The Acute Oral Toxicity of Cottonseed Pigment Glands and Intraglandular Pigments

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Abstract

Acute oral toxicity studies were carried out on cottonseed pigment glands, gossypol, diaminogossypol and gossypurpurin using rats as experimental animals. It was found that both diaminogossypol and gossypurpurin are considerably less toxic than pure gossypol. The pigment glands were more toxic than gossypol and it was concluded that the toxicity of pigments cannot be accounted for entirely on the basis of their gossypol content.

Administration of gossypol with cottonseed oil or *Sterculia foetida* oil, both of which contain cyclopropene fatty acids, increased the toxicity very slightly over that found when gossypol was administered in corn oil. In the case of corn oil and *Sterculia foetida* oil, the difference was statistically significant.

Less gossypol was also found in the feces when the test dose was given in cottonseed oil or *S. foetida* oil indicating a possible effect of the cyclopropene fatty acids in increasing gossypol absorption.

Introduction

COTTONSEED MEAL has been recognized as an excellent protein concentrate for cattle and sheep for many years. With suitable processing controls, cottonseed meal which is also excellent as a source of protein for swine (1) and poultry (2) can be manufactured. Superior quality cottonseed meal for nonruminating animals must be: (1) low in free gossypol and (2) high in protein quality. The reactions of gossypol during processing are related to both of these characteristics of the finished product (3).

Although it has been generally accepted that gossypol is at least a major factor in the toxicity of raw cottonseed, evidence has been presented from time to time which indicates that other factors may also be involved. Schwartze (4–6) found a correlation between raw cottonseed and gossypol content and concluded that the toxicity could be attributed solely to gossypol content. With the availability of cottonseed pigment glands separated from cottonseed kernels, and of pure gossypol, it became possible to evaluate their toxicity. Eagle (7–9) has repeatedly presented evidence which shows that while cottonseed pigment glands are toxic, they show a rather wide range of toxicity (LD-50 values from 925 to 2170 mg/kg body wt) which is not referable to the gossypol content of the glands. This investigator concludes that "the toxicity of cottonseed products cannot be accounted for solely on the basis of analyzed gossypol content" (10).

Boatner (11) isolated a naturally occurring purple colored cottonseed pigment and named it "gossypurpurin." Although Eagle (7) reported that the toxicity of pigment glands did not appear to be related to their content of gossypurpurin, toxicity tests on isolated gossypurpurin have not been reported.

When eggs from hens fed cottonseed meal are kept in storage both the yolks and the whites are likely to become discolored. Typically the yolks are either olive green or dark brown and the whites are pink. Although it has been established that gossypol is a major factor in egg yolk discoloration (12), recently Kemmerer and Keywant (13) found that yolk discoloration was enhanced greatly by the addition of *Sterculia foetida* oil to a ration containing gossypol. This effect was attributed to the presence of a cyclopropene ring fatty acid in the *Sterculia foetida* oil. Cottonseed oil also contains a propene ring fatty acid (malvalic acid) (14). Some cottonseed meals which caused badly discolored eggs no longer did so after the residual oil was extracted from the meal (13). These results suggest that gossypol toxicity in nonruminating animals might also be modified by the presence of a cyclopropene fatty acid.

The present investigation was designed to study the toxicity and physiological effects of gossypurpurin, diaminogossypol and gossypol and to determine to what extent these pigments are responsible for the toxicity of pigment glands. A second purpose was to determine whether the toxicity of gossypol is modified by the presence of oils containing cyclopropene fatty acids such as cottonseed oil and *Sterculia foetida* oil.

Experimental

Isolation of Pigment Glands. A modification of the semipilot plant flotation process described by Spadaro et al. (15, 16) was used for the preparation of the pigment glands. Two kg of hexane-extracted cottonseed meal which had been prepared without heat were ground in a Wiley mill, then sieved on an 80-mesh screen. A mixture of commercial hexane and perchloroethylene was used as the flotation solvent. Preliminary tests indicated that the alcohol which is frequently used as a stabilizer in perchloroethylene causes decomposition of the pigment glands. This difficulty was avoided by washing the perchloroethylene three times with water and then drying over anhydrous sodium sulfate. The pigment glands were collected from the top of the flotation solvent by scooping and siphoning. Excess solvent was removed by filtration on a Buchner funnel and the glands were air dried over night. Further purification was accomplished by sieving the dry glands through a 150-mesh sieve and repeating the flotation process. The collected pigment glands were washed, dried over night and stored according to the method of Boatner et al. (17).

The final yield of pigment glands was 3.1%. The content of gossypol and gossypurpurin was 43.0% and 1.1%, respectively.

Preparation of Diaminogossypol and Gossypurpurin. Diaminogossypol was prepared by a modification of the procedure described by Pominiski et al. (18). Gossypol (1.5 g) was dissolved in 75 ml of chloroform and placed in a three-neck flask. A reflux condenser was fitted to one neck and ammonia gas was intro-

duced through tubes fitted to the other necks. One tube was used to bubble the ammonia into the solution and the other to keep the surface covered with ammonia. After passing the gas for 5 min the solution was heated to boiling and refluxed for 15 min with a continuous flow of ammonia. On cooling in a dark place, a bright yellow crystalline precipitate separated from the solution. Recrystallization from chloroform yielded bright yellow plates which after drying in a vacuum desiccator, melted at 221C. Literature value, 219–221C (18). The yield was 0.64 g.

Gossypurpurin was prepared by a modification of the methods described by Boatner et al. (18, 19) which resulted in improved yields. One hundred g of pigment glands were extracted with chloroform in a Waring blendor for 5 min and the mixture was filtered on a Buchner funnel. The residue of gland walls was re-extracted and washed with chloroform. The chloroform solution was evaporated to dryness and the residue was then dissolved in ethyl ether. The ethereal extract was extracted with 1.5 N ammonium hydroxide containing 5% sodium dithionite to give a total of 400 ml. The separated alkaline extract was allowed to stand overnight in a dark place. A yellow colored solid separated out. 6N HCl was added to the suspension until a pH of 8.4 was obtained. The suspension was then extracted with ethyl ether and the ethereal extract was dried over anhydrous sodium sulfate. Fifty ml of glacial acetic acid were added and the product was heated on a steam bath until all of the ether had evaporated. Acetic acid was evaporated under vacuum and the product obtained was dissolved in a min quantity of chloroform, and then allowed to stand for 2 days in a low actinic flask at 3C. A dark purple crystalline precipitate was obtained upon addition of light petroleum ether to this suspension. The precipitate was separated by filtration and washed with 200 ml of light petroleum ether. An aqueous slurry was made, heated on a steam bath for two hours, filtered, then dried under vacuum in a desiccator over phosphorus pentoxide. The yield was 7.50 g, mp 203C. Literature value, 200–204C (18).

Determination of Acute Toxicity. In all studies, weaned male rats 21 day old were used as experimental animals. The rats were kept on a practical diet in batteries with raised screen floors (10 rats in each) in a well-aerated room until each weighed 160 ($\pm$ 10) g, and then transferred to separate cages. The animals were fasted for 18 hr before administration of the test dose, after which they were permitted free access to food and water, and kept under observation for 7 days or until death.

All test materials were dissolved in warm chloroform. Corn oil was then added to the chloroform solution to make a solution or suspension of the material in oil. The chloroform was then evaporated completely under vacuum using a warm water bath and bubbling nitrogen gas. The volume of corn oil was adjusted so that each rat received the dose in 2 ml of oil. The doses were administered by a stomach tube and the tube was then washed with 1.5 ml of warm water. The method recommended by Bliss (19) was used to calculate the LD-50 values. Rats that did not die were weighed at the end of the experiment. All rats which died were autopsied and the pathological conditions were recorded.

Unless otherwise stated all test materials were administered in corn oil. In some experiments the corn oil was replaced with refined cottonseed oil (commercial cooking oil). Where *Sterculia foetida* oil was used, it was mixed with corn oil in proportions such that the 2 ml of oil used in administering the gossypol contained 20 mg of *Sterculia foetida* oil. All other experimental procedures in these tests were the same as indicated above.

Preliminary experiments were conducted to determine the levels at which the test materials should be administered in order to obtain satisfactory data for the calculation of LD-50 values. The dose levels selected were: for pigment glands, 1.75, 1.50, 1.25 and 1.00 g/kg body wt; for gossypol, 3.50, 2.50, 2.00, and 1.50 g/kg body wt; for diaminogossypol, 4.50, 3.50, 3.00 and 2.00 g/kg body wt; gossypurpurin, 7.50, 5.00, 3.00 and 2.25 g/kg body wt.

The number of rats used at each level of test material is indicated in Table I. For the determination of LD-50 values for gossypol administrated in cottonseed and corn oil with *Sterculia foetida* oil added, the number of rats used were 40 and 32, respectively.

Estimation of Relative Absorbability of Cottonseed Pigments. The trays below the cages of individual rats were covered with wax paper and the feces were collected during a four-day period immediately after the administration of the test dose. The feces were collected in Erlenmeyer flasks containing 2 ml of formalin as a preservative and then made up to a suitable vol with water. Aliquots were taken for analysis after homogenizing the material in a Waring blendor. Total gossypol was determined in the feces from rats fed pigment glands, gossypol and diamino-

TABLE I

Toxicity of Cottonseed Pigment Glands, Gossypol, Diaminogossypol, and Gossypurpurin [a]

Material fed	Dose (g/kg body wt)	No. of rats given the dose	No. of rats which died	% Loss in wt among living	Analysis of feces for material fed [b]	Calculated LD-50 (g/kg body wt)
Pigment glands	1.75	10	10	—f [c]	9.3	1.12 ± 0.30
	1.50	10	8	18.3	9.0	
	1.25	11	6	14.8	8.6	
	1.00	9	1	9.1	4.4	
Gossypol	3.50	8	5	26.1	7.0	2.57 ± 0.25
	2.50	9	5	13.7	7.2	
	2.00	9	3	12.0	6.1	
	1.50	12	0	11.6	3.1	
Diaminogossypol	4.50	10	7	22.6	3.7	3.27 ± 0.22
	3.50	10	6	16.2	4.5	
	3.00	10	4	13.9	4.3	
	2.00	10	1	12.2	4.1	
Gossypurpurin	7.50	10	6	28.5	9.2	6.68 ± 0.11
	5.00	10	3	18.9	7.3	
	3.00	10	1	15.5	4.6	
	2.25	10	0	11.1	3.5	

[a] All materials tested were given in corn oil to male rats weighing 160 ($\pm$ 10) g, and the rats were kept under observation for 7 days or until death.
[b] Total gossypol in feces as % of the initial amount fed in the pigment glands, in gossypol and in diaminogossypol, and total gossypurpurin in feces as % of initial amount of gossypurpurin fed.
[c] Fatal to all rats tested.

gossypol. Gossypurpurin was determined in the feces of rats fed this pigment. The estimates of relative absorbability were made on the assumption that a decrease in the percentage of the administered pigment appearing in the feces reflected increased absorbability.

Analytical Procedures

Total gossypol was determined by the method of Pons et al. (21).

Gossypurpurin was determined according to the procedure described by Boatner et al. (22).

The rat feces were analyzed for blood using the Alvarez and Wright (23) modification of the Gregersen and Boas test.

Results and Discussion

Toxicity of Pigment Glands, Gossypol, Diaminogossypol and Gossypurpurin. (a) *Pigment Glands.*—A summary of the acute oral toxicity studies, the average percentage wt depression, and the average percentage of the test materials which remained in the feces is given in Table I.

The LD-50 value for pigment glands obtained by the use of 40 rats was 1.21 g/kg body wt. This calculation is based on the total wt of the glands. These glands contained 43% of total gossypol. If the calculation were based on the wt of the gossypol, the LD-50 value would be approximately 0.5 g/kg body wt. This amount of pure gossypol does not kill rats.

The amount of gossypol recovered in the feces indicated that the percentage of gossypol absorbed was less at the higher dose levels. All doses caused diarrhea in almost all rats, which ranged from a severe type with higher doses that lasted for the whole time of the experiment or until death, to a mild type with lower doses that lasted for 2–3 days. Analysis for blood in the feces revealed considerable amounts, especially in the case of rats fed the higher doses.

Autopsy of the dead animals revealed consistent hyperemia of the gastrointestinal tract. There was severe inflammation and hemorrhage in the pyloric region of the stomach. The intestines showed congested vessels, inflamed duodenum and in some cases there was severe hemorrhagic enteritis and blood mixed with food and mucilage in the caecum. The liver was light in color and the lobules were prominent. The kidneys were pale in color with congested medulla. The lungs were congested. These results are similar to those reported by Withers and Carruth (24) and Eagle et al. (7).

Subsequent to the experiments reported in Table I, an acetone extract of pigments was prepared and its toxicity determined with 40 rats. The LD-50 value obtained was 0.79.

(b) *Gossypol.*—The LD-50 value for gossypol obtained by the use of 38 rats was 2.57 g/kg body wt. The percent loss in wt among the surviving animals was directly proportional to the dose given. The analysis of the feces showed that 7.0, 7.2, 6.1, and 3.1% of the administered gossypol was not absorbed.

Autopsy of dead rats revealed hemorrhagic gastritis in the stomach, enteritis in the lower part of the duodenum and in some cases, congestions throughout the intestine. The caecum was slightly inflamed. The liver showed prominent lobules, the kidneys showed badly congested medulla, and the lungs were congested.

(c) *Diaminogossypol.*—The LD-50 value for diaminogossypol obtained by the use of 40 rats was 3.27 g/kg body wt. The percent loss in wt among the surviving animals was directly proportional to the dose given. The analysis of the feces for total gossypol showed that 3.7, 4.5, 4.3, and 4.1%, respectively, of the administered dose was not absorbed. Autopsy of dead rats revealed catarrhal gastritis with petechial hemorrhages on the mucous membrane of the fundus portion of the stomach. The duodenum showed catarrhal gastritis with scattered petechiae, in severe cases congestions throughout the intestine were observed. The liver, kidney, and lungs showed symptoms similar to those observed with gossypol.

(d) *Gossypurpurin.*—The LD-50 value for gossypurpurin obtained by the use of 40 rats was 6.68 g/kg body wt. The % loss in wt among the surviving animals was directly proportional to the dose given. The analysis of the feces for gossypurpurin showed that 9.2, 7.3, 4.6, and 3.5%, respectively, of the administered doses was not absorbed. Rats given the 7.5 g dose showed diarrhea throughout the period of the experiment or till death, rats given other doses ranged from mild diarrhea that lasted for 2–3 days to normal stool. Autopsy of dead rats showed that gossypurpurin had stained the mucous membrane of the stomach and intestinal tract with a purple color. The liver, the kidneys, and the lungs showed symptoms which were similar to those observed in the rats fed gossypol.

The general conclusion to be drawn from the data obtained is that although gossypol and gossypurpurin are toxic to rats, yet the pigment glands are much more lethal. This finding may also lead to the conclusion that the toxicity of the cottonseed pigment glands is attributed to some component[s] other than or in addition to gossypol and gossypurpurin. This conclusion is in agreement with Eagle's reports (7–10).

Moore and Rollins (24) studied the ultrastructure of cottonseed pigment glands by the use of the electron microscope and found that the gossypol occurs in the form of tiny spheres of variable diameter. According to the calculations of these investigators 1 g of gossypol would have a surface area of the order of about 8 sq meters. Gossypol in such a physical form could conceivably have considerably greater physiological action due to greater absorption from the digestive tract. That this explanation does not completely account for the difference in toxicity between pigment glands and equivalent amounts of gossypol is indicated by the present finding that acetone extracts of pigment glands are also more toxic than pure gossypol.

Comparing the LD-50 values of gossypol, diaminogossypol, and gossypurpurin, it can be concluded that factor[s] which enhances the reaction:

Gossypol ⟶ Diaminogossypol ⟶ Gossypurpurin in cottonseed meal decreases the toxicity. Boatner et al. (22) found that gossypurpurin increases during the processing and storage of cottonseed meal and obtained evidence that this compound is indeed a reaction product of gossypol.

Effect of Oils Containing Cyclopropene Fatty Acids on Gossypol Toxicity and Absorption. Table II shows the LD-50 values obtained when gossypol was administered in corn oil, in cottonseed oil, and in corn oil with *Sterculia foetida* oil added. The mortality of the rats which received gossypol in cottonseed oil was 70, 60, 30, and 0% for dose levels of 2.50, 2.00, 1.50 g/kg body wt, respectively. The mortality of the rats which received the gossypol in corn oil with added *Sterculia foetida* oil was 85.7, 33.3, 50.0, and 16.6% for the same dose levels as indicated above. As a control, 20 mg of *Sterculia foetida* oil dissolved in 2 ml of corn oil

TABLE II
Effect of Oils Containing Cyclopropene Fatty Acids on Gossypol Absorption and Toxicity

	% of administered gossypol which appeared in the feces				Toxicity LD-50 (g/kg gody wt)
	Doses of gossypol given (g/kg body wt)				
	1.50	2.00	2.50	3.50	
Gossypol administered:					
In corn oil	4.27	6.56	9.67	9.38	2.57 ± 0.25
In cottonseed oil	3.20	4.10	7.30	4.90	2.27 ± 0.21
In corn oil with *S. foetida* oil (20 mg/dose)	1.00	3.83	5.33	4.71	2.10 ± 0.21

was given to each of ten rats. None of these animals died.

Although there was a trend toward greater toxicity when gossypol was administered in cottonseed oil or in corn oil with *S. foetida* oil added, as compared to corn oil alone, the differences were relatively small, and in the case of corn oil and cottonseed oil the difference was not statistically significant. The difference between the toxicity of gossypol in corn oil and gossypol in corn oil with *Sterculia foetida* oil added was statistically significant at the 5% level of probability.

Analysis of the feces for total gossypol (Table II) showed that less gossypol remained in the feces when the test dose was administered in cottonseed oil or *S. foetida* oil. A possible explanation of this finding is that the cyclopropene fatty acids may increase the absorption of gossypol.

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A Kinetic Approach to Detergent Synergism

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Abstract

It is shown that kinetic theory can be applied to the removal of a heterogeneous soil by detergent solutions. By assuming first-order kinetics, const permitting rate comparisons were calculated for the removal of baked P^{32} milk films from stainless steel test discs by NaOH, a nonionic surfactant, and combinations of the two. Values for E* were obtained from Arrhenius plots. The data indicate an abrupt change in the activity of the nonionic detergent occurred as it passed through the cloud point region. Synergism was demonstrated in solutions of NaOH-surfactant maintained below cloud point temps; at higher temps, this synergism was apparently lost. Possible explanations of synergism and detergency as affected by the cloud point phenomenon are discussed.

Introduction

THERE ARE PROBABLY a variety of forces involved in the binding of soils by substrates, and in most cases soil removal is reduced to the problem of decreasing these forces, and supplying sufficient excess energy to overcome an energy barrier and free the soil. The main function of the detergent is to minimize the binding forces, for lack of a more specific term, and to reduce the energy level of desorbed soil particles and thus discourage their redeposition.

The soil desorption undoubtedly involves a variety and series of steps and reactions, some chemical and others largely physical. Because the desorption step demands that the dissolved detergent molecule (or ion, or aggregate) contact the deposited soil or the substrate at the deposition site, the overall effect should be somewhat analogous to a bimolecular reaction which can, after collision, proceed in various directions to a variety of end products. Although there are theoretical objections to treating an undissolved and heterogeneous soil deposit as if it were a single molecular species, it has been demonstrated (1) that the removal of such films can be considered as a process first order with respect to soil (S) and first order with respect to detergent (D):

$$-dS/dt = K(D)(S)$$

It would be ideal to eliminate the effect of soil concentration on this expression by constructing linear plots of (S) as a function of t for various detergent concentrations. For a given value of (S), the tan-

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