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Effect of Fractionation and Treatment on the Acute Oral Toxicity and on the Gossypol and Gossypurpurin Content of Cottonseed Pigment Glands[1,2]

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SINCE the original report (1) on the acute oral toxicity of gossypol and cottonseed pigment glands for rats, mice, rabbits, and guinea pigs, a total of 21 fractionated and variously treated cottonseed pigment gland preparations have been tested for their acute oral toxicity (LD_{50} value) and for their content of extractable gossypol and gossypurpurin; and the acute oral toxicity of six different samples of pure gossypol has been determined.

Experimental

The first three samples of untreated cottonseed pigment glands (1*b*, 2*b*, and 3*a*) used in this study (Table 1) were prepared by the gland flotation process (2, 3) from a single lot of prime cottonseed (1945 crop) which was stored in a silo for about 6 months before processing. Sample 1*b* was a composite of pigment glands prepared from seed processed between March 8 and April 16, 1946; sample 2*b* was obtained from seed processed between May 16 and May 25, 1946; sample 3*a* was made from seed processed between November 18 and December 11, 1946. The separated glands were stored at 7°C. in sealed containers prior to administration. Sample 4*a* of untreated pigment glands was isolated from an entirely different lot of prime cottonseed, 1946 crop, which had been stored for about six months prior to processing in July and August, 1947. This seed was defatted with commercial hexane (boiling point range of 146-158° F.) prior to removal of the pigment glands.

The various fractions (1*a* to 1*h*) were prepared as follows: 100 grams of pigment glands (sample 1*b*) were blended with 400 ml. acetone for 5 min. in a Waring Blendor, the mixture was centrifuged, the supernatant was decanted and the residue was repeatedly washed with small volumes of acetone until the total volume of acetone extract equaled 400 ml. The residue, consisting mainly of gland walls and a small amount of adhering gossypol, was dried in a vacuum desiccator at room temperature. This residue was called the acetone-insoluble fraction (sample 1*h*). To the above acetone extract 400 ml. distilled water was added, the mixture was stirred and centrifuged, and the supernatant was filtered. Most of the acetone in the filtrate was evaporated at room temperature and under vacuum. The water was removed from the remaining portion of this supernatant by lyophilizing to give sample 1*a*. The viscous, oily, reddish-yellow material, resulting from the addition of water to the original acetone extract and recovered by centrifugation, was freed of acetone and water in a vacuum desiccator at room temperature. Then the residue was washed with light petroleum naphtha (boiling point range of 60°-110°F.) until all of the reddish-brown viscous component was removed. The residue, solid and brown in color, was dried overnight in a vacuum desiccator and was sample 1*e*. The light petroleum naphtha wash solutions were combined and evaporated under vacuum at room temperature to give the dark brown, viscous product (sample 1*g*).

A total of 400 grams of pigment glands from the same lot (sample 1*b*) was mixed with 1,600 ml. distilled water at a temperature of 3.5°C. and the mixture was centrifuged at 1,800 r.p.m. at 2°C. The precipitate was composed principally of gland walls and some gossypol (sample 1*d*). The water from the cloudy supernatant was removed at low temperature under vacuum and the dried product was sample 1*c*. A 221-gram portion of the lyophilized product (sample 1*c*) was suspended in 400 ml. water and centrifuged at high speed (10,000 r.p.m.) for 2½ hours at 2°C. The supernatant was decanted and lyophilized (sample 1*f*).

A diagram of the procedures used in the fractionation of the pigment glands and of the products obtained is given in Figure 1.

To determine the effect of heat on the toxicity of cottonseed pigment glands, samples were placed in covered aluminum pans and heated at the temperatures indicated (samples 2*c* and 3*c*). Sample 2*a* was made by grinding some of sample 2*c* in a Wiley Mill through a ½-mm. screen. To ascertain the effect of heat in the presence of water, the pigment gland samples were thoroughly wetted with distilled water and

[1] Report of a study in which certain phases were carried on under the Research and Marketing Act of 1946.

[2] Presented at fall meeting, American Oil Chemists' Society, Chicago, Oct. 31, Nov. 1-2, 1949.

[3] This work was conducted with Swift and Company under a memorandum of understanding with the Bureau of Agricultural and Industrial Chemistry. The preparation of the samples of gossypol and cottonseed pigment glands and the analyses for gossypol and gossypurpurin content were carried out in the Southern Regional Research Laboratory; all of the animal experiments were performed in the Research Laboratories of Swift and Company.

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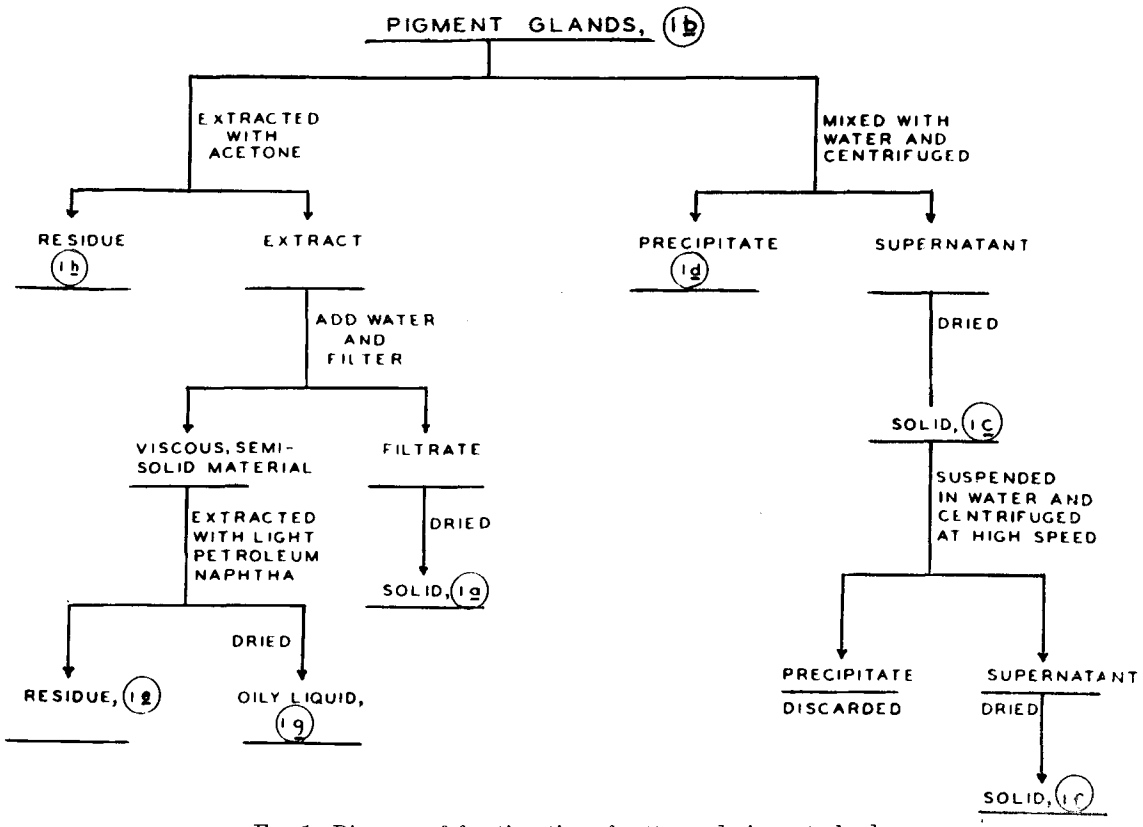

FIG. 1. Diagram of fractionation of cottonseed pigment glands.

heated at 105°C. in covered aluminum containers for 1 hour, dried in a vacuum desiccator over anhydrous calcium sulfate, and then ground to a fine powder (samples 2*e* and 3*d*). To test the effect of extraction the glands were suspended in relatively large volumes of the appropriate organic solvent (500 ml. of the solvent to 200 grams of glands) and allowed to stand overnight at 3.5°C. The insoluble residues were then washed by decantation until the supernatant liquid was practically colorless (samples 3*e* and 3*f*). Samples of untreated cottonseed pigment glands (2*b*, 3*a*, and 4*a*) were stored at 7°C. for periods of 32, 26, and 17 months, respectively, and became preparations 2*d*, 3*b*, and 4*b*.

Six independently prepared samples of gossypol were likewise tested for their toxicity (Table II).

The first sample (A) was prepared by alkaline extraction of diethyl ether extracts of flaked cottonseed meats (4); second and third samples (B and C) were made from diethyl ether extracts of cottonseed pigment glands; and the last three samples (D, E, and F) were prepared from acetone extracts of cottonseed pigment glands as described by Castillon *et al.* (5). The gossypol acetic acid, formed by treating either the ethereal or acetone extracts with glacial acetic acid, was hydrolyzed and the liberated gossypol was recrystallized from a mixture of diethyl ether and light petroleum naphtha. All of the samples of gossypol were identical with respect to melting point, elementary composition, ultra-violet and visible absorption spectra of chloroform solutions, and the absorption spectra of their reaction products with antimony trichloride (6). The absorption spectra of the gossypol samples and their reaction products with antimony trichloride were also identical with those observed for gossypol as it occurs in chloroform extracts of the pigment glands (7). The samples of gossypol were stored at 7°C. in sealed, brown, glass bottles prior to administration. The extractable gossypol content of the pigment glands was determined by the antimony trichloride spectrophotometric method (8, 9), using chloroform solutions prepared from aqueous ethanol extracts (7) of the glands. The content of extractable gossypurpurin in the samples was estimated on the basis of the specific extinction coefficients at 570 mμ. of chloroform extracts of the glands, as previously described by Boatner *et al.* (10).

The acute oral toxicity was determined on male rats (150-220 grams) which had fasted 18 hours, with water, *ad libitum*. Each rat was kept in a separate cage in an air-conditioned animal room maintained at 80° ± 1°F. and ca. 45% relative humidity. The pigment gland (or gossypol) samples were mixed with distilled water and administered as a suspension containing 100 mg. pigment glands (or gossypol) per ml. for most dose levels. Single doses were given by stomach tube on the basis of mg. of pigment glands (or gossypol) per kg. body weight to a group of at least four rats at each dose level, after which all animals were permitted free access to stock diet and water. Calculation of the median lethal dose (that dose which is fatal to 50% of the animals to which it is administered) was made by the method of Reed and Muench (11).

Results and Conclusions

A summary of the acute oral toxicity studies and of the average gossypol and gossypurpurin analyses (average of at least two determinations for each) on a total of 21 preparations from four different samples of cottonseed pigment glands is given in Table I.

TABLE I

Effect of Fractionation or Treatment on the LD_{50} Value and on the Extractable Gossypol and Gossypurpurin Content of Cottonseed Pigment Glands

Sample No.	Fraction or treatment	LD_{50} value	Number of rats used	Content of extractable pigments	
				Gossypol	Gossypurpurin
		mg./kg.		%	%
1*a*	Soluble in water and acetone	ca. 700	25	58	0.0
1*b*	Untreated	925	171	40	1.0
1*c*	Soluble or dispersed in water	955	32	42	0.9
1*d*	Gland walls, insoluble in water	1,170	38	46	1.1
1*e*	Soluble in acetone, insoluble in water and light petroleum naphtha	1,815	31	90	0.4
1*f*	Soluble in water	1,975	23	24	0.5
1*g*	Soluble in acetone and light petroleum naphtha, insoluble in water	>2,000	24	11	0.7
1*h*	Insoluble in acetone	>6,000	34	8	0.1
2*a*	Heated dry for 1 hr. at 105°C. and ground*	930	25	50	....
2*b*	Untreated	1,060	252	38	1.2
2*c*	Heated dry for 1 hr. at 105°C.	1,110	25	48	....
2*d*	Sample 2*b* stored for 32 months at 7°C.	1,140	33	40	0.8
2*e*	Wetted, heated for 1 hr. at 105°C. and ground*	2,400	59	38	0.9
3*a*	Untreated	1,350	92	33	3.2
3*b*	Sample 3*a* stored for 26 months at 7°C.	1,490	32	34	1.7
3*c*	Heated dry for 1 hr. at 103°C.	1,500	62	35	3.2
3*d*	Wetted, heated for 1 hr. at 102°C. and ground*	2,120	30	24	3.0
3*e*	Exhaustively extracted with ethanol	3,200	59	17	1.7
3*f*	Exhaustively extracted with acetone	>6,000	58	4	0.5
4*a*	Untreated, but obtained from hexane-defatted seed	1,775	39	33	1.6
4*b*	Sample 4*a* stored for 17 months at 7°C.	2,290	64	30	1.2

* Grinding was done in a Wiley Mill through a ½-mm. screen.

For each of these four samples the preparations are arranged in order of decreasing toxicity.

The first three samples of untreated cottonseed pigment glands (1*b*, 2*b*, 3*a*) had LD_{50} values of 925, 1,060, and 1,350 mg./kg., indicating a decrease in toxicity with increased length of storage of the original cottonseed. Storage of two of these samples of glands at 7°C. for periods of 26 and 32 months, respectively, (2*d*, 3*b*) caused only slight decreases in their toxicity and little alteration in their content of extractable gossypol but did reduce the content of extractable gossypurpurin.

The pigment glands (4*a*) obtained from hexane-defatted seed suffered a significant decrease in toxicity when they were stored for 17 months at 7°C. (4*b*), but there was very little change in content of either extractable gossypol or gossypurpurin.

The most toxic preparation (1*a*) was the acetone-soluble, water-soluble fraction of the pigment glands. Exhaustive extraction with ethanol (3*e*) reduced the average lethal dose from 1,350 to 3,200 mg./kg., a detoxification to less than half of the original value. Exhaustive extraction with acetone (3*f*) completely detoxified the glands so that doses as high as 6,000 mg./kg. failed to cause fatalities and no LD_{50} value could be obtained. The acetone-insoluble residue (1*h*) was likewise non-toxic. Heating the glands in the absence of water (2*a*, 2*c*, 3*c*) had very little effect on their toxicity. Heating in the presence of water (2*e*, 3*d*) caused a reduction in toxicity to approximately one-half that of the corresponding untreated glands. The toxic material in the glands appears to be readily soluble in water, acetone, and diethyl ether, less soluble in ethanol, and insoluble in petroleum naphtha. It is stable to dry heat but is inactivated by heat in the presence of water.

It may readily be seen that the toxicities of the various individual test preparations are not proportional to their contents of extractable gossypol or gossypurpurin. The most toxic preparation (1*a*) had a gossypol content of only 58% and contained no extractable gossypurpurin. Sample 1*e* containing 90% extractable gossypol was only half as toxic as four samples containing only 58, 50, 42, and 40% extractable gossypol; and despite its high extractable gossypol content it was also considerably less toxic than eight other samples whose extractable gossypol content varied from 33 to 48%. On the other hand, sample 1*b* containing only 40% extractable gossypol was more toxic (LD_{50} 925 mg./kg.) than five samples which contained greater amounts of gossypol (90, 50, 48, 46, and 42%) and whose LD_{50} values were, respectively, 1,815, 930, 1,110, 1,170, and 955 mg./kg. Likewise sample 2*b*, containing only 38% extractable gossypol, was more toxic (LD_{50} 1,060 mg./kg.) than five samples which contained greater amounts of extractable gossypol.

Samples 1*b*, 2*a*, and 1*c*, with approximately the same LD_{50} values (925, 930, and 955 mg./kg.) had extractable gossypol contents, respectively, of 40, 50, and 42%. Again, samples 2*b*, 2*c*, 2*d*, and 1*d*, whose LD_{50} values were 1,060, 1,110, 1,140, and 1,170 mg./kg., respectively, contained 38, 48, 40, and 46% extractable gossypol. In both of these instances there was a wide variation in the extractable gossypol content which was not reflected in the LD_{50} values. Samples 3*d*, 4*b*, and 2*e*, with LD_{50} values of 2,120, 2,290, and 2,400 mg./kg., respectively, had extractable gossypol values of 24, 30, and 38%—a decreasing order of toxicity with an increasing order of extractable gossypol content. Obviously there is no correlation between the content of extractable gossypol or gossypurpurin and the acute oral toxicity of the respective samples.

Acute oral toxicity studies on six different samples of pure gossypol are summarized in Table II. In the case of samples A and B, because of expectation of considerable toxicity, the dose levels used turned out to be well below the median lethal value and there were no fatalities. At the higher dose levels (1,800 to 5,000 mg./kg.) there were large numbers of fatalities and LD_{50} values could be calculated for samples C, D, E, and F. Simultaneous LD_{50} analysis of all six samples gave an overall LD_{50} value of 2,720 mg./kg. for pure gossypol administered by stomach tube to 167 rats. Thus four samples of 100% gossypol proved to be far less acutely toxic to rats than were 17 of the preparations of cottonseed pigment glands shown in Table I, all of which contained considerably less extractable gossypol. The three preparations (1*h*, 3*e*, and 3*f*) which were less toxic than pure gossypol were those involving ethanol and acetone extraction, which have been shown to be methods of detoxification. This relative lack of acute oral toxicity indicated by the high LD_{50} value for single doses of gossypol in the rat is in contrast however to the sub-acute toxicity of unusually small doses of these very same samples of gossypol administered daily to

TABLE II

Toxicity Studies on Various Samples of Gossypol

Sample	LD_{50} value	Number rats used
	mg./kg.	
A	> 600	40
B	>1,600	20
C	3,340	36
D	2,600	27
E	2,800	20
F	2,800	24
All	2,720	167

dogs (12, 13). The effects of small, single, and repeated doses of these same samples of gossypol on the body weight of rats (14) were less marked than was the case for dogs. The symptoms in the rat of intoxication from oral administration of cottonseed pigment glands (and of gossypol in large doses) were immediate diarrhea, anorexia, severe weight loss, prostration, nasal exudation, and hair loss and ulceration at the base of the tail. Post-mortem examination of the rats showed hemorrhagic involvement of the entire gastro-intestinal tract, congestion of the splanchnic organs, distention of the stomach with food, fluid in the abdomen, and pulmonary edema.

All of the foregoing results confirm the original conclusion from acute oral toxicity studies on rats, mice, rabbits, and guinea pigs (1) that the toxicity of cottonseed pigment glands is attributable either to some component or components of the glands other than, or in addition to, gossypol and gossypurpurin, or to some material in the glands which enhances the physiological activity of gossypol.

Summary

Twenty-one preparations from cottonseed pigment glands were tested for their acute oral toxicity in 1,208 fasted rats and for their content of extractable gossypol and gossypurpurin. LD_{50} studies on six samples of pure gossypol were performed on 167 fasted rats. There was no correlation between the toxicity of the various samples of cottonseed pigment glands and their extractable gossypol or gossypurpurin content. Samples containing very large amounts of extractable gossypol were less toxic than many samples with considerably lower extractable gossypol content.

Various fractionation procedures carried out on the same lot of cottonseed pigment glands caused wide alterations in their toxicity, from the extreme of very marked toxicity for the water-soluble, acetone-soluble fraction (LD_{50} ca. 700 mg./kg.) to no detectable toxicity for the acetone-insoluble residue (LD_{50} > 6,000 mg./kg.).

There was a decreased toxicity of subsequently prepared pigment glands with increased time of storage of the cottonseed in a silo. Storage of the pigment glands themselves at 7°C. however had little effect on their toxicity even after 26 and 32 months.

The procedures causing greatest detoxification of cottonseed pigment glands, given in the order of increasing effectiveness, were: heating in the presence of water < extraction with ethanol < extraction with acetone.

In the fasted rat the acute oral toxicity of pure gossypol was less than that of 17 preparations from cottonseed pigment glands having extractable gossypol contents ranging from as little as 24 to as much as 90%.

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Report of the Gossypol Committee April, 1950

During the year nine samples of cottonseed meal were sent out to the committee, and herewith are the results on the work:

	No. 1	No. 2	No. 3	No. 4
T. H. Hopper	0.625	0.077	0.119	0.423
T. L. Rettger	0.630	0.070	0.120	0.355
W. T. Coleman	0.717	0.073	0.137	0.420
V. C. Mehlenbacher	0.630	0.080	0.125	0.430
K. Kuiken	0.600	0.069	0.115	0.375
H. L. Craig	0.613	0.067	0.109	0.368
E. H. Tenent	0.625	0.075	0.119	0.378
Averages	0.634	0.073	0.121	0.393

It is the unanimous opinion of the committee that the present A.O.C.S. tentative method for the determination of free gossypol be made the official method

	No. 5	No. 6	No. 7	No. 8	No. 9
T. H. Hopper	0.072	0.028	0.036	0.058	0.037
T. L. Rettger	0.065	0.030	0.040	0.060	0.042
W. T. Coleman	0.068	0.023	0.024	0.044	0.025
V. C. Mehlenbacher	0.070	0.023	0.032	0.056	0.033
K. Kuiken	0.063	0.026	0.026	0.041	0.028
H. L. Craig	0.060	0.028	0.033	0.054	0.034
E. H. Tenent	0.070	0.031	0.039	0.053	0.039
Averages	0.067	0.027	0.033	0.052	0.031

of the American Oil Chemists' Society and that the committee now be discharged.

W. T. COLEMAN
V. C. MEHLENBACHER
T. L. RETTGER
K. A. KUIKEN
H. L. CRAIG
T. H. HOPPER
E. H. TENENT, chairman