Oxidative and Phosphorylative Activities of Mitochondria Isolated From Cotton Hypocotyls[1]

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Received August 5, 1968.

Abstract. **Mitochondria isolated from 2 strains of cotton plant hypocotyls (*Gossypium hirsutum* L. var. Rex smooth leaf and Rex glandless) were examined for their oxidative phosphorylation activities. Bovine serum albumin at a relatively high concentration was essential in the extraction medium for the isolation of oxidatively active mitochondria from both strains of cotton. Phosphorylation was obtained only with Rex glandless cotton mitochondria. This activity was low in comparison to the mitochondria isolated from soybeans (*Glycine max* L. var. Lee). The endogenous gossypol content was found to be much higher in the Rex smooth leaf tissue than in the Rex glandless tissue. In turn, comparable gossypol differences were found associated with their respective mitochondrial fractions. Exogenous gossypol uncoupled succinate oxidation with active mitochondria isolated from soybeans. Gossypol as a possible uncoupler is discussed and compared to carbonyl cyanide, m-chlorophenyl hydrazone and 2,4-dinitrophenol.**

Metabolically active mitochondria have been isolated and studied in a wide variety of plant tissues (7), but very little work has been reported on cotton mitochondria. Throneberry (13) reported that an extract from cotton tissue inactivated mitochondria prepared from soybean seedlings. He showed that the inclusion of bovine serum albumin (BSA) in the extraction medium facilitated the isolation of active mitochondria from cotton hypocotyls. Later, Throneberry (14) found that the phosphorylation mechanism was probably more sensitive to endogenous inhibitors than were the oxidative enzymes. Plants of the genus *Gossypium*, of which cotton is a member, normally contain several pigments unique to the plant kingdom. Some of the pigments are enclosed in glands that are distributed throughout the above ground part of some strains of cotton. An important component of the glands is gossypol (1,1′, 6,6′,7,7′-hexahydroxy-3,3′-dimethyl-5,5′-diisopropyl-2,2′-binaphthyl-8,8′-dialdehyde), a polyphenolic, toxic, yellow compound (2). Myers and Throneberry (9) reported that gossypol affected mitochondria prepared from sweet potato and beef heart tissues. Gossypol was shown to inhibit oxidative phosphorylation at concentrations which had no effect on oxidation. This prompted the investigation of a glandless strain of cotton, relatively free of gossypol, as a possible source of metabolically active cotton mitochondria.

[1] This investigation was supported in part by a grant from the Cooperative State Research Service, USDA, Washington, D. C.

Materials and Methods

Etiolated hypocotyls from 2 strains of cotton (*Gossypium hirsutum* L. var. Rex smooth leaf and Rex glandless, hereafter referred to as glanded and glandless, respectively) and soybeans (*Glycine max* L. var. Lee) were used as the experimental material. The seeds were surface sterilized with 0.5 % (w/v) sodium hypochlorite. After rinsing thoroughly, the seeds were germinated in paper towels, moistened with 0.1 mM $CaCl_2$, at 30° in the dark. Five-day old hypocotyls were harvested, cut into 3 to 5 cm segments, and chilled at 0°.

A slight modification of the Bonner and Sikes procedure (1) was used for mitochondria isolation. The hypocotyl segments were disrupted in a Waring Blendor at a reduced speed using a volume of extraction medium equal to the weight of the hypocotyls. Unless noted otherwise, the extraction medium for cotton consisted of 0.3 M mannitol, 2 mM cysteine, 5 mM $MgCl_2$, 2 mM EDTA, and 3 % (w/v) BSA, pH 7.2. The BSA concentration was reduced to 0.1 % (w/v) for the isolation of soybean mitochondria. The pH of the suspension was monitored and maintained at approximately 7.2 by dropwise addition of 5 M KOH. The suspension was strained through 4-layers of cheesecloth and centrifuged at 1000*g* for 15 min. The supernatant was centrifuged at 10,000*g* for 15 min. The pellet was resuspended in a volume of 0.3 M mannitol and 5 mM $MgCl_2$, pH 7.2, sufficient to equal the original weight of the hypocotyls and centrifuged at 2500*g* for 10 min; and the supernatant was recentrifuged at 6000*g* for 15 min. The resulting mitochondrial pellet was sus-

pended, with the aid of a glass homogenizer, in approximately an equal volume of 0.3 M mannitol and 5 mM $MgCl_2$, pH 7.2. All of the above procedures were carried out at 0 to 2°.

Mitochondrial nitrogen was determined by either the micro-Kjeldahl method or a nitrogen analyzer. Oxygen uptake was measured either manometrically or polarographically.

In the manometric studies, the reactions were measured at 30°. After equilibration for 15 min, oxygen readings were made at 10 min intervals for 1 hr. The reaction flasks (total volume of 2.5 ml of medium) contained 0.3 M mannitol, 50 mM glucose, 20 mM substrate, 1 mM ATP, 1 mM ADP, 1 mM NAD, 0.5 mM thiamine pyrophosphate (TPP), 0.05 mM cytochrome *c*, 5 mM CoA, 5 mM $MgCl_2$, 0.5 mg hexokinase, and 20 mM phosphate buffer, pH 7.2. Duplicate reaction components were run in test tubes parallel to the reaction flasks. Aliquots were removed from the test tubes and reaction flasks at time "zero" and 1 hr, respectively, for P_i determination using the Fiske and Subbarow method (5). The difference between these 2 phosphate concentrations was used as a measure of the phosphate esterified.

In the polarographic studies, oxygen uptake was measured using a Clark electrode connected to a potentiometric recorder. The reactions were carried out at 25° in 1.5 ml of the reaction medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 5 mM KCl, and 10 mM phosphate buffer, pH 7.2.

The gossypol content of the 2 strains of cotton hypocotyls and their mitochondrial fractions was determined according to the method of Smith (12). The gossypol for the standard curve, as well as for the other gossypol studies, was prepared from gossypol acetic acid (15).

Results and Discussion

Repeated efforts, employing standard techniques for extraction of mung bean (10) and pea root mitochondria (4), resulted in mitochondrial fractions from cotton hypocotyls showing negligible metabolic activity. On the basis of previous reports (4, 10, 13) that the inclusion of BSA in either the reaction mixture or the extraction medium improved the metabolic activity of plant mitochondria, the effect of BSA in the extraction medium on the activity of cotton hypocotyl mitochondria was investigated.

The data in table I show the effect of increasing concentrations of BSA in the extraction medium on the activity of mitochondria isolated from hypocotyls of glanded and glandless cotton. Without BSA in the extraction medium, no oxidative phosphorylation was obtained from either of the 2 strains of cotton mitochondria. On the addition of various concentrations of BSA, oxidative phosphorylation increased to a maximum at a 3 % (w/v) BSA concentration with mitochondria from glandless cotton. No oxidative phosphorylation was observed with glanded cotton mitochondria. Oxygen uptake increased with both strains of cotton mitochondria using increasing concentrations of BSA. This increase, however, was more pronounced with mitochondria isolated from glandless cotton. The maximum P:O ratio of 0.85 compared favorably to that previously reported for cotton (14). Addition of BSA to either or both the washing medium and reaction medium did not appear to improve the metabolic activity of the cotton mitochondrial fractions. This would indicate the possibility of a nonreversible reaction involving an endogenous substance(s), possibly gossypol, occurring in the cotton hypocotyls. This has been reported in other work with gossypol (9). Various

Table I. *Effect of Including BSA in Extraction Medium on Oxidation and Phosphorylation by 2 Strains of Cotton Mitochondria*

Oxygen uptake was measured manometrically with succinate as substrate.

Cotton strain	% BSA	Phosphate esterified	O_2 uptake	P:O
	w/v	*μmoles/mg* N	*μatoms/mg* N	*ratio*
Glanded	0	0.0	8.2	0.00
	1	0.0	13.1	0.00
	3	0.0	14.4	0.00
	5	0.0	17.3	0.00
Glandless	0	0.0	7.1	0.00
	1	10.1	16.9	0.60
	3	15.3	18.0	0.85
	5	10.6	21.2	0.50

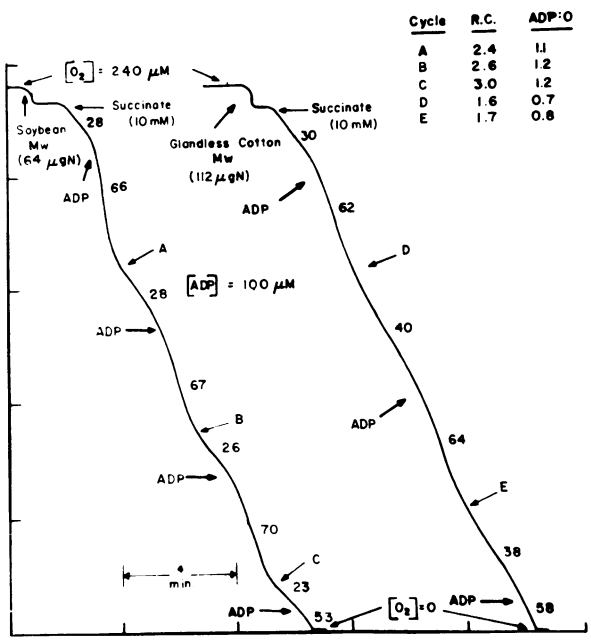

FIG. 1. Polarograph traces showing the oxidative and phosphorylative activities of mitochondria (M_w) isolated from soybean and glandless cotton hypocotyls. Numbers along the traces are rates expressed as mμmoles O_2/min. Additions are shown as final concentrations.

concentrations of polyvinyl pyrrolidone (PVP), used in a manner similar to BSA, improved the metabolic activity of cotton mitochondria, but to a lesser degree than BSA. One of the problems encountered with cotton, compared to soybeans, was the tendency for the mitochondrial particles to aggregate. This was partially overcome by replacing sucrose with mannitol in all of the solutions. Increasing the volume of the extraction medium and washing medium did not seem to improve the aggregation problem.

The oxidative and phosphorylative activities of soybean and glandless cotton mitochondria are compared by polarographic recorder traces as illustrated in figure 1. The oxidation of succinate was stimulated by the addition of ADP with both mitochondrial preparations. Following the nomenclature of Chance and Williams (3), the ADP-stimulated rate is referred to as state 3 and the ADP-limited rate as state 4. The data obtained from glandless cotton mitochondria, compared to soybean mitochondria, showed lower respiratory control ratios (R.C. = state 3 rate/state 4 rate) and ADP:O ratios. This would indicate that oxidation is rather loosely coupled to phosphorylation with cotton mitochondria. The oxidation of succinate by soybean mitochondria increased at least 2.5 times on the addition of ADP, while the increase with cotton mitochondria was only about 1.5 times. Cotton mitochondria had a first state 4 rate oxygen uptake lower than subsequent state 4's. Comparable data have been reported with mung bean mitochondria (6). With soybean mitochondria all state 4's were similar. The ADP:O ratios for soybean mitochondria were close to 60 % of the theoretical value compared to 40 % of the theoretical value for cotton mitochondria. The ADP:O ratio for cotton agrees with the P:O ratio. It is also interesting to note that, per unit mitochondrial nitrogen, the soybean mitochondria were much more active than the cotton mitochondria.

In the manometric studies, inclusion of 10 mM NaF, an ATPase inhibitor had little effect on the P:O ratio and was inhibitory to oxygen uptake. The addition of L-glutamate with succinate to the reaction medium was not stimulatory in the cotton mitochondria studies. It only indicated an additive effect. This additive effect could be due to transamination and/or deamination reactions.

Smith (11) assumed that glandless cotton plants would be free of gossypol, since this compound is associated with resin glands found on some strains of cotton. Nevertheless, he found that gossypol was present in various parts of young seedlings of a glandless variety (Cali. 740). On the basis of this report, the 2 strains of cotton hypocotyl tissues and their respective mitochondrial fractions were assayed for gossypol. The glanded cotton tissue contained almost 30 times more gossypol, on a dry weight basis, than the glandless cotton tissue (glanded 2.13 mg/g and glandless 0.075 mg/g). Results of the gossypol analysis on mitochondrial fractions isolated from the 2 strains of cotton and the effect of BSA are shown in table II. Including BSA in the extraction medium reduced the gossypol content of the isolated mitochondria. However, even with BSA in the extraction medium, the gossypol content of the glandless mitochondrial fraction was at an inhibitory concentration.

Table II. *Effect of Including BSA in Extraction Medium on Endogenous Gossypol Content of the Mitochondrial Fractions Isolated from 2 Strains of Cotton Hypocotyls*

The gossypol concentration in hypocotyl tissue was: glanded 36 μg/mg N and glandless 1.4 μg/mg N.

Cotton strain	Gossypol	
	0 % BSA (w/v)	3 % BSA (w/v)
	μg/mg N	
Glanded	146	56
Glandless	95	35

Since gossypol has been shown to complex with BSA (8), an attempt was made to determine the affinity of BSA for gossypol using a Sephadex gel column. However, since the non-complexed gossypol precipitated on the gel column, it was impossible to quantitate the BSA-gossypol complex relationship.

The effects of gossypol and DNP on oxidation and phosphorylation with glandless cotton mitochondria are recorded in table III. Gossypol concentrations, which had little effect on oxidation, suppressed phosphorylation, thus lowering the P:O ratios. Higher gossypol concentrations resulted in appreciable decreases in oxidation and complete inhibition of phosphate esterification. Similar results have been reported with sweet potato and beef heart mitochondria (9). A similar range in concentrations of 2,4-dinitrophenol (DNP) caused little change in oxygen uptake and phosphate esterification as compared to the control. Perhaps this could be attributed to the inherent gossypol content of the mitochondrial preparation.

Table III. *Effects of Gossypol and DNP on Oxidation and Phosphorylation by Glandless Cotton Mitochondria*

Oxygen uptake was measured manometrically with succinate as substrate.

	Conc	Phosphate esterified	O_2 uptake	P:O
	μM	μmoles/mg N	μatoms/mg N	ratio
Gossypol	0	17.5	38.2	0.46
	1	12.5	45.4	0.28
	5	13.6	37.9	0.36
	10	12.9	38.6	0.33
	50	0.0	14.6	0.00
	100	0.0	1.6	0.00
DNP	0	12.6	26.1	0.48
	1	11.1	27.4	0.41
	5	9.9	32.6	0.30
	10	13.3	36.2	0.37
	50	7.6	23.6	0.32
	100	12.1	24.6	0.49

Figure 2 illustrates the effects of DNP and gossypol on succinate oxidation by soybean mitochondria. When DNP and gossypol were added during the second state 4, succinate oxidation increased. Respiratory rates increased with increasing concentrations of both compounds, reached maxima, and decreased gradually at higher concentrations. The concentrations of DNP and gossypol which caused 50 % stimulation of soybean mitochondrial respiration were 16 μM and 5 μM, respectively. The maximum stimulation of the second state 4 oxidation was obtained at 80 μM of DNP. At this concentration, the respiratory rate approached the second state 3 control. The concentration of gossypol which caused maximum release was 15 μM, with the oxidation of the second state 4 being only about 70 % of the second state 3 control.

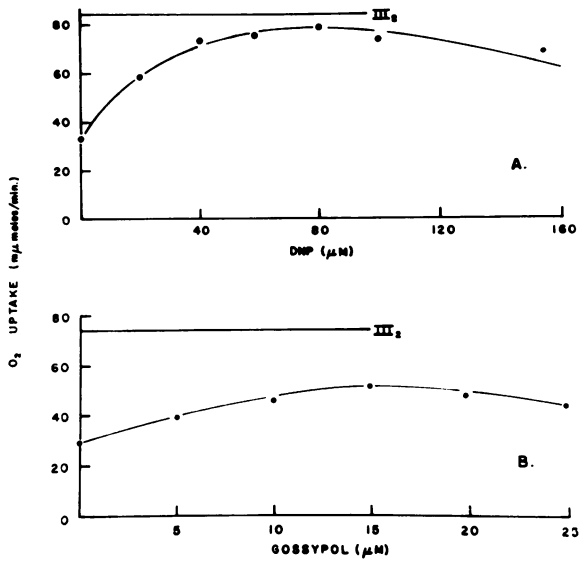

FIG. 2. Effects of DNP (A) and gossypol (B) on succinate oxidation by soybean mitochondria. O_2 uptake was measured polarographically. DNP and gossypol were applied during the second state 4 and the effects were examined on the second state 4 using the second state 3 (III_2) as the control. [Succinate, 10 mM; ADP, 100 μM; mitochondria, 80 μg N (A), 69 μg N (B)].

Figure 3 shows that DNP and gossypol released the oxidation inhibition caused by oligomycin. The second addition of 100 μM ADP did not cause any increase in the respiratory rate of the second state 4 in the presence of oligomycin. Addition of oligomycin to the second state 3 reduced the respiratory rate back to the second state 4 rate. Oligomycin did not inhibit state 4 at the concentrations used here. Oligomycin at higher concentrations reduced the second state 4 rate slightly. In the presence of oligomycin, DNP restored the oxidative rate to the level of the state 3 rate, while gossypol restored the oxidation rate to only about 70 % of the state 3 rate. In a study with 0.3 μM carbonyl cyanide, m-chlorophenyl hydrazone (m-Cl-CCP) the rate was restored to about 90 % of the state 3 rate (data not reported).

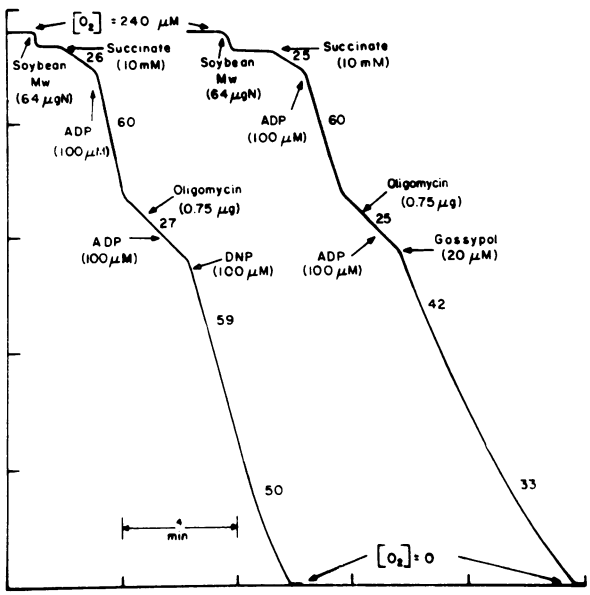

FIG. 3. Polarograph traces showing the inhibition of the second state 3 respiration by oligomycin and reversal by DNP and gossypol using soybean mitochondria (M_w). Numbers along the traces are rates expressed as mμmoles O_2/min. Additions are shown as final concentrations.

These results point out that DNP is a more complete uncoupler of succinate oxidation than either m-Cl-CCP or gossypol and m-Cl-CCP is a more complete uncoupler than gossypol.

Oxidation and phosphorylation of various substrates are shown in table IV. Although specific cofactor requirements for cotton mitochondria were not investigated, the mitochondria oxidized various substrates at generally steady rates for periods up to 60 min using a complete mixture of cofactors. All other substrates tried were oxidized at slower rates than succinate. Reasonably good oxidation rates were obtained with L-malate, α-ketoglutarate, and citrate. Pyruvate gave low rates of oxidation which increased upon the addition of L-malate. The highest P:O ratios were obtained with L-malate, citrate and α-ketoglutarate as oxidizing substrates.

The results presented herein show that it was possible to isolate actively phosphorylating mito-

Table IV. *Oxidation and Phosphorylation with Various Substrates by Rex Glandless Cotton Mitochondria*
Oxygen uptake was measured manometrically.

Substrate	Phosphate esterified	O_2 uptake	P:O
	μmoles/mg N	*μatoms/mg* N	*ratio*
Pyruvate	0.0	3.0	0.00
L-Malate	11.7	11.1	1.05
Pyruvate + L-malate	14.4	17.0	0.85
α-Ketoglutarate	15.4	14.9	1.03
Citrate	13.3	10.5	1.27
Endogenous	0.0	2.7	0.00

chondria from glandless cotton hypocotyls. Difficulties encountered in isolating active mitochondria from glanded cotton were probably due to endogenous gossypol. The action of gossypol on active soybean mitochondria offers evidence that its mechanism of toxicity is probably as an uncoupler.

Acknowledgments

The authors are indebted to Dr. Wayne Sabbe, Dr. Ronald Talbert, and Dr. C. A. Stutte for technical assistance during the course of these studies and in reading the manuscript. Gossypol was supplied by the Southern Utilization Research and Development Division, ARS, USDA, and the cotton strains by Mr. Carl Mooseberg, Cotton Branch Experiment Station, Marianna, Arkansas.

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