GOSSYPOL DEACTIVATION
VIA FUNGAL INTERACTION

by

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INTRODUCTION

The deactivation of gossypol through the intervention of a fungal system is the theme of this thesis. However, a preliminary report dealing with the study of mycotoxicoses will be included since it was from this study that the genesis of the idea of Gossypol Deactivation Via Fungal Interaction evolved.

Mycotoxicoses are the general categories of physiological disorders in warm blooded animals associated with the consumption of toxic compounds produced by molds. These compounds are often referred to as mycotoxins. Many different species of fungi have been implicated in the production of toxic metabolites.

Perhaps the most celebrated occurrence of "mold poisoning" was reported in England about 1960. Approximately 100,000 turkey poults died as a result of the consumption of a ration containing moldy Brazilian peanut meal (1,2). The responsible agent was soon found to be "aflatoxin," the collective term used to designate the toxic metabolites of Aspergillus flavus. Aflatoxin has also been implicated as the cause of hepatic carcinoma in a number of species (3).

Another mycotoxin of possible importance is the metabolite of Fusarium which produces an estrogenic syndrome in swine. In the female, vulvular swelling and other reproductive problems result from low-level long-term ingestion;

on the other hand, acute toxicity leads to uterine prolapse, atrophic ovaries, and abortion in pregnant animals (4).

In a recent study by Scott (as reported by Campbell, 3), ducklings were fed a ration containing 25% moldy corn for 14 days. Individual inoculations with 228 strains of fungi representing 59 species yielded data indicating that 37% of the strains tested were lethal. Using this information, Campbell (3) made the somewhat alarming speculation that of the estimated 100,000 species of molds on earth, 30,000 to 40,000 species may be highly toxic.

Originally, the purpose of this thesis was to examine cottonseed fungi which might be responsible for other mycotoxicoses. In pursuit of this purpose, 47 samples of either glanded or glandless cottonseed were individually inoculated with fungi representing at least 9 different species.

Rations of 50% moldy cottonseed and 50% basal ration (Table I) were fed ad libitum to 98 male weanling rats of the Sprague-Dawley strain (Appendix, Table I). Glanded and glandless cottonseed control groups were also included in the feeding trials. A summary of these findings is shown in Table II.

The glanded, high gossypol control group ate only 53% as much food as the glandless, low gossypol control group; the high gossypol group also lost weight whereas the low

TABLE I Composition of Basal Ration

	Percentage		Percentage
Casein	22.2%	Corn Oil[3]	5.0%
Sucrose	66.0%	L-Lysine	0.4%
Mineral Mixture[1]	4.0%	D,L-Methionine	0.2%
Vitamin Mixture[2]	2.2%		

1 Jones-Foster Mineral Mixture, Nutritional Biochemicals Corporation.

2 Vitamin Dietary Fortification Mixture, Nutritional Biochemicals Corporation.

3 Mazola Oil.

gossypol group made moderate gains. Toxicity was quite evident in the high gossypol group as these animals exhibited tissue dehydration and fluid accumulation in the thoracic and abdominal cavities as well as in the gastrointestinal tract. All animals from the high gossypol group died within 10 days. However, the growth of fungi upon this highly toxic glanded variety alleviated this toxicity (Appendix, Table III).

Feed consumption data for the two groups, inoculated with the various fungi, were essentially the same (11.7 vs 12.0 g/day), whereas such fungal growth upon the high gossypol group actually promoted a 24% greater body weight gain (4.1 vs 3.3 g/rat/day), (Table II). In fact, 12 of the 16 fungi compared in Appendix, Table II, produced a greater body weight gain when grown upon this high gossypol substrate. The toxicity caused by the high gossypol control cottonseed was clearly and significantly reduced when infested with most fungi.

It is interesting to note that unlike the findings of Scott (as reported by Campbell, 3), there is a remarkable lack of toxicity in the large majority of the molds tested (Appendix, Table III). The rats consuming Aspergillus flavus 227*, Aspergillus niger 282, and Aspergillus niger 356 exhibited only slight kidney discoloration when fed on gland-

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* Isolate number.

TABLE II Feed Consumption and Body Weight Gain of Rats Fed Control and Infested Cottonseed (Cf. Appendix, Table I and II).

	Food Consumption (Grams/Day)	Weight Gain (Grams/Day)
Glanded Control	7.4	-0.6
Glandless Control	13.9	3.6
Glanded Infested	11.7	4.1*
Glandless Infested	12.0	3.3*

* 12 out of 16 separate fungal isolates showed greater body weight gain for the glanded substrate.

less cottonseed. Alternaria 351 caused only a questionable occurrence of kidney pyelitis when fed on glanded cottonseed.

The only toxic cultures of apparent signigicance were Diplodia 250 and Diplodia 308. Extensive pyelitis (Plate I) was noted in all of 12 test rats when Diplodia was fed on glanded cottonseed, but only minor and scattered kidney damage was noted when the substrate was glandless cottonseed.

Because this phenomenon had not been previously described in the literature, additional studies were undertaken to reproduce the response so that it could be described in greater detail. However, in these subsequent feeding trials with Diplodia using 108 rats (Appendix, Table IV), not only was pyelitis absent in all cases but also no other pathology could be noticed. Effects of toxicity persisted only in the control groups fed uninoculated glanded cottonseed.

The pathological responses noted in the first trials were not observed in the later trials. Several factors could be responsible for this varied behavior. Fungal isolates of a given strain can easily be lost upon storage or transfer of the culture (5); either major or minor modifications of the substrate can appreciably alter toxin production (6); animal responses are subject to innumerable unknown biological variations; and finally, cottonseed as a substrate is a perfect example of a substrate possessing its own inherent toxicity which can affect not only subsequent animal responses but also the possible inhibition of fungal

PLATE I Pyelitic Rat Kidney (left) Compared With Normal Rat Kidney (right).

growth and toxin production.

Such uncontrolled interactions from a culturing standpoint raise rather obvious objections to testing potential toxin production on a material such as cottonseed. Thus, the original direction of this thesis was changed to focus on the reduction of the apparent gossypol toxicity of cottonseed due to infestation with fungi.

Diplodia 308 was chosen for future studies because of its rather marked effect upon the reduction of gossypol toxicity, its sporadic natural occurrence on cotton plants, and for the possibility that the kidney toxin might be "rediscovered" in subsequent studies.

REVIEW OF LITERATURE

History

Cottonseed, as a harmful and injurious feedstuff, was first described in England by Voelker in 1859. Forty years later, in 1899, while searching for dyes, the Polish chemist Marchlewski isolated and purified a yellow polyphenolic material obtained from a by-product of the alkali refinement of cottonseed oil. He named this yellow material "gossypol"; a contraction of the words phenol and gossypium, the generic name for cottonseed. (As described by Eagle, 7).

Little evidence was brought to light until 1915 when Withers and Carruth (8, 9) claimed a positive correlation between gossypol content and cottonseed toxicity.

Origin and Function

Gossypol is the predominant pigment found in glands which are distributed throughout leaves, stem and root cortices, and floral parts of most cotton varieties (10, 11). Those varieties in which pigment glands are found in abundance are referred to as "glanded"; whereas "glandless" refers to those varieties in which pigment glands are either void or very few in number. In glanded varieties of cottonseed, pigment glands may contain up to 50% gossypol and constitute as much as 3% of the weight of the raw cottonseed kernel. One glandless variety, Bahtim 110, is reported (12) to be totally void of pigment glands and thus free of gossypol.

The cottonseed pigment glands have been studied in detail (13, 14, 15) and appear as darkly colored, distinct morphological entities, spherical or ovoid in shape and 100 to 400 microns in diameter.

The biosynthesis of gossypol in the root of cotton plants was investigated by Heinstein et al. (16) and demonstrated to involve an isoprenoid pathway. Acetate-1-C^{14}, acetate-2-C^{14}, and mevalonate-2-C^{14} were readily incorporated into gossypol which is apparently formed, much like sterols, from two 15-carbon units such as farnesyl pyrophosphate.

The function of gossypol appears to be that of a phytoalexin. Muller (17) and Cruickshank (18) have defined phytoalexins as general antifungal antibiotics produced as a consequence of host-pathogen interactions. Based on these criteria, Bell (19) has shown gossypol to have the following characteristics of a phytoalexin: (i) it forms in all tissues of the cotton plant in response to irritants such as pathogens, metabolic inhibitors, and heavy metal salts, which in turn eventually cause necrobiosis in the affected tissues, (ii) gossypol formation depends on the constituents of living cells since small amounts form in unboiled but not in boiled tissue homogenates, (iii) gossypol acts as a general antifungal antibiotic but has differential toxicity to fungal species (differential toxicity, however, is unrelated to pathogenicity of the species,) (iv) induced gossypol synthesis is restricted to the irritated cells or tissues,

(v) inducibility of gossypol synthesis is not controlled by genes which are known to control the presence of gossypol-containing pigment glands, (vi) and finally the sensitivity of cotton tissues for induced gossypol synthesis is specific; rate and quantity of induced gossypol synthesis in tissues depend on cotton plant genotype, quantity and quality of irritant, and the physiological condition of the tissues. These criteria appear to justify the classification of gossypol as a phytoalexin.

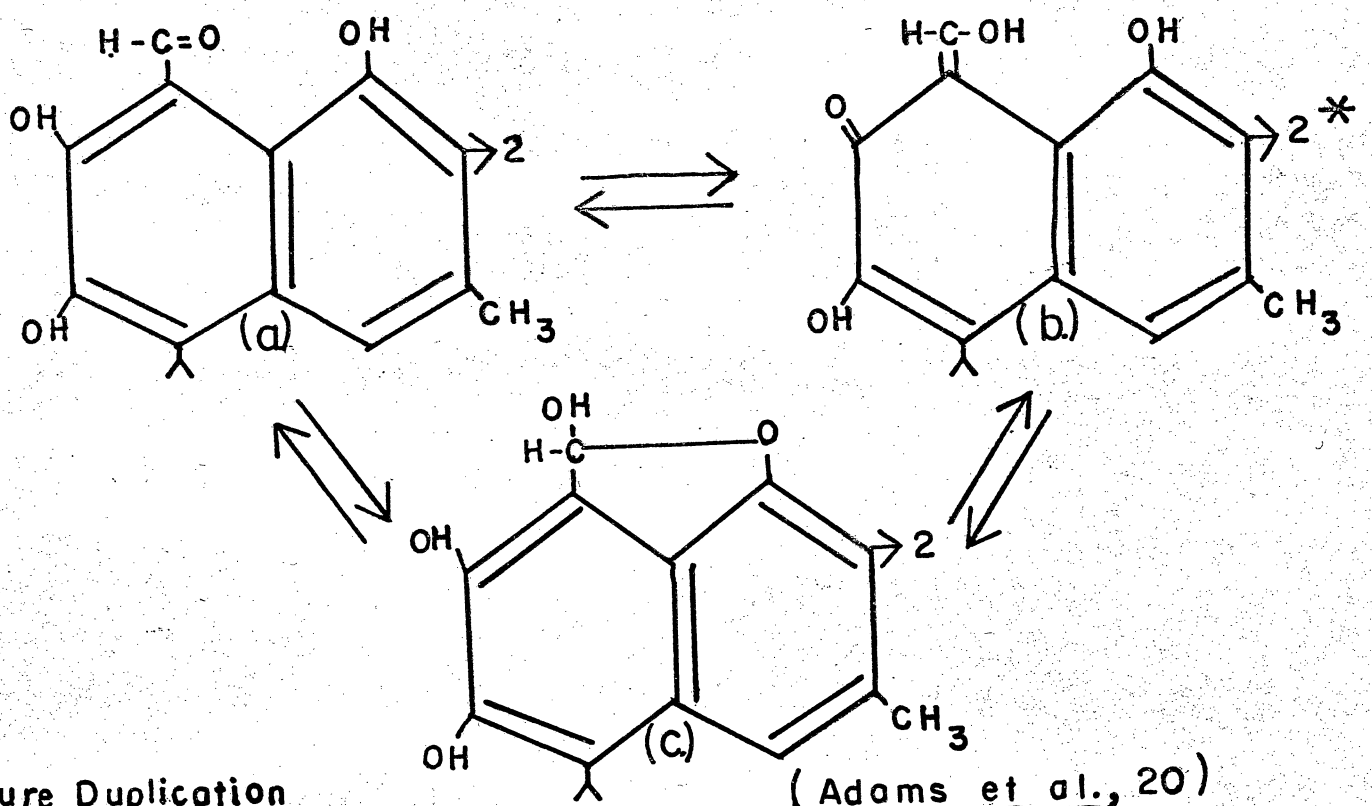
Chemical and Physical Properties

In the monumental work of Adams et al. (20) it was postulated, through the use of gossypol derivatives, that the structure of gossypol could best be represented by three tautomeric forms (Figure I).

Chemical Abstracts has chosen structural form (a) of Figure I to designate the structure of gossypol which has the chemical name 1,1', 6,6', 7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl (2,2'-binaphthalene)-8,8'-dicarboxaldehyde. Subsequent work by Shirley (21) using NMR spectra of gossypol and various gossypol derivatives in different solvent systems has substantiated the existence of the postulated structures (Figure I) as being real and in a dynamic equilibrium.

Dianilinogossypol, the aniline derivative of gossypol, was found by Smith (22) in 1958 to have useful spectrophoto-

FIGURE I TAUTOMERIC STRUCTURES OF GOSSYPOL



* Structure Duplication

(Adams et al., 20)

metric qualities. Using chloroform as the reference solution the absorbance at 440 mu was found to behave according to Beer's law and was therefore valuable for analytical purposes.

Gossypol exists in the cotton plants in two ill-defined forms of chemical combination, "free" and "bound". Free gossypol is determined solely on the basis of solvent extraction. A variety of solvents are commonly used for this purpose, such as ethyl ether, aqueous acetone, and various acetone-water-ether combinations. With this wide variety of accepted solvents it is not surprising that the free gossypol analysis of identical test samples may vary slightly between individual researchers.

Although often inferred in the literature, that portion of gossypol which analyzes as free gossypol does not necessarily have the carbonyl groups free. Any form of bound gossypol, such as certain gossypol peptides, which are soluble in the reagents used in the determination of free gossypol, will analyze as free gossypol (23).

The most probable form of bound gossypol is a gossypol-protein complex with the site of binding being an amino group of the protein bound to the carbonyl group of the gossypol molecule (23).

Pathology of Gossypol Toxicity

It has been demonstrated by Kuiken, Lyman, and Hale (24)

that bound gossypol has no detectable physiological activity even when measured with the very sensitive "egg discoloration on storage" test.

The proportion of free gossypol that is physiologically active has been shown by Bressani et al. (25) to vary considerably. Therefore, the common practice of using the entire free gossypol content as a measure of toxicity should be re-evaluated.

The gross pathology observed in various species of animals due to ingestion or injection of gossypol has been described in detail by a great number of investigators. Some of these adverse effects are: appetite and weight depression (26), dyspnea (27), cardiac muscle degeneration (27), sciatic nerve degeneration (28), edema in pleural, pericardial and peritoneal cavities (27), hemolytic anemia (28), lower serum albumin levels (29), pancreatic enlargement (30), intralobular hepatic necrosis (27), microscopic lesions in spleen and intestine (28), anoxia (26, 28), and death (26, 27, 28).

So universal are the symptoms of weight loss and appetite depression in all species of affected animals that low levels of purified gossypol were once considered therapeutically for the reduction of obesity in man. It was only after a warning in Science by Eagle (31), disclosing the death of experimental animals due to the oral administration of pure gossypol, that this idea was re-evaluated and subsequently

discarded.

Detoxication

Oil Extraction

Due to the high protein value of cottonseed, it is desirable to use cottonseed meal, the by-product of edible cottonseed oil, as a protein supplement for both animal and human consumption. Since the gossypol content and protein value of cottonseed meals vary considerably depending on the method of oil extraction, much attention has been focused on the development of a commercial method of oil extraction which leaves a gossypol-free, high quality protein meal.

King, Kuck and Frampton (32) have recently developed an acetone-petroleum ether-water azeotrope solvent method of extraction which leaves no free gossypol residues in the meal. Also, greater quantities of oil were extracted and the heat-labile epsilon-amino group of lysine was left intact. Unfortunately, due to the time and materials involved, this procedure for the production of a low-gossypol high quality protein supplement is not economically feasible at the present time. In fact, due to this cost, over 50% of all cottonseed meal in the United States today is produced by the screw-pressed method (33).

Heat Treatment

Numerous reports have appeared of varying degrees of successful detoxication of cottonseed by cooking, steaming,

and autoclaving (at least 26 references cited in the reviews by Eagle (34) and Adams et al. (10), the results of which were best summarized by Eagle (7):

> "It has been emphasized that successful detoxification of cottonseed meal by overcooking is in reality a failure if the favorable effects from the decreased toxicity are offset by the unfavorable effect from protein damage in the process."

Alkali Treatment

Eagle, Bialek, Davies and Bremer in 1954 (35) published a comprehensive report on salt and alkali treatments of cottonseed meal. In a series of four tests, three toxic cottonseed meals were treated with 22 aqueous solutions of salts and alkalies and fed to rats. The test results indicated that those rats fed on the cottonseed samples treated with sodium hydroxide showed the least mortality and the highest weight gains. According to these studies, the order of decreasing effectiveness was:

$$NaOH > KOH > NH_4OH > Ca(OH)_2.$$

However, there remained a substantial amount of residual gossypol toxicity in the alkali-treated cottonseed meals.

Protein and Amino Acid Supplementation

The value of the amino acid lysine in relieving gossypol toxicity has been much in dispute. Some investigators (36, 37) have shown a positive correlation between dietary lysine and gossypol detoxication. On the other hand, other investigators (38, 39) have found no reduction in gossypol toxicity

due to dietary supplementation with lysine alone, but detoxication nevertheless did occur when a "lysine rich" protein was used.

The supplementation of cottonseed meal with certain proteins, particularly proteins of high biological values and high lysine content, has been demonstrated to substantially reduce the symptoms of gossypol toxicity (7, 38, 39, 40, 41).

It was postulated by Lyman (38) that free lysine reacts with gossypol in spite of the fact that such a complex can apparently be absorbed as such from the digestive tract; on the other hand, a protein gossypol complex cannot be absorbed.

It should be realized that the addition of high quality protein for the purpose of gossypol detoxication would be prohibitive because of the high cost.

Iron Salts

The dietary use of iron salts, particularly ferrous sulfate, has been shown in a variety of animals (26, 37, 39, 42) to be an effective prophylactic agent in avoiding the symptoms of toxicity commonly associated with the ingestion of gossypol.

The elucidation of the structures of the gossypol-iron chelates, believed to be responsible for the detoxication of gossypol, was effected by Shieh *et al.* (43).

The absorbance of gossypol-iron chelates clearly indicates that the chelate formation increases as iron is added until the ratio of iron to gossypol is 2 to 1. At this point, no further increase in absorption can be accomplished with additional iron. A titration curve supports this conclusion and supplies additional information to show the existence of a chelate also having a gossypol to iron ratio of 1 to 1. It was concluded that the points of binding of the iron-gossypol complex are at the 6,7 and 6',7' positions.

Even though iron salts are highly beneficial in detoxifying gossypol, their use in a practical feeding ration is beset with following difficulties: (i) stored rations containing ferrous sulfate have a very short shelf life due to discoloration and deterioration (44), (ii) dietary requirements for other minerals must be re-established because of interaction with various levels of iron in the diet, (iii) the amount of iron necessary to detoxify gossypol in an animal ration is usually enough for symptoms of iron toxicity to develop. (Wilche *et al.* (45) recommends that up to 600 ppm of iron is necessary in broiler rations to assure complete gossypol deactivation. On the other hand, McGhee *et al.* (46) maintains that, depending on the mineral balance of the diet, as little as 40 ppm of iron can depress the growth rate of chickens.)

Glandless Cotton Plants

There has been much emphasis placed on the development of an essentially gossypol-free cotton plant. This may be the direction of research in which a solution to the gossypol problem will be found. But, it should also be realized that at the same time, such a variety which is free of gossypol is also free of its natural phytoalexin. This leaves the plants highly susceptible to fungal disease and vulnerable to insect infestation. These two problems of gossypol reduction make the glandless varieties undesirable to most planters at the present time.

In conclusion, then, it seems that little progress has been made in the last century concerning the gossypol problem, for cottonseed remains today as it did in the time of Voelker (16) "A harmful and injurious feedstuff."

METHODS AND MATERIALS

General Stock Materials

Preparation and Maintenance of Fungal Cultures

A potato-dextrose-agar (PDA) media (Difco Laboratories) was prepared by autoclaving 50 ml of media at 18 psi and 121° C in cotton-stoppered Roux bottles for 20 minutes. A period of 24 hours was then allowed for the media to solidify and return to room temperature. Inoculations were then made from pure cultures prepared at Beltsville, Maryland (Dr. P. B. Marsh), derived from one cell isolates, by use of the "streak method" of inoculation. Cultures were maintained at room temperature in indirect sunlight.

Spore Suspensions

All spore suspensions were freshly prepared for each experiment in the following manner:

1. fifty ml of ion-free distilled water was added to a Roux bottle containing a fungal culture,
2. a flame sterilized wire loop was then used to gently scrape the surface of the media and thus liberate fungal spores,
3. the spore suspension was poured into a 250 ml glass-stoppered R/B flask to avoid contamination and to allow for convenient shaking.

Aureomycin Solutions

All solutions of aureomycin were freshly prepared to a 0.05% concentration of active ingredient. This was accomplished by mixing 0.2215 g of a 22.57% active aureomycin

(American Cyanamid Company) powder with 100 ml of ion-free distilled water.

Preparation of Cottonseed Meat Supply

A uniform pool of finely chopped cottonseed meats was established by first dehulling a supply of glanded cottonseed* with a Labconco mill and then finely chopping the cottonseed meats with a Waring Laboratory Blendor. The chopped meats were stored in gallon cardboard containers at room temperature.

Experiment I -- Gossypol Binding Via Fungal Interactions As Measured In Vitro

One hundred and thirty samples consisting of 0.25 g of finely ground cottonseed meats from the cottonseed meat pool were placed in individual 8 ounce glass bottles loosely fitted with metallic screw caps to allow for gaseous exchange.

Group Size and Treatment

Group I: The "normal control group" was composed of 20 samples maintained at room temperature and received no treatment prior to gossypol analyses.

Group II: The "water-additive control group" was composed of 10 samples maintained at 5° C and received an initial treatment of 0.25 ml of a 0.05% aureomycin solution and 0.75 ml of distilled water thoroughly mixed into each sample. Further treatment consisted of the addition of 1 ml of distilled water thoroughly mixed into each sample at the end of day 2 and day 6. At the end of day 10, each sample was thoroughly mixed.

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* Variety Acala 4-42-77 (Beltsville, Maryland).

Group III: The "fungally inoculated test group" was comprised of 100 samples maintained at room temperature and received an initial treatment of 0.25 ml of a 0.05% aureomycin solution and 0.75 ml of a spore suspension thoroughly mixed into each sample.
Further treatment consisted of the addition of 1 ml of distilled water thoroughly mixed into each remaining sample at the end of day 2 and day 6. At the end of day 10, each remaining sample was thoroughly mixed.

Gossypol Analyses

Group I: At time 0, 10 samples were analyzed for free gossypol and 10 samples for total gossypol.

Group II: After 15 days, 5 samples were analyzed for free gossypol and 5 samples for total gossypol.

Group III: After 0, 1, 2, 3, 4, 5, 6, 7, 10, and 13 days, 5 samples were analyzed on each of the given days for free gossypol and 5 samples for total gossypol.

Experiment II -- Physiological Activity of Fungally "Deactivated" Gossypol in the Rat

Preparation of Moldy Cottonseed Meats*

Moldy cottonseed meats were prepared in the following manner:

1) 80 g of cottonseed meats from the cottonseed meat pool were placed in each of five autoclaved 1500 ml Erlenmeyer flasks,

2) 80 ml of 0.05% aureomycin solution was added,

3) 10 ml of a Diplodia 308 spore suspension was added and the flask was stoppered with a cotton plug,

4) the contents of each flask were thoroughly mixed by stirring with a flame sterilized glass rod on days 4 and 7,

5) on day 10, the contents of all flasks were placed in a common container and thoroughly mixed,

* Also used in Experiment III.

6) the moldy cottonseed meats were then thinly spread on aluminum foil and dried by forced air at room temperature for 2 days. This returned the cottonseed meats to their original weight.

7) five samples of moldy cottonseed meats were analyzed for free gossypol and 5 samples for total gossypol.

Preparation of Rations

Control Ration: The control ration consisted of 50% basal ration (Table I) and 50% untreated cottonseed meats from the cottonseed meat pool.

Test Ration: The test ration consisted of 50% basal ration (Table I) and 50% of the previously prepared moldy cottonseed meats.

All rations were placed in cardboard containers and maintained at 5° C.

Group Size and Treatment

Three groups of 6 male weanling Sprague-Dawley derived rats were used.

Group I: the "normal cottonseed meat control group" was free-fed the control ration.

Group II: the "moldy cottonseed meat test group" was pair-fed to the Group I rats.

Group III: a second "moldy cottonseed meat test group" was free-fed the test ration.

Daily weight gain and feed consumption records were kept. After the third test day, three rats in each of the three groups were sacrificed and their livers and lungs excised and separately stored at 0° C.

The livers and lungs of the remaining three rats of each group were taken after the sixth test day for the Group III

rats and after the eighth test day for the Group I and Group II rats. All excised organs were frozen until an analysis for total gossypol content was made on each organ.

Experiment III: Physiological Activity of Fungally "Deactivated" Gossypol in the Chicken

Preparation of Rations

Control Ration I: control ration I consisted of 50% poultry "starter ration"* and 50% untreated cottonseed meats from the cottonseed meat pool.

Control Ration II: control ration II consisted of 71% poultry "starter ration"* and 29% untreated cottonseed meats from the cottonseed meat pool. This ration was standardized to the test ration so that it contained an equivalent amount of free gossypol in order to determine if the "free gossypol" resulting from the fungal growth was equivalent to the original free gossypol content.

Test Ration: the test ration consisted of 50% poultry "starter ration"* and 50% moldy cottonseed meats prepared in Experiment II.

All rations were placed in cardboard containers and maintained at 5° C.

Group Size and Treatment

Three groups, each containing 3 white leghorn cockerels, were used in Experiment III.

Group I: these animals were fed ad libitum the control ration I.

Group II: these animals were fed ad libitum the test ration.

* V.P.I. Dept. of Poultry Science Starter Diet A 2-1.

Group III: These birds were fed control ration II offered at the consumption rate of Group II.

Records of body weight change and total feed consumption were kept for each bird. After the second test day, one bird was sacrificed from each group and its liver, gallbladder and pectoral muscles were taken. Identical tissue samples were taken from a second bird of each group after the fourth test day and from the last bird of each group after the sixth test day. All excised organs and tissues were frozen at 0° C until an analysis for total gossypol content was made.

Determination of Free Gossypol in Cottonseed Meats

The Department of Health, Education, and Welfare (47) procedure for the determination of free gossypol in cottonseed meats was used with the following modifications:

1. the Hyflo Super-Cel filter was replaced by a four-inch column of tightly packed glass wool.
2. 200 ml instead of the recommended 100 ml volumetric flasks were used for volumetric measurements.

Determination of Total Gossypol in Cottonseed Meats

Smith's (22) procedure for the determination of total gossypol in cottonseed meats was used with the following modifications:

1. all samples were placed in a water bath for one hour instead of on a porcelain water bath cover for 45 minutes.
2. the Hyflo Super-Cel filter was replaced by a four-inch column of tightly packed glass wool.
3. the bell jar was eliminated since no vacuum was needed with the glass wool filter.

4. 200 ml instead of the recommended 100 ml volumetric flasks were used for volumetric measurements.

5. in the sample dilution a 4 ml aliquot transfer was used.

Determination of Total Gossypol in Rat and Chicken Tissues

Smith's procedure (48) for the determination of bound gossypol in swine tissues was used for the determination of total gossypol in rat and chicken tissues, since no extraction for free gossypol was used as in the original method. Additional modifications included the following:

1. the 10 g sample sizes were reduced to:
   rat livers -- 1 gram
   rat lungs -- weight of organ to a maximum weight of 0.5 gram
   chicken liver -- 1 gram
   chicken muscles -- 1 gram
   chicken gallbladder -- organ weight

2. all tissues were homogenized in a Potter-Elvejhen Tissue Homogenizer.

3. the Hyflo Super-Cel coluum was replaced with a four-inch tightly packed glass wool column.

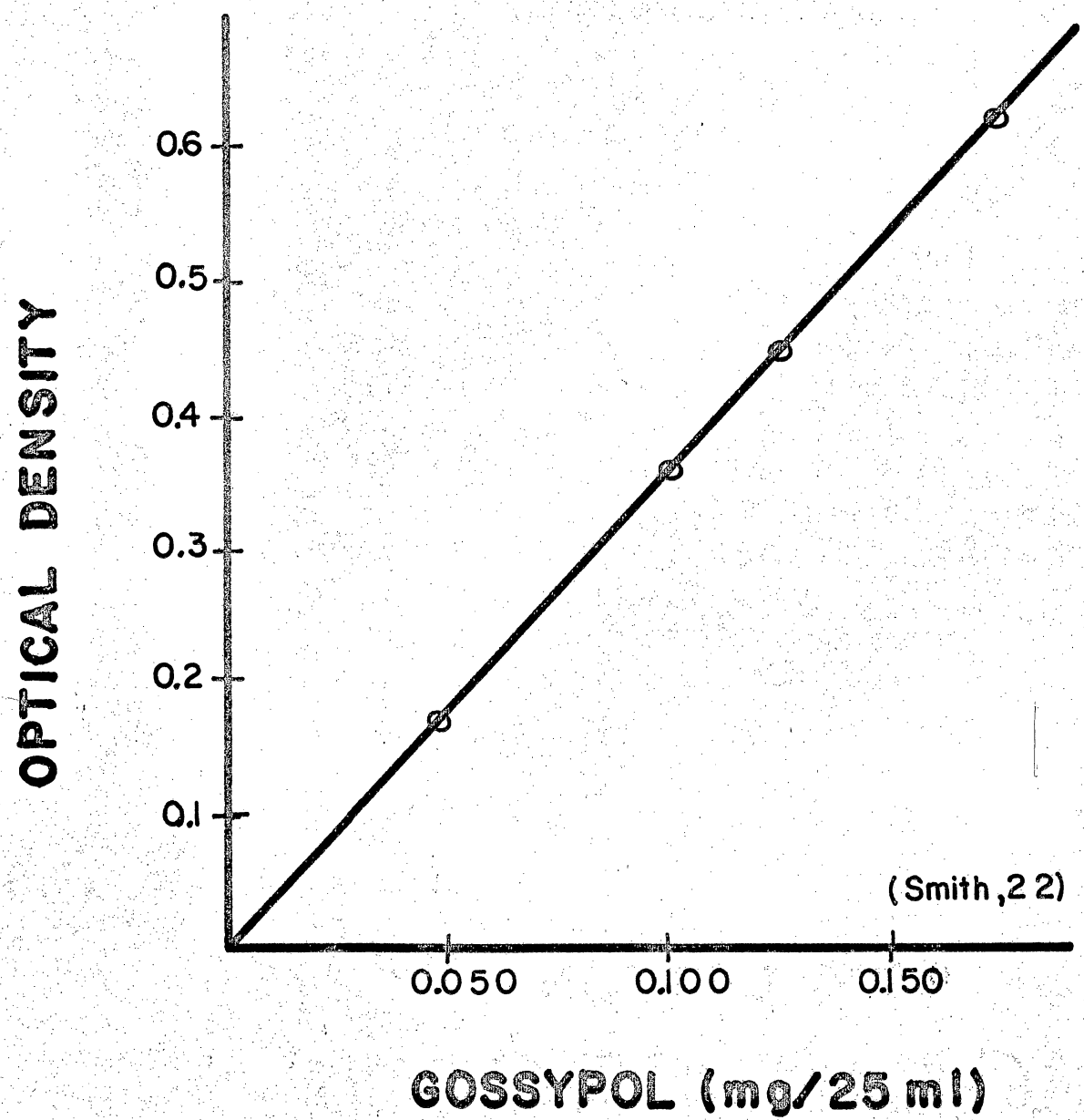
4. normal rat organs, treated in the same manner as the test organs, replaced redistilled hexane as a "blank" in the colorimetric determination of gossypol.

Calculations

1. Total and free gossypol content in cottonseed meats and total gossypol in animal tissues

The final 25 ml aliquots were read at 440 mu and the weight of gossypol in milligrams was determined from a standard curve (Figure II). The following formula is then employed:

FIGURE III CALIBRATION CURVE OF GOSSYPOL AS THE ANILINE DERIVATIVE IN CHLOROFORM (440mu)



$$\frac{I \times Mg \times 100}{Wt \times T \times 1000} = \% \text{ of Total or Free Gossypol}$$

I -- initial sample dilution volume

Mg -- milligrams of gossypol per 25 ml final solution

Wt -- weight of sample analyzed

T -- volume of sample transferred for final dilution.

2. Bound gossypol content in cottonseed meats and animal tissues

The bound gossypol content was calculated by use of the following relationship:

Bound gossypol = Total gossypol — Free gossypol

3. Means and standard deviations of the means

All means and standard deviations of the means were calculated in a regular statistical manner with the exception that bound gossypol means and standard deviations of the means were calculated from total and free gossypol means and not from individual values.

RESULTS AND DISCUSSION

Experiment I

The purpose of this experiment was to examine, by analytical means, the possibility that free gossypol might undergo some type of binding or in some other way be inactivated due to the fungal growth. Three sample groups were required for this experiment.

Group I consisted of non-treated glanded cottonseed meats and was analyzed to establish the quantities of both free and bound gossypol present in "normal" cottonseed meat.

It was found in Group I, which is representative of all normal glanded cottonseed meat samples used in this thesis, that the mean value of total gossypol is 1.11 ± 0.03%, the mean value of free gossypol is 0.91 ± 0.01%, and the calculated mean value of bound gossypol is 0.20 ± 0.03% (Table III).

Group II consisted of water-treated glanded cottonseed meats and was used as a control to measure the effect of water on free gossypol content. In order to prevent bacterial or fungal growth, an environment of 5° C and 0.05% aureomycin was used.

After 15 days of water treatments, the mean gossypol content was found to be 1.06 ± 0.03% total gossypol, 0.73 ± 0.02% free gossypol, and 0.33 ± 0.04% bound gossypol as calculated (Table IV). Thus, the amount of free gossypol that was somehow bound after water treatment is 0.18 ± 0.03%

TABLE III Gossypol Content in Normal Cottonseed Meats

Sample Number	% Free Gossypol	Sample Number	% Total Goss.
1	0.88	1	1.20
2	0.85	2	1.06
3	0.85	3	1.28
4	0.92	4	1.20
5	0.92	5	1.08
6	0.96	6	1.03
7	0.94	7	1.03
8	0.94	8	1.08
9	0.94	9	1.03
10	0.90	10	1.08
$\leq X$ -- 9.10		$\leq X$ -- 11.07	
$\bar{x}$ -- 0.91		$\bar{x}$ -- 1.11	
$S_{\bar{x}}$ -- 0.01		$S_{\bar{x}}$ -- 0.03	

Mean Total Gossypol -- 1.11 ± 0.03%

Mean Free Gossypol-- 0.91 ± 0.01%

Mean Bound Gossypol -- 0.20 ± 0.03% (Calculated from Mean Total Gossypol and Mean Free Gossypol)

TABLE IV Gossypol Content in Water-Treated Cottonseed Meats

Sample Number	% Total Gossypol	Sample Number	% Free Gossypol
1	1.04	1	0.74
2	1.06	2	0.74
3	1.01	3	0.68
4	1.03	4	0.78
5	1.17	5	0.72
$\leq X$ -- 5.31		$\leq X$ -- 3.66	
$\bar{x}$ -- 1.06		$\bar{x}$ -- 0.73	
$S_{\bar{x}}$ -- 0.03		$S_{\bar{x}}$ -- 0.02	

Mean Total Gossypol -- 1.06 ± 0.03%

Mean Free Gossypol -- 0.73 ± 0.02%

Mean Bound Gossypol -- 0.33 ± 0.04% (Calculated from Mean Total Gossypol and Mean Free Gossypol)

of the sample weight. Indeed it would seem that a significant amount of free gossypol undergoes a binding effect that can be attributed to the presence of water. This is in agreement with the findings of Bressani et al. (49), who concluded that of 18 samples of ground cottonseed meats, from different localities, at least 14 samples showed some reduction in free gossypol upon the addition of water.

Group III consisted of water-treated, fungally inoculated glanded cottonseed meats. These were periodically analyzed for changes in free and bound gossypol levels that can be directly attributed to the presence of a fungal system. In order to prevent bacterial growth, 0.05% aureomycin was used.

By the use of sequential gossypol analyses of moldy cottonseed meats (Table V), free gossypol was found to significantly decrease during periods of active mold growth as graphically illustrated in Figure III. To sustain mold growth it became necessary to maintain moist conditions by periodic additions of water equivalent to that added in Group II.

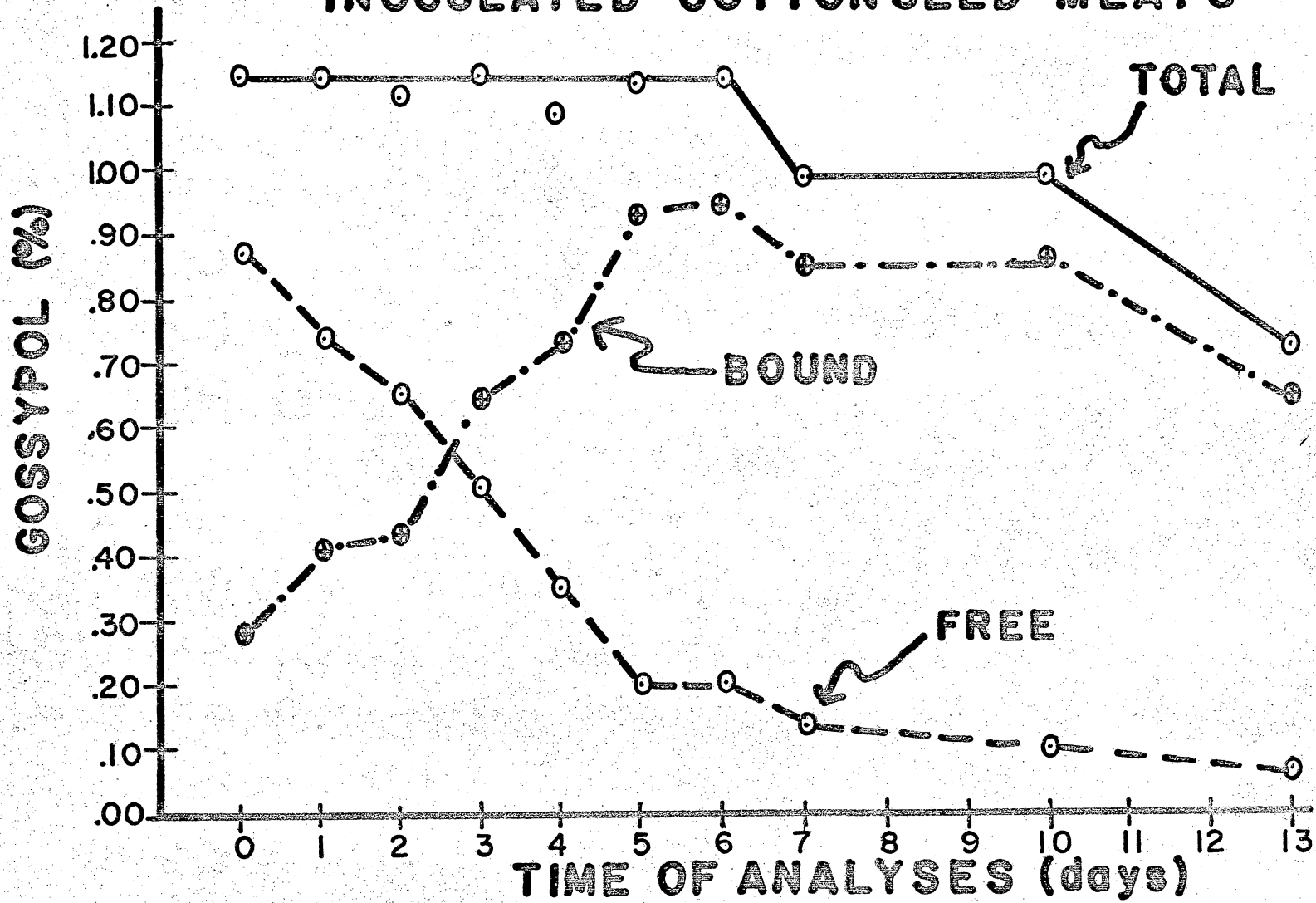
Visible mold growth could not be detected until after the analyses were performed on day 1, but vigorous mold growth was in evidence by the time of analyses on day 2. Therefore, it is reasonable to assume that the reduction of free gossypol to the mean level of 0.73 $\pm$ 0.02%, as analyzed on day 1, was the result of water addition only. The validity of this assumption is strengthened by the previously

TABLE V Mean Gossypol Content of Fungally-Inoculated Cottonseed Meats (Cf. Appendix, Table V and Appendix, Table VI)

Day	Percent Total Gossypol	Percent Free Gossypol	Percent Bound Gossypol
0	1.15 ± 0.03	0.87 ± 0.02	0.28 ± 0.03
1	1.14 ± 0.02	0.73 ± 0.02	0.41 ± 0.03
2	1.10 ± 0.04	0.66 ± 0.01	0.44 ± 0.04
3	1.15 ± 0.02	0.51 ± 0.01	0.64 ± 0.02
4	1.07 ± 0.02	0.35 ± 0.01	0.72 ± 0.02
5	1.12 ± 0.02	0.20 ± 0.03	0.92 ± 0.03
6	1.14 ± 0.01	0.20 ± 0.02	0.94 ± 0.02
7	0.97 ± 0.02	0.13 ± 0.01	0.84 ± 0.02
10	0.98 ± 0.01	0.10 ± 0.01	0.88 ± 0.02
13	0.71 ± 0.02	0.08 ± 0.01	0.63 ± 0.02

Note: Further treatment consisted of the addition of 1 ml of distilled water thoroughly mixed into each sample at the end of day 2 and day 6. At the end of day 10, each sample was thoroughly mixed.

FIGURE III MEAN GOSSYPOL CONTENT IN FUNGALLY-INOCULATED COTTONSEED MEATS



mentioned findings of the Group II water control group, which also had a 0.73 ± 0.02% free gossypol level which was achieved after 15 days of water treatment. It therefore seems improbable that any further reduction in the free gossypol level below that of 0.73% can be attributed to water *per se*.

The role of free gossypol as a bacteriacide was lost after day 6 and a staphlococcus-type organism was identified as the major bacterial contaminant. This was presumably due to the low concentration of free gossypol present in the samples at that time.

Due to the bacterial contamination, it was impossible to determine whether the analytical loss of both free and bound gossypol, after day 6, was due to the action of bacteria, fungi or to the synergistic action of the two. Also, since both free and bound gossypol decreased at the same time, it could not be determined by the conventional methods of gossypol analyses whether the decrease was due to the loss of molecular integrity or to the formation of a bond in which aniline could not substitute.

In any event, it can be concluded that the decrease in free gossypol content from day 1 to day 6 of 0.53 ± 0.2% is due solely to the interaction of the fungal system.

Experiment II

The purpose of this experiment was to determine whether the reduction in free gossypol, observed by the *in vitro* studies of moldy cottonseed meats, would show a corresponding decrease of physiological activity in the rat. In pursuit of this purpose, it was necessary to establish three rat feeding groups: a control group, a pair-fed test group and a free-fed test group. Analyses were run on days 3 and 8* to determine the gossypol concentration in rat livers and lungs. These values were then used to evaluate differences between groups as well as changes within a group. The results are presented in Tables VI and VII.

Group I

Group I rats were free-fed the control ration to establish normal activity. Since the control ration is composed of 50% glanded cottonseed meats from the cottonseed meat pool (Table III), the ration fed to Group I rats contained 0.45% free gossypol.

At the end of day 3, the average daily feed consumption was 1.2 g/rat/day and the average daily weight loss was 3.2 g/rat/day. The average gossypol concentration was 0.098 mg/g in rat lungs and 0.167 mg/g in rat livers.

For days 4 through 8, the average daily feed consumption was 1.1 g/rat/day and the average weight loss was 1.5 g/rat/day.

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* Group III analyses were run on days 3 and 6.

TABLE VI Average Daily Feed Consumption and Weight Changes
(Cf. Appendix, Table VII, IX, and XI)

Rat Group	Days 0 thru 3 (g/rat/day)		Days 4 thru 8 (g/rat/day)	
	Feed Cons.	Weight Change	Feed Cons.	Weight Change
I	1.2	-3.2	1.1	-1.5
II	1.2	-1.6	1.1	-0.5
III	5.7	4.3	7.1*	4.4*

* Days 4 thru 6.

TABLE VII Average Gossypol Concentration and Tissue Content
(Cf. Appendix, Table VIII, X, and XII)

GOSSYPOL CONCENTRATIONS (mg/g)					
	Livers			Lungs	
Group	Day 3	Day 8	Group	Day 3	Day 8
I	0.167	0.075	I	0.098	0.051
II	0.026	0.027	II	0.045	-0.017
III	0.043	0.036**	III	0.032	0.005**

GOSSYPOL CONTENT (mg)					
	Livers			Lungs	
Group	Day 3	Day 8	Group	Day 3	Day 8
I	0.323	0.077	I	0.043	0.017
II	0.052	0.055	II	0.024	-0.010
III	0.138	0.132**	III	0.017	0.002**

** Day 6

On test day 8, the average gossypol concentration was 0.051 mg/g in rat lungs and 0.075 mg/g in rat livers.

It was noted that throughout the feeding trial, feed consumption for Group I and thus gossypol ingestion, remained low but fairly constant; whereas the gossypol concentration in organs decreased from day 3 to day 8. Gossypol concentration decreased 1.9 times in rat lungs and 2.2 times in rat livers. This indicates that, at the level of free gossypol fed, the tissue retention of gossypol in the rat decreased with time. It was also obvious and not unexpected that the gossypol content was higher in rat livers than in rat lungs.

All Group I rats showed the typical symptoms of gossypol toxicity: appetite and weight depression, dyspnea, lack of vigor (Plate II), and post-mortem findings of fluid accumulation in body cavities and intestines (Plate III). During test day 8, the remaining test rats entered a coma which culminated in death. These symptoms in addition to the high gossypol content, in both the livers and the lungs of the test rats, leave little doubt as to gossypol being the toxic agent.

Group II

Group II rats consumed the test ration pair-fed to the consumption of the control group. The test ration was composed of 50% moldy cottonseed meats which were found by

PLATE II Rat Suffering From Gossypol Toxicity

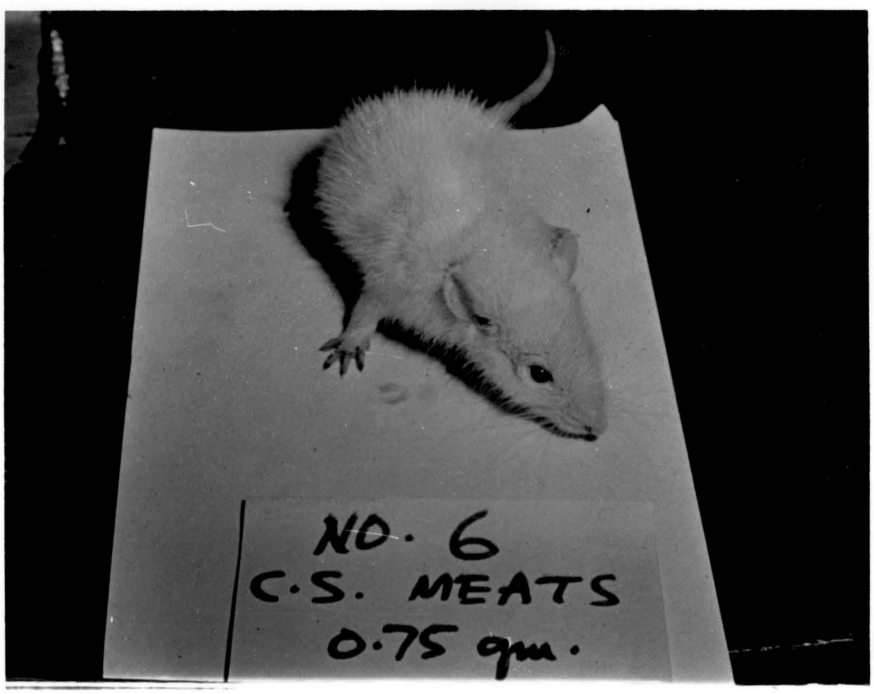
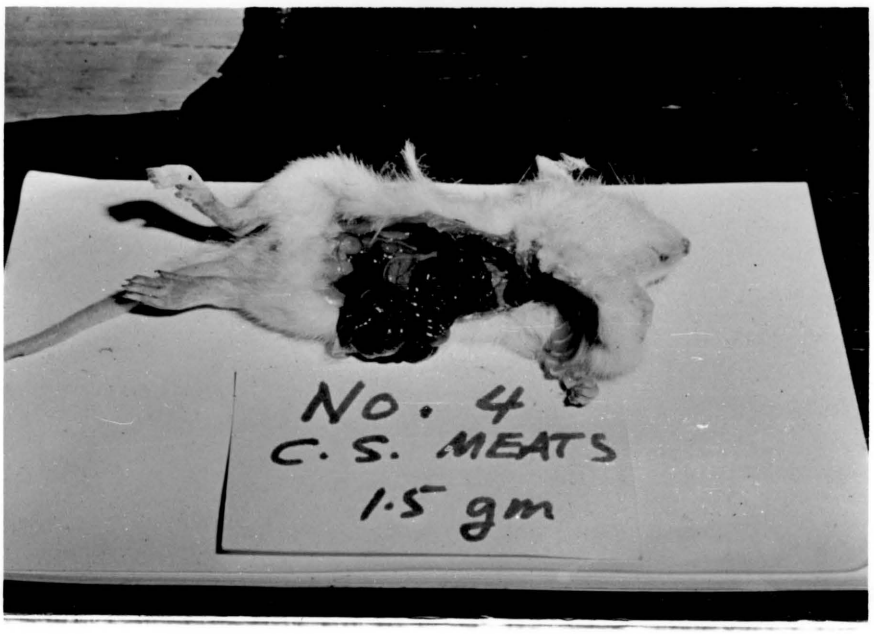


PLATE III Fluid Accumulation in Intestines Due to Gossypol Toxicity in the Rat



analyses to contain 1.14% total gossypol of which 0.52% was free gossypol and 0.62% was calculated as bound gossypol (Appendix, Table XIII). Therefore, the test ration contained 0.26% free gossypol which was only 58% as much free gossypol as in the control ration.

At the end of day 3, the average daily weight loss was 1.6 g/rat/day. The average gossypol concentration was 0.045 mg/g in rat lungs and 0.026 mg/g in rat livers. The average daily weight loss, for days 4 through 8, was 0.45 g/rat/day. On test day 8, the average gossypol concentration was -0.017 mg/g in rat lungs and 0.027 mg/g in rat livers.

It was again noticed, as in Group I, that the concentration of gossypol in rat livers was much higher than in the lungs; also the trend toward decreased tissue retention of gossypol with time was evident in rat lungs. In fact, those rat lungs analyzed on day 8 showed negative values for gossypol concentration. This indicates that not only was the gossypol level low but that the natural components of rat lungs, which react with aniline and thus cause spectrophotometric interference, were lower in the lungs of the Group II rats than in the lungs of rats on a cottonseed free diet used to "zero" the analyses.

Although Groups I and II consumed equal amounts of feed, the test rats lost less than one-half as much weight as did the control rats. In fact, by day 5 the Group II rats

leveled off where weight was neither lost nor gained.

Group II rats showed no symptoms of gossypol toxicity and appeared at all times to be hungry and quite healthy as compared to the control rats (Plate IV).

Group III

Group III rats were free-fed the test ration containing 0.26% free gossypol. At the end of day 3, the average daily feed consumption was 5.7 g/rat/day and the average daily weight gain was 4.3 g/rat/day. The average gossypol concentration was 0.043 mg/g in rat livers and 0.032 mg/g in rat lungs. The average daily feed consumption, for days 4 through 6, was 7.1 g/rat/day and the average weight gain 4.4 g/rat/day. On test day 6, the average gossypol concentration was 0.036 mg/g in rat livers and 0.005 mg/g in rat lungs. A reduction in the tissue retention of gossypol with time was again in evidence as was the significantly higher gossypol content in livers as compared to lungs.

No visible effects of toxicity could be attributed to the ingestion of the test ration (Plate V), in spite of the fact that the food consumption was much greater than the control group.

Discussion

A comparison of the ad libitum feed consumption for the rats of Groups I and III showed that, for the average rat day, more than 5 times as much moldy test ration was

PLATE IV Rat in Coma Due to Ingestion of Cottonseed Meats Compared to Rat Pair-Fed Moldy Cottonseed Meats

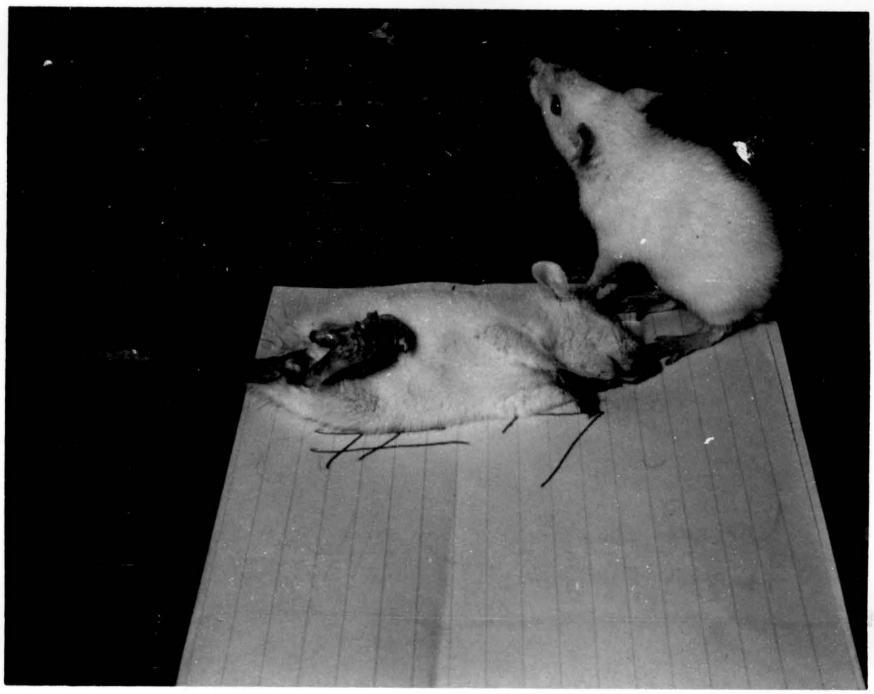


PLATE V Rats Free-Fed Moldy Cottonseed Meats For Six Days

consumed as normal control ration (Figure IV).
In observing the weight changes in each rat group, it was not unexpected that Group III rats with their high feed consumptions would also make moderately high weight gains. A comparison of Groups I and II showed that even though their feed consumptions were identical and both groups lost weight, Group II rats lost less than one-half as much weight as did Group I rats (Figure V). This indicates a clear-cut superiority in the value of the moldy test ration over the control ration.

Although rat Groups I and II were pair-fed, the analytical amount of free gossypol consumed was greater for the Group I rats than for the Group II rats fed the moldy cottonseed ration. This difference was reflected in the concentration of gossypol found in rat livers and lungs of the respective groups (Table VII).

The overwhelming evidence found in the feed consumption, weight changes, animal health, and tissue analyses leads to the conclusion that in fungally inoculated cottonseed the binding of free gossypol, as observed by analytical means in Experiment I, is effective in reducing the physiological activity of gossypol in the rat. In fact, the extent of physiological reduction may not be adequately measured by analyses of free gossypol in the ration after fungal growth. This seems to be the case in Group III rats as compared to the control Group I. Group III rats consumed free gossypol

FIGURE IV AVERAGE CUMULATIVE FEED CONSUMPTION

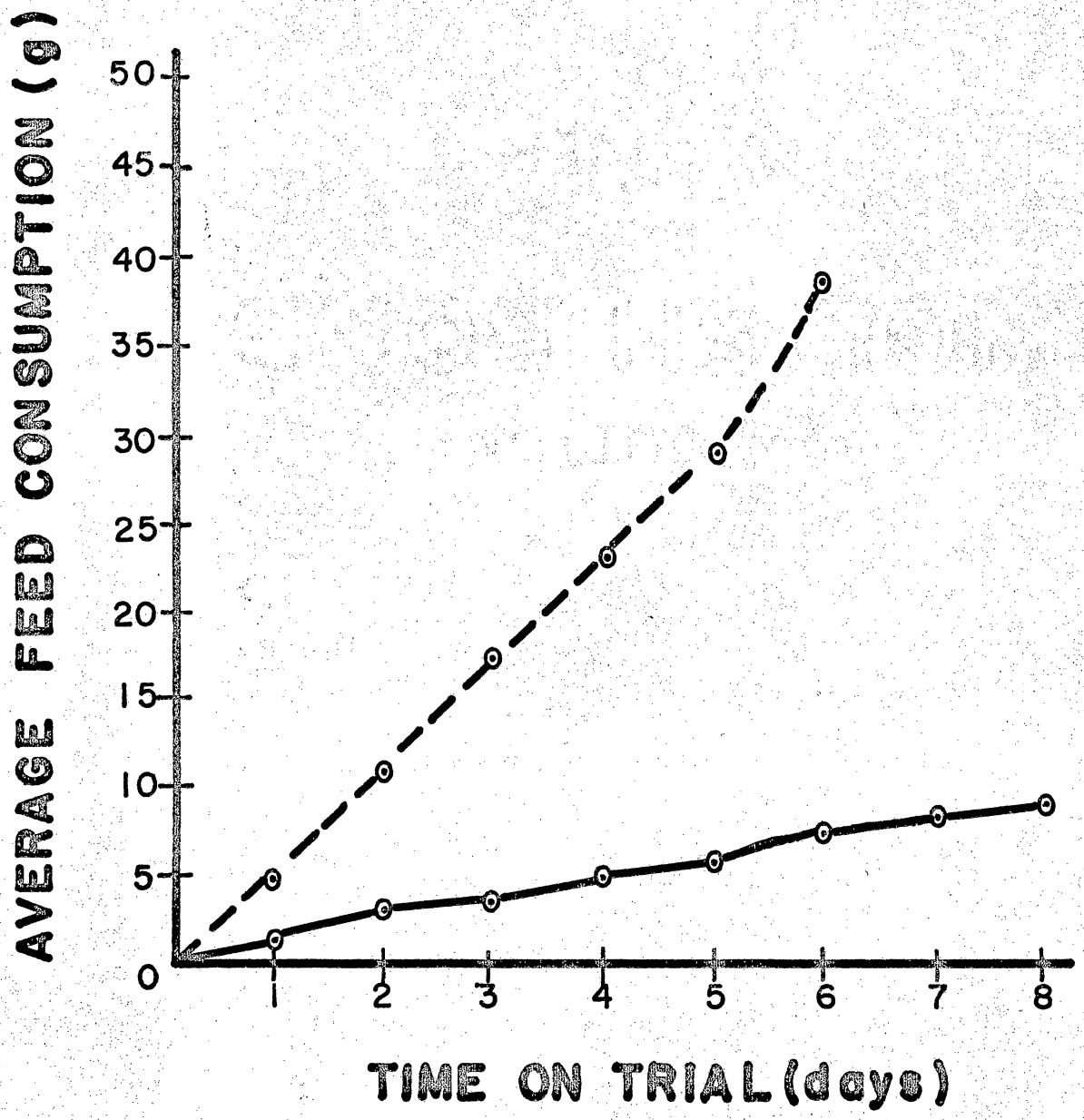
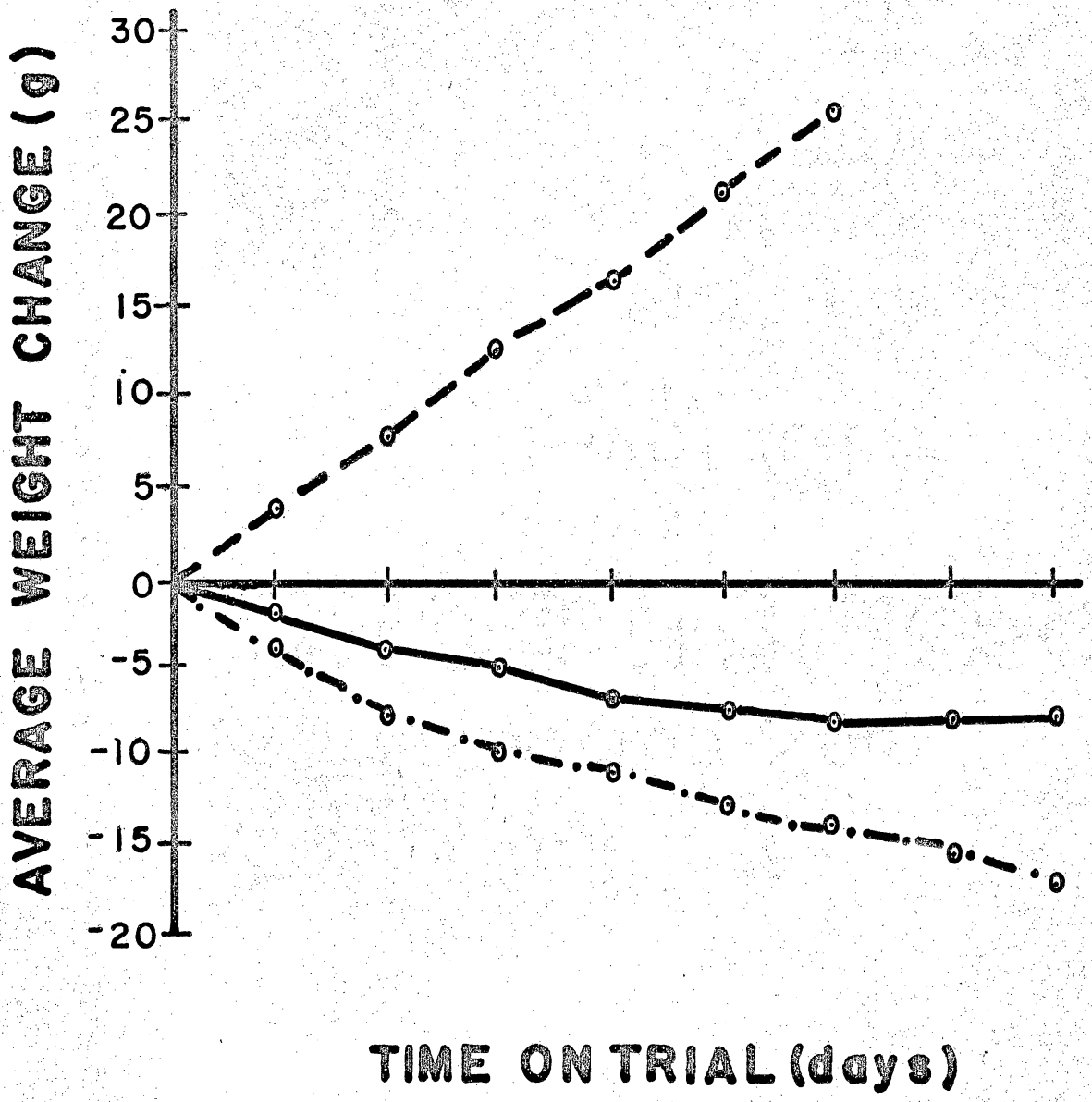
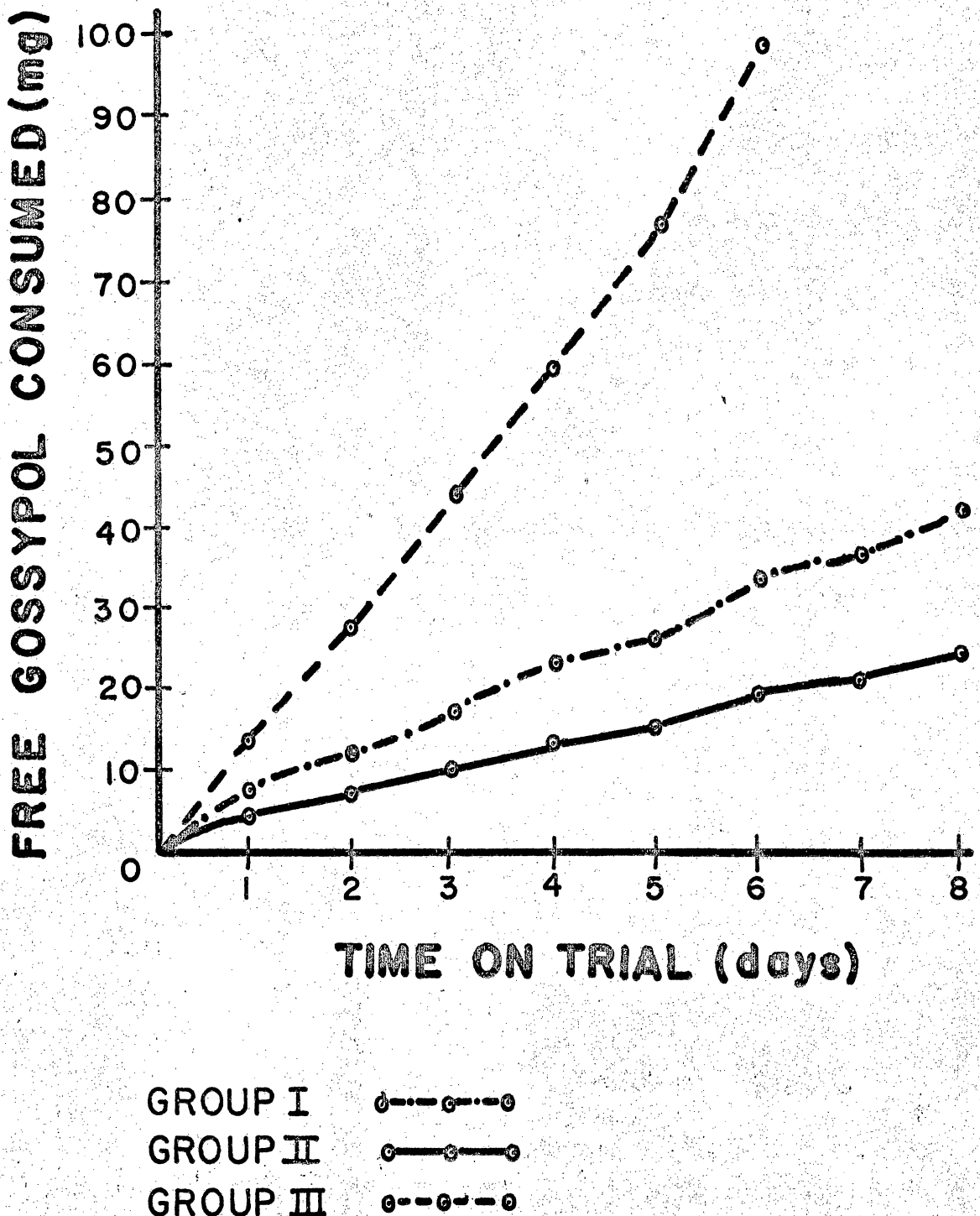


FIGURE V AVERAGE CUMULATIVE WEIGHT CHANGE



at almost 3 times the rate (Figure VI) with approximately one-third the tissue accumulation of gossypol (Table VII) as did the Group I rats. This difference in the toxicologic activity of the free gossypol, perhaps has something to do with feed intake.

FIGURE VI AVERAGE CUMULATIVE FREE GOSSYPOL CONSUMPTION



Experiment III

The purpose of this experiment was twofold: 1) to determine whether fungal growth on cottonseed would decrease the physiological activity of gossypol in the chicken, and 2) to evaluate the correlation of free gossypol content with toxicity in normal and molded cottonseed meats.

Three chicken feeding groups were established: I, a free-fed control group; II, a free-fed test group; and III, a control group which was fed a ration containing the same analyzed level of free gossypol and offered at the same consumption rate as the test group. Analyses were run on days 2, 4 and 6 to determine the gossypol concentration in livers and pectoral muscles and total gossypol content of gallbladders. These analyses were used to evaluate differences between groups and changes within a group. The results are presented in Table VIII and IX.

Group I

This group was free-fed the control ration to establish normal toxicity. Since this ration is composed of 50% glanded cottonseed meats from the cottonseed meat pool (Table III) plus 50% basal ingredients, the free gossypol content of the total ration was 0.45%.

Chicken I was sacrificed on day 2. The feed consumption was 12.5 g/day and the weight loss was 12.5 g/day. The gossypol concentration was 96 mcg/g in the liver and 0.6 mcg/g

TABLE VIII Days On Trial, Feed Consumption, Free Gossypol Consumption and Body Weight Changes
(Cf. Appendix, Table XIV)

GROUP I

Chicken Number	Days On Trial	Feed (g) Consumption	Free Gossypol Consumption	Weight Changes (g)
1	2	25	0.11 g	-25
2	4	35	0.16 g	-64
3	6	95*	0.43 g	-112

GROUP II

Chicken Number	Days On Trial	Feed (g) Consumption	Free Gossypol Consumption	Weight Changes (g)
1	2	22	0.06 g	-28
2	4	138	0.36 g	33
3	6	202	0.53 g	48

GROUP III

Chicken Number	Days On Trial	Feed (g) Consumption	Free Gossypol Consumption	Weight Changes (g)
1	2	22	0.06 g	-34
2	4	98*	0.26 g	-52
3	6	142*	0.36 g	-74

* Large amount of soured feed found in distended crop.

TABLE IX Total Gossypol Concentration in Livers and Pectoral Muscles; Total Gossypol Content in Livers and Gallbladders
(Cf. Appendix, Tables XV, XVI, and XVII).

GROUP I

Chicken Numbers	Gossypol Concentration (mcg/g)		Gossypol Content (mcg)	
	Livers	Pectoral Muscles	Livers	Gallbladders
1	96.0	0.6	1123.0	212.0
2	160.0	1.0	1824.0	214.0
3	136.0	0.8	2462.0	200.0

GROUP II

Chicken Numbers	Gossypol Concentration (mcg/g)		Gossypol Content (mcg)	
	Livers	Pectoral Muscles	Livers	Gallbladders
1	4.0	0	41.0	0
2	16.0	0	251.0	0
3	48.0	0	878.0	0

GROUP III

Chicken Numbers	Gossypol Concentration (mcg/g)		Gossypol Content (mcg)	
	Livers	Pectoral Muscles	Livers	Gallbladders
1	72.0	0.5	1066.0	165.0
2	178.0	1.2	3079.0	232.0
3	210.0	1.6	4011.0	364.0

in the pectoral muscles. The total gossypol content in the gallbladder was 212 mcg.

Chicken II was sacrificed on day 4. The feed consumption was 8.8 g/day and the weight loss was 16.0 g/day. The gossypol concentration was 160 mcg/g in the liver and 1.0 mcg/g in the pectoral muscles. The total gossypol content in the gallbladder was 214 mcg.

Chicken III was sacrificed on day 6. The feed consumption was 15.8 g/day* and the weight loss was 18.7 g/day. The gossypol concentration was 136 mcg/g in the liver and 0.8 mcg/g in the pectoral muscles. The gossypol content in the gallbladder was 200 mcg.

All Group I chickens showed the typical symptoms of gossypol toxicity: appetite and weight depression, dyspnea, lack of vigor, loss of eye-ring color, and erection of the feathers (Plate VI). These symptoms, in addition to the good correlation of gossypol content with body weight loss, leave little doubt as to the presence of gossypol toxicity.

Although liver gossypol concentration appears to be low on day 6, the total content is nevertheless higher than at day 4, since the liver size for the animal on day 6 is larger. The gallbladder content remains at a relatively constant level at all three time intervals. The concentration in the pectoral muscles is so low that the levels do no reflect the gossypol consumption.

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* Large amount of soured feed found in distended crop.

PLATE VI Chicken (Top) Fed Normal Diet vs. Chicken (Bottom) Fed High Free Gossypol Diet

Group II

Group II chickens consumed the test ration ad libitum. The test ration was composed of 50% moldy cottonseed meats which were found by analyses to contain 0.52% free gossypol (Appendix, Table XIII). Therefore, the test ration contained 0.26% free gossypol which was only 58% as much free gossypol as in the control ration offered to Group I.

Chicken I was sacrificed on day 2. The feed consumption was 11 g/day and the weight loss was 14 g/day. The gossypol concentration was 4 mcg/g in the liver. Gossypol could not be detected in either the pectoral muscles or in the gallbladder.

Chicken II was sacrificed on day 4. The feed consumption was 34.5 g/day and the weight gain was 8.2 g/day. The gossypol concentration was 16 mcg/g in the liver. Gossypol could not be detected in either the pectoral muscles or in the gallbladder.

Chicken III was sacrificed on day 6. The feed consumption was 33.7 g/day and the weight gain was 8.0 g/day. The gossypol concentration was 48 mcg/g in the liver. Gossypol could not be detected in either the pectoral muscles or in the gallbladder.

Group II chickens showed no visible symptoms of gossypol toxicity and appeared healthy and alert at all times. No gossypol could be found in either pectoral muscles or gallbladders and only moderate amounts were present in liver

tissues. By day 6, the daily feed consumption of Chicken III was more than twice that of the bird fed the same length of time in Group I. Also, there was a weight gain of 8.0 g/day instead of a weight loss of 18.7 g/day, in spite of the fact that the free gossypol consumption was 23% higher. Even though these data are obtained with an absolute minimum number of birds, the reversal of the toxicity from Group I to Group II is nevertheless very striking.

It is also of some interest that even though the liver gossypol content reached a level of 878 mcg on day 6 in the animal fed the molded cottonseed meats, there was still no gossypol in the gallbladder. Theoretically, there should be at least 50-100 mcg if there were the same liver-gallbladder relationship as observed in Group I. One possible explanation for this divergency would be that the gossypol is of two different types and is therefore metabolized and/or excreted by different pathways. Also, the gallbladder is known to accumulate by-products of hemolyzed blood in the animal intoxicated with gossypol and it appears from these data that the Group I birds were affected by gossypol toxicity whereas the Group II were not.

Group III

Group III birds were offered a control ration containing a level of normal cottonseed such that the resulting free gossypol level was equivalent to the free gossypol level of

the molded cottonseed ration (Test Ration II). They were also offered the ration at a consumption rate equal to the group fed the test ration although it was not possible to accomplish equivalent feed intakes after the second day. This control ration II therefore contained 29% glanded cottonseed meats from the cottonseed meat pool (Table III) to give a level of 0.26% free gossypol in the total ration.

Chicken I was sacrificed on day 2. The feed consumption was 11 g/day of the normal cottonseed test ration and the weight loss was 17.0 g/day. The gossypol concentration was 72 mcg/g in the liver and 0.5 mcg/g in the pectoral muscles. The gossypol content in the gallbladder was 165 mcg.

Chicken II was sacrificed on day 4. The feed consumption was 24.5 g/day* and the weight loss was 13.0 g/day. The gossypol concentration was 178 mcg/g in the liver and 1.2 mcg/g in the pectoral muscles. The gossypol content in the gallbladder was 232 mcg.

Chicken III was sacrificed on day 6. The feed consumption was 23.7 g/day* and the weight loss was 12.3 g/day. The gossypol concentration was 210 mcg/g in the liver and 1.6 mcg/g in the pectoral muscles. The gossypol content in the gallbladder was 364 mcg.

Group III chickens showed much the same visible symptoms of gossypol toxicity as did the Group I chickens.

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* Large amount of soured feed in distended crop.

Discussion

A comparison of the ad libitum feed consumption for Groups I and II showed that, for the average chicken day, more than twice as much moldy test ration was consumed as normal control ration (Table VIII). The fungus in some way appears to improve palatability.

The observations on body weight changes and tissue content of gossypol showed that the Group I animals lost considerable weight and possessed substantial amounts of gossypol in all analyzed tissues. On the other hand, Group II chickens made moderate weight gains and no gossypol could be found in pectoral muscles or gallbladders and only a small amount could be found in liver tissues.

The data on feed consumption, weight change, general appearance and tissue analyses leads to the conclusion that in fungally inoculated cottonseed the binding of free gossypol, as shown in the analyses in Experiment I, is effective in reducing the toxicological activity of gossypol in the chicken.

A comparison of toxicity was made between free gossypol in moldy cottonseed meats and free gossypol in uninoculated cottonseed meats in Group II and III, respectively. Even though both rations contained the same percentage of analyzed free gossypol, Group III chickens failed to achieve the feed consumption of the Group II chickens. Not only was the feed consumption of Group III chickens lower but they all

showed the typical symptoms of gossypol toxicity as expressed in weight loss, lack of vigor, loss of color in the eye-ring, erection of feathers, enlarged gallbladder (Plate VII) and high gossypol content in body tissues. As previously stated, the Group II chickens showed none of the symptoms of gossypol toxicity with the exception of a small concentration of gossypol that was found in the liver tissues.

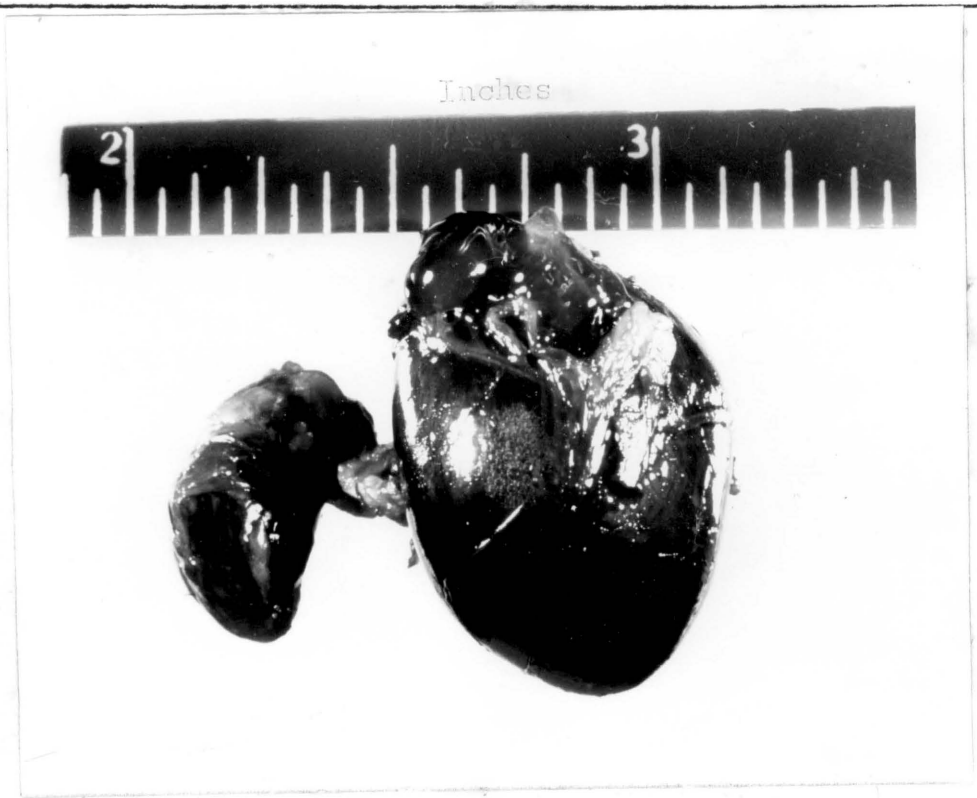
It is therefore abundantly clear that the fungus reduces the overall toxicity of the gossypol normally present in cottonseed. In spite of the fact that the animals in Group II (the molded test group) consumed more "free gossypol", they showed far less toxic symptoms. Obviously, the "free gossypol" in the two rations must have been different.

There are two possible explanations for the poor correlation between free gossypol and toxicity in molded and non-molded cottonseed meats.

The first explanation relies upon the supposition that a fungal system could in some way alter free gossypol such that it would render the molecule physiologically inactive. Possibly, some sort of complex might result. It could further be surmised that this gossypol-constituent complex would be soluble in the solvent used for free gossypol extraction. Thus, this physiologically inactive gossypol complex would analyze as free gossypol.

A second explanation would suppose that free gossypol is composed of two or more closely related compounds, each

PLATE VII Gallbladder From Chicken (Left) Consuming Diet Containing Moldy Cottonseed Meats Compared to Gallbladder From Chicken (Right) Consuming Diet Containing Normal Cottonseed Meats.



possessing different physiological activity. A fungal system could then preferentially react with the most physiologically active of the compounds to form bound gossypol and then leave the least active forms to be analyzed as "free gossypol".

In either case, it is obvious that the free gossypol found in a moldy cottonseed ration does not possess the high level of toxicity as the same amount of free gossypol found in a normal cottonseed ration.

The trend of decreased tissue retention of gossypol with time, as observed in the rat in Experiment I, was not noticed in the chicken. This suggests that the mechanism responsible for the metabolic inactivation of gossypol undergoes a higher degree of stimulation in the rat. Perhaps, this is the reason that the rat enjoys a higher resistance to gossypol than does the chicken.

SUMMARY

The objective of this thesis was to evaluate the ability of fungi to reduce the toxicity of cottonseed. Studies were made by analytically examining the effect on gossypol content as well as toxicologically testing the product by feeding it to rats and chickens.

It was observed by analyses that "free gossypol" was converted to the "bound" form by a fungal system and that only during periods of active mold growth did this conversion take place. Also, it was noticed that, after the "free gossypol" level had undergone a nine-fold reduction, it leveled off, at which time the "total gossypol" started to disappear. Unfortunately, the cause of this disappearance could not be unequivocally evaluated due to the appearance of bacterial contamination at low levels of "free gossypol".

In an effort to relate the analytically measured "free gossypol" to physiological activity, both rats and chickens were used in feeding trials. The parameters used in the evaluations were: feed consumption, weight changes, general health, physical abnormalities, and gossypol accumulation in various body tissues.

In all cases, the symptoms of gossypol toxicity were either dramatically reduced or entirely eliminated. In fact, it was found that the resulting levels of "free gossypol" in moldy cottonseed meats are far less toxic than

equivalent levels of "free gossypol" in normal cottonseed meats. Needless to say, the common practice of measuring cottonseed toxicity by analyzing for "free gossypol" content should be re-evaluated.

Further examination of the observed phenomenon of gossypol detoxication via fungal interaction holds both scientific and economic importance. Its importance lies in the fact that a completely detoxified cottonseed product could be marketed for animal use as a "universal protein supplement". Its use as a human food would, of course, require extensive toxiciological evaluation and, provided safety were indicated, such a product might be used as a valuable source of much-needed protein in the world.

APPENDIX

TABLE I Experimental Plan of Tests for Toxicity of Cotton-Seed Infested With Various Fungi

Fungi	Isolate Number	Substrate (Cottonseed)	Number Of Rats	Days On Trial	Number Of Rat Days
None	-	Basal Ration	14	12	168
None	-	Glanded*	14	7,7,10,10	119
None	-	Glandless**	14	12	168
Aspergillus flavus	227	Glanded	4	12	48
		Glandless	4	12	48
Aspergillus niger	282	Glanded	2	6	12
		Glandless	2	6	12
Aspergillus niger	356	Glanded	4	12	48
		Glandless	4	10	40
Alternaria	351	Glanded	4	12	48
		Glandless	4	12	48
Colletotrichum	215	Glanded	2	6	12
		Glandless	2	10	20
Diplodia	250	Glanded (test I)	4	2	8
		Glandless (test I)	4	2	8
		Glanded (test II)	2	10	20
		Glandless (test II)	2	5	10

* Wild-type variety usually contains 0.3-1.5% total gossypol.

** Developed variety usually contains 0.03 - 0.15% total gossypol.

Continued.

Fungi	Isolate Number	Substrate (Cottonseed)	Number Of Rats	Days On Trial	Number Of Rat Days
Diplodia	308	Glanded (test I)	2	6	12
		Glandless (test I)	2	10	20
		Glanded (test II)	4	6	24
		Glandless (test II)	4	6	24
Diplodia	Florence	Glanded	2	10	20
Fusarium	206	Glanded	2	6	12
		Glandless	2	6	12
Fusarium moniliforme	349	Glanded (test I)	4	6	24
		Glandless (test I)	4	6	24
		Glanded (test II)	2	10	20
		Glandless (test II)	2	7	14
Nigrospora	350	Glanded	4	10	40
		Glandless	2	10	40
Rhizopus	274	Glanded	2	10	20
		Glandless	2	10	20
Rhizopus	344	Glanded (test I)	2	6	12
		Glandless (test I)	2	6	12
		Glanded (test II)	4	2	8
		Glandless (test II)	4	2	8

TABLE II Feed Consumption and Growth Data of Animals in Studies of Table I

Fungi	Isolate Number	Substrate (Cotton-seed)	Total Feed Consumed (g)	Feed Consumed g/rat/day	Total Wt. Gain	Weight Gain g/rat/day
None	-	Basal Ration	2265	13.5	676	4.0
None	-	Glanded	882	7.4	-73.5	-0.61
None	-	Glandless	2335	13.9	606	3.6
Aspergillus flavus	227	Glanded	442	9.2	73	1.5
		Glandless	498	10.4	100	2.1
Aspergillus niger	282	Glanded	128	10.6	21	1.8
		Glandless	112	9.3	46	3.8
Aspergillus niger	356	Glanded	577	12.0	137	2.9
		Glandless	537	13.4	93	2.3
Alternaria	351	Glanded	614	12.8	136	2.8
		Glandless	649	13.5	163	3.4
Colleto-trichum	215	Glanded	118	9.8	37	3.1
		Glandless	210	10.5	41	2.1
Diplodia	250	Glanded (test I)	140	17.5	59	7.4
		Glandless (test I)	133	16.6	56	7.4
		Glanded (test II)	218	10.9	163	8.2
		Glandless (test II)	121	12.1	63	6.3
Diplodia	308	Glanded (test I)	118	9.8	42	3.5
		Glandless (test I)	158	7.9	39	1.9
		Glanded (test II)	263	10.9	117	4.9

Continued.

TABLE II Continued

Fungi	Isolate Number	Substrate (Cotton-seed)	Total Feed Consumed (g)	Feed Consumed g/rat/day	Total Wt. Gain	Weight Gain g/rat/day
Diplodia	308	Glandless (test II)	127	10.6	41	3.4
Diplodia	Florence	Glanded	155	7.8	19	1.0
Fusarium	206	Glanded	133	11.1	47	3.9
		Glandless	127	10.6	41	3.4
Fusarium	349	Glanded (test I)	269	11.2	113	4.7
		Glandless (test I)	298	12.4	119	4.9
		Glanded (test II)	257	12.9	182	9.1
		Glandless (test II)	201	14.4	119	8.5
Nigrospora	350	Glanded	547	13.7	183	4.6
		Glandless	529	13.2	142	3.6
Rhizopus	274	Glanded	199	9.9	35	1.8
		Glandless	181	9.1	33	1.7
Rhizopus	344	Glanded (test I)	143	11.9	20	1.7
		Glandless (test I)	124	10.3	15	1.3
		Glanded (test II)	129	16.1	59	7.4
		Glandless (test II)	127	15.9	58	7.3

TABLE III Pathology Due to Mold Toxicity of Animals in Studies of Table I

Fungi	Isolate Number	Substrate (Cottonseed)	Pathology
None	-	Basal Ration	None
None	-	Glanded	Weight and appetite depression, diarrhea, tissue dehydration, fluid accumulation in intestinal, abdominal, and thoracic cavities, and death
None	-	Glandless	None
Aspergillus flavus	227	Glanded	None
		Glandless	White spots on kidney (1 of 4 rats)
Aspergillus niger	282	Glanded	None
		Glandless	White spots on kidney (1 of 2 rats)
Aspergillus niger	356	Glanded	None
		Glandless	White spots on kidney (2 of 4 rats)
Alternaria	351	Glanded	Questionable pyelitis (2 of 4 rats)
		Glandless	None
Colletotrichum	215	Glanded	None
		Glandless	None
Diplodia	250	Glanded (test I)	Pyelitis (4 of 4 rats)
		Glandless (test I)	None
		Glanded (test II)	Severe pyelitis (2 of 2 rats)
		Glandless (test II)	Kidney hemorrhage (1 of 2 rats)

TABLE III Continued.

Fungi	Isolate Number	Substrate (Cottonseed)	Pathology
Diplodia	308	Glanded (test I)	Severe pyelitis (2 of 2 rats)
		Glandless (test I)	None
		Glanded (test II)	Severe pyelitis (4 of 4 rats)
		Glandless (test II)	None
Diplodia	Florence	Glanded	None
Fusarium	206	Glanded	None
		Glandless	None
Fusarium moniliforme	349	Glanded (test I)	None
		Glandless (test I)	None
		Glanded (test II)	None
		Glandless (test II)	None
Nigrospora	350	Glanded	None
		Glandless	None
Rhizopus	274	Glanded	None
		Glandless	None
Rhizopus	344	Glanded (test I)	None
		Glandless (test I)	None
		Glanded (test II)	None
		Glandless (test II)	None

TABLE IV Diplodia Feeding Trials

Isolate Number	Substrate	Number of Rats	Pathology
250	Glanded Cottonseed	7	None
	Glandless Cottonseed	4	None
	Corn	5	None
	Rice	3	None
276	Corn	2	None
277	Corn	2	None
308	Glanded Cottonseed	53	None
	Glandless Cottonseed	4	None
	Corn	5	None
	Rice	3	None
393	Corn	2	None
394	Corn	2	None
395	Corn	2	None
396	Corn	5	None
	Rice	3	None
397	Corn	2	None
398	Corn	2	None
399	Corn	2	None
		108	

TABLE V Total Gossypol Content of Fungally Inoculated Cottonseed Meats of Experiment I

Sample Number	% Total Gossypol	Sample Number	% Total Gossypol
Day 0		Day 5	
1	1.20	1	1.13
2	1.20	2	1.06
3	1.18	3	1.12
4	1.08	4	1.15
5	1.08	5	1.12
Day 1		Day 6	
1	1.08	1	1.13
2	1.18	2	1.10
3	1.14	3	1.14
4	1.16	4	1.16
5	1.15	5	1.15
Day 2		Day 7	
1	1.06	1	0.91
2	1.08	2	0.96
3	1.04	3	1.00
4	1.24	4	0.94
5	1.06	5	1.03
Day 3		Day 10	
1	1.12	1	1.01
2	1.18	2	1.00
3	1.19	3	1.00
4	1.18	4	0.96
5	1.10	5	0.95
Day 4		Day 13	
1	1.12	1	0.74
2	1.06	2	0.72
3	1.08	3	0.62
4	1.08	4	0.74
5	1.03	5	0.74

TABLE VI Free Gossypol Content of Fungally Inoculated Cottonseed Meats of Experiment I

Sample Number	% Free Gossypol	Sample Number	% Free Gossypol
Day 0		Day 5	
1	0.92	1	0.14
2	0.84	2	0.18
3	0.88	3	0.14
4	0.84	4	0.28
5	0.88	5	0.24
Day 1		Day 6	
1	0.78	1	0.20
2	0.78	2	0.18
3	0.68	3	0.26
4	0.72	4	0.19
5	0.68	5	0.18
Day 2		Day 7	
1	0.68	1	0.12
2	0.66	2	0.16
3	0.64	3	0.12
4	0.66	4	0.13
5	0.64	5	0.14
Day 3		Day 10	
1	0.51	1	0.12
2	0.54	2	0.06
3	0.52	3	0.10
4	0.48	4	0.12
5	0.50	5	0.12
Day 4		Day 13	
1	0.36	1	0.08
2	0.38	2	0.12
3	0.34	3	0.06
4	0.32	4	0.06
5	0.37	5	0.07

TABLE VII Feed Consumption and Weight Losses for the Group I Rats of Experiment II

Rat Number	Feed Consumption	Weight Loss	Rat Number	Feed Consumption	Weight Loss
Day 1			Day 5		
1	1	5	1	-	-
2	2	2	2	0	2
3	2	3	3	0	3
4	1	1	4	-	-
5	1	6	5	2	1
6	2	5	6	-	-
Day 2			Day 6		
1	1	1	1	-	-
2	1	5	2	1	1
3	2	2	3	2	0
4	1	5	4	-	-
5	1	2	5	2	1
6	1	6	6	-	-
Day 3			Day 7		
1	1	4	1	-	-
2	1	2	2	1	2
3	1	5	3	0	1
4	1	5	4	-	-
5	1	1	5	1	2
6	1	1	6	-	-
Day 4			Day 8		
1	sacrificed	-	1	-	-
2	1	0	2	1	1
3	1	1	3	1	2
4	sacrificed	-	4	-	-
5	2	3	5	2	2
6	sacrificed	-	6	-	-

TABLE VIII Gossypol Concentrations in the Organs of Group I Rats of Experiment II

Rat Number	Organ Weight	Mg of gossypol per g of tissue	Mg of gossypol per organ
		LUNGS	
1	0.45	0.076	0.034
2	0.32	0.034	0.011
3	0.29	0.038	0.011
4	0.42	0.124	0.052
5	0.37	0.081	0.030
6	0.42	0.095	0.040
		LIVERS	
1	1.76	0.144	0.253
2	1.00	0.078	0.078
3	1.00	0.082	0.082
4	1.88	0.162	0.305
5	1.13	0.062	0.070
6	2.12	0.194	0.411

TABLE IX Feed Consumption and Weight Losses for the Group II Rats of Experiment II

Rat Number	Feed Consumption	Weight Loss	Rat Number	Feed Consumption	Weight Loss
Day 1			Day 5		
1	1	3	1	-	-
2	2	2	2	0	1
3	2	0	3	0	1
4	1	2	4	-	-
5	1	3	5	2	0
6	2	1	6	-	-
Day 2			Day 6		
1	1	1	1	-	-
2	1	2	2	1	2
3	2	2	3	2	+2*
4	1	3	4	-	-
5	1	2	5	2	1
6	1	2	6	-	-
Day 3			Day 7		
1	1	1	1	-	-
2	1	0	2	1	+1*
3	1	+1*	3	0	0
4	1	2	4	-	-
5	1	2	5	1	0
6	1	1	6	-	-
Day 4			Day 8		
1	sacrificed	-	1	-	-
2	1	3	2	1	0
3	1	3	3	1	1
4	sacrificed	-	4	-	-
5	2	0	5	2	0
6	sacrificed	-	6	-	-

* Weight Gain

TABLE X Gossypol Concentrations in the Organs of Group II Rats of Experiment II

Rat Number	Organ Weight	Mg of gossypol per g of tissue	Mg of gossypol per organ
		LUNGS	
1	0.50	0.068	0.034
2	0.62	-0.008	-0.005
3	0.50	-0.020	-0.010
4	0.60	0.032	0.019
5	0.60	-0.024	-0.014
6	0.55	0.036	0.020
		LIVERS	
1	1.90	0.028	0.053
2	1.90	0.030	0.057
3	2.23	0.026	0.058
4	2.10	0.026	0.058
5	2.10	0.024	0.050
6	1.95	0.024	0.047

TABLE XI Feed Consumption and Weight Gains for the Group III Rats of Experiment II

Rat Number	Feed Consumption	Weight Gains	Rat Number	Feed Consumption	Weight Gains
Day 1			Day 4		
1	8	8	1	-	-
2	3	0	2	5	3
3	3	2	3	6	4
4	3	3	4	-	-
5	7	7	5	7	4
6	5	4	6	-	-
Day 2			Day 5		
1	7	4	1	-	-
2	5	6	2	5	3
3	5	5	3	7	5
4	4	3	4	-	-
5	7	5	5	8	7
6	5	2	6	-	-
Day 3			Day 6		
1	8	6	1	-	-
2	6	4	2	8	5
3	7	5	3	10	5
4	4	3	4	-	-
5	8	5	5	8	4
6	5	6	6	-	-

TABLE XII Gossypol Concentrations in the Organs of Group III Rats of Experiment II

Rat Number	Organ Weight	Mg of gossypol per g of tissue	Mg of gossypol per organ
		LUNGS	
1	0.61	0.036	0.022
2	0.53	0.024	0.013
3	0.32	0.008	0.003
4	0.48	0.012	0.006
5	0.70	-0.016	-0.011
6	0.50	0.048	0.024
		LIVERS	
1	3.60	0.060	0.216
2	2.85	0.034	0.160
3	3.14	0.042	0.132
4	2.50	0.036	0.090
5	3.25	0.032	0.104
6	3.16	0.034	0.107

TABLE XIII Gossypol Content In Moldy Cottonseed Meats Used to Prepare Rations In Experiment II and III

Sample Number	% Total Gossypol	Sample Number	% Free Gossypol
1	1.14	1	0.56
2	1.16	2	0.52
3	1.12	3	0.50
4	1.14	4	0.53
5	1.15	5	0.49
ΣX -- 5.71		ΣX -- 2.60	
$\bar{x}$ -- 1.14		$\bar{x}$ -- 0.52	
$S_{\bar{x}}$ -- 0.02		$S_{\bar{x}}$ -- 0.02	

Mean Total Gossypol -- 1.14 - 0.02%

Mean Free Gossypol -- 0.52 - 0.02%

Mean Bound Gossypol -- 0.62 - 0.02% Calculated From Mean Total Gossypol and Mean Free Gossypol

TABLE XIV Feed Consumption and Weight Changes for the Chickens of Experiment III

GROUP I

Chicken Number	Days On Trial	Feed (g) Consumption	Initial Weight (g)	Final Weight (g)	Weight Change (g)
1	2	25	720	695	-25
2	4	35	475	411	-64
3	6	95*	765	653	-112

GROUP II

Chicken Number	Days On Trial	Feed (g) Consumption	Initial Weight (g)	Final Weight (g)	Weight Change (g)
1	2	22	560	532	-28
2	4	138	680	713	+33
3	6	202	610	662	+48

GROUP III

Chicken Number	Days On Trial	Feed (g) Consumption	Initial Weight (g)	Final Weight (g)	Weight Change (g)
1	2	22	652	618	-34
2	4	98**	754	702	-52
3	6	142**	853	779	-74

* Large amount of undigested soured feed in crop.
** Some undigested soured feed in crop.

TABLE XV Total Gossypol Concentration and Content in the Gallbladders of Experiment III Chickens

Chicken Numbers	Days On Trial	Organ Weight (g)	Corrected* Organ Weight (g)	Gossypol Content (mcg)	Gossypol Concen. (mcg/g)
			GROUP I		
1	2	2.96	2.46	212.0	86.0
2	4	3.01	2.51	214.0	85.0
3	6	2.65	2.25	200.0	89.0
Chicken Numbers	Days On Trial	Organ Weight (g)	Corrected* Organ Weight (g)	Gossypol Content (mcg)	Gossypol Concen. (mcg/g)
			GROUP II		
1	2	0.36	-	0	0
2	4	0.40	-	0	0
3	6	0.42	-	0	0
Chicken Numbers	Days On Trial	Organ Weight (g)	Corrected* Organ Weight (g)	Gossypol Content (mcg)	Gossypol Concen. (mcg/g)
			GROUP III		
1	2	2.21	1.81	165.0	91.0
2	4	3.13	2.73	232.0	85.0
3	6	4.06	3.66	364.0	99.0

* A normal organ weight of 0.4 g was subtracted to get a correction factor.

TABLE XVI Total Gossypol Concentration and Content in the Livers of Experiment III Chickens

Chicken Number	Days On Trial	Gossypol Conc. (mcg/g)	Organ Weight (g)	Gossypol Content (mcg)
		GROUP I		
1	2	96.0	11.7	123.0
2	4	160.0	11.4	824.0
3	6	136.0	18.1	462.0
		GROUP II		
1	2	4.0	10.2	41.0
2	4	16.0	15.7	251.0
3	6	48.0	18.3	878.0
		GROUP III		
1	2	72.0	14.8	66.0
2	4	178.0	17.3	79.0
3	6	210.0	19.1	11.0

TABLE XVII Total Gossypol Concentration in the Pectoral Muscles of Experiment III Chickens

GROUP I		
Chicken Number	Days On Trial	Gossypol Concentration in Pectoral Muscles (mcg/g)
1	2	0.6
2	4	1.0
3	6	0.8

GROUP II		
Chicken Number	Days On Trial	Gossypol Concentration in Pectoral Muscles (mcg/g)
1	2	0
2	4	0
3	6	0

GROUP III		
Chicken Number	Days On Trial	Gossypol Concentration in Pectoral Muscles (mcg/g)
1	2	0.5
2	4	1.2
3	6	1.6

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GOSSYPOL DEACTIVATION VIA FUNGAL INTERACTION

William Lewis Baugher

ABSTRACT

Studies were made by analytically examining the effect on gossypol content as well as toxicologically testing the product by feeding it to rats and chickens.

It was observed by analyses that "free gossypol" was converted to the "bound" form by a fungal system and that only during periods of active mold growth did this conversion take place. Also, it was noticed that, after the "free gossypol" level had undergone a nine-fold reduction, it leveled off, at which time the "total gossypol" started to disappear.

In an effort to relate the analytically measured "free gossypol" to physiological activity, both rats and chickens were used in feeding trials. The parameters used in the evaluations were: feed consumption, weight changes, general health, physical abnormalities, and gossypol accumulation in various body tissues.

In all cases, the symptoms of gossypol toxicity were either dramatically reduced or entirely eliminated. In fact, it was found that the resulting levels of "free gossypol" in moldy cottonseed meats are far less toxic than equivalent levels of "free gossypol" in normals cottonseed meats.

Further examination of the observed phenomenon of gossypol detoxication via fungal interaction holds both scientific and economic importance. Its importance lies in the fact that a completely detoxified cottonseed product could be marketed for animal use as a "universal protein supplement". Its use as a human food would, of course, require extensive toxicological evaluation and, provided safety were indicated, such a product might be used as a valuable source of much-needed protein in the world.