The *in vivo* Effect of Gossypol on Cytochrome Oxidase, Succinoxidase, and Succinic Dehydrogenase in Animal Tissues

L. A. Meksongsee,[1] A. J. Clawson, and F. H. Smith[2]

The *in vivo* effect of gossypol on cytochrome oxidase and succinoxidase activity of the liver tissue of pigs, rabbits, and rats were studied. The heart and kidney tissues of the rabbits were assayed also for the above enzymes. Succinic dehydrogenase activity in the livers of one group of pigs was determined. The results showed no difference in enzyme activity between the gossypol-treated and the control animals for any of the enzymes or tissues studied even though the animals exhibited an extreme degree of toxicity.

Gossypol, a phenolic substance in cottonseed, is toxic to nonruminant animals, especially to pigs and rabbits (Schwartze and Alsberg, 1924; Smith, 1957; Wither and Carruth, 1918). During processing of the seed, gossypol is detoxified probably by combination with certain seed constitutents (Clark, 1928; Lyman *et al.*, 1959; Wither and Carruth, 1918) such as the ε-amino groups of lysine.

Ferguson *et al.* (1959) were unable to demonstrate effects of ingested gossypol on cytochrome oxidase in liver homogenates of the hen, but did show that it reduced the succinic dehydrogenase and cytochrome oxidase activity in liver homogenates of chicks. In their *in vitro* studies using liver homogenates of the normal hen, gossypol markedly reduced succinic dehydrogenase, cytochrome oxidase, and xanthine oxidase activities. Inhibitions of succinoxidase activity encountered by Throneberry (1961) in the preparation of succinoxidase particulate fractions from cottonseed hypocotyls led to *in vitro* studies by Myers and Throneberry (1966) of the effects of gossypol on succinoxidase, cytochrome oxidase, and succinic dehydrogenase. They showed that at certain concentrations of gossypol, activities of these enzymes were inhibited; however, at certain other concentrations, stimulatory effects on succinic dehydrogenase and cytochrome oxidase were observed.

Braham and coworkers (1967) reported that glutamic-oxaloacetic transaminase in the serum of gossypol-fed pigs was increased, but lactic dehydrogenase, leucine amino peptidase, and aldolase were not affected significantly. A significant decrease in hemoglobin and hematocrit values was observed.

Thus, it seemed desirable to determine whether or not the toxicity from ingested gossypol is associated with the activities of certain respiratory enzyme systems, since labored breathing is a clinical symptom of gossypol toxicity. Consequently, a study was undertaken to determine the *in vivo* effects of gossypol on cytochrome oxidase and succinoxidase. Succinic dehydrogenase activity was also determined in one of the experiments. The test animals were pigs, rabbits, and rats.

EXPERIMENTAL PROCEDURES

The composition of the diets fed to pigs in three experiments is shown in Table I. The diets contained 15% protein supplied by corn, cottonseed meal, cottonseed flakes (rolled dehulled cottonseed), and soybean meal. Some of the diets were supplemented with additional lysine. The gossypol was supplied by cottonseed meal or cottonseed meal and cottonseed flakes. The cottonseed meals used contained approximately 0.4% free gossypol and were analyzed for crude protein, crude fat, nitrogen solubility in 0.02*N* NaOH, ε-amino free lysine, and free and bound gossypol (Table I). Nitrogen solubility and ε-amino free lysine indicated that the cottonseed meals were of good quality. In the control diets, the protein was supplied by soybean meal and corn. The diets were lot-fed *ad libitum* to the pigs, which had free access to water.

A total of 53 pigs were used in three experiments. The average starting weights in Experiments I, II, and III were approximately 27, 35, and 18 kg, respectively (Table II). The pigs, randomly assigned to treatment, were fed until those receiving gossypol either reached market weight or showed severe symptoms of toxicity, such as labored breathing, weakness, and emaciation. Those pigs showing gossypol intoxication were continued on treatment until death appeared inevitable within 24 to 48 hr for the development of maximum effects of gossypol on the enzymes studied.

Thirteen young adult rabbits, housed in individual cages, had free access to water and a commercial diet guaranteed to contain crude protein not less than 15%, crude fat not less than 2%, crude fiber not more than 18%, and added minerals not more than 3%. Five of the rabbits were given gossypol as the disodium salt in physiological saline solution intravenously in the ear. The gossypol solution was prepared to contain 25 mg of gossypol per ml and was administered as a single dose of 75 mg to two rabbits and 125 mg to one, and three daily doses of 25 or 50 mg to the fourth and fifth, respectively. A sixth rabbit was fed 12 daily doses of 25 mg by stomach tube. Seven rabbits were used as controls; of these, two were starved for 24 hr and two for 72 hr before sacrifice.

Weanling rats were randomly assigned to a basal diet containing 10% protein, supplied by soybean meal, to serve as controls, or to the basal diet supplemented with 0.14% gossypol acetic acid. The rats were fed *ad libitum* in individual cages for 3 to 6 weeks.

When the pigs showed toxic symptoms such as anoxia, labored breathing, and difficulty in standing or walking, they were killed by stunning, followed immediately by exsanguination. The livers were removed immediately and chilled on ice for assay of enzyme activity and gossypol content. The rabbits and rats were killed by stunning and decapitation. Livers, hearts, and kidneys from the rabbits, and only livers from the rats, were removed and chilled on ice for enzyme assay.

Animal Husbandry and Nutritional Biochemistry Sections, Department of Animal Science, North Carolina State University, Raleigh, N.C. 27607

[1] Department of Chemistry, Kasetsart University, Bangkok, Thailand.

[2] To whom correspondence should be addressed.

Table I. Percentage Composition of Diets (15% Protein) Fed to Pigs

Ingredients	Diets					
	Exp. I			Exp. II		Exp. III
	Control	CSM[a] 5%	CSM[a] 10%	Control	CSM[b] 10%	CSM[b] 15%
Ground corn	79.1	80.0	78.7	80.6	76.8	78.1
Cottonseed meal (CSM)	...	5.0	10.0	...	10.0	15.0
Cottonseed flakes	...	...	...	...	2.9	...
Soybean meal	18.0	11.1	7.4	16.5	7.5	3.0
Defluorinated phosphate	1.0	1.0	1.0	1.0	0.8	1.0
Calcium carbonate	0.9	0.9	0.9	0.9	1.0	0.9
Trace mineral salt[c]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin supplement[d]	0.5	0.5	0.5	0.5	0.5	0.5
Lysine supplement	...	1.0[e]	1.0[e]	...	...	0.25[f]
Free gossypol,[g] ppm (calculated)	0.0	215	430	0.0	600	570

[a] Cottonseed meal contained in percent: Moisture, 9.94; fat, 1.59; crude protein, 39.25; nitrogen solubility, 73.09 in 0.02*N* NaOH; free gossypol, 0.43; total gossypol, 0.98; and ε-amino free lysine, 3.45 g/16 g of nitrogen. [b] Cottonseed meal contained in percent: Moisture, 8.60; fat, 0.70; crude protein, 40.81; nitrogen solubility in 0.02*N* NaOH, 76.42; free gossypol, 0.38; total gossypol, 1.12; and ε-amino free lysine, 3.54 g/16 g of nitrogen. [c] Supplied per kg of diet: Sodium chloride, 4.8 g; zinc, 40 mg; manganese, 30 mg; iron, 10 mg; copper, 3 mg; iodine, 0.8 mg; cobalt, 0.75 mg. [d] Supplied per kg of diet: Vitamin A, 2200 U.S.P. units; vitamin D, 1100 U.S.P. units; vitamin E, 1.1 I.U.; riboflavin, 4.4 mg; pantothenic acid, 10.1 mg; niacin, 22 mg; choline chloride, 5.5 mg; vitamin B_{12}, 22 μg; oxytetracycline, 22 mg. [e] Lysine supplement contained 50% L-lysine. [f] Lysine supplement was L-lysine. [g] Supplied as a constituent of cottonseed meal and cottonseed flakes.

Table II. Performance of Pigs Fed Diets Containing Gossypol

Treatment							
Protein Source	Dietary Gossypol, ppm	No. of Animals	Average Days Fed	Weight		Gain	
				Starting, kg	Final, kg	Total, kg	Daily, kg
			Experiment I				
Soybean meal control	0.0	10	78.2	27.7	79.1	51.4	0.66
Cottonseed meal, 5%	215	6	97.7	25.7	79.5	53.8	0.55
Cottonseed meal, 10%	430	7	52.4	28.7	58.2	29.4	0.56
			Experiment II				
Soybean meal control	0.0	4	31	33.9	55.9	22.0	0.71
Cottonseed meal + 10% cottonseed flakes	600	6	29.7	36.8	47.0	10.2	0.34
			Experiment III				
Soybean meal control	0.0	10	50.9	18.2[a]	41.7	23.5	0.46
Cottonseed meal, 15%	570	10	44.2	18.2[a]	31.1	12.9	0.29

[a] Estimated weight.

One gram of the diced tissue was homogenized in an ice bath with 9 ml of buffer for 2 min according to the method of Hogeboom (1955). The buffer used was *M*/30 phosphate (pH 7.4) in pig Experiment I, 0.0625*M* sucrose in pig Experiment II and the rat experiment, and 0.25*M* sucrose in pig Experiment III and the rabbit experiment. The mitochondria were prepared in 0.0625*M* sucrose in Experiments I, II, and the rat experiment; they were prepared in 0.25*M* sucrose in pig Experiment III and the rabbit experiment. The mitochondria pellets were washed twice by suspension in 2 ml of the sucrose buffer and centrifuged at 20,000 × G for 10 min. The washed mitochondria were suspended in 2 or 5 ml of sucrose buffer and stored on ice until assayed for enzyme activity. The homogenates, from pig Experiments I and II and the rat experiment, and the mitochondria from all experiments were assayed polarographically at room temperature for cytochrome oxidase activity with an oxygraph (Gilson Medical Electronics, Middleton, Wis.) using the reaction mixture of Takemori and King (1965). The cytochrome oxidase activity of the liver mitochondria of pigs in Experiment III was also assayed with a recording spectrophotometer (Gilford Instruments, Oberlin, Ohio) using the reaction mixture of Hogeboom and Schneider (1952). The enzyme activity was measured in nanomoles of O_2 uptake per min per mg of protein by the oxygraph and as the decline in absorbance per mg of protein by the spectrophotometer at 550 mμ. Succinoxidase activity was measured by the oxygraph using the reaction mixture of Schneider and Potter (1943). Succinic dehydrogenase activity was determined spectrophotometrically on the liver mitochondria of the pigs in Experiment III by recording the decline in absorbance at 420 mμ due to the reduction of ferricyanide by succinate (Wang *et al.*, 1956). All of the tissue preparations were tested to determine the optimum amount to be used for most accurate measurement of the enzyme activity. The protein in the tissue preparations was determined by the method of Lowry *et al.* (1951). The free and bound gossypol values (combined values represent total gossypol) were determined by the method of Smith (1965). The data were analyzed statistically.

RESULTS AND DISCUSSION

The performance of the pigs is shown in Table II. Those in Experiments I and II made fair daily gains. The smaller and younger pigs in Experiment III did not perform as well. The pigs consuming gossypol had lower daily gains than did those fed the control diets. Pigs on the high levels of gossypol gave evidence of gossypol intoxication after 5 to 6 weeks by reduced feed intake, listlessness, labored breathing when disturbed, and difficulty in standing. The animals receiving the lowest level of gossypol never showed signs of acute intoxication. Those receiving the intermediate level showed acute intoxication after 6 to 8 weeks. The pigs in Exper-

Table III. The *in vivo* Effect of Gossypol on Cytochrome Oxidase, Succinoxidase, and Succinic Dehydrogenase in Porcine Liver[a]

Animals		Gossypol, ppm			Homogenate		Mitochondria			
					Oxygraph		Oxygraph		Spectrophotometer	
No.	Weeks Fed Avg.	In Diet	In Liver Free	In Liver Total	Cytochrome Oxidase[b]	Succinoxidase[c]	Cytochrome Oxidase[b]	Succinoxidase[c]	Cytochrome Oxidase[d]	Succinic Dehydrogenase[e]
					mmoles O_2 uptake/min/mg protein				Δ abs./min/mg protein × 10	
					Experiment I					
10	11.2	0	15	25	55 ± 3[f]	17 ± 2	96 ± 12	34 ± 3	...	...
6	14.0	215	188	321	49 ± 3	16 ± 2	105 ± 16	27 ± 2	...	...
7	7.5	430	210	396	42 ± 6	14 ± 2	88 ± 9	31 ± 3	...	...
					Experiment II					
4	4.4	0	3	12	58 ± 3	17 ± 2	173 ± 18	44 ± 4	...	...
6	4.2	600	241	392	65 ± 1	17 ± 3	164 ± 10	39 ± 3	...	...
					Experiment III					
10	7.3	0	7	14	...	...	177 ± 17	77 ± 8	2.76 ± .29	1.68 ± .12
10	6.3	570	243	531	...	...	140 ± 24	62 ± 8	3.50 ± .50	1.65 ± .18

[a] The effects are expressed as oxygen uptake in nanomoles and as changes in absorbance per min per mg of protein by the oxygraph and spectrophotometer, respectively. [b] The reaction mixture contained 30 mM phosphate buffer (pH 7.4), 37 μM cytochrome C, 40 mM ascorbic acid neutralized to pH 7.4 with NaOH, and 0.05 to 0.2 ml of tissue preparation in a total volume of 2.4 ml. [c] The reaction mixture contained 55 mM phosphate buffer (pH 7.4), 83 μM $CaCl_2$, 83 μM $AlCl_3$, 62 mM succinate, and 0.1 to 0.2 ml of tissue preparation in a total volume of 2.4 ml. [d] The reaction mixture contained $1.05 \times 10^{-4} M$ cytochrome C (70 to 80% reduced), $0.033 M$ phosphate buffer (pH 7.4), $4 \times 10^{-4} M$ $AlCl_3$, and suitable dilutions of enzyme preparation (0.01 to 0.05 ml) in a total volume of 2.5 ml. [e] The reaction mixture contained $0.1 M$ phosphate buffer (pH 7.8), $0.033 M$ succinate, 2 mM potassium ferricyanide and enzyme preparation (0.02 ml) to make a total volume of 2.5 ml. [f] ± Standard error of the mean.

Table IV. The *in vivo* Effect of Gossypol on Cytochrome Oxidase and Succinoxidase Activity of Homogenates and Mitochondria of Rabbit and Rat Tissues[a]

Treatment	No. of Animals	Liver		Kidney		Heart	
		Cytochrome Oxidase[b]	Succinoxidase[c]	Cytochrome Oxidase[b]	Succinoxidase[c]	Cytochrome Oxidase[b]	Succinoxidase[c]
				Rabbit Homogenates			
Control	3	16 ± 2[d]	15 ± 2	24 ± 6	26 ± 3	77 ± 26	49 ± 19
Control (Starved)	4	22 ± 4	24 ± 7	27 ± 5	25 ± 7	75 ± 16	73 ± 19
Gossypol	6	27 ± 5	15 ± 2	37 ± 7	27 ± 1	80 ± 19	39 ± 8
				Rabbit Mitochondria			
Control	3	72 ± 9	25 ± 4	72 ± 25	42 ± 1		
Control (Starved)	4	95 ± 23	36 ± 5	73 ± 19	39 ± 6		
Gossypol	6	131 ± 42	31 ± 3	92 ± 26	61 ± 9		
				Rat Liver Homogenates			
Control	4	73 ± 7	28 ± 2				
Gossypol	4	74 ± 8	27 ± 6				
				Rat Liver Mitochondria			
Control	4	138 ± 12	56 ± 7				
Gossypol	4	130 ± 13	59 ± 7				

[a] Determined by the oxygraph as nanomoles of O_2 uptake per min per mg of protein. [b,c] Same as in Table III. [d] ± Standard error of the mean.

iment II were larger when put on treatment so the diet was not supplemented with lysine; these pigs made better daily gains than did those in Experiment III, although both groups received about the same level of gossypol. This study was not planned as a study of the effect of gossypol on performance, but rather to determine the effect of gossypol intoxication on the activity of the respiratory enzymes.

The rabbits were very sensitive to gossypol and did not readily consume diets containing it. Some swelling occurred in the ear after intravenous injections. Rats were affected less severely. They showed a rough coat, reduced gains, and an unthrifty appearance.

The results of the enzyme assays are shown in Table III for the pig experiments and in Table IV for the rabbit and rat experiments.

The gossypol content of the pig liver is shown in Table III as free and total gossypol. A product determined as gossypol in the control pigs probably is due to some constituent other than gossypol reacting with the reagent. However, the mean value for this product in the control pigs was less than 8% of that for the livers of pigs which had consumed diets containing 215 or 430 ppm of free gossypol in Experiment I, and was approximately 3% of that for pigs that consumed approximately 600 ppm gossypol diets in Experiments II and III.

Analysis of variance of the data in Table III showed that the differences in activity between the controls and the gossypol-treated pigs were not statistically significant for either cytochrome oxidase or succinoxidase in either the liver homogenates or mitochondria. Similar results were obtained for cytochrome oxidase and for succinic dehydrogenase for the liver mitochondria, in Experiment III, by the spectrophotometric method (Table III).

Analysis of variance of the data summarized in Table IV showed no statistically significant differences between the controls, starved-controls, and the gossypol-treated rabbits in the activity of cytochrome oxidase or succinoxidase in the homogenates of liver, kidney, or heart tissues. Nor were there significant differences between treatments for the

mitochondria of the liver and kidney tissues of the rabbits. Intravenous injection and feeding by stomach tube were used for administering the gossypol, since the rabbits refused a diet containing gossypol. Inasmuch as the rabbits refused to eat commercial rabbit chow after they were injected with gossypol, food was withdrawn from some of the controls. The rabbit fed by stomach tube showed paralysis in the rear legs, indicating the severity of the toxicity.

Analysis of the data for cytochrome oxidase and succinoxidase for the liver homogenates and for the liver mitochondria of rats did not show differences that were statistically significant between the gossypol-treated and the control animals.

Myers and Throneberry (1966) found that gossypol added *in vitro* to the reaction medium, inhibited cytochrome oxidase, succinoxidase, and succinic dehydrogenase activities of sweet potato particulate fractions at gossypol concentrations of $2.5 \times 10^{-4}M$, $7.5 \times 10^{-3}M$, and $2.0 \times 10^{-3}M$, respectively. They found in beef heart mitochondria, at a gossypol concentration of $2.5 \times 10^{-3}M$, succinoxidase was completely inhibited, cytochrome oxidase activity was reduced to one-half, and succinic dehydrogenase was stimulated about 50%. In agreement with the above study, a preliminary *in vitro* experiment showed that a free gossypol concentration of 2.5×10^{-3} M homogenized with rat liver tissue (total volume of 10 ml containing 13 mg of free gossypol and 1 g of tissue) inhibited succinoxidase and cytochrome oxidase. This concentration of gossypol is equivalent to approximately 40,000 ppm on a dry basis, calculated on a dry matter content of 30% for rat liver, which is very much higher than the 531 ppm total gossypol accumulated in the livers of the gossypol-fed pigs in this study (Table III). Smith and Clawson (1965) have been able only to effect in porcine liver an accumulation of approximately 900 ppm of gossypol which is much less than 2.5×10^{-3} M. These results were in harmony with those of Ferguson *et al.* (1959) who found that gossypol did not affect the cytochrome oxidase activity of liver homogenates from mature hens that were given capsules containing pure gossypol and were killed when toxicity symptoms occurred. However, these investigators found that 0.25 mg of gossypol-acetic acid added to a liver homogenate from a normal hen almost completely inhibited succinic dehydrogenase and cytochrome oxidase activities, while endogenous respiration and xanthine oxidase activity were reduced to less than half the original value.

These data showed that cytochrome oxidase and succinoxidase activities in the liver of pigs, rabbits, and rats, and succinic dehydrogenase in pigs were not markedly affected by gossypol *in vivo*, even in animals exhibiting severe toxicity. The level of gossypol in the tissue was not sufficiently high to affect the enzymes studied in the manner observed when large amounts of gossypol were added to these tissues *in vitro*. The presence of bound gossypol in the liver tissue, however, indicates an interaction between gossypol and some constituent of the tissue. This interaction could have resulted from a combination of gossypol with the free ϵ-amino groups of lysine in the protein or enzymes (Lyman *et al.*, 1959), but at a level too low to inhibit enzyme activity.

LITERATURE CITED

Braham, J. E., Jarquin, R., Elias, L. G., Gonzales, M., Bressani, R., *J. Nutr.* **91,** 47 (1967).
Clark, E. P., *J. Biol. Chem.*, **76,** 229 (1928).
Ferguson, T. M., Couch, J. R., Rigdon, R. H., Proceedings of the Conference on the Chemical Structure and Reactions of Gossypol and Nongossypol Pigments of Cottonseed, pp. 131, Southern Regional Research Lab., New Orleans, La., March 19–20, 1959.
Hogeboom, G. H., "Methods of Enzymology," Vol. I, Colowick, S. P., Kaplan, N. O., Eds., pp. 16–18, Academic Press, New York, 1955.
Hogeboom, G. H., Schneider, W C., *J. Biol. Chem.* **194,** 513 (1952).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Lyman, C. M., Baliga, B. P., Slay, M. W., *Arch. Biochem. Biophys.* **84,** 486 (1959).
Myers, B. D., Throneberry, G. O., *Plant Physiol.* **41,** 787 (1966).
Schneider, W. G., Potter, V. R., *J. Biol. Chem.* **149,** 217 (1943).
Schwartze, E. W., Alsberg, C. L., *J. Agr. Res.* **28,** 191 (1924).
Smith, F. H., *J. Amer. Oil Chem. Soc.* **42,** 145 (1965).
Smith, F. H., Clawson, A. J., *J. Nutr.* **87,** 317 (1965).
Smith, H. A., *Amer. J. Pathol.* **33,** 353 (1957).
Takemori, S., King, T. E., *J. Biol. Chem.* **240,** 504 (1965).
Throneberry, G. O., *Plant Physiol.* **36,** 302 (1961).
Wang, T. Y., Tsou, C. L., Wang, Y. L., *Scientia Sinica* **5,** 73 (1956).
Wither, W. A., Carruth, F. E., *J. Agr. Res.* **12,** 83 (1918).

Received for review August 20, 1969. Accepted June 15, 1970. Paper No. 2498 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C. Study supported in part by Public Health Service Research Grant No. AM 07039 from the Institute of Arthritis and Metabolic Diseases, the National Cottonseed Products Association, Memphis, Tenn., and the Rockefeller Foundation, New York, N. Y.