GOSSYPOL TOXICITY IN

FEEDER LAMBS

By

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INTRODUCTION

Gossypol is a yellow, polyphenolic pigment that is found primarily in the glands of cottonseed (*Gossypium spp.*). [1,2] It exists in two forms; *e.g.* "free" and "bound".[2] For many years the free form has been known to be toxic to monogastric animals, whereas ruminants are able to detoxify large quantities of free gossypol by irreversibly binding it to soluble proteins in their rumen and thus, render it nontoxic.[3]

The gossypol content of cottonseed varies widely depending on the species and variety of cotton plant, climatic and soil conditions of the region, water supply and agrotechnical treatment including the amount and composition of fertilizers used.[4] Free and bound gossypol are both in cottonseed during growth and maturation, but the ratios can change naturally during storage, or by the type of processing done to extract the cottonseed oil.[4] All of these factors influence the amount of free gossypol that is in cottonseed meal, the by-product of cottonseed oil extraction. Because of its nutritive and economic value as a feed for livestock, cottonseed meal is widely used as a protein source in a variety of rations.

During the summer of 1985, five separate sheep farms reported death losses in young feeder lambs that had been on cottonseed meal rations. Necropsy and histopathologic findings resembled those of pigs dying of gossypol toxicity. Cardiac lesions were the most significant observation.

Adult ruminants are able to detoxify large quantities of free gossypol. On the basis of clinical and pathologic observations, detoxification of gossypol in young ruminants may be apparently inadequate. This study was designed to determine if free gossypol has detrimental effects on young ruminants. Eight-week-old feeder lambs were used as experimental animals.

The objectives of this study are: 1) to determine whether or not free gossypol is toxic to eight-week-old lambs, 2) to determine the "maximum tolerated dose" of free gossypol in these lambs, 3) to record observations of disease, 4) to record and evaluate clinicopathologic data from blood and serum, 5) to evaluate electrocardiograms taken before and during experimental feeding of gossypol, 6) to characterize gross and histopathologic lesions, 7) to analyze and quantitate liver, kidney and skeletal muscle for the presence of gossypol.

LITERATURE REVIEW

CHAPTER II

GOSSYPOL

Gossypol in the Cotton Plant

Gossypol [1, 1', 6, 6', 7, 7'-hexahydroxy-5, 5'-diisopropyl 3, 3'-dimethyl-(2, 2'-binaphthalene)-8, 8'-dicarboxaldehyde] is a yellow polyphenolic compound found in cotton plants (*Gossypium spp.*); especially concentrated in the seed.[5] Unlike other plant pigments which are more or less evenly distributed throughout the tissues of an organ, gossypol is contained in separate morphologic formations called pigment glands.[4] These can be seen as tiny black dots on the cut surface of cottonseed.[6] They are characteristically ovoid, existing exclusively in the cotton plant and are unevenly distributed throughout the thickness of the cotyledons.[4]

Gossypol glands are found not only in the seed of the cotton plant, but also in the bark of the roots and stalk, leaves, boll

valves, seed hulls, pericarp and flowers. Up to 3.0% gossypol has been found in the root bark but the main concentration is in the seeds. The gossypol content of cottonseeds varies within a very wide range from 0.002 to 6.64%.[4] The gossypol content is affected by the species and variety of the cotton plant (*G. barbadense* is believed to contain more glands and more gossypol than *G. hirsutum*), on climatic and soil conditions of the region, water supply, agrotechnical treatment, amount and composition of fertilizers used and any great changes in plant growth during the vegetative period.[4,7]

The role that gossypol plays in the life of cotton plants is still unknown but it is suspected that the physiologic function of gossypol is connected with its antioxidant properties.[4]

History of Discovery and Isolation

Gossypol, was first isolated by Longmore in 1886 from black cottonseed oil previously treated with lime.[8] He was interested in the pigment as a dye for wool and silk but it proved to be unsuitable for this purpose due to its many impurities.

In 1899, Marchlewski purified and refined this pigment by treating an ether solution of it with acetic acid, thus precipitating gossypol-acetic acid, a product of their interreacation.[8] This method is still used today because the compound it forms is more stable than gossypol alone and is more useful for experimental

purposes. Marchlewski ascertained the polyphenolic nature of the pigment and therefore called it gossyp (ium-phenol) - "gossypol".

As early as 1915, Withers and Carruth demonstrated that gossypol could be removed from unprocessed cottonseed kernels by mild extraction with diethyl ether.[1, 9] Later Carruth, Clark and others showed that the conventional wet cooking of cottonseed prior to oil extraction led to deep seated changes in the gossypol present in the kernels. After cooking, most of the gossypol was insoluble in diethyl ether, but could be extracted in hot aniline. Carruth called this difficult-to-dissolve gossypol, D-gossypol but Clark later suggested the term, "bound gossypol", and postulated that it was formed from the reaction of the aldehyde groups of gossypol and free amino groups of the protein. From this, two concepts gradually formed concerning the gossypol present in cotton seed meal. The portion of gossypol soluble in diethyl ether was termed, "free gossypol", and was assumed to be the same compound in the unprocessed kernels. After removal of free gossypol, extraction with hot analine removed the remaining bound gossypol.[1]

Structure and Physical Properties

Clark determined the molecular formula of gossypol to be $C_{30}H_{30}O_8$ but Adams and his students published papers in 1937-1941 elucidating the structure of the gossypol molecule (Fig 1).[1, 4] The postulation of three tautomeric structures was necessary to explain many of the reactions of gossypol.

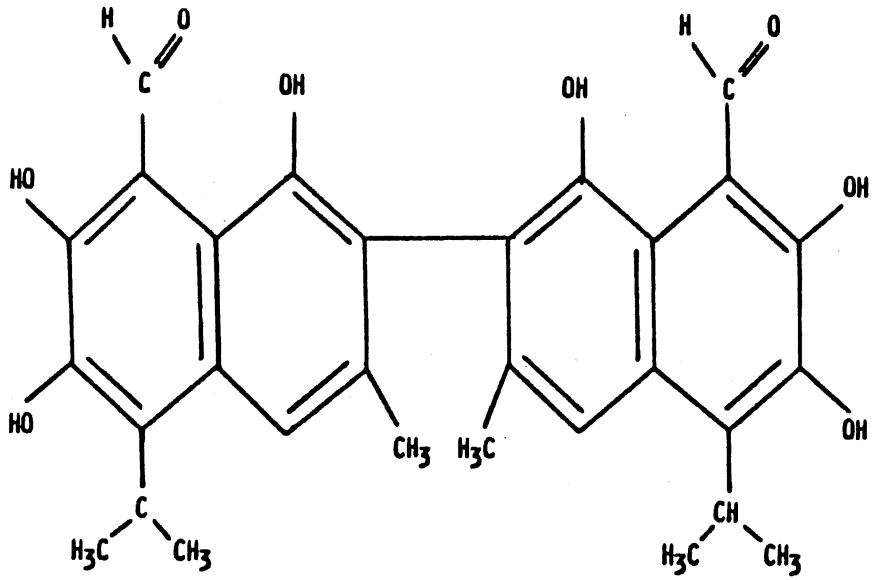

Fig 1 -- The chemical structure of gossypol [1,1^{1},6,6^{1},7,7^{1}-hexahydroxy-5, 5^{1}-diisopropyl 3, 3^{1}-dimethyl-(2,2^{1}-binaphthalene)-8, 8^{1}-dicarboxaldehyde].

Gossypol is a lemon yellow crystalline substance which is soluble in most organic solvents. It contains many polar groups but the presence of two heavy dialkylnaphthalene groups makes it insoluble in water.[1, 4]

Gossypol is a very reactive, unstable compound which is easily oxidized. Because of this, the melting point has not yet been accurately determined. Various investigators have reported figures ranging from 180 to 214°C. It is known to have distinctively different melting points when crystalized in different solvents. Some believe this to be a polymorphism phenomenon.

Other Pigments of the Gossypol Group

There are other pigments which are structurally related to gossypol. Some are in the glands while others are transformation products of gossypol found in cottonseed oil. Boatner reported the presence of at least 15 gossypol pigments or derivatives in extracts of cottonseed or cottonseed oils and meals, but only about 8 have been isolated in a more or less purified form.[8]

A purple pigment that is found in very low concentrations in gossypol glands can easily be separated from gossypol by various absorption techniques. This pigment was named gossypurpurin and is structurally similar to gossypol. Although it is stable in the solid state, it decomposes quickly in solutions and changes to a yellow substance that is not identical with gossypol.[4] Some researchers

believe it to be a product of oxidative transformation of gossypol but this has not been proven. The gossypurpurin content of cottonseed increases during prolonged storage, the amount depending on the temperature and period of storage. An unstable "red gossypol" has been shown to be a mixture of gossypol and gossypurpurin.[8]

Gossyfulvin is an orange pigment found in ether extracts of cottonseed and can be easily converted to gossypol by treatment with acids. It is not an innate pigment of cottonseed but a transformation product of gossypol formed by reactions with proteins or their degradation products. Isolation of this pigment has only been possible when cottonseed has been stored under conditions of high moisture and, consequently, to some extent deteriorated.[4]

"Cottonseed blue" named gossycaerulin was the dark blue color produced when cottonseed meats were heated. During this process the gossypol content decreased simultaneously with the increase in gossycaerulin.

More recently another pigment, named gossyverdurin ("cottonseed green"), was detected in the gossypol glands. This compound was found to be more toxic than gossypol itself but was found in such low concentrations that much speculation was made over the toxicity of gossypol glands being due to more than one component.[4, 10, 11]

Gossypol Transformations During Industrial Cottonseed Processing

The basic processes of vegetable-oil extraction from oil containing raw material, mainly oilseeds, are similar for most

species and details of the processes vary according to the specific properties of the different oilseeds. There are two characteristic properties of cottonseeds that influence processing.

One property is the oil content in the seeds. If it is low, the oil extraction is usually performed by a single operation of pressing (hydraulic or screw pressing) or, more often, solvent-extraction. Soybeans containing about 20% oil are processed by solvent-extraction.

If the oil content is high, two consecutive oil-extraction operations are used: prepressing and solvent extraction. This applies to cottonseed which contains 35-40% oil after removing most of the hulls.

The second property of oilseeds is whether or not the seed coats (cottonseed hulls) are easy or difficult to remove, so that the oil is extracted from the whole seed without previous destruction and separation of the seed coat.

Cottonseed has two other factors that influence the flow sheet of their processing. One is the presence of lint, some of which still remains after ginning and delinting, and the other is the presence of gossypol. Because of toxicity problems in animals (to be discussed later), it is desirable to have low levels of free gossypol in the final product which is cottonseed meal.

Many reactions have an adverse effect on the quality and yield of cottonseed meal. Therefore, limiting undesirable transformations

of the seed components during processing is one of the most important ways of improving both the product quality and the utilization of raw material.[4]

Because of the unique characteristics of gossypol, (very unstable and polyfunctional), it is able to react in various ways. The transformations of gossypol may be divided into two types: changes in gossypol itself by the influence of heat and oxygen in the air; various interactions between gossypol and other cottonseed substances.[4] Each of these transformations can take place not only during the processing of cottonseed and cottonseed oil, but also, to a certain degree, during ripening and storage of the seed. During the traditional extraction of cottonseed oil, the seeds are heated to high temperatures for a relatively long period of time before being mechanically pressed to expel the oil. When the seeds are crushed by rollers, the glands are not destroyed, but fall out of the cotyledons together with their envelopes. Water and polar organic solvents containing water will rupture the envelope and release the pigments.[4] Free gossypol is inactivated by the passage of time by reactions which can occur spontaneously in the meal. The amount of free gossypol present in the meal at any time depends upon the degree to which the glands have been ruptured and the degree of subsequent destruction of gossypol by reaction with other compounds. The term "bound gossypol" has been used to denote gossypol which has reacted during the milling process in such a way as to be converted into a nontoxic molecule.[6]

The covalent binding of gossypol to proteins during cooking of cottonseed kernels is now used extensively to inactivate the toxic action of free gossypol in oil cake and meal. However, complete inactivation of gossypol in cakes and meals can hardly be expected, as even under extremely high moisture and heat treatment (115-130°C for 2 hours at 15-20% moisture in the meals) a large quantity of gossypol still remains in the form of unstable hydrolyzable gossyproteins, from which gossypol may be freed by hot aniline or oxalic acid solution.[4]

The hydraulic press and direct solvent methods usually result in a product containing more than 0.04 per cent free gossypol while the screw press, prepress solvent, and direct solvent methods followed by procedures for chemical inactivation of free gossypol result in meal containing less than 0.04 per cent active gossypol.[6] This is due to the fine grinding of kernels, intense steaming of the ground material before it enters the cookers and subsequent heating to high temperatures during the cooking process.[4] The Soviet Union has strict standards for cottonseed meal which limit their content of free gossypol to not more than 0.02% on a dry matter basis.[4]

During storage of cottonseed, gossypol reacts with other substances contained in gossypol glands and with the proteins of the seed, thus yielding bound gossypol. The higher the moisture content of stored cottonseed and the temperature of self-heating, the larger the amount of bound gossypol, the increase sometimes reaches 11-to-14 fold.[4]

It has been demonstrated that free gossypol as an isolated pure substance loses some of its toxic activity within a few days when mixed in commercial feeds. Furthermore, a high protein level (providing amino groups) in the diet is protective against the effects of gossypol poisoning.[6]

Although these factors may solve some of the toxicity problems, this same binding of gossypol to proteins in turn brings about an equivalent reduction in the nutritive value of the residue protein, in particular lysine.[3,4,6,10,12] As the level of bound gossypol increases, there is a concomitant decrease in the percentage of lysine molecules that have free epsilon amino groups.[13,14]

Milling procedures have been investigated to find commercially feasible methods to insure meal of consistently low gossypol content and retain the high protein value of the raw seed. Certain solvents may be used for extraction which do not require high-heat cooking in advance of extraction, but the most useful of these, hexane, does not extract or cause much inactivation of gossypol. Cottonseed meals prepared by this process may be highly toxic because they typically contain as much as 0.6 percent free gossypol. Laboratory procedures based upon breaking up cotton seed flakes into very fine particles and subsequently removing pigment glands by flotation or differential sedimentation have been successful but have not been utilized commercially. Other factors which limit the effective reduction of toxicity of cakes and meals is the presence of genetically related toxic substances other than gossypol. In 1947, Boatner found that isolated gossypol glands were much more toxic than gossypol itself.[15]

These findings were confirmed by Eagle and many others.[4] The toxic substance localized in the glands was very difficult to inactivate by heat treatment and prolonged storage.

In 1963, Lyman detected and investigated gossyverdurin, whose toxicity is tenfold that of gossypurpurin, almost fourfold that of gossypol and 1.7 times that of the gossypol glands themselves.[8] The comparative toxicities and the content of the separate components of gossypol glands are summarized in Table 1.

It is possible that other highly toxic, but still unknown compounds exist in the glands, which could occur according to the laws of multiplicity of substances formed by biosynthesis in the general formation scheme.[4,8]

TABLE 1--Toxicity of individual identified components of gossypol glands

Substance	Approximate content in glands (%)	Lethal dose (LD_{50}) for rats, (mg/kg)
Gossypol glands	100	1,120
Gossypurpurin	0.15 - 3.0	6,680
Gland residue after acetone extraction	35 - 45	6,000
Gossypol	24 - 40	2,570
Gossyverdurin	2.0	666

PHYSIOLOGICAL ACTION OF GOSSYPOL

Early Studies of Monogastric Gossypol Toxicity

In 1915 Whithers and Carruth extracted from cottonseed kernels the same substance that Longmore and Marchlewski had separated from crude cottonseed oil.[9] They found their material to be toxic to rabbits and published several papers concerning this. It was their work combined with subsequent work done by Schwartze in 1923-26 that made a positive correlation between the toxicity of raw cottonseed and gossypol content, and led to the general belief that the toxicity of cottonseed can be attributed solely to its gossypol content.[11] Once gossypol had been isolated, the content in the seeds could be determined. Various LD_{50} studies on rats, mice, rabbits and guinea pigs were taken using different combinations of gossypol, pigment glands, oil, water, etc.[11]

In 1947 Zucker and Zucker published papers stating that gossypol was an appetite depressant and that purified gossypol had no toxic properties. The widespread publicity relative to the possible use of gossypol in the treatment of obesity in man prompted Eagle to determine the effect of small daily doses on the body weight and food consumption of the dog. He gave four littermates nineteen doses of 0, 50, 100 and 200 mg of gossypol per kg. of body weight over a period of 37 days. On the fourth and fifth day after the last dose, the three dogs receiving gossypol were found dead. These and other dogs he experimented with had lassitude, diarrhea, anorexia and weight loss. Vomiting occurred at the highest dose level. On gross

necropsy there were hemorrhagic intestines, hydrothorax, edema of the lungs, excessive fluid in the peritoneal cavity, hydropericardium and congestion of the splanchnic organs.[11] Eagle did similar studies with rats but concluded that the greater mortality and body weight depressions caused by adding various levels of cottonseed pigment glands to the diet could not be attributed solely to their gossypol content.[11]

Lillie and Bird in 1950 studied the effect of gossypol on chickens and concluded that the reduction in weight gain and deaths proved to be proportional to the amount of gossypol consumed.[4] Five years later Heywang and Bird determined the maximum allowable content of gossypol in chicken rations and found that it should not exceed 0.16-0.20%.[4]

Cottonseed meal has been used as a concentrated source of protein for the feeding of livestock for many years. Toxicity problems were reported as early as 1911 but many of the cases in the past may have been the result of Vitamin A deficiency.[16] It is now known that cottonseed meal is low in lysine and tryptophan and deficient in Vitamin D, carotene (Vitamin A value) and calcium, contains high levels of phosphorus and varying amounts of gossypol.

Most recorded outbreaks of gossypol poisoning refer to pigs.[18] Clinical symptoms and the lesions caused by gossypol were documented by Smith in 1956.[16] During feeding trials on 18 pigs, the most seriously poisoned animals had signs of illness that were typically apparent for 2 to 6 days, or exceptionally as long as one month. The outstanding symptom was always dyspnea, with violent labored respir-

ations which stockmen refer to as thumping. Progressive weakness and emaciation were accompanied by a good appetite almost until death. The gross and microscopic lesions that were described are considered to be characteristic for cumulative poisoning by gossypol.

Gross lesions seen in the majority of the pigs were: hydrothorax, congestion and edema of the lungs, hydropericardium, dilatation of the heart, hydroperitoneum, edema of the lymph nodes, congestion of the liver and kidney and white skeletal muscles. In some of the pigs there was subcutaneous edema, hypertrophy of the heart, edema of the gallbladder and icterus.

Histopathologically, almost all pigs had: congestion and edema of the lungs, degeneration and hypertrophy of the heart, severe congestion and loss of hepatocytes in the liver, congestion of the spleen, congestion of lymph nodes and kidneys with cloudy swelling in the renal tubules.

Most of the lesions were attributed to a progressively failing heart. Microscopic changes in the myocardium, which were interpreted as compensatory hypertrophy, were found to some degree in practically all of the hearts. These changes consisted of increased size or number of muscle nuclei, or both, often accompanied by the presence of muscle fibers of unusually large diameter.

The pale or white skeletal musculature could not be explained microscopically other than by the presence of an abnormal variation in the size of certain fibers, some being atrophied and other hypertrophied.[16]

These lesions were nearly identical to the lesions previously described by West in dogs.[19]

Ruminant Detoxification of Gossypol

It has been known for many years that cottonseed meal containing free gossypol produced toxic symptoms if fed to swine or chicks but relatively large amounts could be fed to ruminants with no sign of toxicity.[3]

Results from injections of free gossypol intravenously in 31 crossbred wethers indicated that if the rumen was by-passed, sheep had pathologic changes similar to those of nonruminants. When gossypol was fed at high levels to sheep in a semipurified diet for 70 days, the sheep did not have internal or external signs of toxicity.[20]

In 1976, Reiser and Fu demonstrated that the mechanism of ruminant detoxification of gossypol was by binding to soluble proteins in the rumen and that the bond was permanent during protein digestion.[3]

Gossypol Toxicity in Adult Ruminants

In the early 1900's ruminants were considered subject to gossypol toxicity in the form of "cottonseed meal injury". However, by 1930, it had been demonstrated that cottonseed meal injury was attributable to Vitamin A deficiency rather than gossypol in the ration. Subsequently, it was not until the 1980s that gossypol toxicity was reported in adult ruminants.[21] Most of the recent

literature still considers gossypol toxicity as a problem that affects only monogastric animals.[8,17,22]

Reasons for the recent recurrence of problems with gossypol and adult ruminants could be: recent trends toward increasing protein in dairy rations, increased feed intake by genetically superior animals and excessive use of cottonseed and cottonseed meal in rations for economic reasons.[21,23]

A feeding trial was done in 1980 by Lindsey, Hawkins and Guthrie to investigate physiologic effects of feeding high levels of cottonseed meal (CSM) to high-producing dairy cows in early lactation and to determine if gossypol toxicity could be demonstrated in mature ruminants consuming cottonseed meal. Twenty-four cows received either soybean meal, screw-pressed CSM, or direct solvent extracted CSM. Reduced milk production and dyspnea during hot weather was the only physical change noted and it was only in the group receiving solvent extracted cottonseed meal. The major physiologic effects were decreased hemoglobin and increased erythrocytic fragility which did not appear to be detrimental unless the animal was subjected to physiologic, nutritional and/or environmental stress.[21]

The recommended amount of whole cottonseed per cow per day is 4-6 pounds.[3] In the spring and summer of 1980, a syndrome which resulted in mortalities up to 10% occurred in six dairies in the Phoenix area. All herds in which death occurred were being fed high levels (6-10 lb/head/day) of ammoniated cottonseed. This same amount had been fed in previous years without problems. The levels of

gossypol in the cottonseed from these dairies ranged from 1700 to 6800 ppm in ammoniated seed versus 9100 ppm in untreated seed.

One herd that had no deaths attributable to gossypol toxicity did have a severe reproductive problem. Gossypol has been shown to be an effective male contraceptive in both laboratory animals and male human subjects by decreasing sperm motility and sometimes complete arrest of spermatogenesis. It has also caused irregular estral cycles, decreased incidence of pregnancy and a reduction in the number of viable embryos in female laboratory animals. Because of this, it is conceivable that gossypol may also be responsible for reproductive problems in ruminants.[2,5,7,24,25] The lesions in the dairy cows mentioned above resembled those of swine with gossypol toxicity and were accompanied by a severe abomasitis. Some cows had blood in their urine. Gossypol was not found in the milk. Tissue levels of gossypol can be found in most cows fed cottonseed products, it is not diagnostic without concurrent clinical or postmortem indications of gossypol toxicity.[2]

Gossypol Toxicity in Young Ruminants

A study was done in 1958 by Hollon, *et al.*, to determine the responses of young calves to starters containing cottonseed meals produced by different methods and containing different concentrations of free gossypol.[26] Their observations substantiated the prevalent view that young calves were more susceptible to gossypol toxicity than older calves. On the basis of the relationship of age to the tolerance of free gossypol ingestion, the responses corresponded to

the functional development of the rumen. They conceived that free gossypol became diluted in the rumino-reticular contents and either complexed with ruminal constituents or disintegrated to such an extent that the rate and degree of absorption of free gossypol was reduced.

From the studies mentioned above, a recommendation of not more than 0.01%, (the level tolerated by fattening-growing pigs), free gossypol in the total diet be fed to young calves, particularly those under four months of age.[26]

In 1975, an article written by Rogers, Henaghan and Wheeler, suggested that cottonseed products may be highly toxic to preruminal calves and lambs because the protective mechanism of the rumen in binding gossypol to proteins, was absent or poorly developed.[18] There have been numerous reports of gossypol toxicity in calves, but gossypol toxicity in lambs has not been reported in this country.[18,27]

Although it would seem that the recommended level of free gossypol in the diet of young lambs would be similar to that of young calves, there has never been a reported case of gossypol toxicity in adult sheep. Therefore, lambs may be more resistant than calves.

Because more lambs are placed in feedlots today than ever before and nursing lambs are often placed on creepfeeders, there is an increased usage of concentrate and thus cottonseed and cottonseed meal as an economical protein source.

During the summer of 1985, five farms in Oklahoma lost 3-4 month old lambs that were on cottonseed meal as their protein source. They

had lesions resembling those seen in pigs that died from gossypol toxicity.[28] The most characteristic lesions were seen in the heart which are unique to very few known toxins which will be discussed in the following section.

GOSSYPOL AND METALS

As early as 1913, Withers and Brewster reported that iron was an antidote to cottonseed meal toxicity. They believed that gossypol and iron formed an insoluble complex which prevented the gossypol from being absorbed from the gastrointestinal tract.[11]

In 1965, iron given as ferrous fumarate supplement to cottonseed meal rations for swine prevented the 100% toxicity (50% mortality) which occurred in pigs receiving unsupplemented diets.[29]

Menual, in 1922, using sheep erythrocytes found that gossypol prevented the release of oxygen from oxyhemoglobin and *in vitro* caused lysis of erythrocytes.[30] He postulated that gossypol produced death by reducing the oxygen carrying capacity of the blood. Studies in fowl supported this conclusion.[31] From these experiments it was believed that the beneficial effects of iron were the result of its value in hematopoiesis.[6]

In 1980, Lindsey, Hawkins and Guthrie reported lower hemoglobin levels, increased erythrocytic fragility and increased extravascular hemolysis in dairy cows on cottonseed meal rations. These findings led them to speculate that the hemolytic activity of gossypol caused anemia rather than a gossypol-induced iron deficiency.[21]

The inactivation of gossypol with mineral salts has been studied and it was determined that gossypol reacts as a strong dibasic acid and forms neutral disodium and dipotassium salts. The sodium, potassium, calcium and barium salts are soluble in water, whereas the lead and iron salts are insoluble. Sodium gossypolate reacts with

ferrous ions in a 1:1 molar ratio and it was postulated that the perihydroxyls (1-hydroxyls) of gossypol are the site of reaction with ferrous ions. The same site was believed to be the combination point when gossypol reacts with the ferric ion in a 1:1 molar ratio. The addition of calcium ions to soluble ferrous-gossypol chelates renders them insoluble and suggests that a similar occurrence may take place during the biological synergism of calcium and iron to inactivate gossypol.[8]

GLANDLESS COTTONSEED

Cottonseed oil is the most valuable of the cottonseed products. Almost all of the cottonseed oil produced in the United States is used in the manufacture of edible products such as salad oil, margarine and shortening; but because of the gossypol pigments which are quite resistant to removal, almost 25% of crude cottonseed oil cannot be refined and is not acceptable for the manufacture of high-quality shortening. Because of this, many processors operate their mills under conditions that confine most of the gossypol pigments to the cottonseed meal. The presence of gossypol presents two problems that can not be easily separated. The presence of high levels of free gossypol causes unfavorable physiologic effects and the reaction between gossypol and protein during processing reduces protein quality, especially by decreasing the biological availability of lysine.

Cottonseed meal is the second largest U. S. source of vegetable protein concentrate. However, it is not a product of uniform quality and has wide variability in gossypol content. Because cottonseed flour is being investigated as a protein supplement in human foods, the levels of gossypol in body tissues would be an important finding.

It seems that a glandless cottonseed would solve all the detrimental attributes of cottonseed products, but in the last decade it has become apparent that the pigment gland contents may provide resistance to certain insects, decreasing the need for insecticides.

Because of this, the glandless variety of cotton has never become widely cultivated.

MYOCARDIAL NECROSIS IN LAMBS

The list of myocardial diseases that affect animals is long, but very few cardiotoxins with known etiologies have been reported in sheep.[32]

Focal myocardial necrosis is frequently observed as an incidental finding on microscopic examination in a variety of diseases, and its immediate pathogenesis can seldom be ascertained. The lesions have been thought to have an ischemic basis and have been accounted for by the normal pattern of end-capillary anastomosis in the myocardium. Myocardial degeneration, mineralization and necrosis are most common in the subendocardial myocardium because this area undergoes the greatest intramyocardial tension during systole and is the most likely to be ischemic. Although variations in the microscopic appearance of cardiomyocytic necrosis have been seen, few conclusions can be drawn when attempting to associate the differing microscopic patterns with particular causes.[22]

Nutritional vitamin E-selenium deficiency causes necrosis of myocardial and skeletal muscle. In lambs, pale myocardial lesions have been seen grossly in the subendocardial myocardium of the right ventricle and throughout the skeletal musculature. Histologically, areas of myocardial damage have hyaline necrosis with or without accompanying mineralization, macrophage invasion and areas of stromal collapse with fibrosis. Loss of distinct striations are noted in heart and skeletal muscle with swollen and fragmented fibers.[32,33]

Monensin toxicity in lambs has been well documented. Both heart and skeletal muscle are affected as evidenced by vacuolation and intracellular edema of muscle cells. These are followed by segmental necrosis, interstitial fibrosis and macrophage infiltration in areas of severe necrosis. Skeletal muscle is affected more severely than cardiac muscle.[34,35]

Myocardial necrosis has been described in horses as a result of blister beetle poisoning.[36] Deaths in lambs due to blister beetle poisoning have been reported and confirmed by the presence of cantharidin in their rumen contents, but heart lesions have not been documented.[37]

Several poisonous plants are known to cause cardiac lesions but fortunately these can be ruled out in a feedlot situation unless the hay contains large quantities of weeds. Bracken fern (*Pteridium agilinum*), white snakeroot (*Eupatorium rugosum*), coffee senna (*Cassia occidentalis*) and lantana (*Lantana camara*) have all been reported to cause myocardial necrosis in ruminants.[22,38,39] Similar myocardial necroses have been reported in swine and horses.

CHAPTER III

MATERIALS AND METHODS

Animals and Facilities

As a pilot study, five 10-week old preconditioned (access to grain since birth) lambs, were obtained to determine if free gossypol given orally at a dose rate of 817 mg/hd/day was toxic to lambs. After obtaining positive results on the pilot study, twenty more 8-week old preconditioned lambs were obtained from another source. The lambs were divided randomly into groups of five. The breed and sex of each animal in the five groups are presented in Table 2. Group E is the pilot group.

During the 30-day course of the study, the lambs were kept in three large stalls which were bedded with wood shavings. Some members of Group A (control) were kept in each stall with members of other groups. Their diet consisted of water, free choice alfalfa hay and a free choice pelleted ration that was composed of: 50% ground corn, 35% ground alfalfa hay, 10% soybean meal, 5% molasses.

TABLE 2--Signalment and amount of gossypol received in each group of lambs

Group A control no gossypol		Group B 45 mg gossypol		Group C 136 mg gossypol		Group D 409 mg gossypol		Group E 817 mg gossypol	
Breed	Sex	Breed	Sex	Breed	Sex	Breed	Sex	Breed	Sex
R	W	R	W	R	W	R	W	R	F
R	W	R	W	RS	F	RS	W	R	F
R	W	RS	W	R	W	R	W	D	F
RDS	F	R	W	R	W	R	W	D	W
RS	W	RDS	W	RS	W	RS	W	R	F

R = rambouillet; D = dorset; S = suffolk; W = wether; F = female.

Five days before the project began, the lambs were wormed with fenbendazole and vaccinated against tetanus and *Clostridium perfringens* Type D. They received individual metal ear tags and were weighed initially and weekly. The lambs were observed at least twice a day for appearance and signs of general health. Lambs in Group A did not receive gossypol, but otherwise were treated identically to the other groups.

Gossypol

The free gossypol used in this experiment was donated by the U.S.D.A. Southern Regional Research Center in New Orleans, Louisiana. It was a 95% pure extract in a 1:1 molar addition complex of gossypol with acetic acid. This complex is more stable than gossypol alone and disassociates in solution.[40] The free gossypol, which is a yellow powder, was weighed to the nearest milligram[a] and placed in individual gelatin capsules, size 00.

Dosing Regimen

The quantity of free gossypol administered daily to each group of lambs was based on the various levels of free gossypol determined

[a]Mettler PR700 top loading balance, Mettler Instrument Corp., Anaheim, California.

to be present in commercial feeds and on the fact that 8-week-old lambs eat approximately one pound of grain per head per day.[17]

Since free gossypol is measured in feed in parts per million (ppm); 100 ppm = 100 mg gossypol/kg of feed. One kg = 2.2 pounds, therefore 45.4 mg of gossypol was needed per pound of feed to equal 100 ppm gossypol. Therefore, 45 mg of gossypol is approximately equal to one pound of feed containing 100 ppm gossypol, 136 mg of gossypol, 409 mg of gossypol is approximately equal to one pound of feed containing 900 ppm gossypol and 817 mg of gossypol is approximately equal to one pound of feed containing 1800 ppm gossypol. Table 2 lists the amounts of free gossypol given daily to each lamb in each group for 30 days.

The gelatin capsules were placed in a small-animal rubber tipped pill gun and given orally to the lambs each morning. The starting dates for each group were staggered so that experimental treatment of each group would terminate on consecutive days rather than all on the same day.

Blood Collection

Blood samples were obtained by jugular venipuncture on all lambs the day before treatment began and once a week during the experiment. The blood was placed in heparinized tubes for hematologic examination and clot tubes for serum chemistries. All blood samples were taken directly to the laboratory for immediate analysis.

White blood cells (WBC), hemoglobin (Hb), hematocrit (Hct), total protein (TP) and differential white blood cell counts were done on the whole blood.

Total bilirubin (Bil T), creatinine phosphokinase (CPK), creatinine kinase isoenzyme (CKMB), glutamic pyruvic transaminase (SGPT), glutamic oxalacetic transaminase (SGOT), lactic dehydrogenase (LDH) and liver lactic dehydrogenase (LLDH) were the serum chemistries that were analyzed.[a]

Electrocardiograms

Electrocardiograms were recorded on ten lambs prior to the experiment and once a week until the project was completed. Four lambs were from the control group and 6 lambs were from treated groups. The recordings were made while the lambs were suspended in an upright position using the Bipolar Standard Limb Leads on a Honeywell AR-6 Simultrace Recorder.

[a] DuPont ACA^{R} Automatic Discrete Clinical Analyzer, E.I. DuPont de Nemours and Co. Inc., Clinical and Instrument Systems, Wilmington, Delaware.

Gross Pathology

Systematic necropsies were done on each sheep when they died or after euthanasia with sodium pentobarbitol intravenously at the end of 30 days.

Since Group E was done as a pilot study and all of the lambs died before 30 days, many of the tissues which did not have any lesions visible by light microscopy in 100% of the lambs of Group E were omitted from the list for the remaining 20 lambs. The following tissues were taken from each lamb in Group E; tongue, esophagus, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum near the ileocecocolic junction, cecum, spiral colon, distal colon, diaphragm*, serratus ventralis muscle, trachea, diaphragmatic and cardiac lobe of the lungs*, kidney*, adrenal, bladder, cerebrum*, cerebellum*, pituitary, trigeminal ganglion, spleen*, pancreas, ovary and uterus or penis, thyroid, thymus, eyeball, salivary mandibular gland, parotid salivary gland, buccal mucosa and mediastinal, mesenteric and thoracic lymph nodes. The left ventricular papillary muscle*, right ventricular papillary muscle*, left atrium*, interventricular septum*, wall of left and right ventricle and any area that appeared unusual were taken from the hearts.

Sections from the right*, left* and caudate* lobe of the liver were taken.

All tissues were fixed in buffered 10% formalin except the eyeballs which were fixed in Bouin's fixative.

The tissues followed by an asterisk (*) were taken from Groups A-D.

The liver, one kidney and a large piece of semimembranosus muscle were taken from each lamb and frozen for toxicologic analysis.

The wet weights of the heart, lungs, and liver were obtained from Groups A-D.

Pericardial fluid was measured and evaluated for color and consistency in each lamb.

Histopathology

Sections for histopathologic examination were embedded in paraffin (processed in a Lipshaw Trimatic automatic tissue processor[a]), cut 4 to 6 micrometers thick, stained with hematoxylin and eosin in a Fisher automatic stainer.[b] The severity of lesions, particularly in the heart, liver and lungs were graded as none, mild, moderate or marked (explained in detail in the results).

Photomicrographs of histologic lesions were taken with an automatic camera[c] capable of exposing negatives. The negatives were printed for preliminary evaluation by contact exposure and selected photomicrographic enlargements were reproduced for this publication.

[a] Lipshaw, Detroit, Michigan

[b] Fisher Scientific, Pittsburgh, Pennsylvania.

[c] Orthomat^{R}-W, 35 mm, E. Leitz, Inc. Instrument Div., Rockleigh, New Jersey.

Special stains that were used included: Phosphotoungstic acid hematoxylin stain (PTAH) for striated muscle, Hall's stain for bile and Perl's stain for iron.

Gossypol Extraction and Quantitation of Tissues

The liver, kidney and semimembranosus muscle were extracted and analyzed for levels of free and bound gossypol using the technique described by F. H. Smith for swine livers.[41] The extraction and analyses procedures can be found in Appendix A.

CHAPTER IV

RESULTS

Clinical Observations

The clinical observations will be discussed separately for each group. Table 3 is a record of the amount of weight gained by each lamb throughout the experiment.

Group A (Control) appeared clinically healthy and in good condition throughout the experiment. An average gain was 0.3 kg/day during the 30 day trial.

Group B received 45 mg/head/day of free gossypol. The amount of gossypol per kg (of lamb) is calculated on Table 3. Averaged gain was 0.27 kg (bodyweight)/head/day during 30 days and all appeared clinically healthy throughout the experiment except #4. During the final week of the experiment, he appeared depressed and was much more easily restrained than in previous weeks. He gained only 0.06 kg per day, compared to the rest of this group which averaged a gain of 0.32 kg per day. He did receive more gossypol per kg of body weight than

TABLE 3--Amount of gossypol (mg) received per Kg of body weight (BW), initial weight, final weight and average daily gain (ADG)

Group	Animal	gossypol mg/kg BW	Initial Weight (kg)	Final Weight (kg)	ADG (kg)
A	1	0	15.9	24.1	0.3
	2	0	15.5	26.8	0.4
	3	0	18.2	27.7	0.3
	4	0	17.3	27.1	0.3
	5	0	18.2	30.0	0.4
	Ave	0	17.0	27.1	0.3
B	1	2.5	18.2	25.2	0.2
	2	2.1	21.8	32.3	0.4
	3	2.9	16.0	24.6	0.3
	4	3.8	11.8	13.6	0.1
	5	2.6	17.3	29.1	0.4
	Ave	2.8	16.9	25.0	0.3
C	1	8.3	16.4	23.6	0.2
	2	12.5	10.9	22.3	0.4
	3	7.9	17.3	30.0	0.4
	4	7.9	17.3	28.9	0.4
	5	12.5	10.9	25.0	0.5
	Ave	9.4	14.6	25.9	0.4
D	1	50.0	8.2	*	0.9
	2	30.0	13.6	23.6	0.4
	3	20.5	20.0	*	-0.1
	4	25.0	16.4	*	0.3
	5	24.3	16.8	*	0.3
	Ave	30.0	15.0	-	0.4
E	1	33.3	24.6	*	0.3
	2	33.3	24.6	*	-0.1
	3	43.8	18.6	*	0.0
	4	39.1	20.9	*	0.2
	5	44.9	18.2	*	0.0
	Ave	38.9	21.4	-	0.1

* Died before day 30.

kg = kilograms; Ave = average.

the other lambs in his group but the amount of gossypol received *versus* the amount of weight gain was inconsistent in all groups (Table 3).

Group C received 136 mg/head/day of free gossypol and appeared clinically healthy throughout the experiment. They averaged 0.38 kg of gain per head/day.

Group D received 409 mg/head/day of free gossypol. At the end of the first week, all lambs appeared healthy and had good appetites. By the end of the second week, it was obvious that they were not consuming as much grain as the other groups of lambs. Their weight gain was also decreased (Table 3). On day 19, lamb #1 was found dead. He had received 18 daily doses of 817 mg of free gossypol. This was the smallest lamb in the entire trial. On day 21, lamb #4 appeared depressed. Compared to the other lambs, this group had extremely wet frothy discharges from their noses. On day 24, lambs #3 and #4 appeared gaunt. Lamb #3 was inactive and laid down much of the time. These two lambs became more and more depressed. Lamb #3 died on the morning of day 25 and lamb #4 was found dead that evening with red froth exuding from his nose. Each lamb had received 25 doses of gossypol. Lamb #5 was found dead on day 28 and lamb #2 was found dead on day 30. These two lambs never appeared clinically ill. Their deaths were unexpected. They gained 0.35 kg/day. The controls gained 0.34 kg/day.

The lambs in Group E received 817 mg/head/day of free gossypol. Their average weight was greater than the rest of the lambs (Table 3) because they were the "pilot group" which was done in the fall. Fall

lambs were scarce and the only lambs that could be obtained were 10 weeks old. On day 9, lamb #5 was found dead in his stall with froth exuding from his nostrils. He had received only 8 doses of gossypol and was not observed to be clinically ill. By day 10, lamb #3 preferred to be recumbent but would get up if approached. The overall per capita consumption of feed had decreased. Lamb #1 was observed to be dyspneic after mild exercise on day 11. On day 12, lamb #1 was found dead and lamb #4 was observed to run around the stall once, fall over, gasp and die. On day 13, lambs #2 and #3 were alert but were not consuming much feed. By day 14, both lambs were noticeably easier to catch and lamb #2 laid down much of the time. Both lambs appeared quite gaunt and weak on day 15. They were icteric and their urine was red (Fig 2). Lamb #3 acted as if it fainted when it was caught on day 17. When lying down it was observed to spread his front legs widely apart in front of its body. On the morning of day 18, both lambs appeared very uncomfortable and kept getting up and lying down. Lamb #2 died at 11:30 A.M. and lamb #3 was euthanatized that afternoon so that proper samples could be taken for future electron microscopic studies of the heart and liver.

In summary, all of the lambs in Groups D and E died during the 30 day experiment. Lamb #4 in group B was the only other lamb observed to be clinically ill. The percent deathloss (Groups A-E) in relation to the milligrams of gossypol received per kilogram of body weight is illustrated in Figure 3. There appeared to be two separate clinical syndromes. One was characterized by a chronic onset of depression, anorexia, dyspnea and death while the other was an acute death without premonitory signs.

Fig 2--Red urine from lamb #2 in Group E.

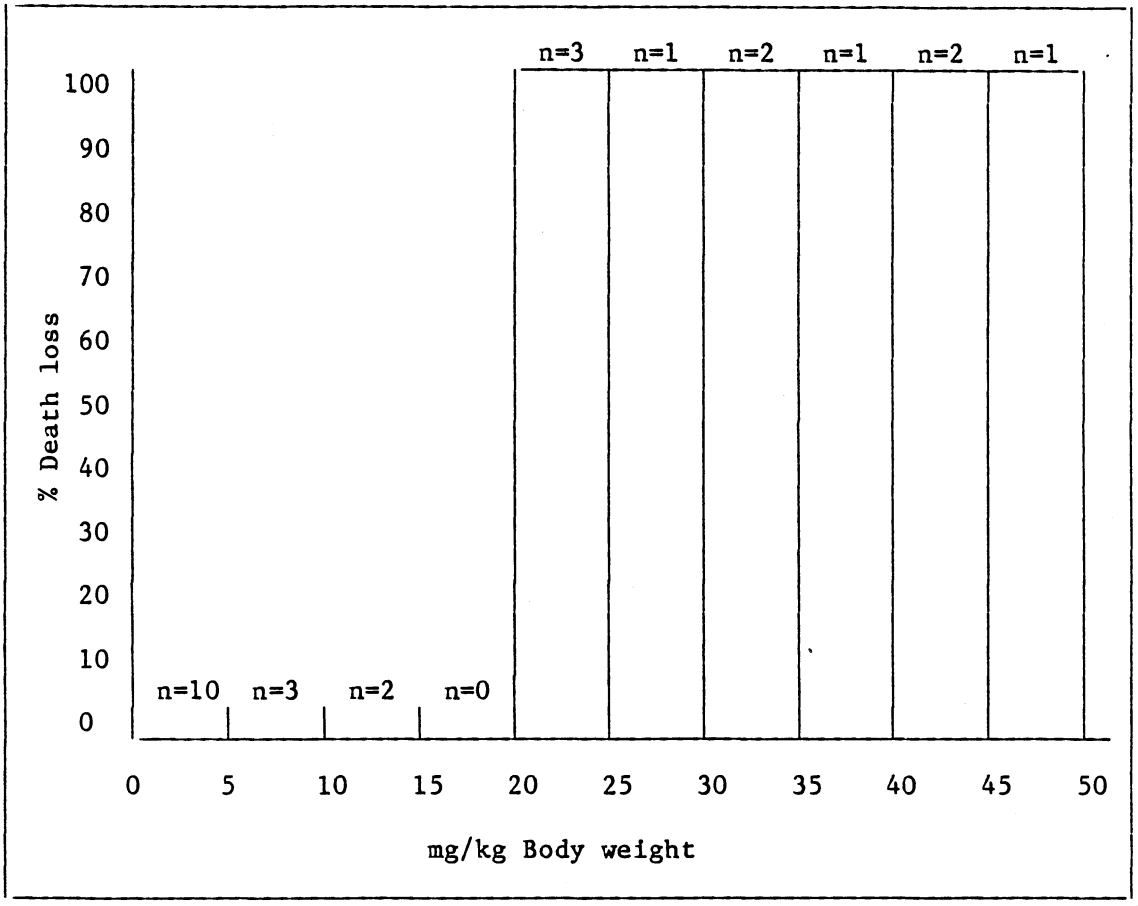

Fig 3--Percent death loss in lambs in relation to the amount of gossypol received (mg/kg body weight) daily, during a 30 day feeding trial.
n = number of lambs

Clinicopathologic Observations

Hematologic examination

Mean hematologic values for each group are presented in Table 4 for each sample collection period. There were no significant differences in the values for each group. A total red blood cell (RBC) count was not done because the small size of ovine erythrocytes caused functional problems in the automatic counter.

Clinical chemistry

Total bilirubin values are presented in Table 5. The control lambs (Group A) had values between 0 and 0.02 mg/dl. Lamb #1 in group C had a value of 12 mg/dl on week 4 which brought the group average above normal. Only 2 lambs were tested in Group E, but their values were well above the control values after the first week and they continued to rise.

Table 6 lists the average weekly values for creatine kinase M-chain B-chain isoenzyme (CKMB), serum glutamic pyruvic transaminase (SGPT), serum glutamic oxalacetic transaminase (SGOT), lactic dehydrogenase (LDH) and liver lactic dehydrogenase (LLDH).

The weekly averages of CKMB for the control lambs (Group A), ranged from 1.2-6.4 international units (I.U.). Group B greatly exceeded those levels on week 3 with an average of 21.6 I.U. Group C exceeded the control values on weeks 1 and 2 only. Group D never exceeded the control values. The levels from Group E fluctuated from 0 to 74 I.U.

TABLE 4--Group mean hematologic values

	Week	Group A	Group B	Group C	Group D	Group E
WBC	0	9.6	13.5	11.4	9.8	10.2
(x 10-3)	1	10.1	13.2	10.5	9.7	11.6
	2	9.4	13.1	10.2	10.3	†
	3	10.4	11.7	9.7	12.1*	†
	4	10.3	14.7	15.5	†	†
Hb	0	12.7	12.9	12.3	12.0	13.8
(g/dl)	1	12.4	12.3	11.8	11.6	14.7
	2	12.3	12.7	11.6	11.6	†
	3	12.0	11.5	11.2	11.0*	†
	4	11.5	11.8	11.2	†	†
Hct	0	37.4	39.4	34.8	36.0	42.0
(%)	1	37.1	37.1	35.0	34.1	43.0
	2	37.2	39.0	34.7	35.2	†
	3	36.2	35.0	34.3	35.9*	†
	4	35.5	36.3	34.7	†	†
TP	0	5.9	6.1	5.9	5.4	6.7
(g%)	1	6.1	6.1	6.1	5.7	6.6
	2	6.0	6.0	6.1	6.0	†
	3	6.1	5.8	6.0	6.2*	†
	4	6.0	6.1	6.1	†	†

* Some of the animals in this group died. This value is the average of the remaining animals. † All animals in the group died.

WBC = white blood cell; Hb = hemoglobin; Hct = hematocrit; TP = total protein.

TABLE 5--Average weekly total bilirubin values

Week	Group A	Group B	Group C	Group D	Group E
0	0	0.13	0.06	0.25	ND
1	0	0	0	0.04	0.84*
2	0	0	0.10	0.07	7.55*
3	0	0.16	0.19	0.32	24.68*
4	0.19	0.02	2.40	0.10*	†

* Some of the lambs in the group have died. † All of the lambs in this group have died.

ND = not determined.

TABLE 6--Average of weekly CKMB, SGPT, SGOT, LDH and LLDH values

	Week	Group A	Group B	Group C	Group D	Group E
CKMB	0	6.4	2.0	9.6	2.8	18.0
	1	4.8	2.8	13.6	1.6	21.2
	2	3.2	2.4	4.8	3.6	9.6*
	3	5.2	21.6	1.2	0.8*	22.0*
	4	1.2	3.2	3.6	0.0*	†
SGPT	0	0.0	1.0	0.8	3.6	5.2
	1	0.4	0.4	2.0	3.2	11.6
	2	0.0	0.4	2.8	3.2	38.0
	3	0.0	2.8	7.6	2.5*	†
	4	0.0	0.0	2.0	0.0*	†
SGOT	0	98.8	129.8	113.6	115.2	104.8
	1	104.8	111.6	115.6	112.0	217.2
	2	93.4	126.8	108.8	115.6	623.0*
	3	128.8	121.2	111.2	163.5*	871.0*
	4	127.6	122.8	128.4	108.0*	†
LDH	0	465.0	365.0	438.0	489.0	429.0
	1	456.0	430.0	484.0	518.0	592.0
	2	436.0	402.0	430.0	472.0	1300.0*
	3	450.0	360.0	461.0	428.0*	1600.0*
	4	411.0	350.0	480.0	540.0*	†
LLDH	0	27.6	17.2	24.4	30.4	ND
	1	32.4	32.0	32.0	27.2	4.0*
	2	22.4	18.0	27.6	14.8	37.2*
	3	23.6	18.8	24.0	16.8*	334.0*
	4	20.4	11.6	44.8	87.0*	†

* Some lambs in the group have died. † All lambs in this group have died.

CKMB = creatine kinase M-chain B-chain isoenzyme, SGPT = serum glutamic pyruvic transaminase, SGOT = serum glutamic oxalacetic transaminase, LDH = lactic dehydrogenase, LLDH = liver lactic dehydrogenase.

The SGPT values for control lambs (Group A) ranged from 0 to 0.4 with Groups B, C and D never averaging above 7.0 I.U. Only Group E had a significantly higher average of 38 I.U. on week 2.

The average SGOT values for control lambs (Group A) ranged from 93.4 to 128.8 I.U. The majority of the values from the other groups were within this range. Group E exceeded it on weeks 1, 2 and 3 with lamb #3 in this group having a value of 1200 before he died.

The average LDH values for Groups A, B, C and D were all within a range of 350 to 540 I.U. Group E had marked elevations in weeks 2 and 3 as high as 1300 to 1600 I.U.

The control values for LLDH were between 20.4 and 32.4 I.U. Group D had an average of 86 I.U. on week 4 and Group E had a marked increase to 334 I.U. in week 3. The rest of the values for each group were close to the control values.

The individual CPK values are given on Table 7. The values in Group A ranged from 90-245. The lowest reading the DuPont automatic clinical analyzer could obtain for this enzyme was a 10. This was considered to be zero and results were written as <10. This occurred in Groups B, C and D, in weeks 3 and 4. In contrast, the lambs from Group E had CPK values that rose as great as 1195 I.U. The CPK values in Group E were never below 85 I.U.

Urinalysis

A complete urinalysis was done on lambs #2 and #3 from Group E on day 16 of the experiment. In both lambs, the significant findings were the clear red color which was predominantly hemoglobin, and the 3+ blood and bile values.

TABLE 7--CPK values

	Group A						Group B						Group C						Group D						Group E					
Week	1	2	3	4	5	Ave	1	2	3	4	5	Ave	1	2	3	4	5	Ave	1	2	3	4	5	Ave	1	2	3	4	5	Ave
0	245	140	105	130	125	149	145	120	70	75	115	105	230	175	130	70	115	146	170	190	145	100	175	156	140	270	295	125	290	224
1	180	135	60	125	90	118	210	110	190	70	120	140	275	140	155	95	235	180	145	100	110	85	155	119	125	90	340	85	160	160
2	90	65	95	110	65	85	<10	<10	190	105	135	90	<10	15	105	<10	<10	30	240	130	15	10	175	114	D	1195	325	D	D	760
3	200	180	170	155	135	168	25	<10	25	<10	35	21	95	35	15	<10	125	56	D	<10	15	80	30	27	D	500	700	D	D	600
4	122	117	167	107	122	127	117	72	122	32	52	69	382	62	42	52	67	121	D	D	D	95	<10	53	D	D	D	D	D	D

CPK = creatinine phosphokinase; D = died; < = less than or equal to.

Electrocardiograms

Bipolar Standard Limb Lead II was used for the measurements, since this lead demonstrates the earliest recognizable electrocardiographic pattern changes. No significant changes between the control group and the treated group were noted for the amplitude (height) or duration of the P wave, QRS segment and the QT segment of the electrocardiograms (Table 8). The most easily recognized change which occurred was a significant ($P < 0.05$) increase in the amplitude of the T wave (Fig 4). The duration of the S-T segment was also significantly decreased ($P < 0.05$) in the gossypol treated lambs. There was no signficant difference in the heart rates of the control versus the treated lambs.

Gross Pathology

All of the lambs in Groups A-C were euthanatized at the end of the 30 day experiment. All of the lambs in Groups D and E died before the 30 day research trial was completed.

The wet weights of the heart, liver and lungs were determined from Groups A-D (Table 9). Treated lambs had heart weights greater than the control group. Pericardial fluid was observed for color, consistency and amount (Table 10).

All lambs contained adequate amounts of body fat. No parasites were found. No significant lesions were found in Group A (control).

TABLE 8--Average electrocardiogram measurements

	mV			mSec		
	P	QRS	T	QRS	ST	QT
Control, n=4	0.19	0.72	0.29*	53	107*	247
Treated, n=6	0.12	0.60	0.69	48	73	250

* Significant difference between control and treatment groups ($P < 0.05$).

mV = milivolts; mSec = miliseconds; n = number of animals tested.

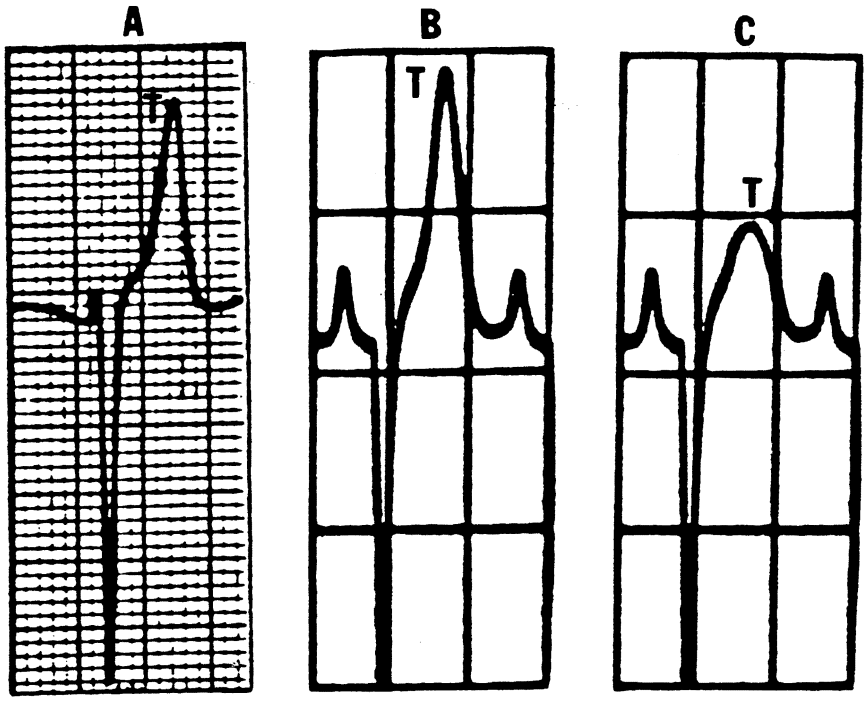

Fig 4--Typical, narrow, symmetrical, tall and peaked T waves (lead II) of hyperkalemia in man (A), are quite similar to those seen in lambs produced by gossypol intoxication (B). Control lambs had T waves of lower amplitude (C).

TABLE 9--Average wet weights of heart, liver and lung

	Lung (% BW)	Heart (% BW)	Liver (% BW)
Group A	1.24	0.54	2.15
Group B	1.67	0.66	2.58
Group C	1.39	0.64	2.36
Group D	3.11*	0.93*	2.97

* Values are significantly different from groups A, B and C ($P < 0.05$).

BW = body weight.

TABLE 10--Amount, color and consistency of pericardial fluid

		Amount (ml)	Color	Consistency
Group A	1	2.5	clear yellow	watery
	2	10.0	" "	"
	3	4.5	" "	"
	4	3.0	" "	"
	5	12.0	" "	"
	Ave	6.4		
Group B	1	12.0	clear yellow	watery
	2	18.0	" "	"
	3	10.0	" "	clotted when exposed to air
	4	8.5	" "	watery
	5	8.0	" "	"
	Ave	11.3		
Group C	1	8.0	clear yellow	clotted when exposed to air
	2	6.0	" "	watery
	3	7.0	" "	"
	4	9.0	" "	"
	5	11.0	" "	watery with several small gelatinous clots (0.5 x 0.15 x 0.5 cm.)
	Ave	10.5		
Group D	1	10.0	clear red	thick with fibrinous clots
	2	17.0	clear red	watery
	3	20.0	red tinged clear yellow	watery
	4	15.0	clear red	watery
	5	8.0	dark red	watery
	Ave	14.0		

Ave = average.

Group B had no lesions except for increased quantities of pericardial fluid in several lambs (Table 10) and white streaks in the myocardium of three lambs.

Several cardiac changes were noted in lambs from Group C. Both atrioventricular (AV) valves in one lamb were edematous, thickened and had thickened margins. All of the lambs in this group had multiple white streaks 3 cm long X ½ cm wide and white patches 2 cm X 2 cm X 1 cm in the epicardium. These white streaks often extended into the myocardium.

One lamb in Group D was mildly icteric, having yellow mucous membranes and sclerae. An excessively wet crusty nose, eyes and mouth was noted in another lamb. A diffuse distribution of pale skeletal musculature occurred in 3 lambs. Except for the three observations just mentioned, the remaining findings were noted in every lamb in this group. Red-tinged froth exuded from the nostrils and filled the trachea and bronchi. Rumens were full. Peritoneal cavities contained between 150-750 ml of dark red to flocculent red fluid. Thoracic cavities contained 75-500 ml of fluid ranging from translucent-to-dark red with or without a fibrinous clot. Lungs were heavy and wet with marked widening of interlobular septa. Hearts had a pale parboiled mottled appearance with multiple white streaks 2-4 cm X ½ cm X 1 cm and white patches 2 cm X 3 cm X 1.5 cm in the endocardia, myocardia and epicardia. Livers were mottled brown-burgundy and had rounded margins.

The lambs in Group E had the greatest variability of grossly visible changes. Externally, the only abnormal findings were icterus noted in the sclerae of two lambs. These lambs were extremely

icteric internally (Fig 5). White froth was found in the tracheas and major airways of two lambs. The lungs ranged from normal (1), to emphysematous (2), congested (2), and edematous (2). White patches and white streaks were noted on all of the hearts with one heart having a roughened epicardium over some of the white patches. Four of the hearts were obviously pale. One lamb had red-tinged peritoneal fluid. A distinct lobular pattern could be seen in 3 of the livers while the other 2 were golden, friable and had rounded margins. Two enlarged spleens were noted, 2 lambs had dark red urine and 2 lambs had moderate amounts of blood in intestinal lumina.

The amount of pericardial fluid in the treated lambs was significantly higher than in the non-treated lambs. Only pericardial fluid in Groups D and E contained fibrin or had a red color.

Histopathology

To avoid redundancy of descriptions, the lesions in the lung, liver and heart were classified into four groups: none, mild, moderate, or marked. These classifications will be described and then the findings in each group will be presented (Table 11).

Mild lesions in the lung were minimal amounts of congestion and alveolar edema (Fig 6).

Moderate lesions were blood-tinged fluid in the airways; edema and congestion resulting in widened intralobular septa; focal areas of atelectasis and vascular congestion with perivascular edema.

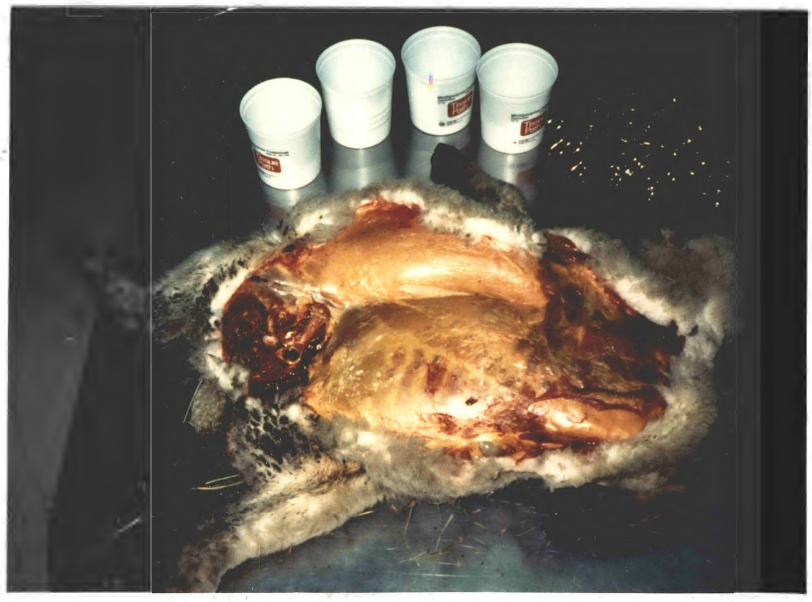

Fig 5--Severe, extremely marked icterus noted in Lamb #3 in Group E.

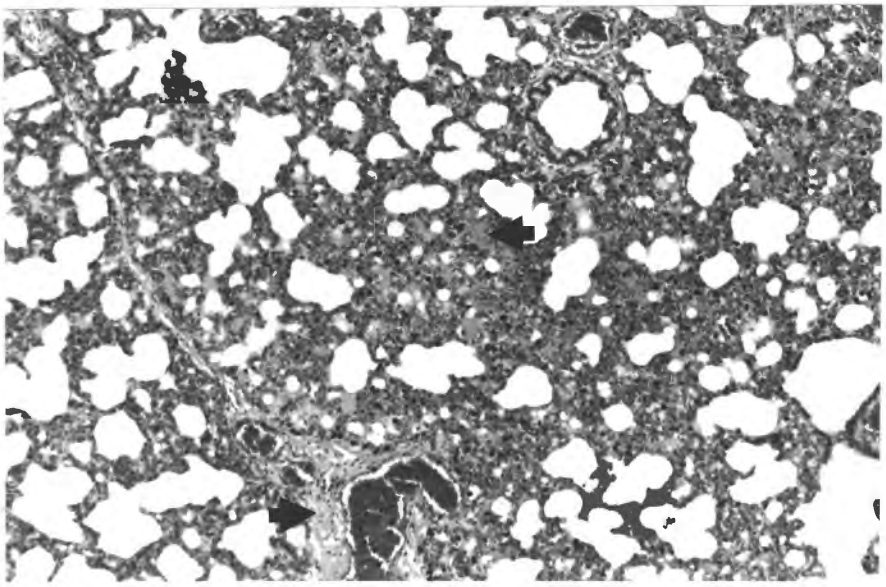

Fig 6-- Mild pulmonary lesions; congestion (➡) and edema (➡).

TABLE 11--Histopathologic classification of cardiac, hepatic and pulmonary lesions

Group	Lamb	Cardiac	Hepatic	Pulmonary
A*	1	none	none	none
	2	none	none	none
	3	none	none	none
	4	none	none	none
	5	none	none	none
B*	1	mild	none	none
	2	moderate	none	none
	3	mild	none	moderate
	4	mild	mild	none
	5	mild	mild	none
C*	1	mild	mild	none
	2	mild	none	mild
	3	mild	mild	none
	4	mild	mild	none
	5	mild	none	none
D†	1	mild	moderate	marked
	2	marked	marked	mild
	3	marked	marked	none
	4	mild	moderate	moderate
	5	mild	marked	marked
E†	1	moderate	mild	marked
	2	marked	PMA	marked
	3	moderate	mild	moderate
	4	marked	marked	marked
	5	moderate	PMA	moderate

* All of the lambs in this group were euthanatized. † All of the lambs in this group died.

PMA = postmortem autotysis.

Marked pulmonary lesions were an overall increase in the severity of the moderate lung lesions (Fig 7).

Mild hepatic lesions (Fig 8) were slight midzonal or centrilobular hepatocellular cytoplasmic clearing or mild acute cellular swelling (cloudy swelling). Some hepatocytic nuclei were in eccentric locations within the cytoplasm of cells in affected areas. Vacuolization of pyknotic centrilobular hepatocytes was observed.

Moderate hepatic lesions included hepatocellular cloudy swelling around central veins (Fig 9) progressing to necrosis. Congestion of the portal triads, and pyknosis, karyorrhexis and karyolysis of hepatocytes were also evident. Perivascular edema was present and the overall density of the hepatic cells was decreased. Fatty change was not present. Some portal triads were hypercellular due to the presence of numerous reticuloendothelial cells.

Marked hepatic lesions were severe fatty change throughout the entire liver which decreased the overall density tremendously (Fig 10), edema in the connective tissue of portal triads, walls of portal veins and hepatic arteries and pronounced bile retention. Intracanalicular bile plugs were more numerous and larger around triads. Centrilobular hepatocellular necrosis consisting of pyknosis and karyorrhexis in some livers, while others had severe centrilobular congestion.

All of the treated lambs had either mild, moderate or marked cardiac lesions. Cardiac lesions were not observed in the controls.

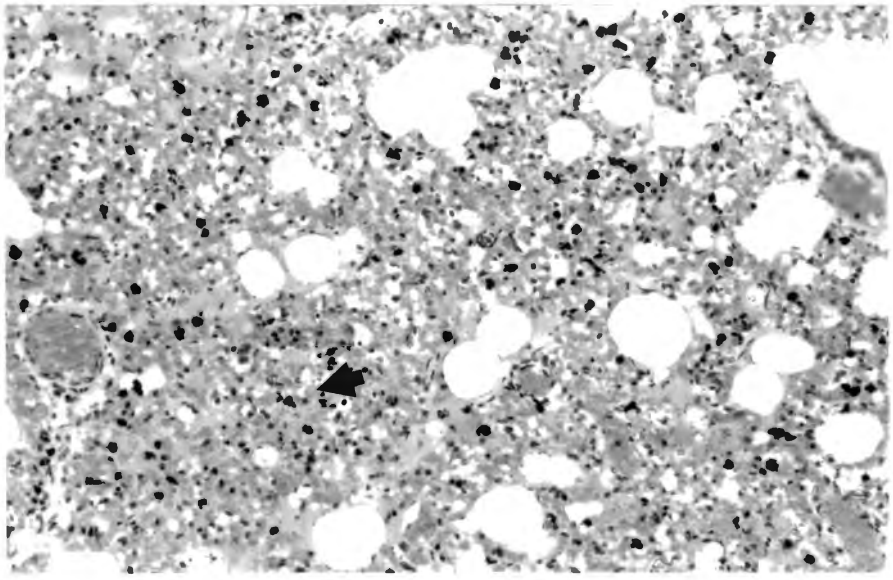

Fig 7--Marked pulmonary congestion, edema and necrotic cellular debris (➡).

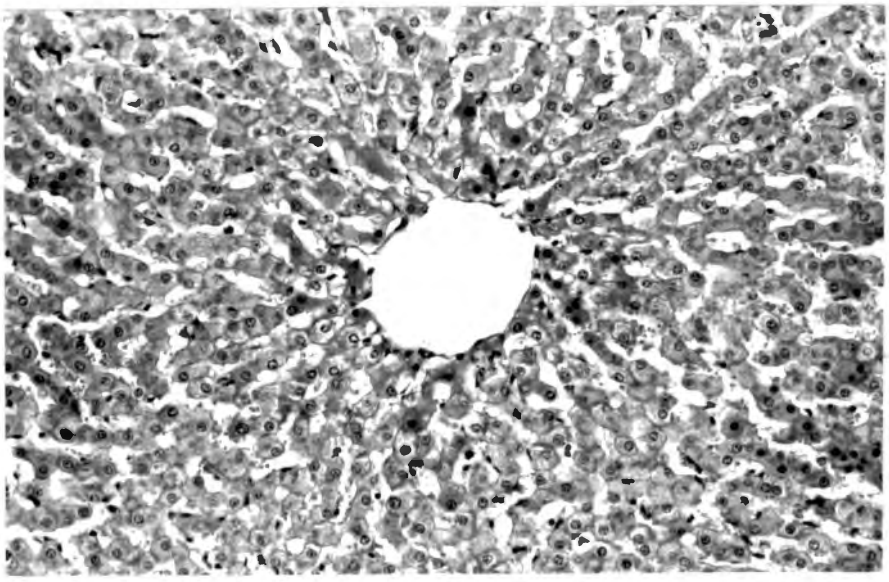

Fig 8--Extremely mild hepatocellular degeneration with pyknotic nuclei and darker cytoplasm around central vein.

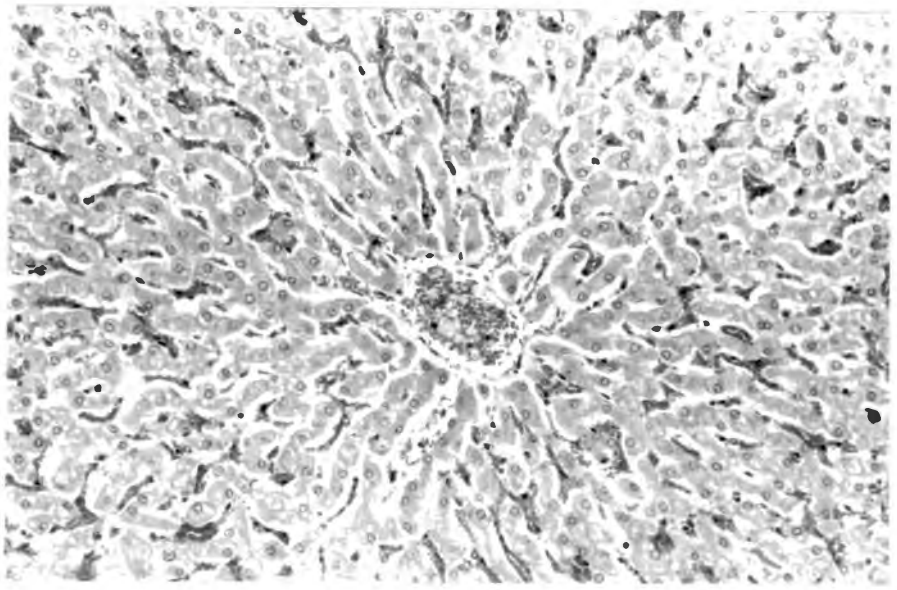

Fig 9--Moderate hepatic cloudy swelling around central veins, necrosis, congestion of portal triads, pyknosis, karyorrhexis and karyolysis.

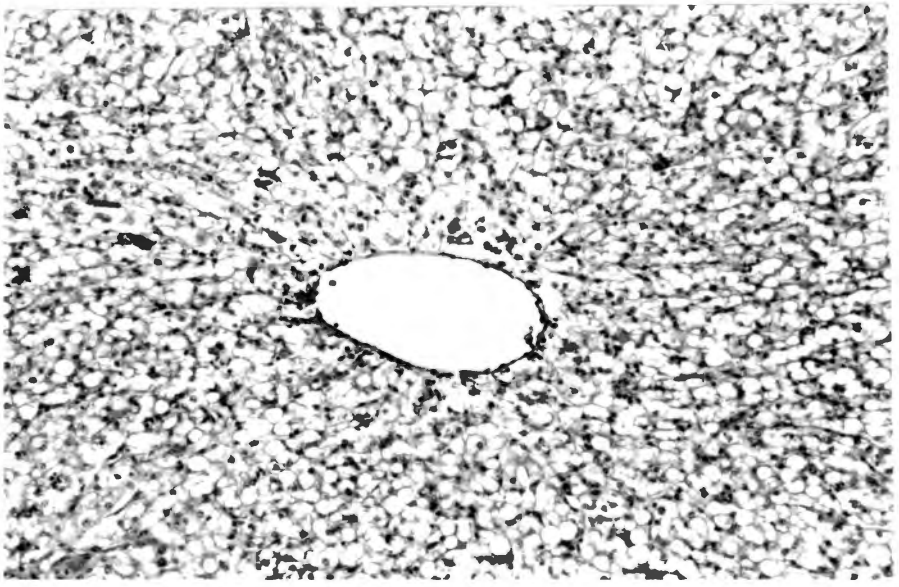

Fig 10--Marked hepatic fatty change.

Mild cardiac lesions were small foci of hypercellularity (Fig 11) often around or adjacent to arterioles and venules. The cells consisted of macrophages, lymphocytes, reticuloendothelial cells and proliferating sarcolemmal tubule cells. Nonstaining perivascular edema was another common finding. Nuclear rowing Fig (12), hyperchromatic nuclei and enlarged hyperchromatic nuclei with very blunt ends were present (Fig 13). Focal areas of necrosis and degenerating fibers which were vacuolated and/or replaced by macrophages and reticuloendothelial cells were seen adjacent to normal muscle fibers. Some fibers were separated by edema.

Only 20% of the treated lambs were classified as having moderate heart lesions. These lesions were generally larger and more numerous areas with morphologic changes similar to but more severe than those with mild heart lesions. Muscle fibers with loss of cross and longitudinal striations were more numerous and the proliferation of sarcolemmal tubule cells was more prominent (Fig 14).

Marked cardiac lesions were more severe and areas of cellular proliferations were more numerous and much larger than those in the previously described categories. Focal areas of necrosis were extensive and pronounced in most tissue sections with the most severe lesions being in the subendocardium. Many remaining fibers had foamy vacuolated sarcoplasm with pyknosis and karyorrhexic nuclei. On cross section, perinuclear clearing, known as "Ring Binden" was present (Fig 15). Focal areas of hemorrhage and lacy reticulated cells were often seen around vessels.

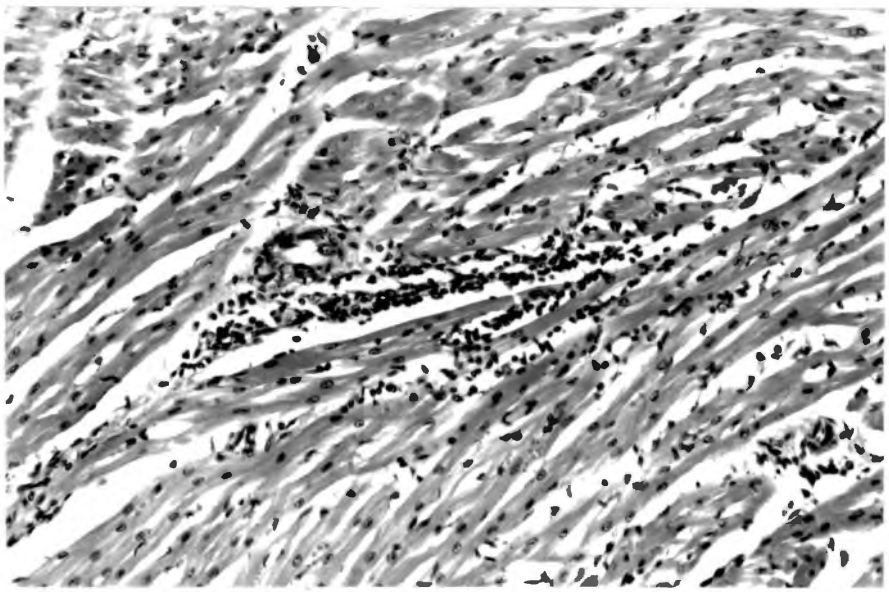

Fig 11--Mild cardiac focal areas of hypercellularity.

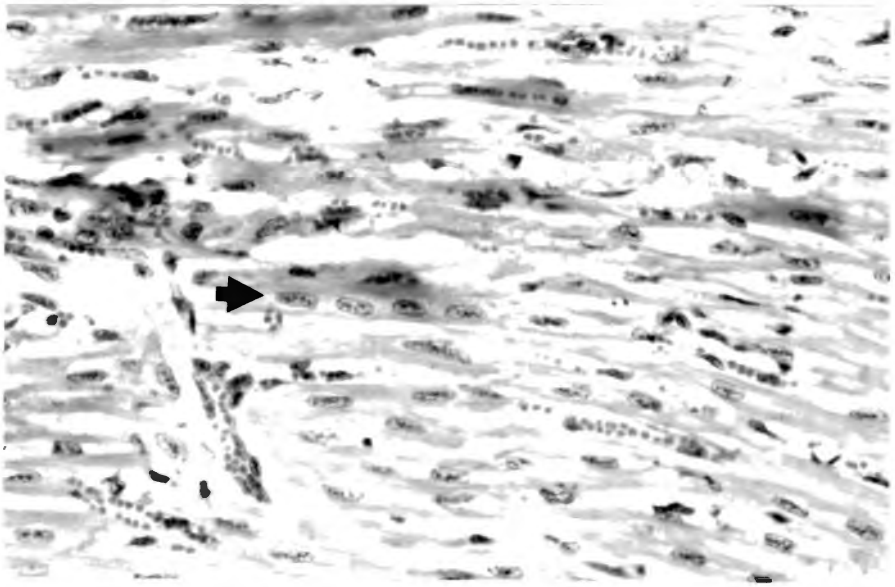

Fig 12--Nuclear rowing (➡), vacuoles and sarcoplasmic strands with loss of cross striations.

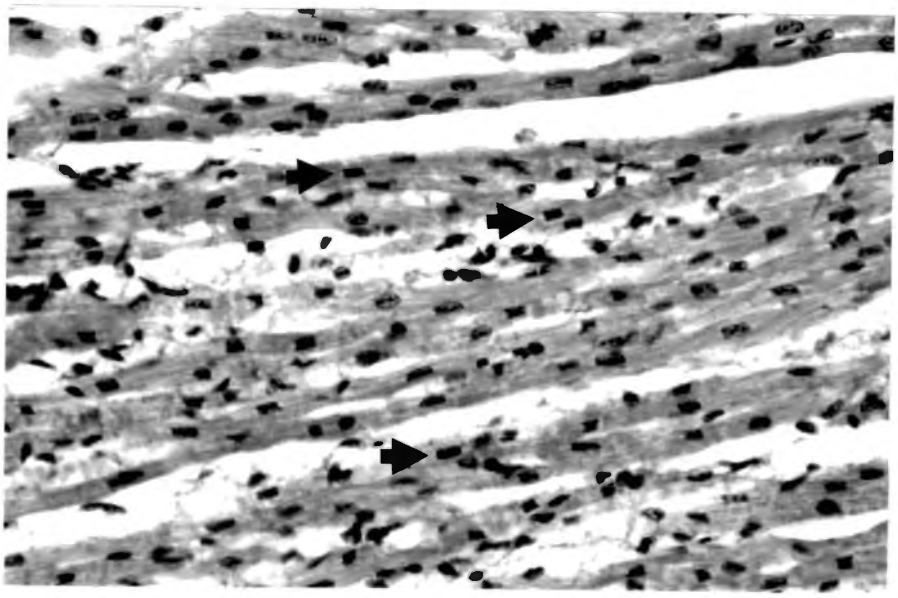

Fig 13--Endomysial edema, swelling of fibers with vacuolation and sarcoplasmic stranding. Note hyperchromatic nuclei with blunt ends (➡).

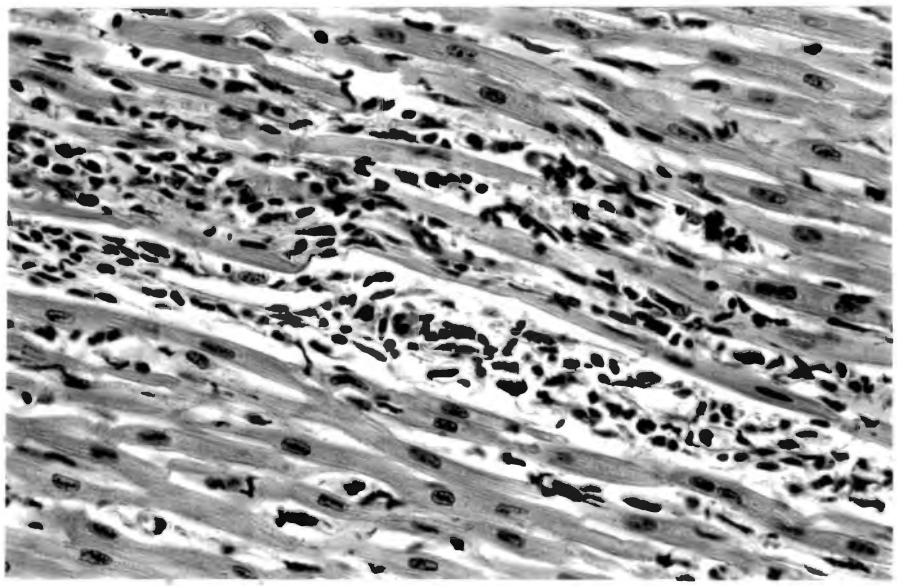

Fig 14--Moderate loss of longitudinal and cross striations in muscle fibers with adjacent fibers unaffected.

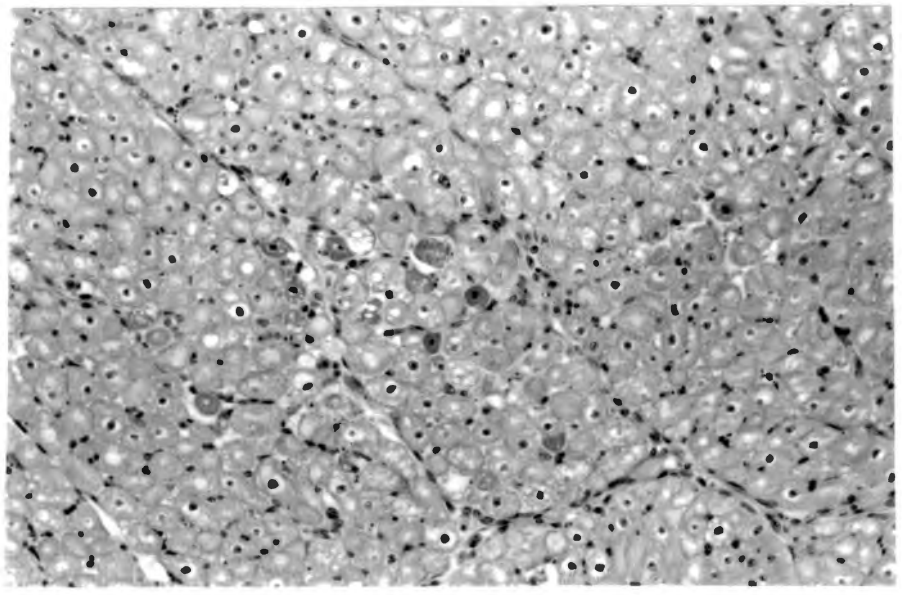

Fig 15--Cross section of cardiac muscle showing "Ring Binden" formation.

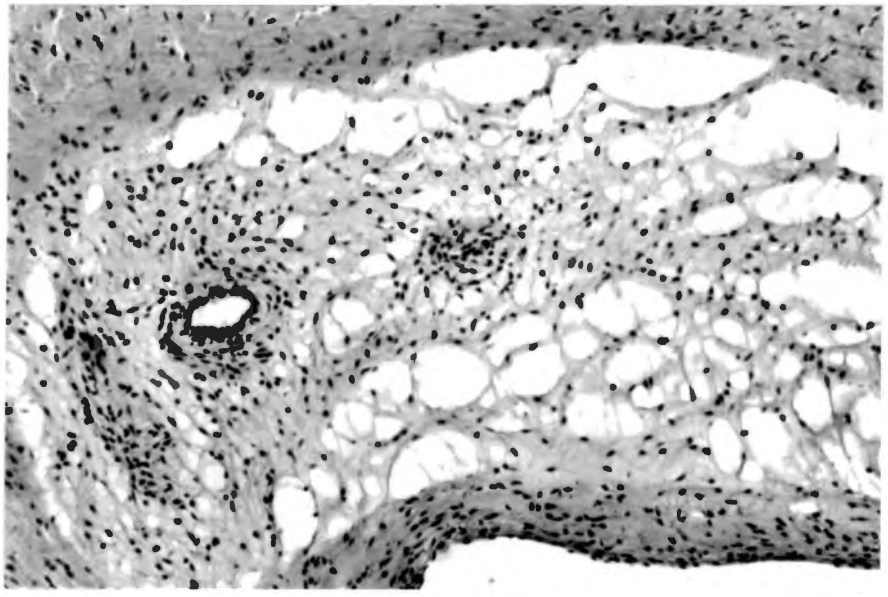

Fig 16--Edematous left atrioventricular valve.

An edematous left AV valve (Fig 16) was one of the most significant cardiac lesions noted in Group B. Vacuoles were seen in the gray matter of the brain in lambs #4 and #5 from this group.

Vacuoles in the white matter of the brain were seen in lamb #1 from Group C. This lamb also had proteinaceous fluid in Bowman's space of the kidney.

The atria seemed especially affected in Group D. Lesions were seen in the epi-, myo- and endocardium. There was extensive postmortem degeneration in two of the livers. One sarcocyst was found in the heart of lamb #5; there was no inflammatory response to the sarcocyst.

Some edema and vacuolization was seen in the brains from several lambs in Group E. Bilateral spongiform degeneration was seen in the red nucleus of lamb #3 (Fig 17).

Toxicology

Gossypol (free or bound) was not found in kidney, liver or skeletal muscle of the control lambs. Levels of free and bound gossypol in liver, kidney and skeletal muscle from 25 lambs is presented in Table 12. There is a significant ($P < 0.05$) increase in the level of free and bound gossypol in relation to the amount of gossypol each group received. The livers contained significantly ($P < 0.05$) higher levels of gossypol than the kidneys. The kidneys contained significantly ($P < 0.05$) higher levels of gossypol than the muscles. While the kidneys from Groups D and E contained significantly higher levels of gossypol than Group B and C, there were no

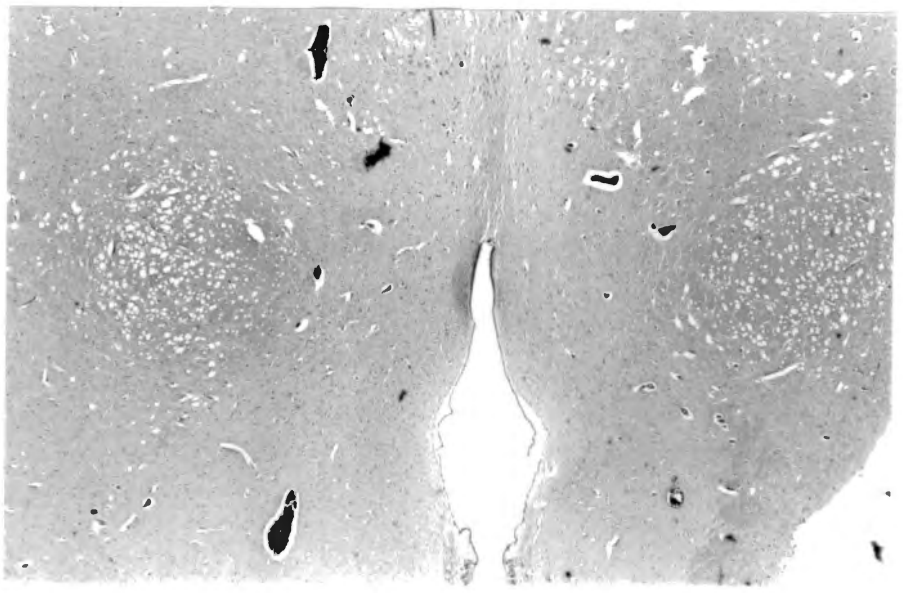

Fig 17--Bilateral spongiform degeneration in the red nucleus of Lamb #3 in Group E.

TABLE 12--Average amounts of free and bound gossypol found in liver, kidney and muscle

	Free (ppm)			Bound (ppm)		
	Liver	Kidney	Muscle	Liver	Kidney	Muscle
Group A*	7.23†	3.90†	4.57	4.15†	1.39†	1.84
Group B	12.21†	3.39†	1.18	21.33†‡	5.19†‡	2.75
Group C	18.42†	7.17†	3.61	40.35‡	5.64†‡	1.79
Group D	85.84‡	13.97‡	2.60	91.25§	16.83‡§	3.04
Group E	145.14‡	24.52‡	8.38	141.63‖	31.68‖	7.03

* Since this group is a control, these values are considered to be non-specific background readings. Values with different superscripts are different ($P < 0.05$).

ppm = parts per million (mg/kg).

significant differences in gossypol values in the muscles from any groups. Significant levels of gossypol were not found in any skeletal muscles from any of the groups.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Most of the current literature considers gossypol toxicity to be a disease of the past. This is because regulations on swine feed no longer allow levels of free gossypol to exceed 0.01% in the ration and ruminants are considered to be relatively safe from gossypol toxicity.[17,22] This belief may be the reason that sudden deaths in lambs are usually diagnosed as "overeating", and chronic labored breathing is treated as pneumonia. Necropsies are usually not done. When tissues are collected at necropsy and sent for histopathology, the myocardium is rarely included. On the basis of this study, the principal tissue that was damaged was the myocardium and this was also the tissue that suffered primary damage. All of these factors may be why gossypol toxicity in lambs has not been reported previously. The first confirmed case of gossypol toxicity in lambs was diagnosed at the OADDL in the summer of 1985 and more have been recognized since then. This apparent increased incidence could have been due to the increased use of cottonseed in feeds or the growing conditions and processing conditions could have caused an increase in

the gossypol that was normally present in the feeds. Because cottonseed is such an excellent source of protein in livestock feeds, it is important to note that from 1975 to 1984, the price of cottonseed ranged from $89.70 to $103.00 per ton. In 1985, this price dropped to $59.50 per ton, making cottonseed one of the most economical and widely used sources of protein in the animal feed industry.[42]

The inability to detoxify gossypol in an immature rumen is the primary cause of gossypol toxicity in feeder lambs. Other factors may influence the degree of gossypol that becomes bound. The amount of iron available in the ration could be an important factor. The more iron in the feed, the more free gossypol may become bound to iron and thus be detoxified. Younger feeder lambs may still be nursing. Milk contains little iron. The rapidly dividing erythrocytes in these younger animals would probably use any available iron in their production. Older animals would probably not have the same demand for iron because their erythrocytes are dividing less rapidly and thus more iron may be stored. This stored iron may be a factor influencing the degree of binding of gossypol.

The cardiomyopathy found in 100% of the treated lambs is the one outstanding feature of gossypol toxicity. The two syndromes seen were manifested clinically as: (1) chronic labored breathing, and (2) sudden death. Both can be attributed to cardiac failure. Fifty per cent of the lambs that died in this study had a chronic disease syndrome with gradual onset of labored breathing, depression, decreased appetite, decreased weight gain and general unthriftiness. Lamb #4 in Group B also had these signs but did not die during the 30

day trial. Pulmonary edema that could be seen grossly and microscopically caused labored breathing. The fluid build up in the lungs was believed to be the direct result of a progressively failing heart. This was supported grossly by increased myocardial weight in treated *versus* controls and was indicative of cardiac hypertrophy. Lamb #4 in Group B also had an extremely high percentage of heart weight to body weight. Sudden deaths, also seen in 50% of the lambs that died were often seen in the most active, robust lambs in the group. One lamb actually ran around the stall as he usually did when approached and simply fell over and died; it was as if his heart had suddenly failed. Group D had a higher percentage of cardiac weights to total body weight than Groups A through C.

Since all of the lambs that died were from Groups D and E, it was difficult to compare the sudden death syndrome to the chronic labored breathing syndrome. Many of the lambs in Group E died before much data could be obtained. There were not as many serum enzymes taken in Group E, and they were not taken as frequently. Tissue weights and ECGs were not done in Group E. The criteria that could be compared (necropsy and histopathology) had no significant differences between the lambs that died suddenly and the lambs that had antemortem signs of chronic labored breathing.

Necropsy findings on the treated lambs (Groups B-E) were indicative of cardiomyopathy with white streaks, white patches and pale myocardia being observed. Thickened, edematous atrioventricular valves were evident in some of the lambs. Excessive pericardial, thoracic and peritoneal fluids were considered to be indicative of

cardiac failure that resulted in pulmonary edema and hepatic congestion.

Histopathology confirmed the grossly observable cardiomyopathy. All treated lambs had mild-to-marked myocardial lesions. In all instances these could be described as multifocal areas of macrophage, lymphocytic and reticuloendothelial cell hypercellularity that were usually in close proximity to vessels. The macrophages and reticuloendothelial cells in the affected areas were considered to be an inflammatory reaction resulting from myocardial damage due to the toxin. Other areas were hypercellular because of proliferation of nuclei of myocardial fibers and nuclear rowing. These changes were considered to be attempts at repair and regeneration. In severely affected animals, focal areas of necrosis with loss of cross and longitudinal fibers were evident. Perivascular edema and edema between some fibers was evident and would account for the increased wet weights of the hearts in the treated groups.

Of the treated lambs, 60% had mild cardiac lesions, 20% had moderate myocardial lesions and 20% had marked cardiac lesions. Although Group B was the group that received the smallest amount of free gossypol, myocardial lesions in this group were more numerous than those of Group C. Even when the gossypol was calculated on a per kilogram basis, all of the lambs in this group received less gossypol per kilogram of body weight than Group C. This result cannot be explained on the basis of this experiment. All of Group B had mild cardiac lesions except for lamb #2 which had moderate lesions. All of Group C had very mild cardiac lesions. Three lambs

in Group D had moderate cardiac lesions and two lambs had marked myocardial lesions. Group E had the greatest variability of lesions. Lambs #2 and #3 had marked cardiac lesions but the remaining lambs only had mild lesions. However, lambs #2 and #3 lived 6-10 days longer than the other lambs in the group and had more time to develop lesions. Lamb #5 was found dead on day 9 after receiving only 8 doses of gossypol; myocardial lesions were minimal.

Vacuolization and regenerative attempts without neutrophils all indicate a toxin specifically targeting the musculature of the heart. This helps to rule out infectious causes of cardiomyopathy but is not specific for confirmation of gossypol toxicity. Skeletal muscle lesions have been seen consistently in Vitamin E/Selenium deficiency and monensin toxicity in lambs. Although pale skeletal musculature was noted grossly in several lambs, no significant microscopic lesions could be found in the skeletal musculature.

Serum levels of the isoenzyme that is most specific for the heart, (CKMB), did not correlate to the lesions seen in the various groups. Instead of gradually increasing because of cardiac damage, it seemed to increase and decrease unexpectedly. The higher baseline CKMB value for Group E cannot be explained. Unexplained variations in the level of this enzyme were similar to variations in levels of CPK. Creatine phosphokinase activity of serum is usually increased in diseases of the heart, skeletal muscle and brain. These tissues contain large quantities of this enzyme and extremely mild injury is all that is needed for release into the blood. A plausible explanation for this enzyme (and possibly CKMB) to be decreasing,

especially when tissue damage can be confirmed histopathologically, is for it to be inhibited. Inhibition is the only plausible explanation as to why the control group never had decreased CPKs, but Groups B, C, and D all had some lambs with levels $\leq$10 by week 3. This inhibition may occur only at certain levels, which may explain why the CPK values went back up during week 4. If overwhelmed with a substrate such as gossypol, inhibition may not occur at all. Possible theories could be: 1) the inhibitor is an antibody against gossypol, 2) gossypol may be complexed with an antibody or a smaller molecule and inhibit CPK, 3) gossypol may be a part of a complex formed in the rumen or serum that inhibits CPK, 4) there could be a competitive or non-competitive inhibition relationship between gossypol (or a gossypol induced substrate) and CPK, 5) gossypol breakdown products could be the inhibitors.

Creatine phosphate and creatine phosphokinase, the enzyme which synthesizes and degrades it, are important compounds in the storage and transfer of chemical energy within cells. When food from the diet is oxidized, its potential energy is trapped as a high-energy compound which may be utilized immediately or stored in the cell until it is needed to perform mechanical work or to drive some energy-requiring metabolic reaction. The most common of the "energy transfer" compounds is adenosine triphosphate (ATP). The energy for muscular contraction and for many other metabolic reactions is supplied by the hydrolysis of the terminal pyrophosphate bond of ATP to yield adenosine diphosphate (ADP) and an inorganic phosphate ion. There is only a small amount of ATP in muscle cells - about 6 u

mole/gm, which is sufficient to sustain muscle contraction for only a short period of time. Additional ATP can be produced fairly quickly by partial degradation of glucose liberated by hydrolysis of stored muscular glycogen, but with the onset of a sudden burst of intense muscle contraction, cells of striated muscle require some form of "stored" energy which can be quickly converted to ATP. The high-energy compound which serves to store energy in the muscle cells is creatine phosphate which has a high-energy guanidophosphate bond. ATP can be quickly resynthesized by the one step transfer of the phosphate group of creatine phosphate to ADP. This reaction is catalyzed by the enzyme creatine phosphokinase. Creatine kinase M-chain B-chain is an isoenzyme of CPK but is specific for the cardiac muscle. If these enzymes (CPK and CKMB) are inhibited, the storage and transfer of chemical energy (ATP) within the myocardial cells cannot take place.[43] This could be the cause of a chronic deterioriating physical condition and pulmonary edema or a sudden death syndrome.

The variations in the electrocardiographic patterns of these lambs strongly resembled those found in swine after gossypol intoxication.[44] The increased amplitude of the T wave and shortening of the S-T segment found in these two species, resemble electrocardiographs produced by increased concentrations of potassium (hyperkalemia) in man.[45] From studies on gossypol and its effect on electrocardiograms in swine and work published by other scientists, Smith suggested a shift in intracellular water, with a change in

intracellular potassium concentration as a cause of the electrocardiographic aberrations. It was suggested that the relative concentration of electrolytes in the extracellular fluid may be of importance in the production of electrocardiographic changes. A change in the relative concentration of the extracellular and intracellular fluid potassium, or other factors which interfere with the movement of potassium through the cell membrane during muscular contraction and relaxation could produce electrocardiographic changes. Any type of injury may permit a migration of potassium to the cell surface, equalizing the potassium concentrations on either side of the cell membrane. Electrocardiographic alterations may result from binding of gossypol to the free epsilon amino group of lysine or to phospholipids in the cytoplasmic membranes, and may alter cellular permeability. The electrocardiographic signs of myocardial injury may, in fact, represent a local change in potassium concentration, probably in the subendocardial layer.[46]

Electrocardiographic alterations were very prominent in treated *versus* control lambs. As suggested for swine, these alterations may lead to a loss of electrical rhythm, resulting in cardiac failure. More work needs to be done in this area with vector analysis and measurement of other parameters such as central venous pressure and intracarotid pressure. Serum potassium also needs to be measured to determine if there is a correlation to the amplititude of the T-wave on the ECG.

Free and bound gossypol levels in the heart were only measured from only one lamb in Group E, and the heart of this lamb was found

to contain 55.21 ppm free gossypol and 36.6 ppm bound gossypol. The fact that gossypol accumulates in the heart does not explain why this organ seems to be targeted even more specifically than the liver which accumulates even higher levels of gossypol. In this same lamb, the liver contained 256.55 ppm free gossypol and 144.35 ppm bound gossypol.

Pulmonary edema was a prominent and consistent finding in almost all of the lambs that died (Groups D and E). The degree of pulmonary edema was the one factor that was negatively correlated to the amount of weight gained. As a general rule, the level of free gossypol given per kilogram of bodyweight did not significantly alter the amount of weight gained in the majority of the lambs. With the exception of lamb #4 in Group B, the lambs in Groups B, C and D had weight gains similar to the controls. There was an obvious decrease in weight gain in those lambs affected by chronic labored breathing (pulmonary edema).

Microscopically, in the treated lambs, 40% had no pulmonary lesions, 10% had mild pulmonary lesions, 25% had moderate pulmonary lesions and 25% had marked pulmonary lesions. Only one lamb in Group B had pulmonary lesions, but they were marked. Group C also had only one lamb with pulmonary lesions, and they were mild. Group D had two lambs with moderate pulmonary lesions and three with marked pulmonary lesions. The pulmonary lesions in Group E ranged from none-to-marked.

Thirty-five percent of the treated lambs had mild hepatic lesions, 10% had moderate hepatic lesions and 25% had marked hepatic lesions.

Grossly, many of the livers had rounded edges and were mottled. A few of the livers were obviously pale and icteric. Most of the gross lesions in the liver were believed to be secondary to those in the heart. Additionally, some changes in the liver could probably be attributed to pulmonary edema with resultant hypoxia or could have been due to direct toxicity of gossypol.

The majority of these lesions were indicative of cardiomyopathy with passive congestion of the liver. Broad categories of causes of hepatocellular degenerative changes in the liver that lead to necrosis are generally considered to be hypoxia, metabolic disturbances and toxicities. Degenerative changes specifically alluded to are cloudy swelling, hydropic degeneration and fatty change (all seen in various degrees in the livers of lambs given high does). Gossypol did accumulate to higher levels in the liver than in other tissues. This accumulation may be the best diagnostic test in the future to determine gossypol toxicity. The yellow gossypol pigment may have actually stained the tissues and caused the icteric appearance.

The serum enzymes tied together some of the previous findings but also created new questions. In healthy sheep, if liver conjugation and excretion are normal, the levels of total serum bilirubin range from 0.0-0.6 mg/dl. The fact that Groups C, D and E exceeded these values, could coincide with hepatic lesions that were seen histopathologically. The amount and severity of lesions were greater in these groups.

Other serum enzymes which are used as indicators of hepatic necrosis (SGPT, SGOT, LLDH and LDH), rarely exceeded values recorded

for healthy sheep in any of the groups. These enzyme levels increase after hepatocellular destruction. This could indicate that the hepatic lesions found in this study may be secondary to another condition such as hypoxia or a failing heart.[43]

Group E had levels of SGOT, LLDH and LDH that exceeded those of the control lambs. This could also support the idea of accumulation of gossypol in the liver to toxic levels. In week 4, Group D also greatly exceeded control values for LLDH.

Serum enzymes would have been more helpful if they had been taken every day or every other day in Group E. Many of these lambs died before the second set of samples were taken. The first lamb in this group died on day 8 of the experiment. All of the lambs were dead by day 17.

The exact cause of gossypol-related hemoglobinuria, such as that seen in Group E, is unknown. In calves and cows, gossypol, in high doses, has been reported to cause hemolysis of red blood cells.[2,18] There have been previous correlations in the literature of gossypol and erythrocyte fragility.[21] More research needs to be done in this area.

The generalized pale musculature seen in some of the lambs could not be explained. There was insufficient histopathologic or serum enzymatic evidence to suspect a toxic myopathy. The lack of gossypol accumulation in skeletal muscle may be a partial explanation.

Vacuoles seen in the gray matter of the brain in lambs #4 and #5 in Group B and in the white matter in lamb #1 in Group C were believed to be an artifact of fixation and processing rather than a

true lesion. However, the edema, vacuolization and bilateral spongiform degeneration (in the red nucleus) seen in some of the lambs in Group E were believed to be true lesions. More research needs to be done in this area.

At this time, gossypol toxicity may not be definitely diagnosed by histopathology alone. The toxicologic analysis of free and bound gossypol in tissues may be useful if more work is done on field cases with comparisons to research lambs. History of cottonseed in the diet, gossypol analysis of the feed, more than one animal affected, lack of other toxic plants, no monensin detected after feed analysis, characteristic gross and microscopic lesions, and lack of evidence of any other disease processes which cause similar signs and lesions may be necessary to confirm a diagnosis of gossypol toxicity.

From this study, it is concluded that free gossypol, even as low as 0.01% (100 ppm) of a grain-concentrate ration, is detrimental to 8-10 week old lambs. With this conclusion, and previous evidence in young calves, cottonseed meal cannot be recommended as a protein source for young ruminants because of their inability to convert free gossypol to the bound form in an immature rumen.

CHAPTER VI

Summary

Twenty-five lambs, 8-10 weeks old, were divided into 5 groups of 5 each. They were given free gossypol orally in doses of 0, 45, 136, 409, 817 mg respectively, once a day for 30 days. All of the lambs in the 2 higher dosage levels died by the end of 30 days. There were two distinct syndromes. One was sudden death in an apparently healthy animal, and the other was chronic dyspnea and depression. Hematologic surveys did not reveal any unusual findings. Values of some of the serum chemistries were unusual with CPK and possibly CKMB values diminishing almost consistently in treated lambs to zero or very low values. The ECG's were remarkably similar to those of swine poisoned with gossypol and suggest the possibility of hyperkalemia as seen in man. Hyperkalemia interferes with the normal conduction system of the heart resulting in death. Gross pathologic findings in the 10 lambs that died were similar, with excessive pericardial, pleural and thoracic fluid, pulmonary edema, white patches and streaks on the heart and tan-to-golden livers with rounded edges.

Histopathologically, all treated lambs had cardiac lesions that varied from hypercellularity to necrosis. Most of the treated lambs also had hepatic congestion, necrosis or fatty change. Pulmonary edema was a prominent finding in lambs that died and some that were euthanatized. Toxicologic analysis of free and bound gossypol in the liver, kidney and skeletal muscle revealed virtually no gossypol accumulation in the muscle. Moderate levels accumulated in the kidneys and even higher levels accumulated in the liver, relative to the dose received.

This study proved that gossypol is a potent cardiotoxin in young feeder lambs. Since even the lowest level of gossypol caused cardiac lesions in these lambs, it could not be recommended as a protein source in the ration of young lambs.

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Bound Gossypol Extraction and Analysis

Reagents:

a. Aniline: freshly distilled, water clear

b. Solution B: same as for free gossypol

c. Hexane: redistilled

Procedure:

1. Transfer the tissue residue remaining from the free gossypol extraction from the Buchner funnel to a sheet of white paper.

2. Remove the layer of Hyflo Super-Cell from the tissue residue with a spatula without disturbing the tissue residue.

3. Chop the tissue residue with scissors, pulverize and transfer to a 250 ml. ground glass stoppered Erlenmeyer flask.

4. Moisten the residue with 10 ml of solution B.

5. Then add 2 ml. of the freshly distilled aniline to the flask, and mix until all of the residue is moistened with the mixture.

6. Place the flask on the metal surface of a steam bath, not in direct contact with the steam, and heat gently for 45 min. with occasional shaking to prevent overheating the tissue on the bottom of the flask.

7. Remove the flasks from the steam bath and immediately add 20 ml. of glass beads (6 mm diameter) and add 50 ml. of redistilled hexane from a pipet while swirling the flask in a pan of hot water (60-70°C) to expel the air.

8. Insert the glass stopper tightly with a twisting motion to prevent leakage.

9. Shake vigorously for 1 hour.

10. Filter the extract through paper into a 50 ml. volumetric flask.

11. Read the color intensity as absorbance at a wavelength of 440 mu using hexane as the reference solution.

12. If necessary because of color density, dilute suitable aliquots to 25 ml. with hexane.

13. Determine the gossypol content of the tissues from a standard absorbance - conc. curve prepared from pure gossypol.

14. Prepare a standard absorbance conc. curve for bound gossypol by dissolving 0.0250 g. of pure gossypol in 10 ml. of ethyl ether and making the solution to 100 ml. with hexane.

15. Transfer 10 ml. of this to a 100 ml. volumetric flask, dilute to 100 mls. with hexane and mix.

16. In triplicate, and at ml. intervals, transfer a series of aliquots ranging from 1-8 ml. into 25 ml. volumetric flasks.

17. Reserve one flask of each group as a reference.

18. To the remaining flasks, after diluting the 1 to 4 ml. aliquots to 5 ml. with hexane, add 0.5 ml. of aniline and heat gently on the steam bath for 45 min.

19. Allow to cool, and make references and samples to 25 ml. with hexane, being careful not to contaminate the references with aniline.

20. After mixing, read the absorbance using the appropriate reference solution at a wavelength of 445 mu.

21. Plot absorbance vs. conc. in ug gossypol/25 ml. and calculate the extinction coefficient based on a volume of 25 ml.

22. More reading and calculations in article.

Free Gossypol Extraction and Analysis

Reagent:

a. Aniline: Reagent, freshly distilled, water clear.

b. Acetic acid: Glacial reagent.

c. Ethyl ether: USP or purified for fat extraction, must be peroxide free.

d. Solution A: 95% Ethanol + 0.2 ml. glacial acetic acid/liter.

e. Solution B: A 60% ethanol water solution prepared by diluting 715 ml. of 95% ethanol to 1 liter with distilled water and subsequently adding 200 ml. of ethyl ether and 0.2 ml glacial acetic acid.

f. Hyflo Super-Cel: To remove iron, boil 100 g. of Hyflo Super-Cel with 600 ml. of distilled water and 50 ml. of conc. HCl for 15 min., filter through a paper in a Buchner funnel and wash with distilled water. Repeat the acid treatment, wash and dry.

Procedures:

1. Keep tissues frozen until analyzed.

2. Just before analysis - partially thaw and grind in a food chopper.

3. Mix thoroughly and place in a glass bottle to prevent moisture changes.

4. Transfer 10 g. of ground tissue to the water jacket jar.

5. Add 50 ml. of 95% ethanol (solution A) and 20 ml. of ether.

6. Homogenize for 2 min. (while blending jar is surrounded by ice water to prevent heating).

7. In the meantime, put a 5.5 cm. filter paper in a size 1 Buchner funnel. After applying a vacuum, pour a suspension of 2-3 g. of Hyflo Super-Cel in 15 ml. of 95% ethanol on the paper.

8. After washing the jar cap and blades of the homogenizer with Solution B delivered from a wash bottle, suspend 2-3 g. of Hyflo Super-Cel in the homogenate and filter through the prepared Buchner funnel.

9. Thoroughly wash the tissue residue, which will serve as the sample for bound gossypol, with small portions of Solution B to remove all of the free gossypol.

10. Combine filtrate and washings are not <120 ml and >130 ml.

11. Using a flask calibrated to contain 130 ml., dilute the filtrate to 130 ml., mix and refilter a portion of the solution through Wat. No. 1 filter paper if turbid.

12. Transfer 10 ml aliquots in triplicate to 25 ml. volumetric flasks, one which will be a reference (standard).

13. To the two other flasks add 0.5 ml. freshly distilled aniline and heat them on the surface of the steam bath, not directly over the steam for 40 min. to convert the gossypol to dianilinogossypol.

14. Cool flasks to room temperature and add 2 ml. of ether to replace that lost during heating and sufficient Solution B to bring the volume to 25 ml.

15. Mix the contents of the flasks, determine the absorbance at a wavelength of 445 mu, using the aliquot containing no aniline as the reference solution.

16. Determine the gossypol content of the tissue from a standard absorbance - conc. curve prepared from pure gossypol.

STD Curve:

1. Prepare the std curve by dissolving 0.0250 g. of pure gossypol in 10ml. of ethyl ether.

2. Dilute to 100 ml. w/Solution B and mix.

3. Transfer 10 ml. aliquot to 100 ml. volumetric - dilute to volume with Solution B.

4. In triplicate and at ml. intervals, pipet a series of aliquots ranging from 1-8 ml. into 25 ml. volumetric flasks.

5. Reserve one flask containing an aliquot at each volume level as a reference.

6. Dilute the remaining aliquots to at least 10 mls with Solution B.

7. Add 1.0 ml. freshly distilled aniline and heat on a steam bath gently for 40 min.

8. After cooling, dilute all solutions, including the references to 50 ml. with Solution B.

9. Avoid contaminating the reference solutions with aniline!

10. Mix well and using the appropriate reference solution, determine the absorbance at a wavelength of 445 mu.

11. Prepare the standard curve by plotting absorbance vs. conc. in ug. of gossypol 25 ml. More in article on how to accomplish this.

12. The 95% ethanol-ether mixture extracts the free gossypol and reduces the moisture content to a suitable level for the bound gossypol estimation. The ether dissolves the tissue fat, preventing solution turbidity and the ether lost during vacuum filtration must be replaced.

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