Effect of Gossypol on Some Oxidative Respiratory Enzymes[1, 2]

Byron D. Myers[3] and G. O. Throneberry

Department of Botany and Entomology, Agricultural Experiment Station, New Mexico State University, University Park

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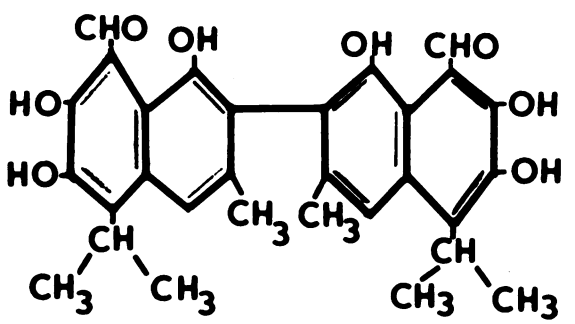
Summary. Gossypol was examined in relation to its effect on certain enzymes and enzyme complexes associated with the tricarboxylic acid cycle and the electron transport system. Succinic dehydrogenase and cytochrome oxidase activity from sweet potato was completely inhibited by gossypol at 7.5×10^{-3} M and 2.0×10^{-3} M, respectively. Succinoxidase activity of the same preparations was fully inhibited at a lower concentration, 2.5×10^{-4} M. This concentration did not affect either succinic dehydrogenase or cytochrome oxidase, the primary and terminal enzymes of the succinoxidase complex. The nature of the intermediate step or steps inhibited at this concentration is not yet known. Gossypol was further shown to inhibit phosphorylation at concentrations having no appreciable effect on oxidation. Inhibition in general was not reduced by increased substrate concentrations in the enzyme systems examined, with the exception of cytochrome c for cytochrome oxidase. Bovine serum albumin was partially effective in reducing gossypol inhibition, provided that it was present before enzyme exposure to gossypol.

Gossypol, (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl [2,2′-binapthalene]-8,8′-dicarboxaldehyde), is a pigment produced in specific glands distributed throughout the cotton plant.



Gossypol

Because of its toxicity to nonruminant animals and therefore its significance to cottonseed products industries, it has been the subject of considerable investigation regarding its chemical, physical and toxicological properties. Much of this work has been reviewed and summarized (1, 12).

The specific action of gossypol on various metabolic systems, as a basis for understanding the physiological mechanism of its toxicity, has however received little attention. It has been reported to interfere with certain proteolytic enzymes (7). Brief reference has been made to inhibition by gossypol of endogenous respiration, xanthine oxidase, succinic dehydrogenase, and cytochrome oxidase of chicken liver homogenates (4). The present junior author reported that mitochondrial preparations from cotton seedling stems were nonfunctional with regard to oxidation and phosphorylation unless bovine serum albumin (BSA) was included in the isolation medium (19, 20). It may be surmised that gossypol and allied compounds were at least partially responsible for this observed inhibitory effect.

This report presents results from determinations of the effects of pure gossypol on activity of certain enzymes or enzyme complexes associated with the tricarboxylic acid cycle and the electron transport pathway.

Materials and Methods

All assays were made on subcellular particulate preparations from sources purchased in local markets. Sweet potato and beef heart tissue was disrupted in a Waring blendor and avocado tissue was ground in a mortar. Blending times and speeds were 10 seconds at low speed and then 50 seconds at high speed for sweet potato and 30 seconds and then 90 seconds for beef heart. The medium used contained 0.4 M sucrose and 0.1 M phosphate at pH 7.0.

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[3] Present address: P. O. Box 1302, University Park, New Mexico.

Tissue-volume ratios were 1:1.5 for sweet potato, 1:2.5 for avocado, and 1:5 for beef heart. Homogenates were first strained through cheesecloth and then cleared of cellular debris by centrifugation for 5 minutes at 500 to 1000 × *g*. Particulate fractions were obtained by centrifugation as follows: 15 minutes at 15,000 × *g* for sweet potato, avocado, and beef heart (for -ketoglutarate, malate, pyruvate and isocitrate oxidations); 1 hour at 104,000 × *g* for beef heart (for succinoxidase, succinic dehydrogenase, and cytochrome oxidase measurements). All particulate fractions were washed by resuspension in at least 40 ml medium followed by recentrifugation. Final suspension was in appropriate quantities of the same medium. All procedures were carried out at near freezing temperatures.

Enzymic determinations were based on O_2 uptake at 30° in the presence of appropriate substrates, using standard manometric techniques. Rates were corrected for endogenous activity by subtracting activity, if any, of nonsubstrate blanks. The procedure for measuring oxidation of tricarboxylic acid cycle intermediates was that reported for succinate (19). Cytochrome oxidase was measured in the presence of cytochrome c reduced with hydroquinone. Succinic dehydrogenase activity was measured by the phenazine methosulfate method (17). Oxidative phosphorylation was determined over a 30-minute interval, employing a hexokinase-glucose trapping system (20). Inorganic phosphate was measured by the method of Fiske and Subbarow (5). Gossypol was made up in 0.5 N NaOH, and then diluted and adjusted to about pH 7.5.

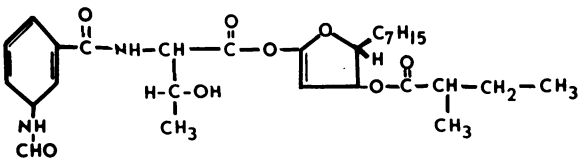
Duplicate reaction vessels were used in all experiments, and almost all experiments were repeated one or more times. Those done once only are so noted. Results from replicate experiments usually agreed to within about 15 %; where differences beyond this level were found, additional experiments were done for verification. Values reported are means of the results obtained.

Gossypol was supplied by the Southern Utilization Research and Development Division of the USDA, and estimated by them to be 99+ % pure. Biochemicals were obtained from Nutritional Biochemicals Corporation.

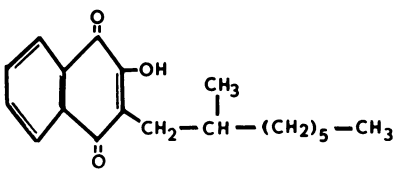
Results and Discussion

The effect of gossypol on activity of succinoxidase, succinic dehydrogenase, and cytochrome oxidase of preparations from sweet potato is shown in figure 1. At sufficiently high concentrations, gossypol was completely inhibitory to each enzyme or complex. This is in agreement with its reported inhibition of succinic dehydrogenase and cytochrome oxidase (4), although concentrations were not reported. The fully inhibitory levels found here were about 7.5×10^{-3} M, 2.0×10^{-3} M, and 2.5×10^{-4} M for succinic dehydrogenase, cytochrome oxidase, and succinoxidase, respectively. At 2.5×10^{-4} M, which almost completely blocked succinoxidase activity, neither succinic dehydrogenase nor cytochrome oxidase was inhibited. Decreasing concentrations were successively less inhibitory to succinoxidase; no inhibition was found at less than 8.8×10^{-6} M. It is thus evident that within a specific concentration range gossypol inhibits some critical enzyme step between the succinate substrate level and the terminal oxidative enzyme, with no interference of electron transport on either side of the step.

The action of gossypol in this respect resembles that of antimycin A (11, 15), SN 5949 and other napthoquinones (3), and 2-heptyl-4-hydroxyquinoline-*N*-oxide (9). Its action differs from that of



ANTIMYCIN A



SN 5949

these materials, however, in that both cytochrome oxidase and succinic dehydrogenase were inhibited at a sufficiently high concentration and further that cytochrome oxidase was more sensitive than was succinic dehydrogenase. Antimycin A has been reported to have no effect on cytochrome oxidase (2, 11) and to inhibit succinic dehydrogenase at only very high concentrations (2). Similar results were reported for various napthoquinones (3). Further, a higher concentration of gossypol is required for inhibition of succinoxidase than is required for inhibition by antimycin A and napthoquinones. The location and nature of the intermediate step apparently blocked by gossypol, at concentrations noninhibitory to succinic dehydrogenase and cytochrome oxidase, has not yet been examined.

Succinoxidase, succinic dehydrogenase, and cytochrome oxidase activities of a beef heart preparation were also examined for their response to 1 concentration of gossypol. The differential effect observed for succinoxidase of sweet potato and its initial and terminal steps was exhibited also in this preparation. However, the general level of sensitivity in the beef heart enzymes was lower at an equivalent gossypol concentration. At 2.5×10^{-3} M, which severely inhibited all 3 systems in the sweet potato fractions, succinoxidase was fully blocked, cytochrome oxidase was inhibited about 50 %, and succinic dehydrogenase

apparently was stimulated about 50 %. This difference in response between the 2 tissue sources might be related to permeability characteristics of the mitochondrial membranes, or possibly to tissue-inhibitor ratio, as with antimycin A (11). No attempts were made to test this possible relationship with this tissue source. Further determinations with various gossypol concentrations will be required to establish a level for selective action on succinoxidase components in this and other tissue. The stimulation of succinic dehydrogenase, also observed occasionally with cytochrome oxidase at certain gossypol concentrations, is not readily explainable.

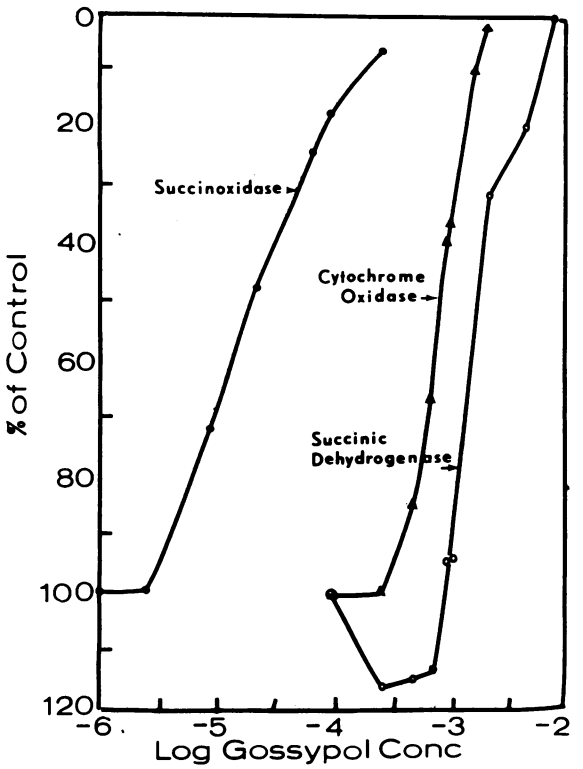


FIG. 1. Effect of various gossypol concentrations on succinoxidase, succinic dehydrogenase, and cytochrome oxidase activity in sweet potato particulate preparations. Reactions were carried out in a final volume of 2.8 ml containing 200 mM sucrose, 50 mM phosphate at pH 7, and (a) 20 mM succinate, 1 mM $MgSO_4$, 0.5 mM ATP, and 32.5 μM cytochrome c for succinoxidase, (b) 20 mM succinate, 50 mM KCN at pH 7, and 2.3 mM phenazine methosulfate for succinic dehydrogenase, (c) 65 M cytochrome c and 20 mM hydroquinone for cytochrome oxidase. All flasks contained 0.2 ml 20 % KOH in center wells.

Oxidation of some other organic acid intermediates and NADH as affected by gossypol was also examined. The results shown in table I indicate that in general these systems were inhibited in about the same manner as was succinoxidase, though probably to a lesser extent at equivalent gossypol levels. No attempts have been made to compare gossypol sensitivity of individual enzymatic steps to that of the entire complex. At the highest gossypol concentration used, NADH oxidation was apparently less sensitive than were the oxidations of the other intermediates. Such an effect was reported for malate but not NADH oxidation with antimycin A inhibition (11). Further work is required to determine if the effect observed here with NADH is due to enzyme source or to specific enzymatic mechanisms.

Table II shows the effect of gossypol on oxidative phosphorylation. At gossypol concentrations which had little or no effect on oxidation, phosphorylation was suppressed about 50 % or more, resulting in lowered P/O ratios. At higher concentrations, causing appreciable decreases in O_2 uptake, phosphate esterification was essentially completely inhibited. Such partial dissociation of phosphorylation from oxidation has been reported for antimycin A and 2-heptyl-4-hydroxyquinoline-*N*-oxide in sweet potato mitochondria (6) and benzoquinones in beef heart mitochondria (18). The results shown here also agree in principle with the observation that phosphorylation in cotton stem particulate fractions was more sensitive to apparent toxic materials in the cotton tissue than was oxidation (20).

Since BSA has been shown to complex with gossypol (10) and to have some protective action in the extraction of mitochondrial fractions from cotton seedling stem tissue (19, 20), the effect of added

Table I. *Effect of Gossypol on Oxidation of Various Substrates by Particulate Preparations*

Reaction mixture: 2.8 ml containing 200 mM sucrose, 50 mM phosphate at pH 7, 1 mM $MgSO_4$, 0.5 mM ATP, 32.5 μM cytochrome c, 20 mM substrate (0.94 mM for NADH), 0.54 mM NAD (none for NADH, 0.43 mM NADP for isocitrate), 0.77 mM TPP for pyruvate only, 0.7 mM "sparker" malate for pyruvate only, 40 mM glutamate for malate only. All flasks contained 0.2 ml 20 % KOH in center wells.

Substrate	Enzyme source	Gossypol conc (M)			
		2.2×10^{-3}	2.2×10^{-4}	2.2×10^{-5}	2.2×10^{-6}
Ketoglutarate	Beef heart	—100 %	—68 %	—33 %	—10 %
Pyruvate	" "	—100 %	—69 %	0 %	0 %
Isocitrate	" "	—100 %	—24 %	—20 %	...
"	Avocado	— 94 %	—70 %	— 6 %	0 %
NADH	"	— 58 %	—26 %	—11 %	...
Malate*	Beef heart	—100 %	—86 %	—69 %	—11%

* Only 1 experiment.

Table II. *Effect of Gossypol on Oxidative Phosphorylation by Particulate Preparations*

Reaction mixture: 2.8 ml containing 200 mM sucrose, 25 mM phosphate at pH 7, 20 mM substrate, 1 mM $MgSO_4$, 0.5 mM ATP, 32.5 μM cytochrome c, hexokinase (approximately 60 units/flask), 20 mM glucose, 0.54 mM NAD for α-ketoglutarate only. All flasks contained 0.2 ml 20% KOH in center wells.

Substrate	Gossypol conc (M)	μatoms O_2 uptake	μmoles phosphate esterified	P/O
Succinate	0			
(Sweet potato)		16.7	8.4	0.50
	4.4×10^{-5}	13.6	4.6	0.35
	4.4×10^{-5}	5.6	0.2	0.04
α-Ketoglutarate				
(Beef heart)	0	18.6	25.8	1.39
	2.2×10^{-5}	19.4	12.9	0.66
	2.2×10^{-4}	9.3	1.8	0.19

BSA on gossypol inhibition of succinoxidase activity was determined. BSA reduced inhibitory action when present before gossypol was added (table III). It did not afford complete protection, however, even at the highest (4%) BSA concentration. No reversal of inhibition was found where BSA was added 30 minutes after enzyme exposure to gossypol. Reif and Potter reported that about half protection from antimycin A inhibition was obtained with crystalline BSA (14), about the same extent of protection found here for gossypol. They also found that inhibition by antimycin A was reversed by BSA added 10 minutes after readings were begun (14). No such reversal was evident in the present work with gossypol, but length of exposure of enzyme to inhibitor as well as purity of BSA used here (fraction V powder) are factors which should be considered in comparing the results. It is possible, however, the site and type of inhibition by gossypol is different than that of antimycin A (8, 13).

Inhibition by gossypol of cytochrome oxidase of digitonin-treated and nontreated particulate preparations from sweet potato was examined. Digitonin treatment, adapted from that of Simon (16), was accomplished during the final suspension step by homogenizing in the presence of 8.2 mM digitonin for the specified period, followed by dilution with suspending medium to 1.8 mM digitonin. This treatment generally resulted in about 20% greater cytochrome oxidase activity than in comparable untreated preparations. Inhibition by gossypol was consistently less in the digitonin-treated preparations, even though digitonin increases permeability of mitochondrial membranes (table IV). A possible explanation is that digitonin treatment resulted in greater amounts of cytochrome c penetrating the mitochondrial membrane and that gossypol was bound by this increased cytochrome c content, perhaps similar to cytochrome c binding of a napthoquinone that was reported by Reif and Potter (14).

Attempts were made to determine the influence of varying levels of substrate and cytochrome c on the response to gossypol. Increased concentrations of succinate or cytochrome c did not alter inhibition of succinoxidase of sweet potato, nor did increased amounts of succinate influence inhibition of succinic dehydrogenase. Thus, there did not seem to be any evidence of a competitive relationship between gossypol and these materials in the systems examined.

Table III. *Effect of Bovine Serum Albumin on Inhibition of Succinoxidase of Sweet Potato by Gossypol*

Reaction mixture: Same as in figure 1 for succinoxidase, with BSA added as indicated.

Gossypol conc (M)	BSA conc	Treatment	Inhibition
4.5×10^{-3}	0	--------	100%
"	1%	Added before gossypol	77%
"	2%	" " "	47%
"	4%	" " "	60%
2.5×10^{-4}	0	--------	100%
"	1%	Added before gossypol	67%
"	2%	" " "	40%
"	2%	Added 30 min after gossypol	100%
"	4%	" before gossypol	43%
1.0×10^{-4}	0	--------	36%
"	2%	Added before gossypol	11%
"	2%	" 30 min after gossypol	29%

Table IV. *Effect of Digitonin Treatment on Inhibition of Cytochrome Oxidase of Sweet Potato by Gossypol*

Reaction mixture: Same as in figure 1 for cytochrome oxidase, with 0.9 mM digitonin as indicated.

Gossypol conc (M)	Inhibition		
	No digitonin treatment	Digitonin treatment	
8.8×10^{-4}	68%	(30 sec)*	29%
6.6×10^{-4}	44%	(")	20%
8.8×10^{-4}	47%	(60 sec)	24%
6.6×10^{-4}	31%	(60 sec)	24%
8.8×10^{-4}	53%	(120 sec)	33%
6.6×10^{-4}	31%	(")	13%

* Time refers to period of exposure of particulate fraction to 8.2 mM digitonin before dilution.

There was, however, an apparent reduction in level of inhibition of cytochrome oxidase when the cytochrome c concentration was increased. This might be due to a binding action of cytochrome c for gossypol as has been reported for a napthoquinone (14). The same effect was not evident in succinoxidase inhibition by gossypol, however, where cytochrome c was added also. Reduced enzyme concentrations resulted in greater sensitivity of succinoxidase and succinic dehydrogenase to inhibition by gossypol. This effect was more pronounced for succinoxidase than for succinic dehydrogenase. This interaction between gossypol and tissue level is another characteristic in common with inhibition by antimycin A (11, 14).

Certain aspects of inhibition by gossypol are evident from the results presented here. The toxic nature of this material might be explained by the inhibitory action reported here and in prior literature. Gossypol, to some extent like antimycin A and some napthoquinones, apparently possesses the characteristic of selective intervention at some enzymatic step or steps in the electron transport pathway. While some of the results obtained indicate that gossypol causes inhibition of an antimycin A nature and therefore inhibits at the same site, other results indicate some basic differences between the 2 which could indicate inhibition at a different site. Further work is necessary to make a critical evaluation of gossypol in this respect. In particular, the effect of gossypol on cytochrome-mediated steps, intermediate reductases and perhaps quinone or vitamin K associated steps need to be examined to further characterize the specificity of inhibition. While the present results offer some evidence that the mechanism of toxicity of gossypol is related to the potent inhibition of electron transport through the succinoxidase complex, gossypol might also possess certain characteristics of value for the study of enzymatic reactions associated with electron transport.

Literature Cited

1. Adams, R., T. A. Geissman, and J. D. Edwards. 1960. Gossypol, a pigment of cottonseed. Chem. Rev. 60: 555–74.
2. Ahmad, K., H. G. Schneider and F. M. Strong. 1950. Studies on the biological action of antimycin A. Arch. Biochem. 28: 281–94.
3. Ball, E. G., C. B. Anfinson, and O. Cooper. 1947. The inhibitory action of napthoquinones on respiratory processes. J. Biol. Chem. 168: 257–70.
4. Furguson, T. M., J. R. Couch, and R. H. Rigdon. 1959. Histopathology of animal reactions to pigment compounds; chickens. Conf. on the chemical structure and reactions of gossypol and nongossypol pigments of cottonseed, Proc. National Cottonseed Products Association, Inc. Dallas, Texas. p 131–41.
5. Fiske, C. H. and Y. Subbrow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375–400.
6. Hackett, D. P., B. Rice, and C. Schmid. 1960. The partial dissociation of phosphorylation from oxidation in plant mitochondria by respiratory chain inhibitors. J. Biol. Chem. 235: 2140–44.
7. Jones, D. B. and H. C. Waterman. 1923. Studies on the digestibility of proteins in vitro. IV. On the digestibility of cotton globulin and the affect of gossypol upon the peptic-tryptic digestion of proteins. J. Biol. Chem. 56: 501–11.
8. Keilin, D. and T. E. King. 1960. Effect of inhibitors on the activity of soluble succinic dehydrogenase and on the reconstitution of the succinic dehydrogenase-cytochrome system from its components. Proc. Roy. Soc. B152: 163–87.
9. Lightbown, J. W. and F. L. Jackson. 1956. Inhibition of cytochrome systems of heart muscle and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-hydroxyquinoline-*N*-oxides. Biochem. J. 63: 130–37.
10. Lyman, C. M., B. P. Baliga, and M. W. Slay. 1959. Reactions of proteins with gossypol. Arch. Biochem. Biophy. 84: 486–97.
11. Potter, V. R. and A. E. Reif. 1952. Inhibition of an electron transport component by antimycin A. J. Biol. Chem. 194: 287–97.
12. Proc. of the conference on the chemical structure and reactions of gossypol and nongossypol pigments of cottonseed. 1959. National Cottonseed Products Association, Inc. 618 Wilson Building, Dallas, Texas.
13. Pumphrey, A. M. 1962. Studies on the electron transfer system. XLV. Some effects of antimycin on cytochrome b. J. Biol. Chem. 237: 2384–90.
14. Reif, A. E. and V. R. Potter. 1953. Studies on succinoxidase inhibition. I. Pseudoirreversible inhibition by a napthoquinone and antimycin A. J. Biol. Chem. 205: 279–90.
15. Simon, E. W. 1957. Succinoxidase and cytochrome oxidase in mitochondria from spadix of *Arum*. J. Exptl. Botany 8: 20–35.
16. Simon, E. W. 1958. The effect of digitonin on the cytochrome c oxidase activity of plant mitochondria. Biochem. J. 69: 67–74.
17. Singer, T. P. and Edna B. Kearney. 1957. Determination of succinic dehydrogenase activity. Methods Biochem. Analy. 4: 307–33.
18. Smith, A. L. and R L. Lester. 1961. The inhibition of oxidation and phosphorylation in beef heart mitochondria by benzoquinones. Biochem. Biophys. Acta 48: 547–51.
19. Throneberry, G. O. 1961. Isolation of metabolically-active subcellular particles from etiolated cotton seedling hypocotyls using bovine serum albumin in preparative medium. Plant Physiol. 36: 302–09.
20. Throneberry, G. O. 1962. Factors affecting oxidative phosphorylation by subcellular particles isolated from cotton seedling hypocotyls. Plant Physiol. 37: 781–84.