Gossypol Detoxication by Fungi

Abstract. *An unidentified* Diplodia *isolate representative of several fungi tested and grown for 13 days on gossypol-containing cottonseed reduced the amount of free gossypol by 90 percent and detoxified the cottonseed for weanling rats and chickens. Residual free gossypol was also less toxic than equivalent amounts of normal free gossypol.*

The use of cottonseed meal as a source of edible protein either as feed for monogastric livestock or as an ingredient in food blends for humans has been limited by its content of gossypol, a toxic polyphenolic compound (*1*). No practical way of avoiding this difficulty is available despite many attempts (*1*). We have observed that several fungi, when grown on cottonseed, reduce its toxicity to rats (*2*). The most effective fungus studied is a strain of *Diplodia*.

This study was undertaken to determine the effect of *Diplodia* growth on gossypol in cottonseed. Spore suspensions were prepared by adding 50 ml of deionized, distilled water to a Roux bottle containing a 2-week-old culture of *Diplodia* on potato-dextrose agar (Difco) (*3*). Cottonseed meats were prepared by dehulling whole cottonseed (*4*) with a Labconco mill, and then fine-chopping in a Waring Blendor. Individual samples were prepared by adding 0.25 g of ground meats to 8-oz jars loosely fitted with screw caps and adding either the spore suspension or the sterile, deionized, distilled water. Aureomycin solutions (0.05 percent) were added to inhibit bacterial growth. One hundred samples were inoculated with 0.75 ml of spore suspension and 0.25 ml of Aureomycin solution and incubated at 27° ± 2°C for periods of 0, 1, 2, 3, 4, 5, 6, 7, 10, and 13 days. In addition, ten uninoculated controls were similarly prepared with 0.75 ml of sterile, deionized, distilled water and 0.25 ml of the Aureomycin solution; these were maintained at 5°C to prevent growth of fungi normally present. All samples were assayed for total gossypol according to the method of Smith (*5*) and for free gossypol according to the *Food Additives Analytical Manual* method (*6*). Bound gossypol was calculated by difference.

No fungal growth was evident until the second day (Fig. 1). At least some reduction in free gossypol may have been due to addition of water because the 10 control samples showed a reduction from 0.91 ± 0.01 percent to 0.73 ± 0.02 percent. [This effect was not due to any dilution of gossypol by water; analyses were made on the total sample and expressed on the basis of initial weight (0.25 g)]. This effect of water on free gossypol content agrees with the observations of Bressani *et al.* (*7*) who showed that, of 18 samples of ground cottonseed meats, 14 were reduced in free gossypol upon the addition of water. Free gossypol content was reduced to 0.08 ± 0.01 percent after 13 days. Most of the reduction was evident within the first 6 days (0.20 ± 0.02 percent), when bound gossypol started to decrease from its highest amount (0.94 ± 0.02 percent) to a final level of 0.63 ± 0.02 percent on day 13.

To determine whether the reduction in free gossypol content would result in decreased toxicities, Sprague-Dawley–derived male weanling rats (Dublin Laboratory Animals, Dublin, Virginia) were fed a diet containing one part of a nutritionally adequate basal diet mixture (*8*) and one part cottonseed incubated for 10 days with the same isolate of *Diplodia*. Here more cottonseed was prepared than was used above. Reduction in free gossypol was not as great, final amount being 0.52 percent, most probably because of far lesser penetration of substrate by the fungus. The resulting 1 : 1 dietary mixture contained 0.26 percent free gossypol; it was fed *ad libitum* to six rats (group I). Group II consisted of six rats similarly fed uninoculated control cottonseed (0.90 percent free gossypol) with the basal mixture, resulting in a dietary free gossypol content of 0.45 percent. Total gossypol of each cottonseed sample was 1.13 percent. The six rats of group III were fed the same diet as group I but were pair-fed with the control animals of group II. Daily weight gain and food consumption were recorded (Table 1).

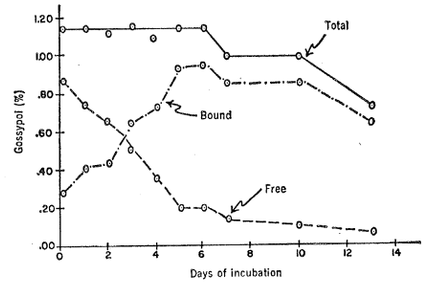

Fig. 1. Percent free, bound, and total gossypol of ground cottonseed meats inoculated with *Diplodia*. Ten samples for each point.

Animals fed normal, uninoculated cottonseed (group II) exhibited characteristic symptoms of gossypol toxicity such as appetite and weight depression, dyspnea, lack of vigor, and fluid accumulation in body cavities and intestines, and were very near death on day 8. Animals pair-fed the same amount of total gossypol (group III) lost less weight and only appeared hungry on day 8; no pathological symptoms were observed. On the other hand, a striking difference was observed in animals fed moldy cottonseed (group I). In spite of their sixfold greater consumption of total gossypol compared to animals of group II, they made moderate gains and maintained a healthy appearance.

To measure tissue accumulation of gossypol, three rats from each group in the above experiment were killed on day 3, and three rats of groups II and III were killed on day 8. Because of the unexpectedly ravenous consumption and subsequent depletion of the molded diet, the remaining three animals of group I were killed on day 6. Tissue analyses of lungs and livers for total gossypol were made according to Smith (*9*) with the following modifications. Reduced sample sizes (1 g for liver and 0.5 g for lungs) were used; all tissues were homogenized in a Potter-Elvehjem homogenizer, a 4-inch glass wool col-

Table 1. Change in body weight, food consumption, and tissue accumulation of gossypol in male weanling rats fed equal parts of a basal diet and either *Diplodia*-inoculated or uninoculated ground cottonseed.

Gossypol consumption (mg/day)		Weight change (g/day)		Feed consumed (g/day)		Liver gossypol (mg/g)		Lung gossypol (mg/g)	
Total	Free	Days 0–3	Days 4–8*	Days 0–3	Days 4–8*	Day 3	Day 8*	Day 3	Day 8*
		Group I Diplodia *inoculated, ad lib feeding*							
36.5	17.7	+4.3	+4.4	5.7	7.1	0.043	0.036	0.032	0.005
		Group II uninoculated, ad lib feeding							
6.5	5.5	−3.2	−1.5	1.2	1.1	0.167	0.075	0.098	0.051
		Group III Diplodia *inoculated, pair-fed with group II*							
6.5	3.1	−1.6	0.5	1.2	1.1	0.026	0.027	0.045	0.0

* Group I rats killed after 6 days.

umn replaced the Hyflo Super-Cel filtration column, and normal rat tissues treated in the same manner as the test tissues were used instead of the hexane "blank" in colorimetric determination. It was assumed that "total" gossypol content of tissues reflects intake of the "free" gossypol of the diet since it has been demonstrated that only the "free" form of gossypol is absorbed and is responsible for the toxicity (*10*).

The concentrations of gossypol in these tissues (Table 1) clearly reflect the toxicity symptoms and growth performance data. The highest concentrations were observed in the group fed the uninoculated cottonseed, which, in turn, produced the most dramatic toxicity symptoms. The reduced toxicity of the inoculated cottonseed appears to be due in part to reduction of free gossypol and in part to lower toxicity of residual free gossypol. Although it would appear that the extra feed intake could have afforded protection for the residual gossypol, additional studies using Leghorn cockerels have demonstrated that the intake of equal dietary levels of free gossypol obtained before and after fungal growth results in no demonstrable toxicity for the gossypol remaining after fungal growth. Furthermore, tissue gossypol accumulation, feed intake, body weight change, and gross symptomatology for these cockerels confirm the observations made with rats.

Such detoxication may have industrial application provided a nontoxic fungus could be chosen to prepare the fermented cottonseed meal; or, perhaps a cell-free fungal extract, lacking toxic factors, might be prepared which would serve as the feed additive used to detoxify the gossypol of cottonseed.

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References and Notes

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3. This was an unidentified species of *Diplodia* supplied to us for our earlier work (*2*) by P. B. Marsh, ARS, USDA, Beltsville, Md.
4. Acala Variety 4-42-77 kindly supplied by P. B. Marsh, ARS, USDA, Beltsville, Md., in the dehulled form.
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8. Basal mixture, in percentages: sucrose, 66.0; casein, 22.2; Jones-Foster mineral mixture, 4.0; vitamin-diet fortification mixture, 2.2; corn oil, 5.0; L-lysine, 0.4; and DL-methionine, 0.2. Mineral and vitamin mixtures were prepared by Nutritional Biochemicals Corp.
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11. These studies represent a portion of a thesis submitted as partial fulfillment of requirements for M.S. degree by W.L.B. Financial support was provided by USDA contract agreement No. 12-14-100-8433(34) and Virginia State Project No. 200068.
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Chromatin and Histones: Binding of Tritiated Actinomycin D to Heterochromatin in Mealy Bugs

Abstract. *The degree of actinomycin D binding to DNA in chromatin is dependent upon the state of repression of chromatin. Living cells bind three times more tritiated actinomycin to euchromatin than to genetically inactive heterochromatin. Extraction of histone results in a general increase in tritiated actinomycin binding and in a ratio of the uptake in heterochromatin to that in euchromatin approaching unity.*

We describe here the uptake of tritiated actinomycin D in genetically active and inactive chromatin in both living cells and cells from which the histones have been extracted. The work was done on the cells of the male mealy bug *Planococcus citri* (Homoptera, Coccoidea). Many of the cells in this organism contain condensed interphase chromatin in the form of five readily visible heterochromatic chromosomes (H, the paternally derived haploid set). The five heterochromatic chromosomes are late replicating (*1*) and genetically inactive (*2*), the block occurring in DNA-directed RNA synthesis (*3*). The five euchromatic chromosomes (the E chromosome set) are the genetically active chromosomes.

Premeiotic gonial cells within whole testes, which readily incorporate tritiated uridine (*3*), were incubated with tritiated actinomycin D (Schwarz; specific activity, 3.38 c/mmole). Living cells and cells previously fixed and squashed were used, the former to study the uptake of actinomycin D in vivo and the latter to determine the degree of actinomycin D binding to DNA after alteration of the relation between the DNA and its associated proteins.

Unfixed living testes were incubated in 0.2 ml of tritiated actinomycin D in Ringer solution for 2 to 4 hours. Care was taken to prevent residual labeled material from being taken up passively after fixation. The testes were flooded with unlabeled Ringer solution for ½ hour to dilute the ratioactivity and wash the unbound tritiated actinomycin D out of the tissue prior to fixation. The testes were first transferred to slides previously coated with gelatin and then squashed in either 45 percent acetic acid or 10 percent neutral buffered formalin. Tissue squashed in formalin was fixed in formalin for an additional 5 minutes. All slides were washed in distilled water and dried in air. Actinomycin D (0.05 μg/ml) will suppress at least 90 percent of the RNA synthesis in premeiotic testis cells after a 2-hour incubation (*3*). In this series of experiments, we used not less than 0.74 μg/ml of tritiated actinomycin D.

Table 1 (items B and C) shows a comparison of living testes which were incubated with 5 μc/ml of tritiated actinomycin but were subject to different fixation techniques after squashing. The data indicate that the amount of labeled actinomycin retained in the euchromatin after formalin fixation is seven times greater than that retained after acetic acid fixation. Camargo and Plaut (*4*) suggest that 45 percent acetic acid will break hydrogen bonds and that this may account for differences in retention of the tritiated actinomycin D. Nevertheless, it is significant that the ratio of uptake in euchromatin to that in heterochromatin remained the same regardless of fixative. The relatively low uptake in the live cells (Table 1, item A) probably resulted from the use of trititiated actinomycin D with a low specific activity in the early experiments.

The relative difficulty with which tritiated actinomycin D was bound to the condensed, heterochromatic chromosome set can be observed in Table 1 and in Fig. 1A. The ratio of uptake of tritiated actinomycin D into the euchromatic and heterochromatic chromosomes of living cells was 3.2–3.4 to 1. Since the amount of DNA is the same in these two sets of chromosomes (*5*), these results indicate that heterochromatin has fewer available binding sites for actinomycin D than euchromatin.

In the second series of experiments, tissue was squashed and fixed prior to incubation according to a procedure