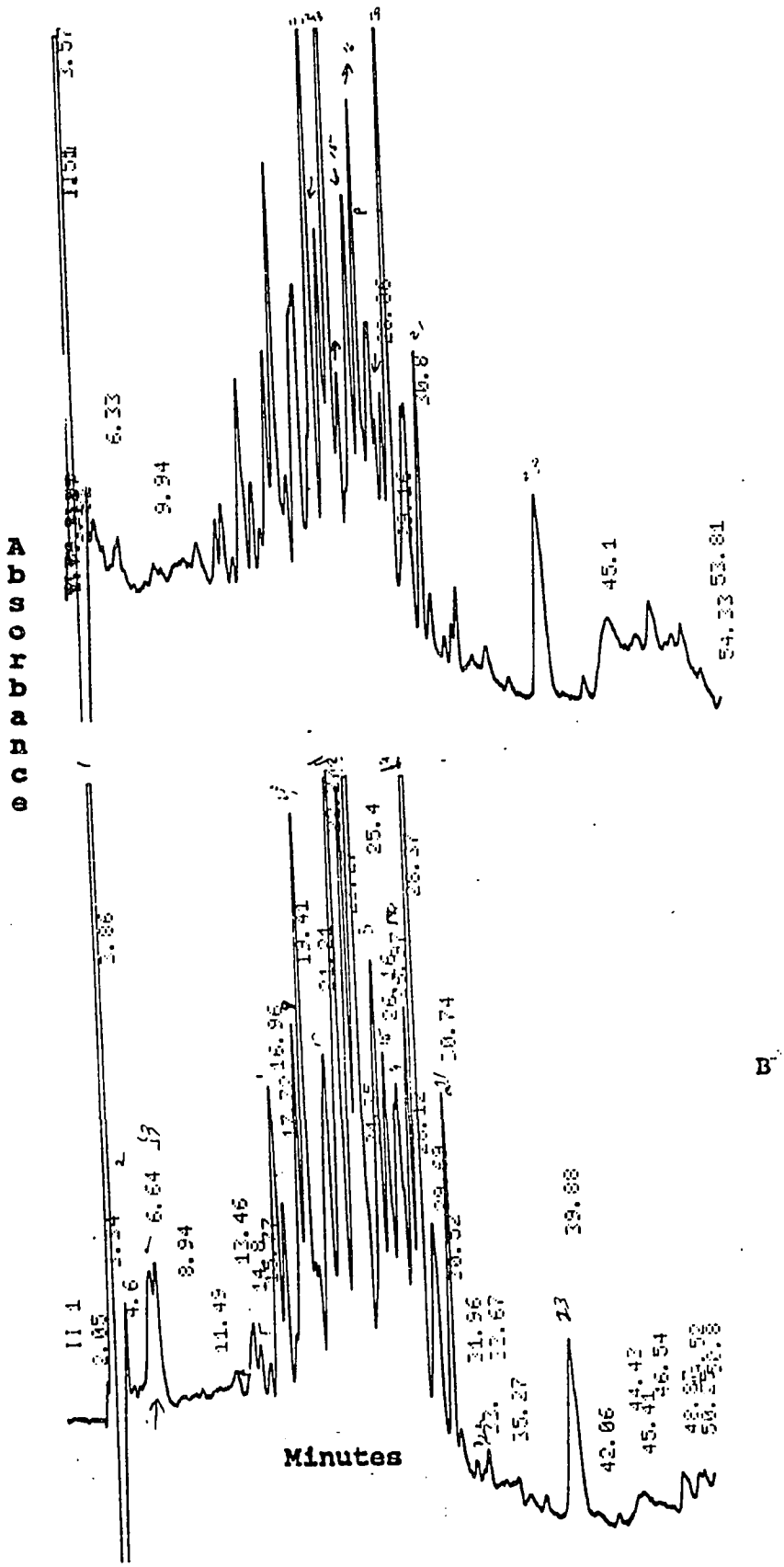


Figure 20

HPLC of LD-M_4 tryptic digest without gossypol. *Gradient 90 water:10 acetonitrile: 0.075 TFA to 40 water: 60 acetonitrile:0.075 over 60 minutes. 215nm wavelength. Attenuation = 32. Flow rate= 1ml/min. Chart speed = 0.1 cm/min.*

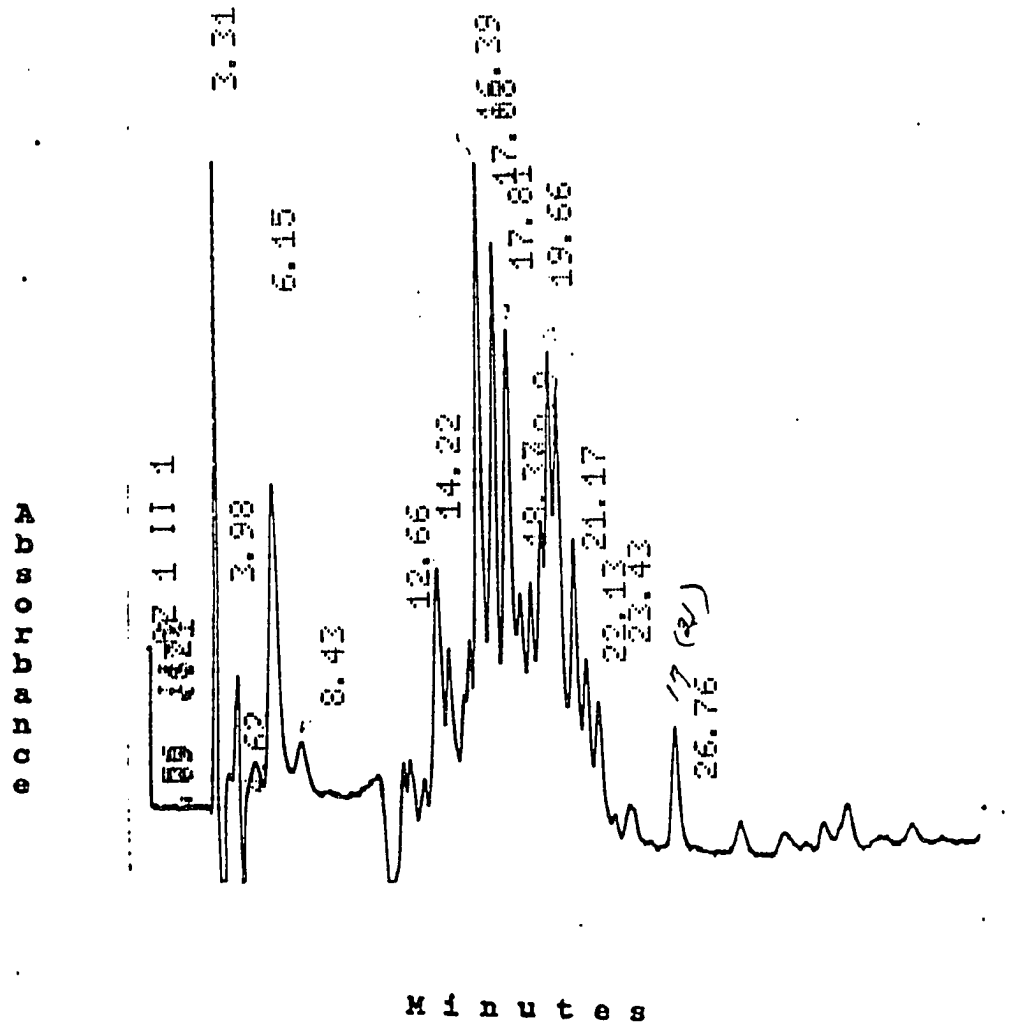
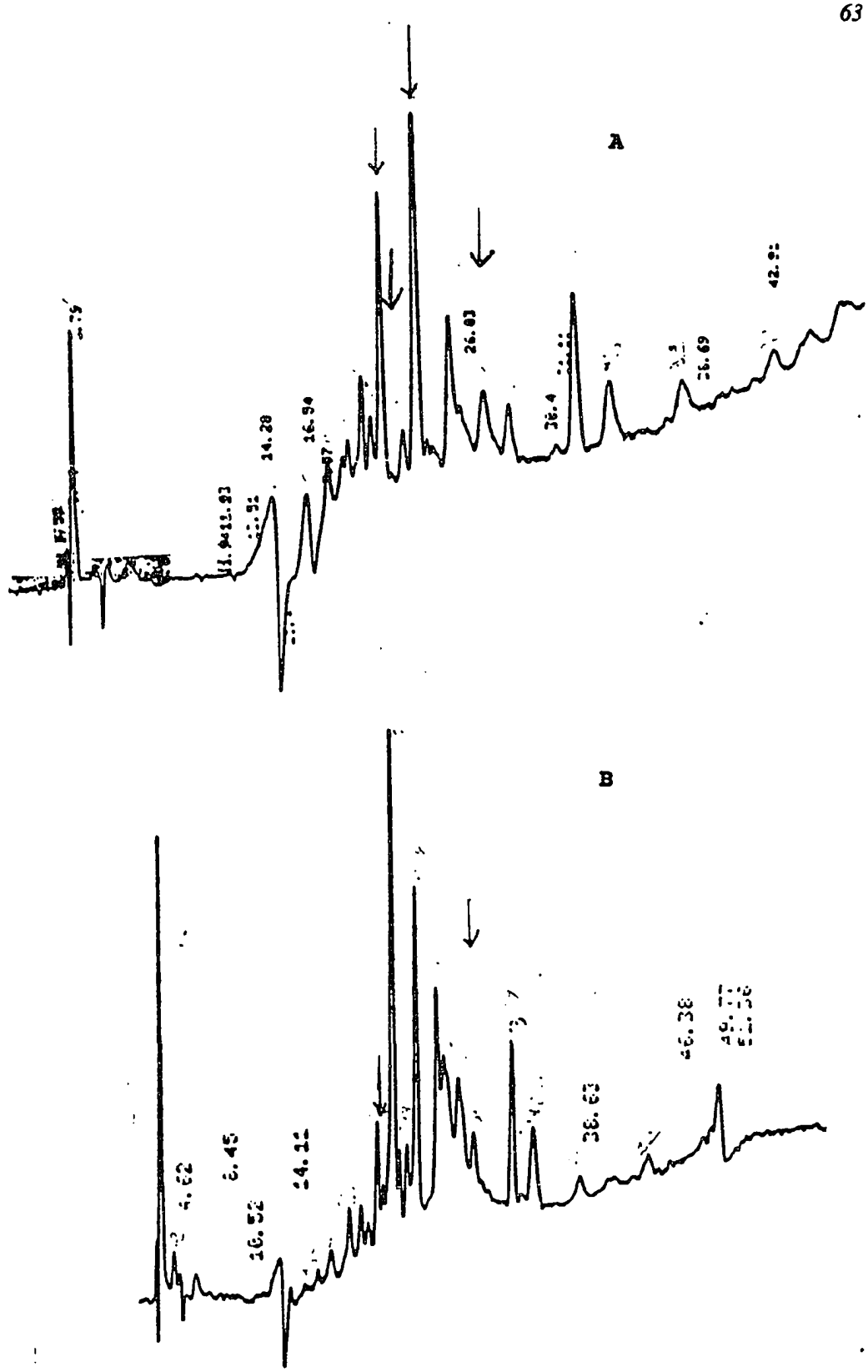


Figure 21

An HPLC chromatogram of the LD-M_4 tryptic digest after the addition of 0.7 μM (A) and 39 μM (B) unlabelled gossypol. *Peaks 11, 13, and 14 were altered. Gradient 90 water:10 acetonitrile: 0.075 TFA to 40 water: 60 acetonitrile:0.075 over 60 minutes. 215nm wavelength. Attenuation = 32. Flow rate= 1ml/min. Chart speed = 0.1 cm/min.*



LD-M_4. Figure 20 shows a chromatogram of LD-M_4 without gossypol treatment and Figure 21 shows LD-M_4 peptide fragments treated with 0.7 μM and 39 μM unlabelled gossypol. On these chromatograms the same number of peaks were observed but there was some shifting of peaks (Figure 21). Most obvious was the shift in peaks 11 and 14, and the severe decrease or loss of peak 13 when compared to the untreated enzyme. Between 0.7 μM and 39 μM gossypol the height of peak 11 decreased, peak 13 disappeared, and peak 14 height increased slightly.

These alterations were observed on two or more successive HPLC separations with column washing between each separation. Although it is likely that these peak changes may be simply due to the gossypol itself, such peak alterations seem to suggest conformational disturbance by the addition of gossypol.

E. Amino acid analysis of altered HPLC LD-X peptide fragments after treatment with unlabelled gossypol

A quantitative amino acid analysis was performed on HPLC fractions that appeared to be altered after incubation with unlabelled gossypol before the HPLC separation. Peak fractions were obtained from gossypol treated LD-X and LD-M_4 peptide fragments. The LD-X peaks were obtained from the 0.01 μM and 0.05 μM gossypol treated LD-X. The LD-M_4 peaks were obtained from the 0.7 μM and 39 μM gossypol treatment. Although there was evidence of alterations in the HPLC chromatogram after exposure to gossypol the characteristics of these peaks could not be reliably determined from the amino acid analysis results derived in this study. The relatively large amounts of arginine in the LD-M_4 0.7 μM gossypol eluates suggest that the peptide was not completely digested or that there was coelution of peptide fragments. All selected fragments contained relatively large quantities of glycine, serine and glutamate which have been reported [102] to often be contaminants in the Picotag analysis. This is due to environmental factors such as dust during the collection and drying of

the samples, and impurities in reagents. This contamination frustrated efforts to estimate the amino acid sequences of the tryptic peptide fragments because the sample quantities were very low when compared to the standards and controls. Given such, the chromatograms of the amino acid analysis offered limited information.

Amino acid analyses were run on peak numbers 9,10,11,12,13,15 and 16 from the .05 μM gossypol treated LD-X; peaks from 0.1 μM gossypol treated LD-X were numbers 8 thru 19; peak numbers 8 thru 16 for 7 μM gossypol with LD-M_4, while those obtained from 39 μM gossypol with LD-M^4 were 8 thru 19. Tables 4 - 9 show the amino acid present in each peak normalized to 100 picomole. All peaks contained low amino acid concentrations that ranged from 0.5 to 2 nM. The amino acid chromatograms obtained were unremarkable from peak to peak for the LD-X and the LD-M_4 for most peak fractions, although there were differences in the quantities of certain amino acids within simultaneous peaks at the different gossypol concentrations. There was also an amino acid peak that was not identified as a specific amino acid eluate from most of the enzyme peptide samples at a retention time from 9.18 to 9.20 minutes. This peak could be a derivative of gossypol or a gossypol-amino acid conjugate. Figure 22 shows a chromatogram of the PITC derivatized amino acid standards and control unknowns, and Figure 23 shows a typical peak sample.

Table 4

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENTS

Amino acid	Peak 8 LD-M_4		Peak 9 LD-M_4		LD-X	
	Gossypol Treatment (μM)					
	39	0.7	0.7	39	0.05	0.1
Asp/Asn	8.5	5.1	7.2	5.0	2.7	6.8
Glu/Gln	13.5	13.4	9.3	10.5	10.1	15.5
Ser	14.6	12.5	14.2	14.8	8.5	14.9
Gly	15.5	19.6	20.5	26.8	17.5	19.5
His	1.2	-	3.4	-	-	-
Arg	2.0	3.2	2.1	4.9	21.7	1.8
Thr	5.1	4.3	3.1	3.6	5.8	3.9
Ala	6.5	6.5	5.8	5.8	15.9	5.4
Pro	6.0	4.4	5.1	3.9	3.3	3.0
Tyr	4.6	3.1	5.1	3.6	2.7	2.4
Val	5.7	5.1	7.3	4.3	4.3	7.6
Met	.3	.3	.7	-	-	-
Cys	.5	1.0	.3	1.0	-	-
Ile	5.9	4.1	6.3	4.1	2.2	4.4
Leu	5.3	9.1	3.2	6.9	3.9	9.0
Phe	2.2	4.1	2.4	3.1	-	2.1
Lys	2.8	4.2	3.8	3.1	1.2	3.7

Values normalized to 100 picomoles

Table 5

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENTS

Amino Acid	Peak 10				Peak 11			
	LD-M_4		LD-X		LD-M_4		LD-X	
	Gossypol Treatment (μM)							
	0.7	39	0.05	0.1	0.7	39	0.05	0.1
Asp/Asn	7.3	5.5	2.5	8.0	9.9	3.9	3.0	6.6
Glu	10.3	15.0	9.6	10.3	4.6	11.3	10.0	12.7
Ser	11.4	14.2	7.0	12.3	16.1	16.7	7.3	13.0
Gly	15.1	21.4	19.4	14.3	13.0	26.7	16.1	25.1
His	1.7	.8	-	2.3	1.8	-	-	-
Arg	1.4	1.7	21.0	.8	-	2.7	28.6	3.2
Thr	4.8	3.1	3.6	4.3	3.6	1.4	8.3	13.4
Ala	5.4	6.4	15.2	4.3	5.8	6.2	12.9	5.8
Pro	5.0	3.4	3.2	4.3	7.9	3.9	3.0	2.9
Tyr	4.5	1.1	2.9	4.9	5.3	4.4	1.9	1.9
Val	9.9	6.6	4.5	10.9	6.7	5.2	2.8	-
Met	.4	.9	-	-	1.6	-	-	-
Cys	.3	3.4	-	-	3.2	-	-	-
Ile	5.2	5.3	2.6	6.5	4.3	4.2	1.7	3.7
Leu	7.2	6.1	6.7	7.9	10.0	6.6	3.4	6.8
Phe	3.2	3.1	-	3.2	2.1	3.9	-	2.5
Lys	6.4	2.2	1.8	5.6	7.7	3.3	.9	2.4

Values normalized to 100 picomoles.

Table 6

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENTS

Amino Acid	Peak 12				Peak 13			
	LD-M_4		LD-X		LD-M4		LD-X	
	Gossypol Treatment (μM)							
uM	0.7	39	0.05	0.1	0.7	39	0.05	0.1
Asp/Asn	7.1	1.8	4.8	6.1	5.1	3.4	-	5.2
Glu/Gln	5.5	8.1	16.4	8.5	4.7	11.3	6.4	8.2
Ser	16.0	11.2	12.1	11.4	17.6	19.0	11.4	6.5
Gly	16.3	26.2	25.6	18.2	19.5	36.4	27.4	17.5
His	-	-	-	1.2	1.5	-	-	.6
Arg	1.6	3.3	22.0	2.1	.6	-	14.2	4.4
Thr	3.4	4.6	-	4.9	4.6	-	5.1	5.9
Ala	6.2	8.0	19.1	6.7	4.9	9.3	13.4	8.6
Pro	5.8	8.5	-	4.5	3.5	-	3.7	3.7
Tyr	3.5	4.5	-	3.4	3.5	-	-	4.4
Val	10.9	6.0	-	8.6	7.4	5.6	9.3	7.4
Met	.7	-	-	-	.1	-	-	-
Cys	.2	2.9	-	-	.4	-	-	-
Ile	7.0	4.0	-	6.6	10.5	3.9	-	6.1
Leu	9.6	6.3	-	11.8	9.4	8.4	7.1	16.5
Phe	3.1	3.3	-	2.9	2.6	-	-	1.4
Lys	3.0	2.1	-	3.0	4.0	3.8	1.8	3.4

Values normalized to 100 picomoles.

Table 7

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENT PEAKS

AAmino acid	Peak 14				Peak 15			
	$LD\text{-}M_4$		LD-X		$LD\text{-}M_4$		LD-X	
	Gossypol Treatment (μM)							
	0.7	39	0.05	0.1	0.7	39	0.05	0.1
Asp/Asn	6.1	2.1	ND	9.7	12.6	8.2	5.1	8.4
Glu/Gln	5.6	7.8		11.8	10.4	19.5	13.3	8.2
Ser	9.9	16.0		8.7	9.2	18.1	11.8	14.9
Gly	16.0	27.6		12.7	16.0	22.1	23.7	16.8
His	-	-		1.7	.7	2.6	-	1.3
Arg	5.8	3.7		2.8	.5	5.0	11.0	.9
Thr	6.5	4.8		3.7	4.7	3.8	4.6	5.4
Ala	11.0	8.3		6.6	6.5	5.1	9.5	8.7
Pro	4.5	5.3		3.7	3.9	3.2	2.5	3.1
Tyr	4.9	4.8		3.4	4.2	2.1	3.0	3.4
Val	6.6	4.7		6.4	7.1	3.3	3.6	6.9
Met	.2	-		2.9	.6	.5	-	.9
Cys	.8	1.7		.8	.4	1.0	-	.8
Ile	6.9	3.1		8.1	8.3	1.3	3.0	5.3
Leu	11.0	5.1		8.4	9.3	1.1	6.0	9.2
Phe	1.6	3.2		3.2	2.2	1.9	-	2.5
Lys	3.0	2.0		5.3	5.4	1.1	2.8	3.7

Normalized to 100 picomoles.

Table 8

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENT PEAKS

Amino acid	Peak 16 LD-M_4		Peak 16 LD-X		Peak 17 LD-M4	
	Gossypol Treatment (μM)					
	0.7	39	0.05	0.1	0.7	39
Asp/Asn	7.7	4.4	ND	9.7	8.4	8.0
Glu/Gln	6.7	11.8		9.0	6.4	8.4
Ser	14.3	19.5		12.2	15.3	14.3
Gly	15.6	27.2		15.8	12.4	15.2
His	1.9	-		1.2	1.7	3.3
Arg	.6	2.8		2.7	.3	1.0
Thr	5.2	2.2		5.3	5.8	3.9
Ala	9.3	6.8		6.6	9.2	7.7
Pro	4.0	4.1		4.6	5.0	3.5
Tyr	4.9	5.4		3.8	4.0	3.6
Val	7.0	3.6		5.0	5.9	7.0
Met	.3	-		2.5	.5	1.2
Cys	.3	2.4		.6	.5	.9
Ile	5.7	1.5		6.7	7.9	6.6
Leu	10.2	4.6		7.8	4.3	9.5
Phe	3.5	2.1		3.3	3.1	2.6
Lys	2.9	1.7		3.2	2.1	3.3

Values normalized to 100 picomoles.

Table 9

AMINO ACID ANALYSIS OF PEPTIDE FRAGMENT PEAKS

Amino acid	Peak 18 LD-X		Peak 19 LD-X	
	Gossypol Treatment (μM)			
	0.05	0.1	0.05	0.1
Asp/Asn	8.9	6.3	3.1	5.8
Glu/Gln	7.0	9.5	12.4	8.4
Ser	14.9	14.9	17.0	14.3
Gly	17.6	20.3	22.0	19.8
His	3.9	2.5	1.4	-
Arg	.8	-	.8	5.2
Thr	2.7	2.9	1.9	4.3
Ala	4.8	6.3	4.0	9.3
Pro	3.2	3.5	2.3	3.1
Tyr	5.5	5.3	2.5	3.7
Val	4.3	8.5	7.1	6.8
Met	.3	.8	.5	-
Cys	.6	.9	.3	2.7
Ile	6.7	5.0	7.4	4.2
Leu	12.6	7.5	13.9	7.6
Phe	4.0	2.7	2.1	2.9
Lys	1.6	3.1	1.4	1.8

Values normalized to 100 picomoles.

Figure 22.

HPLC chromatogram of PITC derivatized amino acid standard and controls.

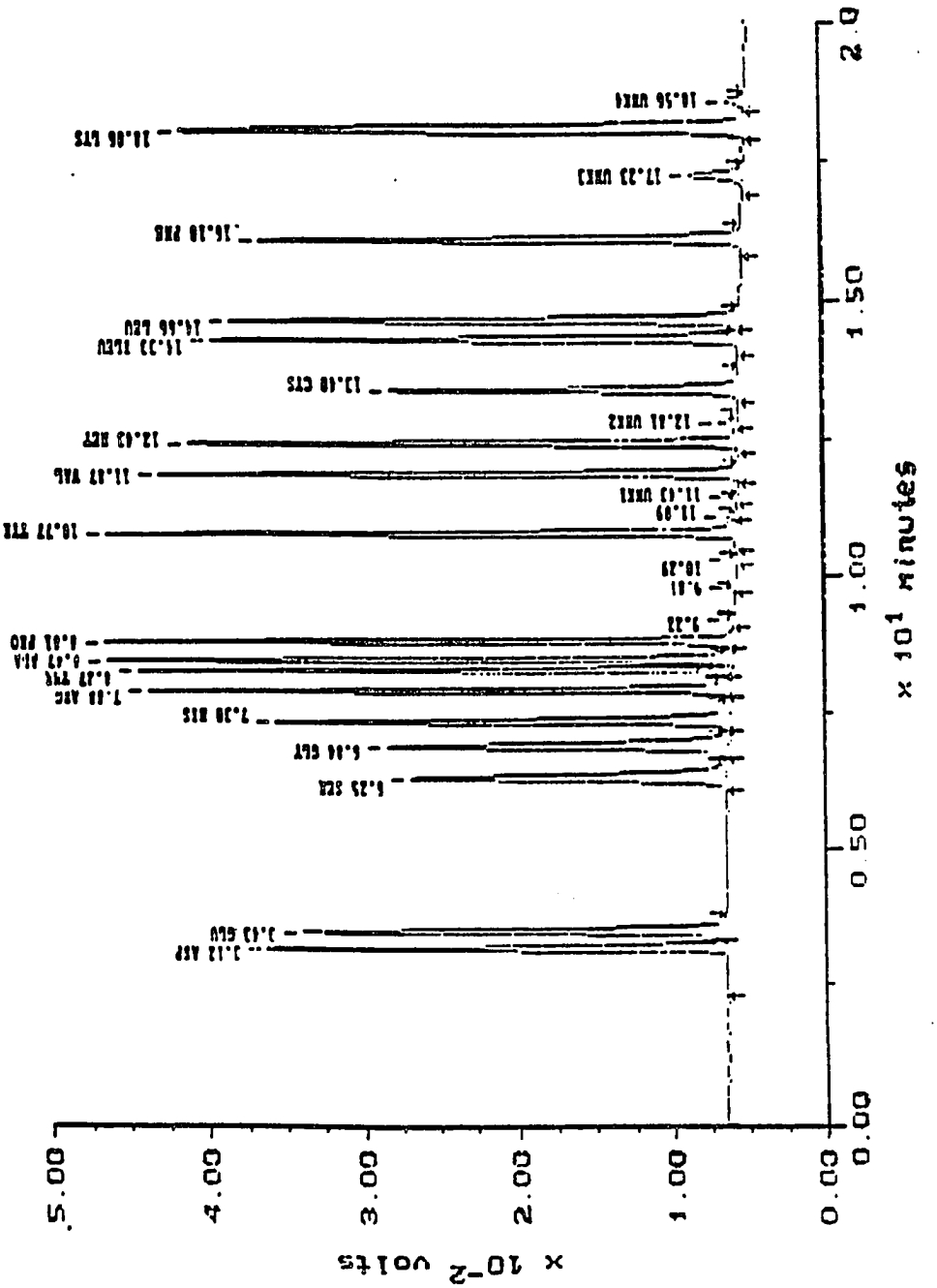
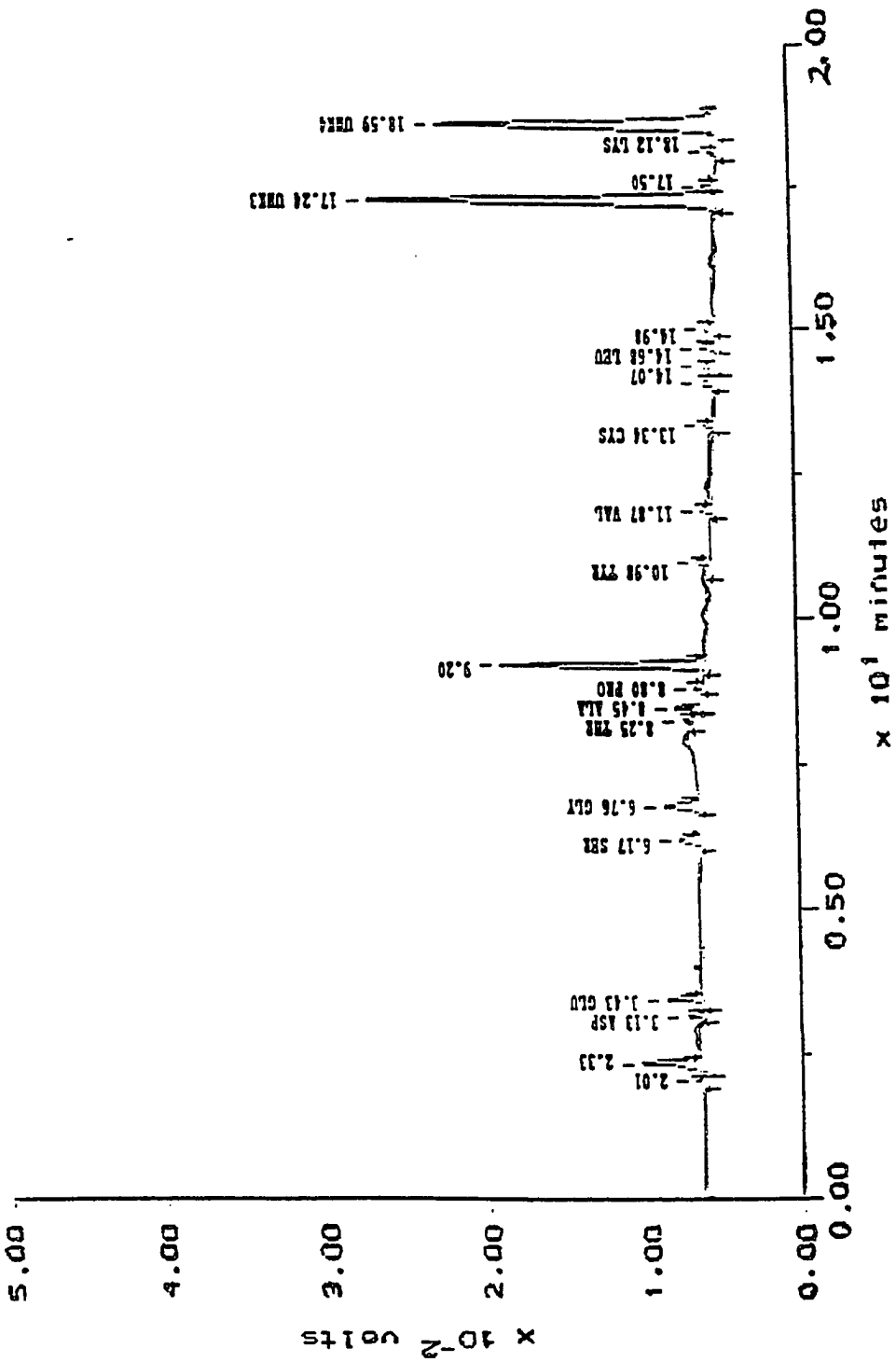


Figure 23

A chromatogram of amino analysis of a peak fraction from trypsin digested LD-X. Unknowns 1, 2 and 3 were controls.



F. Gossypol binding with native LD-X

In one set of experiments designed to observe the binary interaction of LD-X with gossypol, increasing amounts of undigested mouse LD-X were incubated with constant amounts of ^{14}C-gossypol and in the reverse. In another series of experiments increasing amounts of ^{14}C-gossypol were added to a mixture of constant amounts of unlabeled gossypol and constant amounts of LD-X.

The LD-X binding of gossypol was assessed after the addition of gossypol by immunoprecipitation and direct counting of filter bound ^{14}C. The immunoprecipitate was either collected on glass or nitrocellulose filters, or by decanting and counting the supernatant then calculating the difference between this count and the total counts added as the precipitate bound ^{14}C-gossypol. The difference in binding in the absence of LD-X was assumed to be non-specific binding and this value was subtracted from all values obtained after the addition of the enzyme. Non-specific or non-saturable binding was relatively high in all binding assays. Much of this could be attributed to hydrophobic binding to the surface of the polystyrene assay tubes.

When increasing amounts of undigested LD-X were added to a constant amount of ^{14}C-gossypol, the bound ^{14}C radioactivity was proportionately increased as the LD-X concentration increased from 0 to 50 nM with a sigmoidal curve that plateaued between 15 nM to 50 nM LD-X (Figure 24). In experiments where various concentrations (0, 1, 3, and 6 μM) of unlabelled gossypol and a constant amount of ^{14}C-gossypol were added to increasing amounts of mouse LD-X (0 to 100 nM) the LD-X bound ^{14}C activity began to decrease or became saturated at all levels of unlabeled gossypol with between 10 to 15 nM LD-X (Fig 25). The enzyme was saturated between 30 and 40 nM by 6 μM gossypol and the curve became parabolic. The stoichiometric ratio of the binding was 1395 M gossypol per mole LD-X.

Figure 24

The binding of undigested mouse LD-X with ^{14}C-gossypol. *Increasing amounts of undigested mouse LD-X were added to a constant amount of ^{14}C-gossypol in three different experiments. The respective concentrations of ^{14}C-gossypol added were 8 nM +++, 10 nM ..., 25 nM * * * . The 10nM curve represents results from three different experiments. Each point in each experiment represents duplicate or triplicate data.*

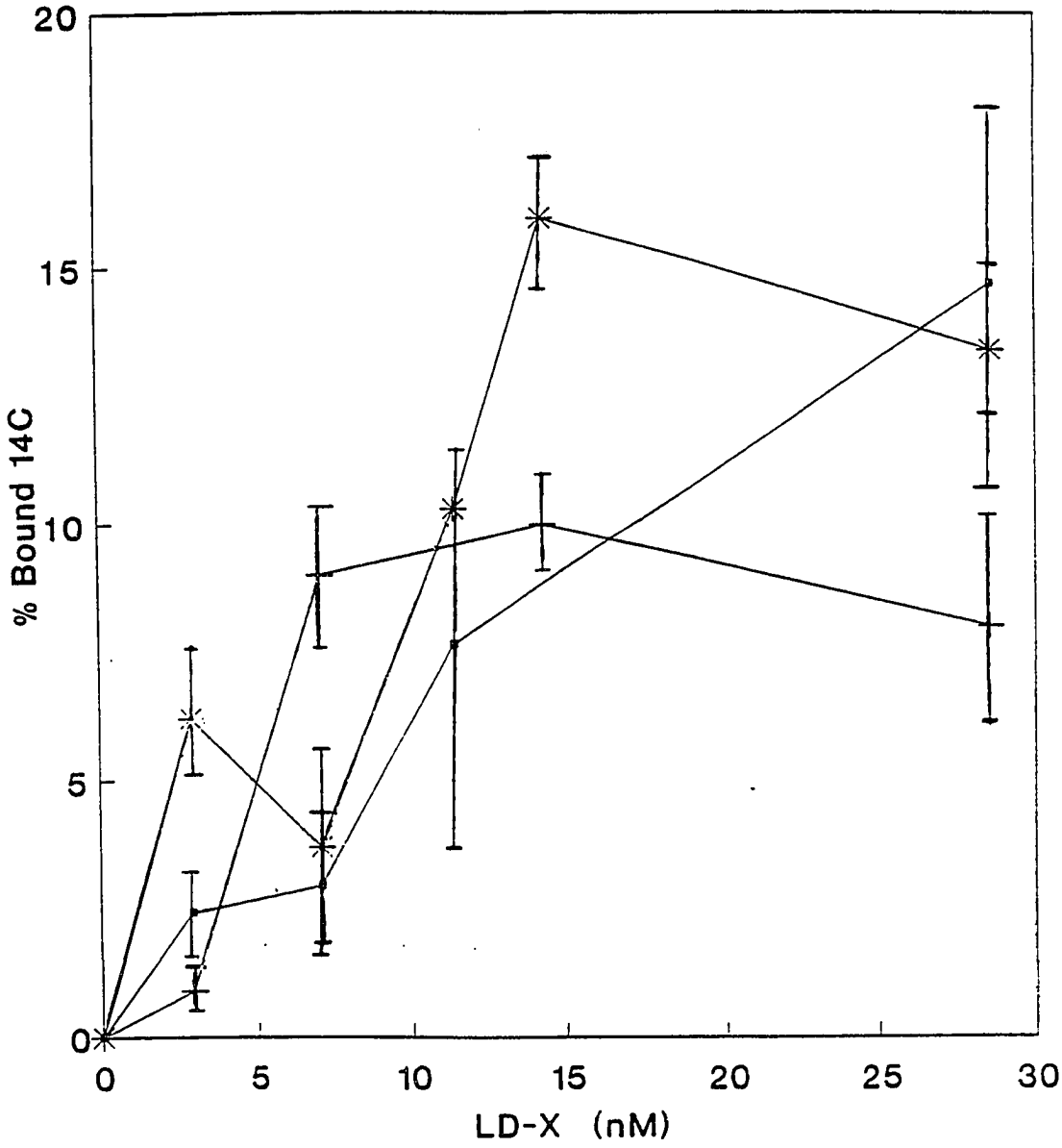
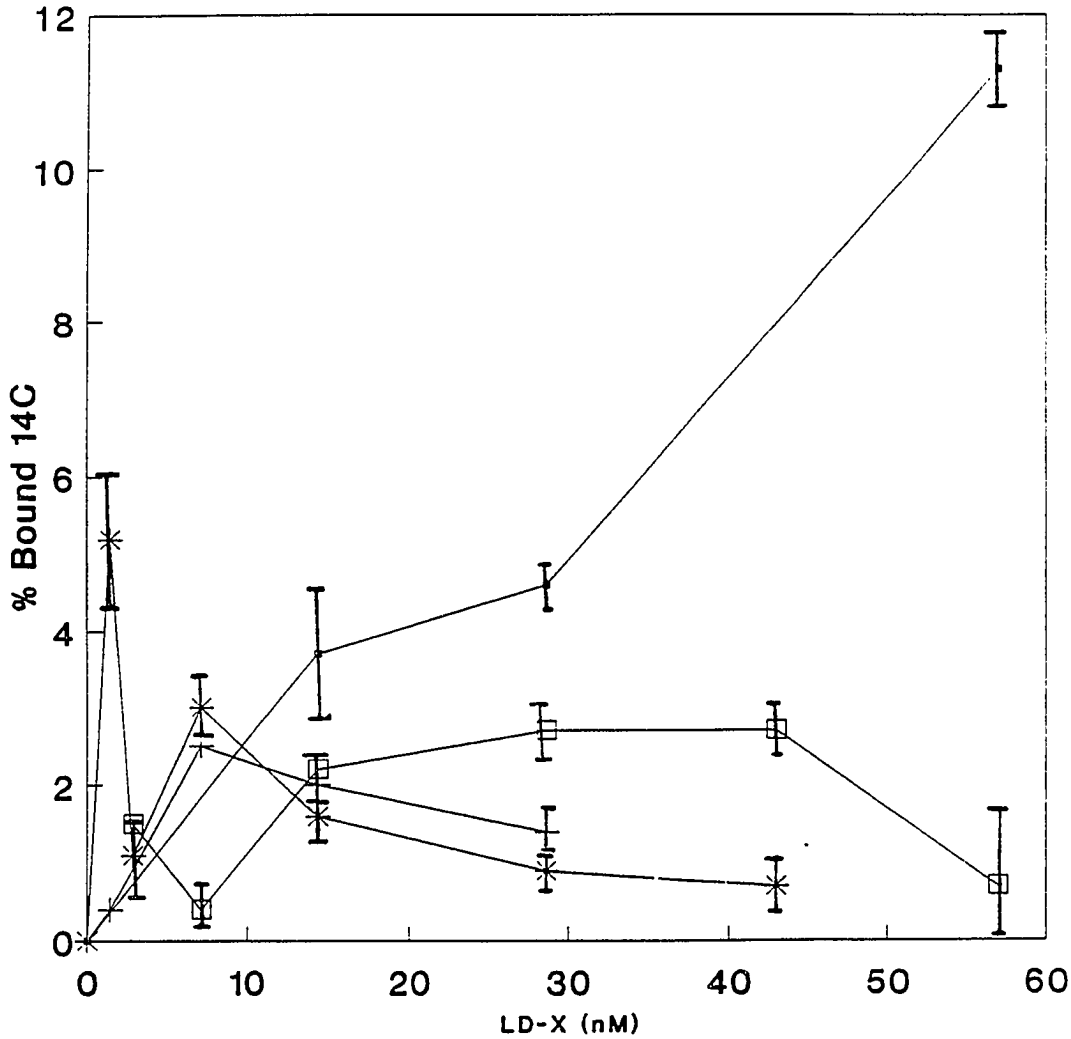


Figure 25

Competitive binding of labeled and unlabeled gossypol to undigested mouse LD-X. *A constant amount of ^{14}C-gossypol was added to increasing amounts of mouse LD-X (0 to 100 nM) and varying concentrations of unlabeled gossypol. No unlabeled gossypol ..., 1μM + + +, 3 μM * * *, 6 μM □ □ □. Each point represents the mean of triplicate values.*



When a constant amount (0.9 nM and 1.8 nM) of native LD-X was incubated with increasing concentrations of ^{14}C-gossypol (0 to 20 nM) there was a proportional increase in LD-X/^{14}C binding. No plateau was observed at these levels. Figure 26 is a best-fit linear plot of pM gossypol binding values using one nanamole LD-X. Figure 27 is a plot of pM gossypol binding at 0.89 nM and 1.79 nM LD-X . Data from both binding assays were used to calculate the K_D [18]. The mean K_D calculated from such binding was 1.3×10^{-7} M $\pm$ 0.39×10^{-7}, n=7.

The treatment of tryptic digested LD-X with increasing amounts of ^{14}C-gossypol showed a relative increase in ^{14}C binding to the digested enzyme and were similar to that of the undigested enzyme. Although the digested protein is unlike the intact enzyme, these experiments were performed to determine if any of the peptide fragments showed preferential binding to gossypol.

In experiments where 0 to 3 μM unlabeled gossypol were added to the trypsin digested LD-X before the addition of ^{14}C-gossypol the relative binding of ^{14}C-gossypol decreased as the concentration of unlabeled gossypol increased. From 0 to 0.75 μM unlabeled gossypol the percent bound ^{14}C increased, but thereafter up to 1.0 μM (519 μg) the percent bound ^{14}C began to decrease; suggesting that the LD-X/gossypol binding sites were saturated around 0.75 μM unlabeled gossypol with 100 μg (7.1×10^{-10}M) of the digested LD-X protein. The stoichiometric ratio of this binding was 1056 M gossypol per mole LD-X digest. This compares to the stoichiometric binding ratio of 1395:1 observed with the undigested enzyme.

Figure 26

Plot of average binding of 14C-Gossypol to LD-X before trypsin digestion. *Increasing concentrations of ^{14}C-gossypol (0 to 50nM) were added to a constant amount of mouse LD-X (1 nM). The K_D values were calculated from this data and the data in figure 27. Each point represents triplicate values. Slope = 0.75 and $K_D = 1.3 \times 10^{-7}$M.*

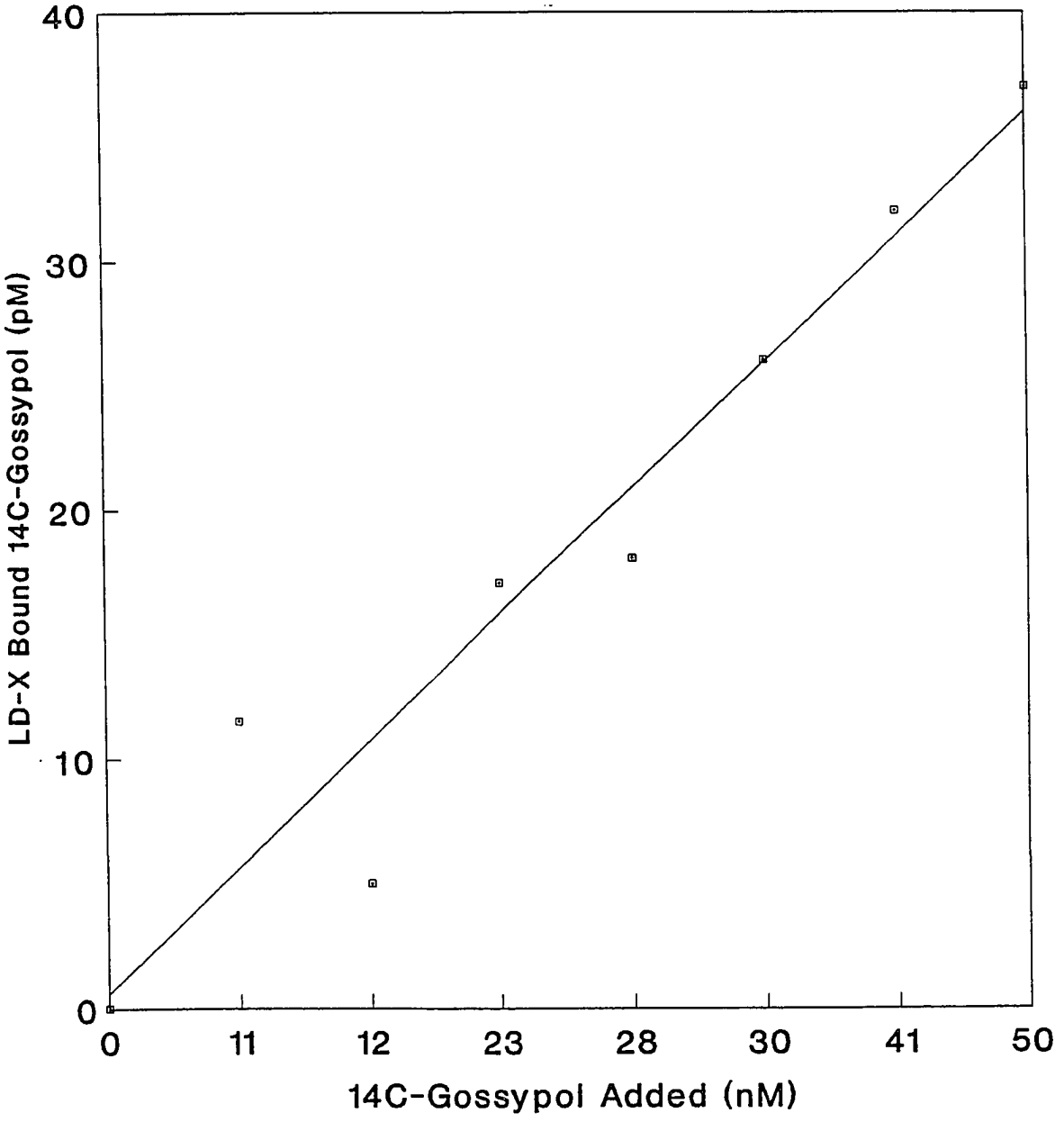


Figure 27

Binding Assay of Increased amounts of ^{14}C-gossypol added to constant amounts of undigested mouse LD-X. *Labeled gossypol (0-18 nM, specific activity 1.67 μM/μCi) was added to 0.89 nM (●-●-●) and 1.79 nM (+ + + +) mouse LD-X. The K_D was calculated from these values.*

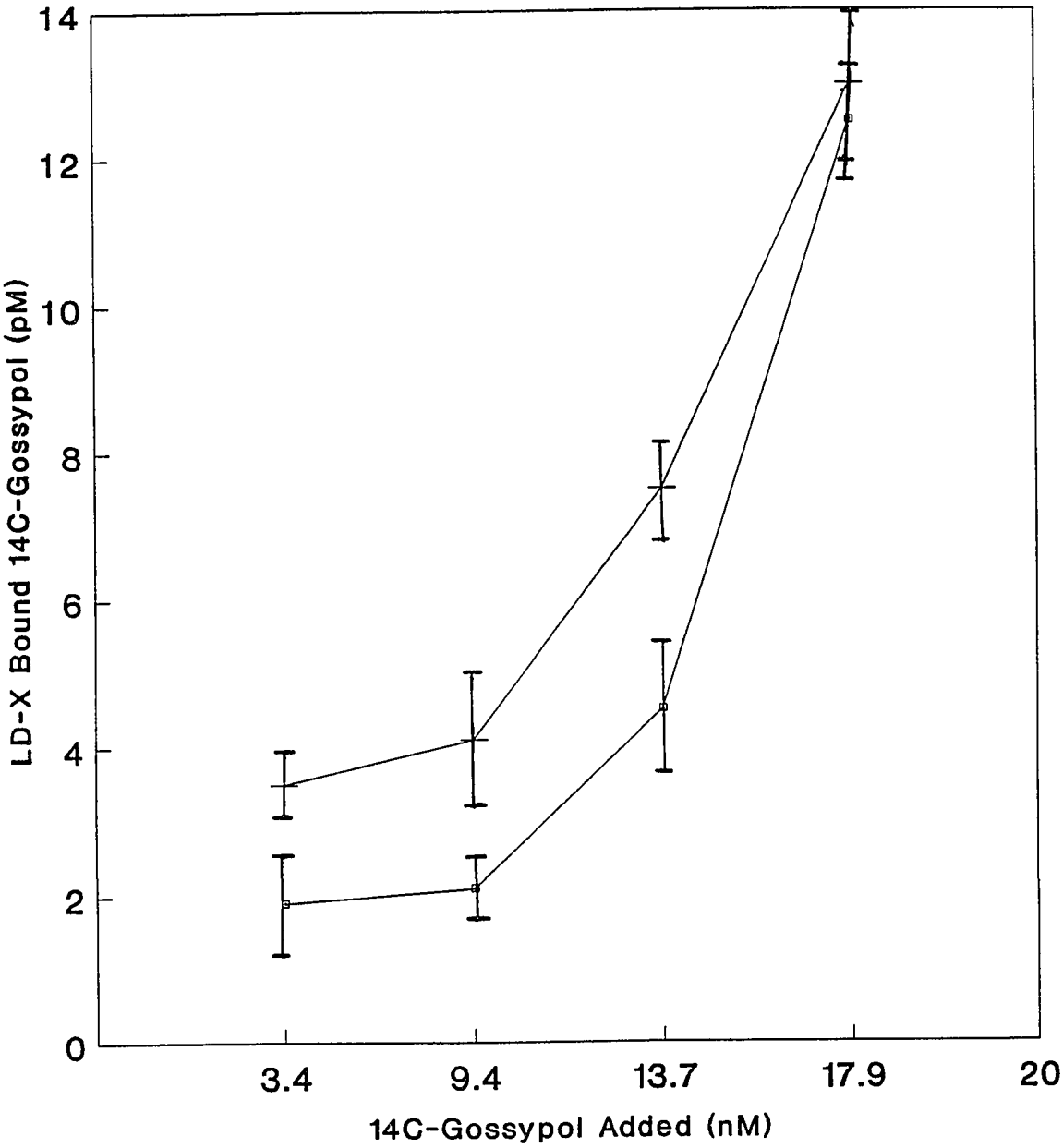
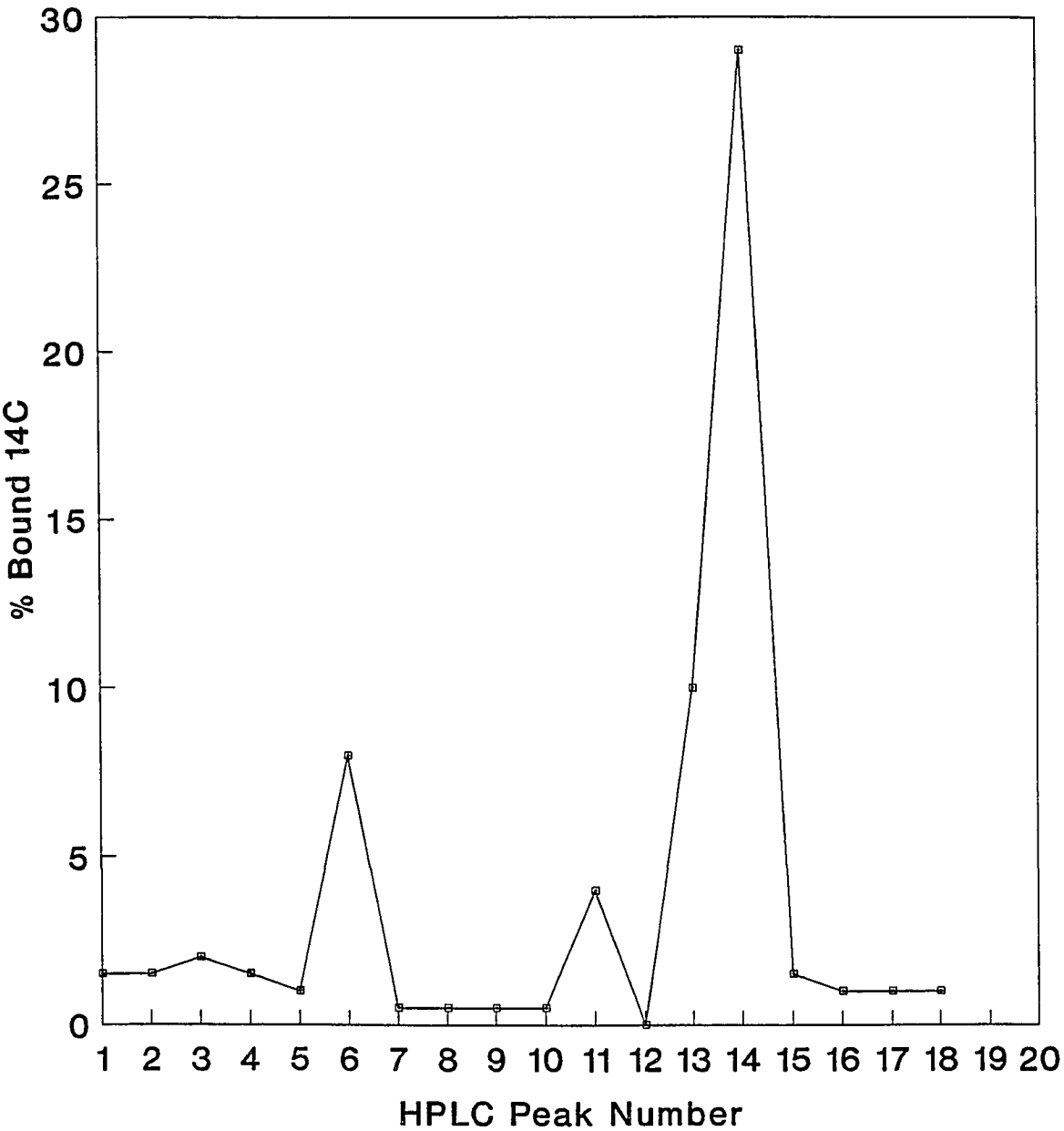


Figure 28

The binding of ^{14}C-gossypol to peak fractions obtained after HPLC separation trypsin digested mouse LD-X. *Although the tryptic fragment with maximum ^{14}C binding is located in the most hydrophobic region peak 6 appears to be hydrophilic.*



G. ^{14}C-Gossypol binding to HPLC LD-X peptide fragments

An experiment in which 0.15 μM ^{14}C-gossypol was added to each peak fraction collected during HPLC separation of tryptic digested LD-X revealed that some peak fractions did not bind with the labelled gossypol, and that the maximum 14C binding was not only in the most hydrophobic fragments. The maximum ^{14}C binding was observed in peak 14 (28% B). The relative but lower binding was also observed in peak 13 (10% B), peak 11 (4% B) and peak 6 (8% B) of the chromatogram (Figure 28).

H. Testicular cell fractions

Since the method used to isolate the testicular cells was not an elutriation method as described by Meitsrich [73, 96] and Den Boer [103, 104] pure spermatogenic cell populations were not anticipated. The crude adaptation of a velocity sedimentation separation technique that was utilized only yielded fractions of mixed cell suspensions. Although a marked number of flagellated spermatozoa were observed in F2 and F3 fractions, no spermatozoa were counted when cell counts were calculated for cultivation. Cell structure was observed while counting and determining viability, and 24 hours post incubation. All cell fractions contained 90% to 95% viable cells. Periodic acid Schiff-hematoxylin stained smears prepared of each cell suspension were observed with a light microscope at 1000x magnification. F2 fraction contained approximately 5% very large cells with prominent nucleoli, 35% large cells with thick chromatin patterns (spermatocytes), 60% medium to small cells with dense chromatin pattern (spermatids). The F3 fraction contained predominantly small round cells (55%) which were assumed to be round spermatids along with around 35% elongated spermatids, fewer medium round cells (10%) and occassional large epithelioid cells (1%) that may have been Sertoli cells. Cells in F4 fraction were predominantly small and medium cells with distinct

chromatin and some elongated cells.

Based upon staining properties and cellular description [73, 74, 95] the spermatogonia are cells with a spherical nucleus and clear light staining cytoplasm; the primary spermatocytes are the largest spermatogenic cells with spherical nucleus and prominent chromatin pattern; secondary spermatocytes are about half the size of primary spermato- cytes with distinct chromatin; the oval spermatids appear as small round cells; while the elongated spermatids clearly show thick short extensions from the cells. Sertoli cells have indistinct chromatin with prominent nucleolus and voluminous cytoplasm. Therefore, the F2 fraction contained possibly equal populations of primary spermatocytes, secondary spermatocytes and round spermatids; the F3 being round spermatid and elongated spermatids; while the F4 contained a predominance of spermatids (Table 10).

I. ^{14}C-Gossypol and testicular cell binding studies

Data collected from three batches of testicular cell suspensions showed no clear delineation as to which of the three cell fractions bind with ^{14}C-gossypol to the greatest extent; which is not surprising since the cell fractions were mixed. However, the data generated was of some general interest with regard to the intracellular location of gossypol in testicular cells and where it is complexed with LD-X. From 50% to 78% of the added ^{14}C-gossypol is directly bound to the solubilized cell pellet or membrane components in all cell fractions when 0.5 to 1 x 10^6 cells were cultivated from 18 to 24 hours at 32°C in Eagles MEM at pH 7.2. after 30 to 60 minutes exposure to the labeled gossypol, and after washing the cell pellet with 4.0mL PBS before solubilization with the Triton X-100 (Table 11). As the concentration of ^{14}C-gossypol increases the direct ^{14}C binding increased (Figure 29) at the concentrations utilized (0.001 μM to 0.146 μM). Exposure of the cells to the same

Table 10

Testicular Cell Suspensions

Cell Suspension	Spermatocytes (%)	Spermatids (%)	
		Round	Elongated
F2	35	60	5
F3	10	55	35
F4	10	75	5

$n=3$

Table 11

Testicular Cell Binding

^{14}C-Gossypol	*F2*		*F3*		*F4*	
nM	*S*	*P*	*S*	*P*	*S*	*P*
27	*1855*	*2050*	*ND*	*ND*	*945*	*5055*
42	*1481*	*2064*	*1016*	*1532*	*1644*	*3435*
71	*2559*	*4014*	*2733*	*3308*	*3720*	*6763*

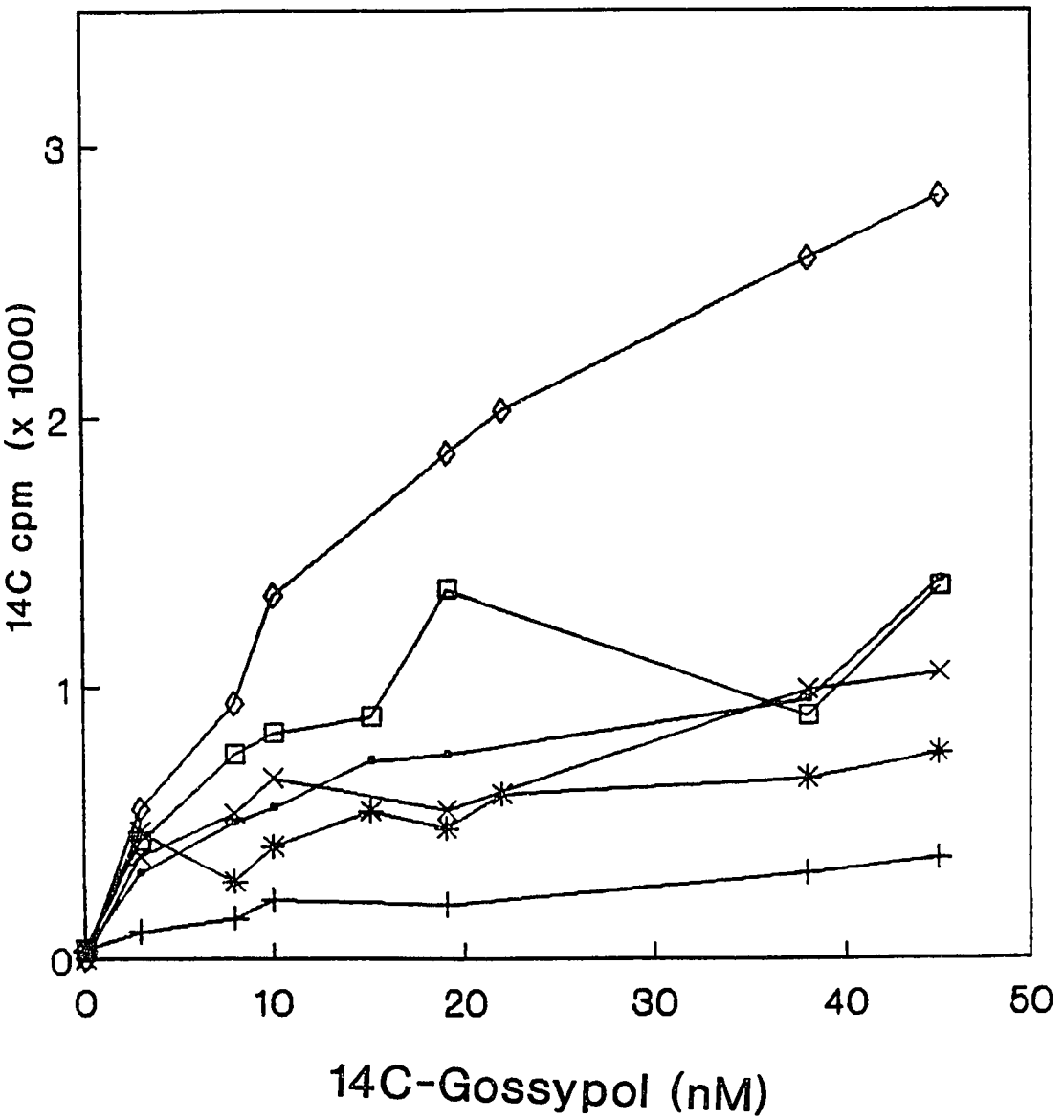
S = ^{14}C retained in supernatant after lysis.

P = ^{14}C retained pellet after lysis.

n=3

Figure 29

Mouse testicular cell binding to ^{14}C-gossypol. *Aliquots of lysate and solubilized pellets from testicular cell suspensions that were cultivated for 24 hours at 32°C and then treated with ^{14}C-gossypol (0 to 100 nM, specific activity = 1.67 μCi/uM) for 30 minutes. The larger percentage of the ^{14}C was retained in the (pellet) membrane fractions (78%) . Pellet: F4 □□□□, F3 x x x, F2 ♦ ♦ ♦. Lysate: F4●-●-●-, F3 +++++, F2 ****.*



to the same concentration of labelled gossypol at 30, 45 and 60 minutes showed an increase in ^{14}C binding with time, but the difference between the 45 and 60 minutes treatment did not appear to be significant (Figure 30).

Conversely, of the total %B ^{14}C in the pellet and the lysate, a higher percentage of the ^{14}C was bound to LD-X in the lysate than in the solubilized pellet after immunoprecipitation of the solubilized cell pellet and the lysate with anti-mouse LD-X γ-Ig at all ^{14}C-gossypol doses. The immunoprecipitation of the binding of the F2 cell lysate to anti-mouse LD-X γ-Ig was linear to a maximum mean percent bound ^{14}C-radioactivity (%B) of 30.8% at 27 nM ^{14}C-gossypol and began to decrease to a mean percent binding of 17.4% B around 100 nM ^{14}C-gossypol (Figure 31, Table 12). A similar initial binding curve, though with less binding, was observed in the pellet. The curve seemed to plateau at the highest concentrations. If the percent bound ^{14}C in the pellet is assumed to be nonspecific binding the mean maximum lysate LD-X ^{14}C-gossypol binding was 24% B at 27 nM ^{14}C-gossypol. These results suggest that the cells bind less gossypol as the gossypol concentration increases, perhaps due in part to a concomitant saturation of available binding sites or that there is a diminution of cell viability with increased concentrations of the labeled gossypol.

The F3 cell lysate and pellet LD-X to ^{14}C-gossypol binding gave curves resembling that the F2 fraction, except that the maximum percent binding occurred around 40 nM ^{14}C-gossypol with a mean of 23% B with the assumption that the 9.3% pellet bound activity is non-specific binding (Figure 32, Table 13). This may imply that this cell population has a lower affinity or all available binding sites are not occupied. A less likely possibility may be that there were more cells in this fraction.

Figure 30

Mouse testicular cell lysate and solubilized pellet binding after incubation with the same amount of ^{14}C-gossypol for 30, 45 and 60 minutes. *After 45 minutes there was little difference in the percent bound ^{14}C in the pellet fraction, but the percent bound ^{14}C activity in the lysate almost doubled as the concentration of the labelled gossypol increased. F4 Lysate: 30 ..., 45 +++, 60 ***. F4 Pellet: 30 □ □ □, 45 x x x, 60 ◆ ◆ ◆.*

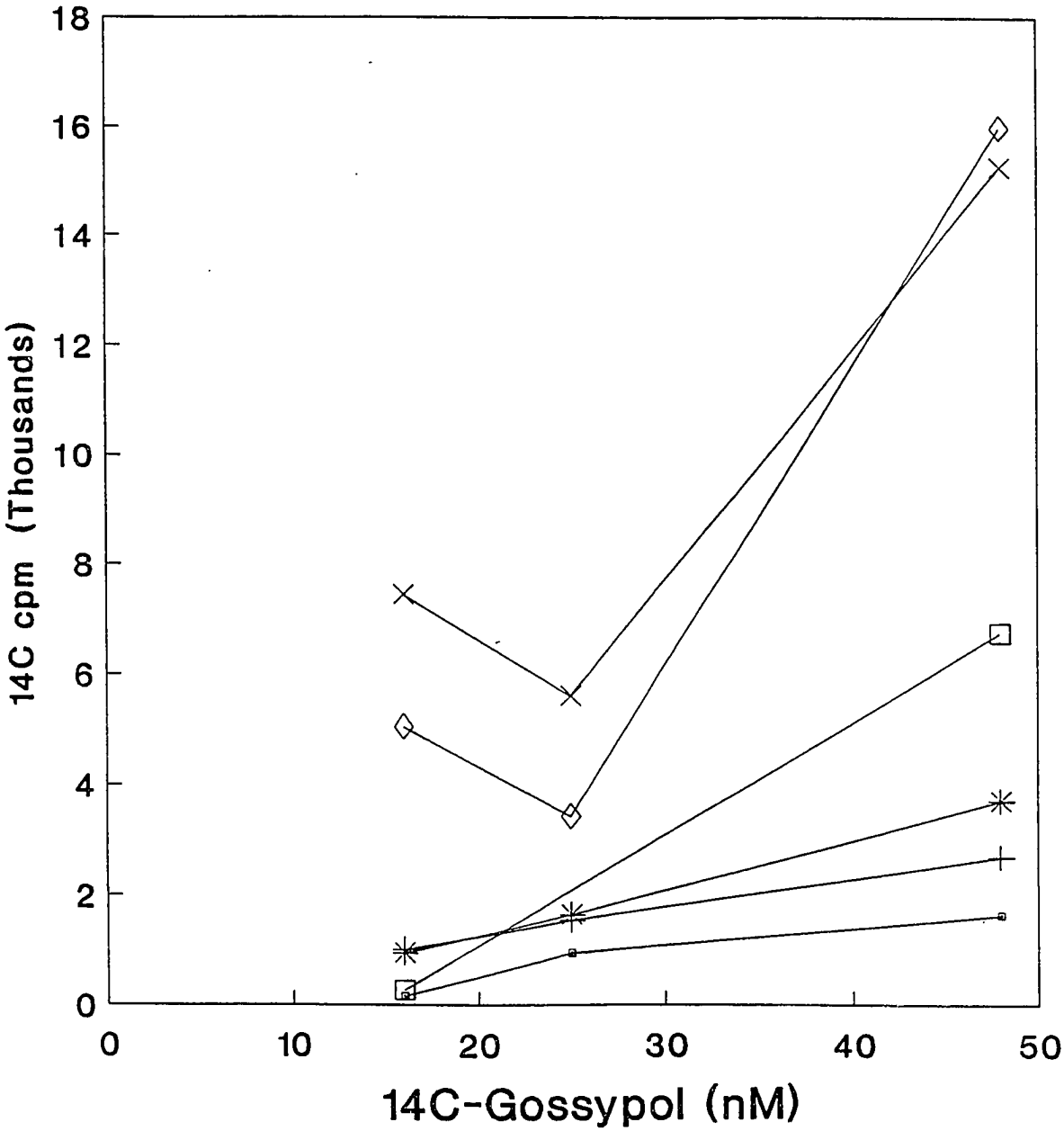


Table 12

Mouse Cell LD-X ^{14}C-Gossypol Binding

Cell Suspension	^{14}C-Gossypol nM	Lysate %B	Pellet %B
F2	27	30.8	6.8 ±1.9
	42	26 ±8	8.5 ±3
	49	27.2	7.2
	64	27	8.5
	71	28.8 ±1	21.4 ±16
	97	17.4 ±3.4	8.0 ±2.3
	146	25	6.6

Summary of experiments wherein "fraction 2" cell suspension of mouse testicular cells was incubated with ^{14}C-gossypol for 60 minutes at 32°C in Eagles MEM with 2% FCS.

Figure 31

Mouse testicular cell suspension (F2) LD-X binding to ^{14}C-gossypol. *Testicular cell suspension F2 lysate and pellet labelled gossypol binding after immunoprecipitation with anti-mouse LD-X. Although the predominance of the total ^{14}C-gossypol was bound to the pellet (membrane), the relative LD-X bound radioactivity was higher in the lysate than the pellet. F2 lysate 1 . . ., F2 lysate 2 + + +, F2 lysate S * * *, F2 pellet 1* □ □ □*, F2 pellet 2 x x x, F2 pellet S* ◆ ◆ ◆. .

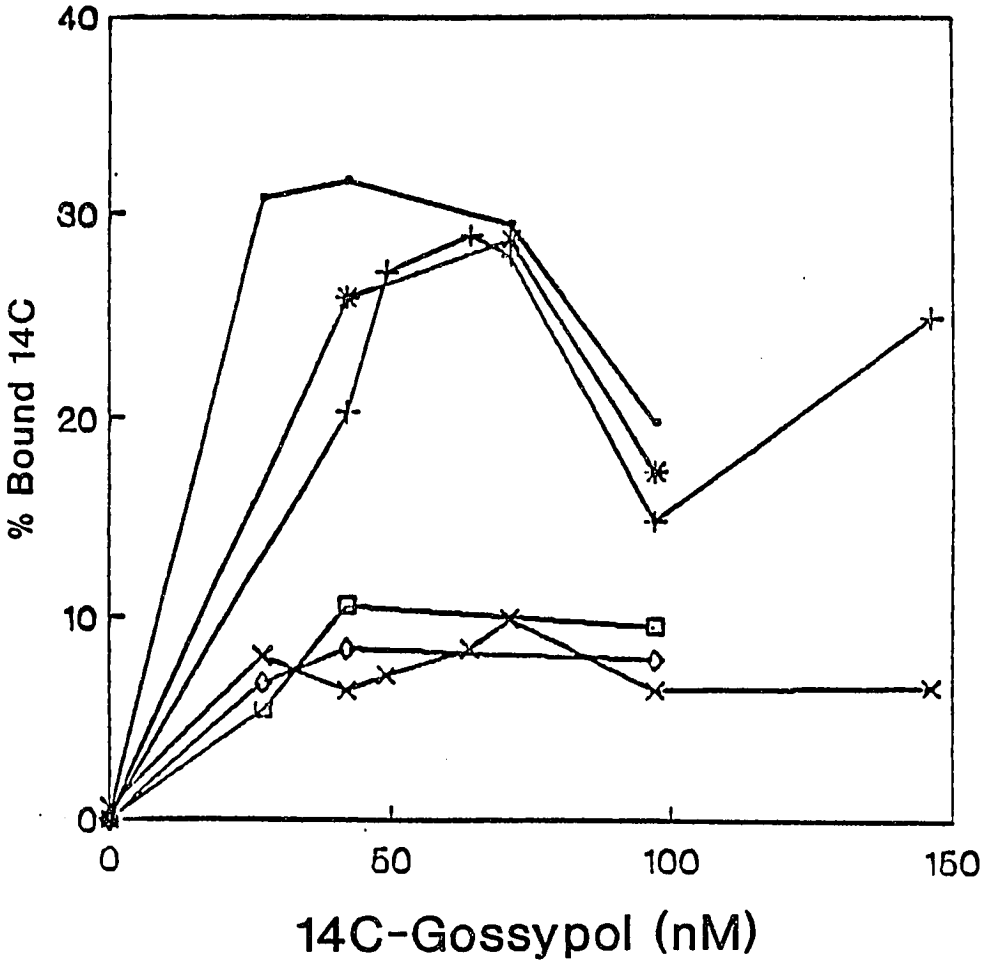


Table 13.

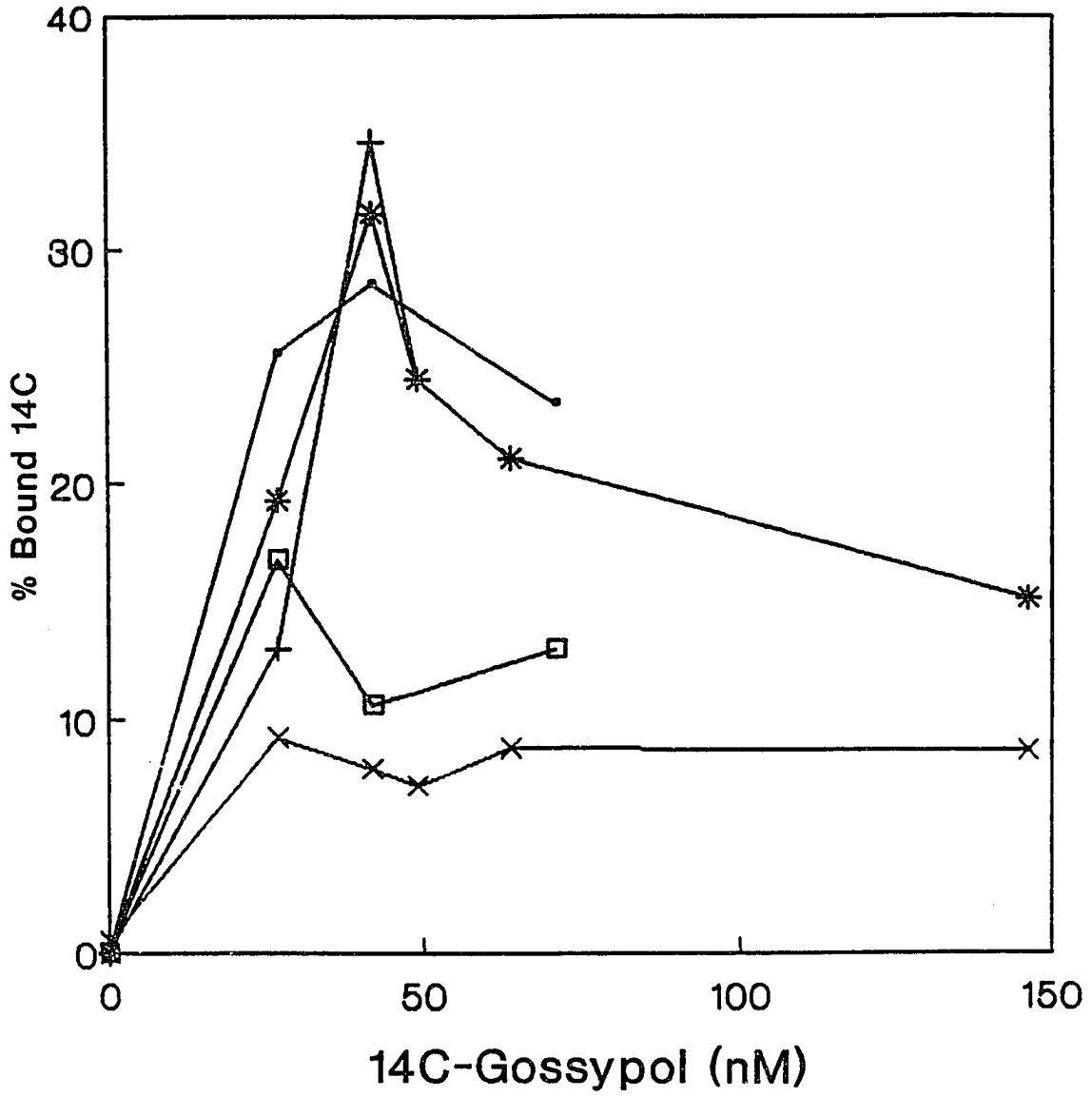
Testicular cell LD-X ^{14}C-Gossypol Binding

Cell Suspension	*^{14}C-Gossypol nM*	*Lysate %B ^{14}C*	*Pellet %B ^{14}C*
F3	*27*	*19.3±8.9*	*13.0±5.4*
	42	*31.6±4.2*	*9.5±1.9*
	49	*24.5*	*7.2*
	64	*21.1*	*8.8*
	71	*23.5*	*13*
	146	*15.2*	*8.7*

Summary of two experiments wherein "fraction 3" cell suspension of mouse testicular cells was incubated with ^{14}C-gossypol between 60 minutes at 32°C in Eagles MEM . Each concentration was run in triplicates.

Figure 32

Mouse testicular cell suspension (F3) LD-X binding to ^{14}C-gossypol. *Testicular cell suspension F3 lysate and pellet ^{14}C binding after LD-X immunoprecipitation. F3 lysate 1 . . ., F3 lysate 2 + + +, F3 lysate S * * *, F3 pellet 1 □ □ □, F3 pellet 2 x x x.*



The F4 cell lysate LD-X binding and pellet binding again gave maximum binding at between 40 and 50 nM labelled gossypol, a parabolic decrease at higher concentrations in the lysate and a sigmoidal binding in the pellet (Figure 33). The comparative LD-X binding between the pellet and lysate was also predominantly in the lysate (Table 14).

Figure 34 shows the mean binding activities for each cell suspension. Gossypol appears to saturate the F2, F3 and F4 lysate fractions more rapidly than the pellet gossypol fractions, as indicated by the plateau at the higher levels of gossypol.

Table 14.

Testicular Cell LD-X ^{14}C-Gossypol Binding

Cell Suspension	*^{14}C-GOSSYPOL nM*	*Lysate %B*	*Pellet %B*
F4	*3.6*	*6.4± 1.4*	*11.6± 1.6*
	9	*7.3± 5.9*	*12.5± 4.9*
	18	*15 ± 14*	*13.1± 5.4*
	27	*27.8± 17*	*11.5± 7.4*
	42	*26.3± 22*	*14.1± 6.2*
	71	*17.7± 5.7*	*14.5± 1.9*
	146	*16*	*17.8*

n=3

Figure 33

Mouse testicular cell suspension (F4) binding to ^{14}C-gossypol. *Testicular cell suspension F4 lysate and F4 pellet ^{14}C bound activity after LD-X immunoprecipitation. F4 lysate 1 . . ., F4 lysate 2 * * *, F4 pellet 1 + + +, F4 pellet 2 □ □ □.*

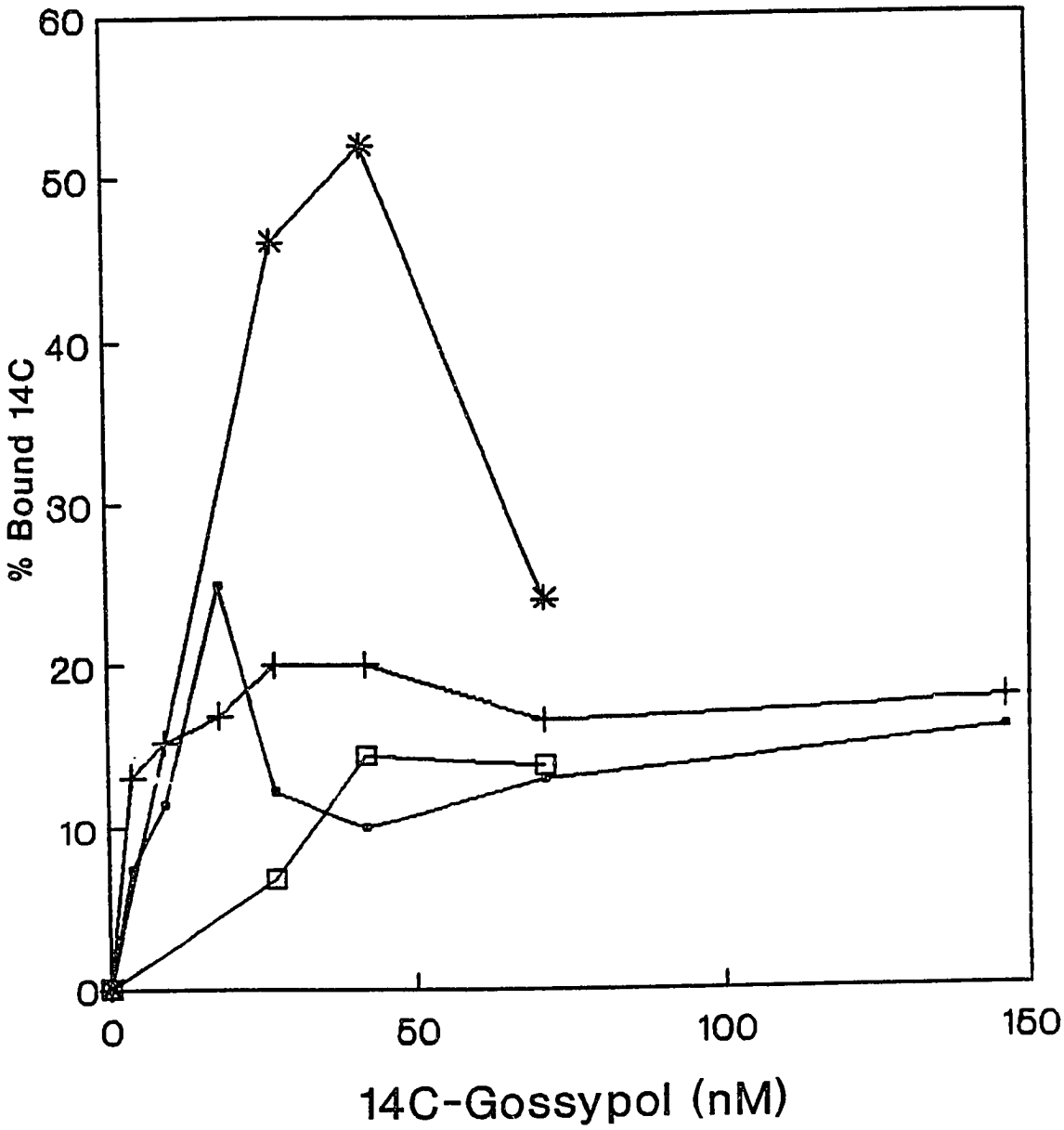
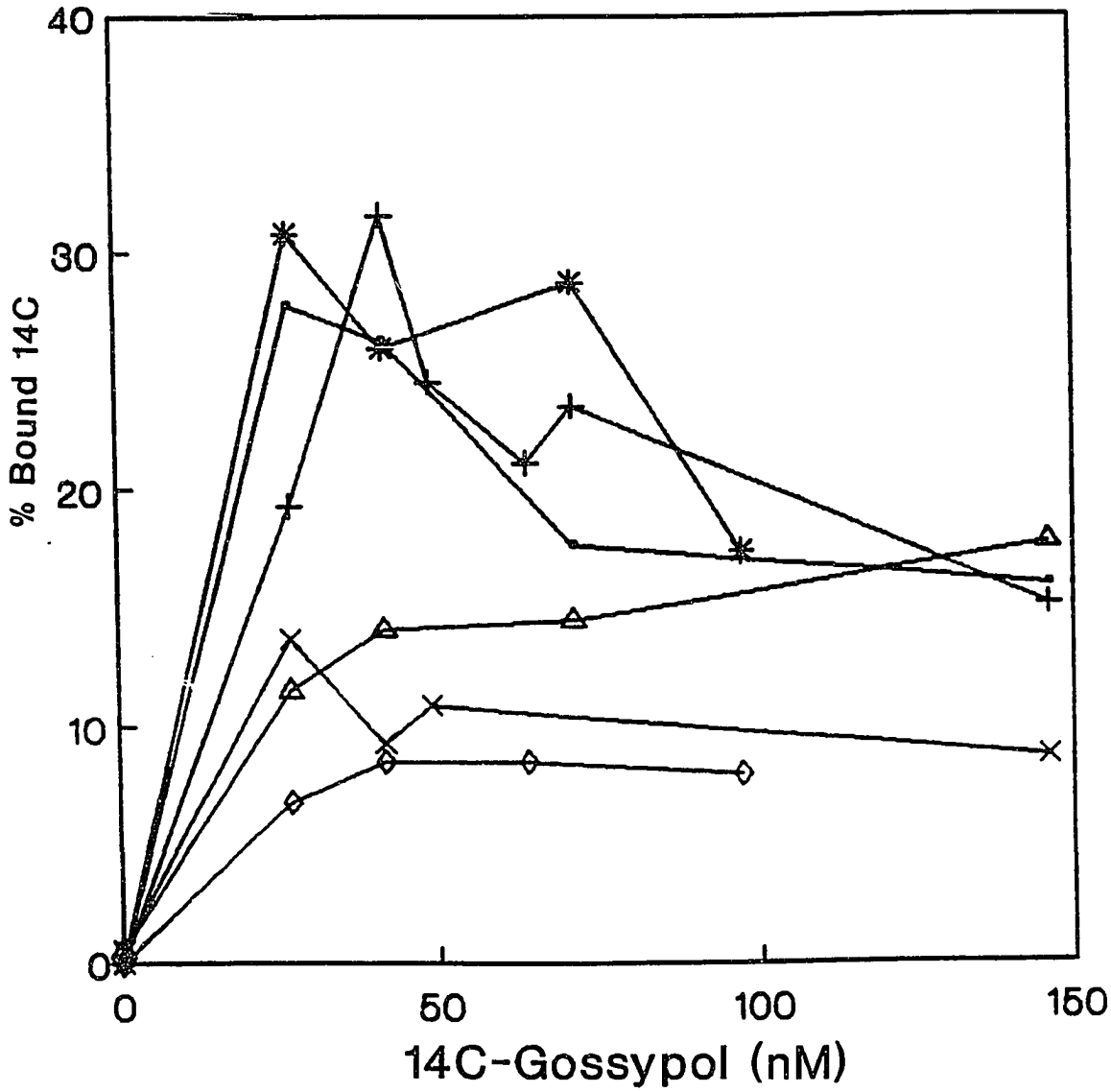


Figure 34

Summary of LD-X specific binding *for in cytosolic and membrane fractions of F2, F3 and F4 spermatogenic cell fractions. A relative larger percent* ^{14}*-C was bound to the supernatant than the pellet fractions of all cell suspensions. Lysates: F2 * * *, F3 + + +, F4 . . ..Pellet: F2 ♦ ♦ ♦, F3 x x x, F4 ▵ ▵ ▵.*



CHAPTER IV

DISCUSSION

The evidence that gossypol inhibits LD-X is unequivocal although the type and the extent of inhibition varies with substrates or coenzymes. Gossypol was reported to inhibit LD-X noncompetitively with respect to NAD [13, 81], and competitively with respect to NADH [104]. Gossypol also showed a mixed inhibition of LD-X with respect to either pyruvate or lactate [13]. In the competitive inhibition, the gossypol combines with free LD-X in a manner that prevents NADH binding. That is, the gossypol and NADH are mutually exclusive because of their competition for the same coenzyme binding domain of the active site. The non-competitive inhibition of LD-X by gossypol implies that the inhibitor has no effect on NAD binding, and vice versa. The difference in structure between NADH and NAD is the presence of a positively charged pyridinium ring on the NAD molecule. This may indicate that gossypol preferentially binds to the LD-X coenzyme binding domain competitively with NADH because it requires a more hydrophobic environment. When competing with NAD, pyruvate, or lactate, gossypol interacts with functional groups other than those in the NAD or substrate binding domains of the LD-X active site, such as the NAD binding domain in the hydrophobic loop and alpha-D helix region or some other nonhomologous site. These findings imply that there may be more than one mode of inactivation of spermatogenic LDs by gossypol, and perhaps one of these is by a mechanism that is common to its inhibition of other oxido-reductases, and perhaps another is unique to LD-X.

The objective of the binding studies executed in this project was to investigate the binding interactions of the binary complex of LD-X and gossypol, with the hope of further illumination on the mechanism by which gossypol specifically or non-specifically inhibits LD-X or its general mode of interaction with spermatogenic cells. Such binding studies could presumably ascertain if these interactions were mainly covalent, hydrophobic or both, and would provide data to explain why such an altered enzyme structure could obstruct productive coenzyme and/or substrate binding, and thus enzyme inactivation. However, while the results of this investigation of binary interactions between the LD-X and gossypol indicate definitive molecular alterations in enzyme structure, they do not indicate whether gossypol binds exclusively covalently or non-covalently with the enzyme. Given the conditions of the binding experiments such interactions appear to be both covalent and hydrophobic interactions, and each may be a significant factor in the inhibition of LD-X by gossypol.

Digestion of native mouse LD-X and chicken LD-M_4 revealed less than the expected number of peptide fragments on HPLC separation and fewer fragments than reported by others [68-70, 72, 76, 77]. This was due to either incomplete digestion of the enzyme or the loss of small fragments in the desalting process. Wheat and Goldberg [72] obtained 26 peptide fragments upon HPLC separation of trypsin digested mouse LD-X after desalting on sephadex G-10 and using a triple pump delivery of a linear gradient from 10% acetonitrile : 90% water with 0.1 % trifluoroacetic acid (TFA) in the system to 60% acetonitrile : 40% water with 0.1 % TFA in the system. They were interested in identifying antigenic determinants in the LD-X in order to develop antibodies to specific idiotypes on the LD-X subunit. Four such peptides were identified and chemically synthesized after amino acid sequencing. Pan et al. [78] identified 34 of 36 expected tryptic peptides after cleaving carboxymethylated mouse LD-X with cyanogen bromide and trypsin.

The 190 to 400 nm UV/visible spectral scans of the peptide eluates obtained after desalting the tryptic digest of mouse LD-X and chicken LD-M_4 were similar, and the addition of gossypol resulted in a second broad absorption peak between 240 to 260 nm as the concentration of gossypol increased. The broadness of this second peak is attributed to the presence of gossypol, as well as its degradation products, given that at least seven degradation products of this compound have been identified [34] by thin layer chromatography and four such by-products were obtained by HPLC [81] when monitored at 254 nm wavelength. The mouse anti-LD-X γ-Ig reacted with both the native and the tryptic digested LD-X fragments, but it did not cross react with the chicken LD-A_4.

The incubation of the individual tryptic digested LD-X peptide fragment eluates with labeled gossypol were performed to observe preferential gossypol binding with the peptide fragments. After the addition of a ^{14}C-gossypol to HPLC peak eluates, it was observed that gossypol complexed predominantly with the most hydrophobic peaks (larger R_f) suggesting non-ionic, hydrophobic or covalent binding. Even though gossypol contains two active aldehyde groups, it is known to be a hydrophobic compound, relatively insoluble in water and quite soluble in ethanol, ether and dimethylformamide. The hydrophobic nature of gossypol may be attributed to its naphthalene rings.

Exposure of both LD-X and LD-M_4 to gossypol resulted in altered HPLC chromatograms for each isozyme; but these peak alterations were dissimilar. Such peak alterations suggest definite molecular modifications in the peptide fragments due to their binding with gossypol and/or its degradation products. The altered peptide fragments observed with both native and trypsin digested LD-A_4 and LD-X suggest that gossypol binds to peptide fragments of both native and tryptic digested LD eluates. However, the evidence that gossypol itself has reducing properties also implies that the altered and/or additional HPLC peaks observed after

treatment with gossypol could have resulted from conformational changes that lead to increased cleavage sites for trypsin via the reducing action of gossypol, as well as gossypol interactions with these digested fragments or a combination of the two mechanisms. In solution, gossypol could display its action as a reducing agent and open or unfold the enzyme molecule to expose or conceal sites that are essential for substrate and or coenzyme binding. This could be accomplished by hydrophobic interactions at the fourth carbon of the pyridinium ring and hydrogen binding at the amide residue of the nicotinamide moiety of the NAD molecule that shifts the pyridinium ring. This shift could result in an orientation and position that would not permit the covalent linkage at the fourth carbon, which is required for the catalytic activity of the enzyme [78, 79].

^{14}C-labeled gossypol was employed to study the type of binding between LD-X fragments and gossypol, presuming that the covalent binding would be more stable under the conditions of these experiments than hydrogen binding and hydrophobic binding. LD-X was incubated with unlabeled and labeled gossypol or both. This was followed by tryptic digestion, during which binding of gossypol to the enzyme at saturated and non-saturated concentrations was observed. When constant amounts of ^{14}C-gossypol were added to increasing concentrations of LD-X/unlabeled gossypol mixture, the amount of ^{14}C-gossypol binding should decrease if the LD-X binding sites for gossypol are fully saturated with unlabeled gossypol. If LD-X/-unlabeled gossypol binding was not saturated, the percent bound ^{14}C-gossypol should increase until its binding sites are saturated, at which time the binding should reach a plateau. Under such conditions, a dissociation constant of K_d of 1.3 x 10^{-7} M compared to a K_d of 1 x 10^{-7} M with albumin [28] and a K_d of 1 x 10^{-8} M with Arbacia sperm [105] was obtained.

Quantitative amino acid analysis was performed on some of the eluates containing altered peaks after mixing gossypol with LD-X and LD-M_4, and had been stored at -70°C up

to one year. This analysis was performed on one occasion in one batch. The results suggest possible contamination with serine, glycine and glutamate/glutamine in all peaks. Quantities of arginine in one set of peaks were in excess of one part arginine in arginine-containing fragments, also suggesting contamination. The excess arginine further supports the incomplete tryptic digestion of LD-X since trypsin cleaves peptides at the carboxyl end of arginine and lysine. If complete tryptic digestion had been accomplished each peptide fragment would have contained the same quantity of arginine or lysine. Amino acid sequence of isolated peptide fragment(s) from the altered HPLC chromatograms would have been useful in assessing the region(s) of the LD-X molecule most affected by gossypol. However, the author's limited resources prevented this endeavor. Future extensive studies in this regard would certainly add more definitive answers as to the nature of these altered fragments. Without such information, the domain locations of presumed altered fragments could not be determined.

Since the catalytic domain is shown to be more heterologous [68, 69, 77], any sperm specific differences would most likely be expected to occur in fragments that include residues from this domain. The coenzyme binding loop is homologous for LD-A_4 and LD-B_4 in mammals, birds and fish at positions 103 and 106; but there is some non-homology in the LD-X. Rat and mouse LD-X differ at positions 103 and 106 [77]. In the mouse, the aspartic acid, a negative charged residue, at position 106 is replaced by alanine, a non-polar residue, which should result in distinctly different types of molecular interactions and thus orientation. Likewise, Goldberg and Wheat attributed the reduced affinity of rat LD-X for anti-mouse LD-X antibodies to threonine as residue 103 in mouse LD-X compared to the smaller side chained serine at this position in rat LD-X [72].

Gossypol should bind covalently via its carbonyl groups with tryptic fragments at least at the arginine and lysine terminals through their free amino groups, along with similar groups of glutamine and asparagine. Its quinoid metabolites such as gossypolone may also interact irreversibly with the amine groups to form a redox system with its corresponding hemiquinone leading to free radical production [56]. However, superoxide free radical formations have been noted in rat liver and kidney microsomes, but not in testicular tissue [56]. Others [25, 107, 108] have also suggested that the aldehyde group is essential for gossypol's antispermatic affect, particularly sperm immotility, in human and hamster spermatozoa. Gossypol derivatives without aldehyde [25] and analogues with the aldehyde covalently bound to long chain alkyl groups [109] still exhibited antifertility properties. In fact, enhanced antifertility effects observed with the long chain alkyl gossypol analogues suggest significant hydrophobic interactions with macromolecules [109], perhaps like LD-X, though the effect on the binding of such analogues on LD-X activity has not yet been reported.

Similar studies of the interaction between the binary complex of gossypol and NAD/NADH are needed to completely resolve the question of possible binding interactions of gossypol and LD-X and its contribution to the anti-spermatogenic properties. If binding of gossypol to the enzyme results in conformational changes in the coenzyme binding domain, which is common to all pyridine nucleotide coenzyme requiring enzymes, its mode of action in antifertility is unlikely to be distinct from its toxic actions. Gossypol may bind in the active site such that inadequate positioning and orientation occurs, thus preventing the proper binding of coenzyme or substrate molecule, or both. Based upon the observations of this investigation, such alterations are assumed not to be the distinguishing features that make gossypol interactions with LD-X unique from the somatic LD isozymes. It must be noted that in this study only the enzyme-gossypol binding was studied before and after tryptic digestion, while

the enzyme-coenzyme-gossypol or ternary complex presents a somewhat different molecular environment. The recognition that the conditions of this study are not the same as in vivo,where the substrate and the coenzyme form a binary complex which then interacts with the enzyme to form a ternary complex [79], is also ever present, as is the fact that the presence of the coenzyme appears to protect LD-X from inactivation by gossypol [81].

Stephen et al. [13, 14] postulated that gossypol binds to the relatively more hydrophobic loop of LD-X after a comparative kinetics study of inhibition of purified monkey (Cynomolgus) spermatozoan LD-A_4, LD-B_4 and LD-X by gossypol. Its K_i for LD-X with respect to pyruvate and NADH was significantly lower than that of the LD-A_4 and LD-B_4 isozymes. The K_i for LD-X was an average of 2.5 μM compared to 10 μM and 24 μM for LD-A_4 and B_4 respectively, suggesting that LD-X is more sensitive to gossypol inhibition than the somatic isozymes. A mixed type inhibition was observed for each isozyme with respect to lactate and pyruvate. Competitive inhibition was observed with respect to NADH, but non-competitive inhibition was observed with NAD. The competitive inhibition of LD by gossypol with respect to NADH indicates that gossypol lowers the affinity of enzyme for NADH, but does not affect the rate of conversion of product once bound to the enzyme. The non-competitive inhibition of NAD binding indicates that gossypol does not lower the affinity for the coenzyme in this direction but makes the enzyme-substrate complex non-productive [13]. Thus, binding of gossypol to LD-X inhibits the binding of NADH, but not the binding or release of NAD, perhaps disrupting a vital mitochondrial-cytoplasmic reducing shuttlein spermatozoa and spermatids. In such a case, the NADH could be exhausted more rapidly than its formation.

If gossypol binds covalently at the NAD/NADH domain of LD-X, this mode of inhibition may be common to other pyridine nucleotide dependent enzymes, or it may be a

unique binding feature since the nucleotide-binding fold has been shown to be dissimilar in detail from one dehydrogenase to another [69, 70]. Gossypol may bind with or occupy portions of the pyridine nucleotide binding domain by hydrophobic or covalent interactions when looking at the coenzyme domain. The findings of the present study reveal that gossypol binds favorably to LD-X peptide fragments that elute in the hydrophobic region, as well as the hydrophilic region; and support the possibility that gossypol may also inhibit LD-X activity through hydrophobic interactions that may result in intramolecular maneuvers which can lead to a tilting of the unique hydrophobic loop in the coenzyme domain.

The selective inactivation of LD-X by gossypol as a possible mechanism of its male antifertility effect was further probed through in vitro observations of the relative amount of gossypol binding to LD-X in testicular cell suspensions which contained a predominance of spermatids and spermatocytes isolated from adult male mice to glean whether a significant amount of the gossypol would be bound to this sperm specific isozyme. The desired velocity sedimentation method for isolating these cells is elutriation because of the wide volume difference in the various spermatogenic cells. However, the unavailability of an elutriator rotor required an alternate method namely mechanical, enzymatic dissociation and centrifugation. Mechanical dissociation of testicular tissue selectively damages Sertoli cells, Leydig cells, spermatogonia and young primary spermatocytes and also removes some cytoplasm and flagella of elongated spermatids [96]. Further treatment of a mechanical cell suspension with trypsin and DNase completely destroys the damaged cells except the late spermatids, with tubular fragments and free germ cells removed by passage through the stainless steel and nylon mesh [96]. The LD-X activity is unaffected by this type of suspension preparation [95, 96]. Thus, seminiferous tubules were freed from the interstitial tissue matrix by collagenase treatment and agitation to obtain the F3 cells, while the trypsin and DNase

should have destroyed most contaminating cells, resulting in a predominance of spermatids in the F4 cell suspension [95]. Those cells with the highest number of spermatids (F3 & F4) were expected to bind the larger percentage of the labeled gossypol, since these cells are reported [65] to contain a higher concentration of LD-X than spermatocytes. If the LD-X was the major target of gossypol binding, one would also expect the greater percentage of this compound to bind to the anti-LD-X/LD-X complex. Likewise, since LD-X has been shown to be found mainly as a cytosolic enzyme, a predominance of the ^{14}C-gossypol would be complexed in the cytosolic fractions of each cell suspension. Neither was the case. The total percent bound ^{14}C in the F2 suspension was higher than the F3 and the F4 cells. The F2 suspension was a filtrate of mechanically treated cells without enzymatic treatment and conceivably contained a greater number of intact spermatozoa, as the spermatozoa were not counted in the cell suspensions. A greater amount of ^{14}C-gossypol (78%) was found in all pellet or membrane fractions than the lysate or cytosolic components, and the average maximum percent bound (%B) of the total ^{14}C-gossypol bound to the lysate (cytosol) of all three cell suspensions was only 35 $\pm$ 14%. However, of the total ^{14}C-gossypol bound to LD-X in each of the membrane and cytosol fractions, albeit small, a larger amount was bound to the cytosol fraction. Thus, since most of the gossypol that is bound to or within the spermatogenic cell is not bound to LD-X, this compound must interact predominantly with other spermatogenic membrane and cytosolic components. With the numerous other possible nucleotide enzyme targets in the enzyme milieu of the cell, such interactions could be pivotal in several cellular defects attributable to gossypol.

These results suggest that gossypol is mainly membrane-associated and agree with Corin et al [2, 29] who used a murine erythroleukemia cell line (MELC) wherein 95% of the ^{14}C-gossypol uptake was found in the membrane fraction at subsaturated (5 μM) and saturated

(30 μM) levels of gossypol. It also appeared that gossypol uptake was not coupled to the energy generating system of these cells, after observing the inhibitory effect of sodium cyanide on the oxygen uptake and ATP synthesis without appreciable inhibition of gossypol uptake by the MELC. Sato et al [106] observed a 58% to 74% uptake of ^{14}C-gossypol (10 μM) by Arbacia sperm within one minute of exposure and a steady state was reached and plateaued at 60 minutes. The specific number of binding sites for gossypol on these sperms was calculated to be 8 x 10^8 sites per sperm cell. This large number of binding sites on the spermatozoa appears to exceed the number of receptors for the average polypeptide hormones indicating that membrane-gossypol interaction may be through covalent bonding with a variety of determinants on or within the membrane.

Albumin has been shown to have a protective effect when cells or LD-X are exposed to gossypol [1, 29, 109]. The gossypol-albumin binding ranged from 63% to 93% in the present study wherein the labeled compound was incubated with the native enzyme in a phosphate buffered saline. Others have observed that the addition of 0.1% albumin or fetal calf serum decreased the uptake of gossypol by nontesticular cells as well as sperm cells. A recent histochemical study [108] in which peroxidase-antiperoxidase was used to localize albumin in the testes of adult golden hamsters reported findings that may be important in an alternate explanation of why the gossypol cytotoxic and antispermatogenic effects on spermatogenic cells might differ. Albumin was localized to varying degrees in interstitial tissue and seminiferous tubules. Localization in seminiferous tubules varied at different and similar stages of maturation with reactivity present and absent in the same stages. Spermatogonia and spermatozoa were the most frequent albumin-localized cells; round and elongated spermatids gave moderate reactions, while all spermatocytes showed no localization of albumin. Abundant albumin was evenly distributed in the extracellular space of interstitial tissue, on the

surface of Leydig cell and in the Sertoli cell. The authors [108] of this study concluded that (1) albumin-like proteins serve as a mediator of a paracrine relationship between Sertoli cells and spermatogenic cells and (2) that this protein also mediated structural changes within the cell membrane. This could partially explain the greater vulnerability of spermatids, spermatocytes and spermatozoa [18] with increasing levels of gossypol. The fact that albumin or albumin-like proteins are more abundant in spermatogonia may serve to protect these cells by competitively binding with gossypol. Likewise albumin located in the extracellular spaces in the interstitial tissue limits the amount of gossypol that is free to permeate the cells in this compartment; but spermatocytes, being devoid of albumin, are vulnerable to relatively larger amounts of gossypol.

The differential cytotoxic effect of gossypol on various carcinoma and melanoma cell lines has shown gossypol to have its greatest cytotoxic effect on fast growing, anaerobic cell lines [101]. The cell death was attributed to a blockage in energy production, since DNA synthesis was unaffected. Conversely, the more resistant cells were the slow growing cell lines that contained high levels of LD isozymes with electrophoretic mobilities that could include LD-X.

Membrane perturbance as observed by Reyes [55] on the artificial phospholipid membranes (liposomes) may be the universal mechanism by which gossypol inhibits membrane and glycolytic enzymes. This could be due predominantly to non-specific hydrophobic interactions with membrane bound proteins or membrane phospholipids, and to a lesser extent its specific effect on inorganic anionic transport.

Observations on the effect of gossypol on the metabolism of rat spermatids through its effect on the ATP, lactate, and pyruvate levels, lactate oxidation to carbon dioxide and oxygen consumption by these cells [21, 22, 65] seem to support gossypol's role as a mitochon-

drial uncoupler in spermatids. There was a biphasic response to gossypol on lactate metabolism [21] and mitochondrial respiration [65] as the concentration of gossypol increased from 1 to 10 μM. The low levels of gossypol stimulated lactate utilization (an LD-X requiring pathway), but as the concentration increased to 10 μM this property was suppressed. This is confirmed by similar findings of others [64] with liver mitochondria and gossypol action as proton carrier in lipid monolayer and bilayer membranes [55]. These findings suggest the mitochondria to be the primary target of gossypol. Yet the time elements with regard to cellular uptake of gossypol and its extracellular binding may be critical in its combined role of enzyme inhibition and mitochondrial or plasma membrane interference, given that most recent investigations implicate disturbances in membrane function or enzyme inhibition [2, 55, 65].

One would also expect the LD-M_4 and LD-H_4 isozymes to bind gossypol in a manner similar to LD-X if the intra-molecular bonding with the inhibitor is uniform. Haspel et al. [2] studied the effect of gossypol (10 μM) on nine membrane related parameters in erythrocyte membranes and only observed the inhibition (>95%) of inorganic anion exchange (phosphate, sulfate and chloride) and the affected membrane proteins corresponding to that of band 3, the inorganic anion exchanger transmembrane protein in the erythrocyte membrane. It also showed that 80% of the cell-bound gossypol was mostly attributable to gossypol binding to membrane lipid components and that the remaining 20% binding was specific or covalent binding. The other membrane parameters wherein no suppression was evident included spontaneous hemolysis, osmotic fragility, cell volume, cholinesterase activity, hexose transport, nucleoside (uridine) transport, ouabain-sensitive inorganic cation transport, and ouabain-insensitive inorganic cation transport. The lack of effect on the ouabain (a powerful inhibitor of Na/K ATPase) sensitive and insensitive transport indicates that cation transport is not

inhibited by gossypol. Here again, gossypol was shown to be a specific membrane active agent causing a stoichiometric conformational change in band 3 protein [2].

Srivastava et al. [109] observed an increase in the total testicular and epididymal LD activity after treating adult rats (30mg/kg) and hamsters (20 mg/kg) with gossypol for seven weeks, while other glycolytic enzymes (hexokinase, pyruvate kinase and phosphofructokinase) were unchanged. The mitochondrial or tricarboxylic acid cycle enzymes, malate dehydrogenase and succinate dehydrogenase, were also inhibited. Since the LD-X activity was not assayed along with the total LD activity, its activity cannot be assessed, even though it is the major LD isozyme in testicular tissue. It is possible that such an increase in total LD activity could result from the unique intramitochondrial localization of LD-X [110] or a compensatory elevation in the production of somatic isozymes in an attempt to provide the shuttle role of LD-X.

Based upon the findings of the current investigation and the work of others [2, 3, 4, 22, 25, 32, 110, 111] a model of gossypol/LD-X interactions in testicular cells is envisioned to be an adaptation of Corin's [29] and Sonenberg's [25] model in non-testicular cells and is illustrated in Figure 33. Most of the gossypol that binds the testes is located in the albumin or albumin-like proteins localized extracellular spaces. The unbound gossypol traverses the plasma membrane where its binding is probably by hydrophobic interactions with membrane lipids and covalent binding with serine- containing phospholipids [107], as well as membrane proteins through covalent binding and hydrophobic interactions. The gossypol that enters the cell may then interact similarly with organelle membranes, while a relatively small amount of gossypol remains in the cytosolic arena and an even smaller amount binds to LD-X. The inactivation of LD-X may very well be the main event leading to the antifertility properties of gossypol, since LD-X is the predominant LD isozyme in the secondary spermatocytes,

spermatids and spermatozoa, as well as the key enzyme in the ultimate utilization of lactate/pyruvate as the key energy source.

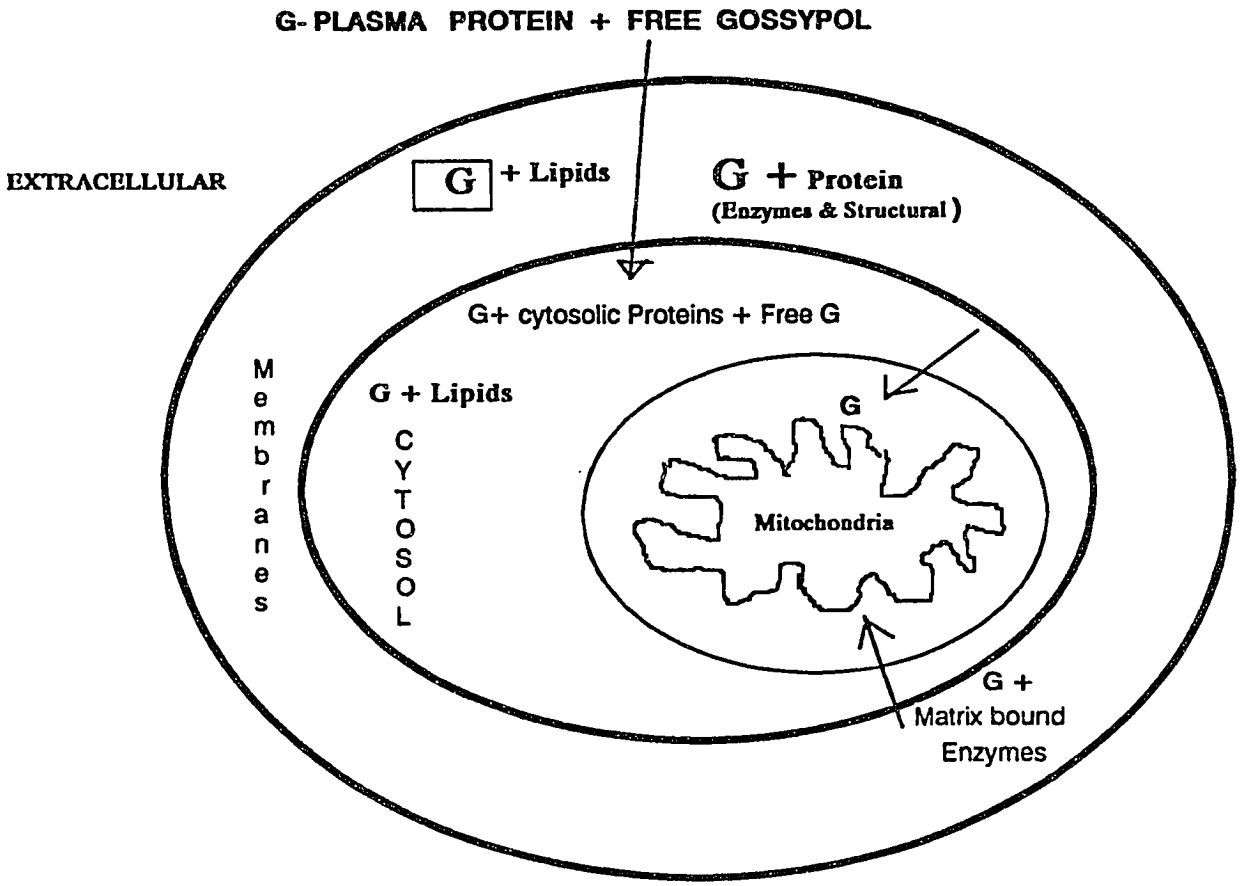
Thus, this study along with several others [5, 55, 61, 62,107] concludes presumptively that gossypol's role as an antispermatogenic compound has not been shown to be unique from its universal effect. The compound interacts with membrane and/or cytosolic components of most cells. This compound's usefulness as a contraceptive agent may be limited perhaps to its use in exogenously contained antispermatogenic agents such as topical spermacidal formulations. Based upon the drug uptake in rat testicular fluid after intravenous injection of a gossypol-liposome formulation, Wang et al. [111] speculated the possibility of liposome encapsulated gossypol formulation that would facilitate rapid entry of the drug across the blood-testis barrier yet reduce its toxicity.

Finally, further studies of the amino acid sequence of altered peptide fragments as well as spectroscopic studies such as nuclear magnetic resonance, mass spectrometry and infrared spectroscopy of these peaks might yield more explicit revelations of unique chemical bondings that can be attributed to one gossypol effect as distinct from another.

Figure 35

A Model of gossypol binding in spermatogenic cells. *Most of the gossypol (G) binds to plasma proteins just as in non-testicular cells. Membrane components are the major target for gossypol binding in spermatogenic cells, mainly through hydrophobic interactions and covalent binding. The free compound enters the spermatogenic cells and interacts predominantly with membrane components. Only a small amount binds to cytosolic components. Within the cell, gossypol may interact with cytosolic enzymes, structural proteins and lipids. It may also permeate membrane-bound organelles such as the mitochondria. Gossypol's antispermatic properties may be incidental to these general properties, since LD-X makes up as much as 80% of spermatogenic LD isozymes.*

Gossypol Binding in Spermatogenic Cells



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. . . . . *Autobiographical Statement*

Patricia Brown Ravenell was born on May 24, 1939 in Atlanta, Georgia. In 1962 she received the Bacholor of Science degree with a major in Biology and minor in Chemistry from Bennett College of Greensboro, North Carolina. While a student at Bennett she was inducted in the Alpha Kappa Mu Honor Society and the Beta Kappa Chi Scientific Honor Society. Upon graduation from Bennett, Patricia was awarded a Ford Foundation Fellowship. In 1964 she completed a Master of Science degree in medical technology at Wayne State University, Detroit, Michigan. She has earned over one hundred continuing education units in clinical laboratory sciences, education and administration. A National Institutes of Health (NIH) Minorities Access to Biomedical Careers (MARC) Fellowship afforded a three year academic (1987-1990) leave for the pursuit of the PhD in Biomedical Science at Old Dominion University.

Professionally, she has been a clinical and research laboratorian or a teacher. From 1964 to 1970 she worked as a research assistant in biochemistry and immunochemistry at Abbott Laboratories, North Chicago, Ilinois. After marriage to a Navy man, she moved to Norfolk and assumed a position as a medical technologist until 1974. In 1974 through a research training program she was invited to teach and revise a technology program at Norfolk State University, Norfolk, VA. Patricia served as director of the medical technology until 1987 when she took academic leave. In 1981 she was selected as "Teacher of the Year" in the School of Health Related Professions and Natural Sciences. She has also received several faculty incentive awards and co-authored a $250,000 research grant funded by NIH funded from 1983 to 1987. Today she continues to serve on the faculty at Norfolk State University.