ISOLATION OF METABOLICALLY-ACTIVE SUBCELLULAR PARTICLES FROM ETIOLATED COTTON SEEDLING HYPOCOTYLS USING BOVINE SERUM ALBUMIN IN PREPARATIVE MEDIUM [1, 2]

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Subcellular particles capable of oxidizing various members of the tricarboxylic acid cycle have been isolated from a number of plant sources, as evidenced by the numerous references in various reviews (6, 8, 9, 11). As far as the author is aware, there have been no reports of such particulate preparations being obtained from the cotton plant. In view of the demonstration of particulate activity from a rather wide range of other plant species and tissues, it was assumed that similar preparations could be obtained from cotton plants. A metabolically-active fraction from this source was desired to use in a study of some physiological aspects of Verticillium wilt. The present report accounts some of the difficulties that were encountered in the isolation attempts and lists the results that were obtained.

MATERIALS & METHODS

SOURCE OF TISSUE: Cotton seeds (*Gossypium hirsutum* L. var. Acala 1517C) were surface sterilized with 0.5 % sodium hypochlorite and rinsed thoroughly with tap water and then distilled water. They were then planted in trays of pasteurized sand in the dark. After 5 to 7 days, when the seedlings were about five to seven inches tall, hypocotyls were harvested and cut into segments 1 to 1.5 cm in length and chilled before they were used. When soybean (*Glycine max* (L.) Merr. var. Dorman) hypocotyls were used, the seedlings were grown and treated in the same manner.

PREPARATION OF PARTICULATE FRACTIONS: The preparative procedure followed that of Bonner and Millerd (3), Millerd (14) and Switzer and Smith (17), except when tissue was disrupted by blending. The extraction medium was basically 0.4 M sucrose-0.1 M Na_2HPO_4-KH_2PO_4 at pH 7.0. These concentrations and pH were held constant when other materials were included. The hypocotyl segments, in 25 to 50 gram lots, were processed in the following volumes of medium per gram tissue: 1 ml for mortar grinding or 5 ml for blending, 0.8 to 1.6 ml for washing the pellets, and 0.2 ml for final suspension. Blending time was 30 seconds at line voltage. A loose fitting power-driven glass homogenizer was used to resuspend the pellets. All operations were conducted at temperatures as near freezing as possible. Final suspension was always in the basic sucrose-phosphate medium.

TECHNIQUE OF MEASUREMENT: Oxygen consumption was measured at 30° C using standard Warburg technique (19). As a general rule, 0.7 ml particulate preparation containing 0.2 to 0.5 mg nitrogen was used in each flask. Total nitrogen was determined by the micro-Kjeldahl method, distilling into 4 % boric acid. The reaction mixture contained sucrose, phosphate (pH 7.0), $MgSO_4$, ATP, and cytochrome c. Substrate, glutamate, and DPN were added where indicated. The total volume in each flask was 3.0 ml, including 0.2 ml 20 % KOH in the center well. All reaction components were placed in the main compartment. The particulate preparation was added just before the flasks were placed on the manometers and the entire system was equilibrated to temperature for 10 minutes. Readings were made at 10 minute intervals for 70 minutes. Oxygen consumption rates, with air as the gas phase, were calculated for the 20 to 50 minute interval and are reported on a nitrogen basis. In instances where rates were not linear the 20 to 50 minute period was usually the most stable portion of the curve. All rates reported are net for substrate oxidation corrected for endogenous respiration. In almost all instances, duplicate flasks were used for each determination and the averages are reported.

Cytochrome c, ATP (99 %+, Disodium, Chromatographed), DPN (Chromotographically Pure), and BSA (Fraction V Powder) were obtained from Nutritional Biochemicals Corp.

RESULTS & DISCUSSION

FRACTIONS PREPARED WITH SUCROSE-PHOSPHATE & ADDITIVES OTHER THAN BSA: The data in table I show that somewhat standard techniques used for other plant material were not adequate for isolating an active succinoxidase particulate preparation form etiolated cotton seedlings. Repeated efforts using a sucrose-phosphate medium, used for extraction of

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mung bean (3, 14) and soybean (17) particles, resulted in fractions from cotton hypocotyls showing no more than negligible succinoxidase activity. This was true with either mortar or blender preparations. Additional trials, data not reported, showed that sucrose alone, variations in sucrose and phosphate concentrations, variations in pH, and alteration of centrifugal forces during centrifugation had no advantageous effects. Various additives included in the extraction medium and sometimes in the washing medium were of little value in enhancing the succinoxidase activity of the preparations. Cysteine and reduced glutathione (7, 16) and EDTA[3] (13, 15, 18), reported to enhance the activity of some preparations, caused minor increases in activity. PVP, also suggested as having some advantageous effect (20), did not essentially improve the preparations. Tris buffer, succinate, gelatin, and egg albumin were of equally small value or less.

The data in table II help to explain why the above mentioned preparations contained very low or no succinoxidase activity. When cotton and soybean fractions were prepared identically, according to Switzer and Smith (17), the soybean preparations generally exhibited $Qo_2(N)$'s in the range of 400 to 600 while the cotton preparations were inactive or very nearly so. Soybean succinoxidase activity decreased very rapidly when aliquots of the cotton fractions were added to flasks containing the soybean system. As shown in table II, inhibition was still very pronounced even when the amount of added cotton preparation was reduced to less than half.

[3] Abbreviations used in text: EDTA (ethylene diamine tetra-acetic acid, disodium salt), PVP (polyvinylpyrrolidone), BSA (bovine serum albumin), tris (trishydroxmethylamino methane), ATP (adenosine triphosphate), DPN (diphosphopyridine nucleotide).

TABLE I

EFFECT OF VARIOUS ADDITIVES IN GRINDING & WASHING MEDIUM ON SUCCINOXIDASE ACTIVITY OF ETIOLATED COTTON SEEDLING HYPOCOTYL PARTICULATE FRACTIONS*

ADDITIVE IN GRINDING MEDIUM**	ADDITIVE CONC	METHOD OF PREPARATION	ADDITIVE IN WASH MEDIUM**	$Qo_2(N)$
None		Mortar	None	16
		Blender	None	10
Cysteine	0.005–0.1 M***	Mortar	None	21–40
	0.04 & 0.1 M	Mortar	Cysteine	21, 40
	0.16 & 0.3 M	Blender	None	53, 55
	0.3 M	Blender	Cysteine	36
EDTA	0.1 M	Mortar	None	22
		Mortar	EDTA	18
		Blender	None	62
	0.05 M	Blender	None	0, 0
	0.1 M	Mortar	No wash	0
Tris	0.1 M	Mortar	None	25
		Mortar	No wash	0
		Blender	None	0, 0
PVP	5 % (w/v)	Blender	None	19
	10 %	Mortar	None	31
		Mortar	PVP	17
Reduced glutathione	0.004–0.12 M***	Mortar	None	36–55
	0.031 M	Blender	None	70
Succinate	0.1 M	Mortar	None	23, 34
		Blender	None	0
		Mortar	Succinate	35
Gelatin	0.5 % (w/v)	Blender	None	13
	1 %	Mortar	None	0
Egg Albumin	2 % (w/v)	Blender	Egg alb.	38

* Reaction mixture contained 0.2 M sucrose, 0.05 M phosphate (pH 7.0), 10^{-3} M $MgSO_4$, 5×10^{-4} M ATP, 3.25×10^{-5} M cytochrome c, and 0.02 M succinate.

** Grinding and washing medium was 0.4 M sucrose-0.1 M phosphate, pH 7.0. Additives included in this medium at concentrations indicated.

*** Indicates range of concentrations tried.

TABLE II

EFFECT OF ADDING PORTIONS OF COTTON FRACTIONS TO A SOYBEAN SUCCINOXIDASE SYSTEM*,**

FRACTIONS USED***	SOYBEAN-COTTON RATIO IN FLASKS	% INHIBITION OR STIMULATION
SB + C	1-1	−55 to −99 % (Avg = −77 %)
	1-0.71	−62, −92 %
	1-0.43	−40, −80, −82 %
	1-0.14	−2 %
SB + C-S	1-1	−26, −20, +47 %
SB + C-B	1-1	−29, −43, −61 %
SB + C-B-S	1-1	+11 to +46 % (Avg = +27 %)
	1-0.71	+12 %
	1-0.43	+6 %
SB +C-B-P	1-1	+2 to −44 % (Avg = −19 %)
	1-0.71	−15, −25 %
	1-0.43	−5, −15 %
	1-0.14	−2 %
SB + C (Tissue mixed)	1-1	−68 %
SB + C (Extracts mixed)	1-1	−89 %

* Soybean and cotton fractions prepared identically by grinding in a mortar with sucrose-phosphate, followed by one washing and finally suspending in same medium.

** Reaction mixture same as in table I.

*** Abbreviations used in table: SB, soybean particulate fraction; C, cotton particulate fraction; C-S, supernatant from first high speed spin of cotton extract; C-B, boiled (10 min) cotton particulate fraction; C-B-S, supernatant from boiled cotton particulate fraction after centrifugation; C-B-P, resuspended sediment from boiled cotton particulate fraction after centrifugation.

The cotton fractions undoubtedly contained a toxic material or materials that acted on the soybean system when the two were mixed. It seems reasonable that the enzyme systems of the cotton fractions were similarly inhibited during the preparative procedure, explaining why little or no succinoxidase activity could be measured. Cotton seedlings apparently contain the inhibiting substance in an inactive form or

TABLE III

SUCCINOXIDASE ACTIVITY OF COTTON PARTICULATE PREPARATIONS WHEN BSA WAS INCLUDED IN EXTRACTION MEDIUM*,**

EXPERIMENT NO.	METHOD OF PREPARATION	Qo_2(N) AT BSA CONC (w/v)				
		0.5 %	1 %	2 %	3 %	5 %
5-4	Blender			167		
5-6	"		119			
5-16	"			142	198	
	Mortar				75	
5-11	Blender		147			
5-20	"		204	232		
5-23	"	188			301	
5-25	"					221
5-30	"	85	128	171	187	228
5-31	Mortar	18	30	48	63	72
5-10	Blender			167		
	" (1-3 Tissue-volume ratio)			98		
6-15***	Blender		303	534		

* Fractions washed once with sucrose-phosphate and suspended in same medium.

** Reaction mixtures same as in table I.

*** Fraction washed once with BSA solution, then with sucrose-phosphate alone, suspended in same medium, 0.043 M glutamate present in reaction mixture.

TABLE IV

EFFECT OF ADDING EQUAL PORTIONS OF BSA-PREPARED COTTON FRACTIONS TO SOYBEAN SUCCINOXIDASE SYSTEM*

FRACTIONS USED**	METHOD OF COTTON PREPARATION	BSA CONC (W/V)	EFFECT ON SOYBEAN SYSTEM
SB+C	Blender	1 %	−32, −57 %
SB+C	Blender	2 %	−14, −50 %
	Blender	2 % (Blend & wash)	−30 %
SB+C-S	Blender	2 % (Blend & wash)	+22 %
SB+C	Blender	3 %	−30 %
	Mortar	3 %	−54 %

* Soybean fractions prepared as in table II, cotton fractions prepared as in table III, reaction mixtures same as in table I.
** Abbreviations same as in table II.

confined to vacuoles and as such it does not affect the cytoplasmic particles. Upon cell disruption, however, the material is released and exerts its toxic effect. Some of the material becomes rather tightly bound to the particles during isolation since washed particle preparations retained their toxic activity.

Further data in table II shows that aliquots of the supernatant from the cotton extraction process were variable in their toxic action and that boiling the various fractions reduced but did not eliminate the inhibitory effect. Further work is needed to characterize the toxic principle.

FRACTIONS PREPARED WITH SUCROSE-PHOSPHATE PLUS BSA: The report of Price and Thimann (15) that inert proteins, particularly BSA, had some stabilizing and enhancing effect on particulate preparations led to the inclusion of BSA in the present work. Their work with pea stems showed that this material exerted some effect if added to the washing medium and to the reaction mixture but had no effect in the extraction medium. In the present work, it was used in the extraction process but not in the reaction mixtures.

The data in table III show that considerably higher succinoxidase activity was found in cotton preparations when BSA was added to the grinding medium than was found previously with other additives. Endogenous respiration ranged from none in most cases to less than 5 % of substrate-induced respiration. In general, higher BSA concentration led to more active preparations. This is particularly evident in the results from experiments 5-30 and 5-31 where direct comparisons were made between BSA-concentration effects. These two experiments also show that blender preparations were superior to mortar preparations. This was probably due in part to the greater amount of BSA per unit of cotton tissue in the blender method. Where a blender was used, a 1:3 tissue:volume ratio was inferior to a 1:5 tissue:volume ratio at the same BSA concentration. However, a comparison between the 5 % BSA mortar preparation and the 1 % BSA blender preparation shows that the mortar method produced a less active

TABLE V

EFFECT OF INCLUDING 2 % BSA IN WASHING MEDIUM ON SUCCINOXIDASE ACTIVITY OF FRACTIONS PREPARED BY BLENDING IN SUCROSE-PHOSPHATE-2 % BSA*, **

TREATMENT	Qo_2 (N)
Washed once with S–P (sucrose-phosphate)	120
" twice " "	96
" once with BSA, once with S–P	199
" twice " " " " "	211
Washed once with S–P	297
" twice " "	292
" once with BSA, once with S–P	639
" twice " " " " "	662
Washed once with S–P	167
" " " BSA, once with S–P	256
Washed once with S–P (1–3 tissue volume ratio)	98
" " " BSA, once with S–P	105

* Reaction mixtures same as in table 1.
** 2 % BSA on w/v basis.

preparation, though the two methods had the same ratio of tissue to total BSA present. It is possible that the violent blender action led to a more rapid association between the BSA and the toxic material. The mechanism whereby BSA exerts its protective action is unknown but it is assumed that it combines in some way with the toxic material released in the grinding process. Thus, the inhibiting substance is not free to act on the particles. It is clear that BSA exerts much more than a stabilizing effect on cotton preparations. Higher BSA concentrations were required, however, than those used by Price and Thimann (15) for pea particles.

Further evidence that BSA suppressed the inhibitory action is shown in table IV. Aliquots from cotton fractions prepared with BSA were less toxic to the soybean succinoxidase system than were similar aliquots from non-BSA preparations, already noted in table II. The inhibitory effect was not completely removed when BSA was used but it was considerably reduced. This coincides with the higher succinoxidase activity of cotton fractions themselves when prepared with BSA, as already noted.

The data in table V show that the protective action of BSA is increased when included in the washing medium as well. After blending in 2% BSA (in sucrose-phosphate), a further wash of the particles with this same medium, before a final wash with sucrose-phosphate, essentially doubled the succinoxidase activity of that where only sucrose-phosphate

TABLE VI

EFFECT OF VARIOUS COFACTORS & CONDITIONS ON SUCCINOXIDASE ACTIVITY OF COTTON PARTICULATE FRACTIONS PREPARED BY BLENDING & WASHING WITH SUCROSE-PHOSPHATE-2% BSA

TREATMENT	$Q_{O_2}(N)$	% COMPLETE SYSTEM*, **
Without magnesium	680	95
Without cytochrome c	394	70
	402	71
	468	75
	403	88
	345	68
1.62×10^{-5} M cytochrome c	472	103
6.50×10^{-5} M cytochrome c	509	111
3.25×10^{-5} M cytochrome c	754	99
Without cytochrome c, without glutamate	229	37
	132	25
Without glutamate	400	56
	384	50
	286	46
	154	29
	163	31
Without ATP, without glutamate	325	45
	150	20
	109	21
Without ATP	667	93
	691	95
2.5×10^{-4} M ATP	720	99
1.0×10^{-3} M ATP	711	98
With DPN (1 mg/flask)	432	89
pH 6.0	376	50
pH 8.0	511	67
10^{-2} M Malonate	41	7
10^{-3} M "	269	46
10^{-4} M Cyanide	80	14
10^{-5} M "	317	54
10^{-6} M "	604	103

* Complete system contained 0.2 M sucrose, 0.05 M phosphate (pH 7.0), 5×10^{-4} M ATP, 3.25×10^{-5} M cytochrome c, 0.043 M glutamate, and 0.2 M succinate.

** A control containing the complete system was run for each preparation and experiment. Endogenous respiration in these preparations was less than 5% of substrate-induced respiration.

was used to wash the particles. A second BSA wash resulted in minor increases in activity. This decided stimulation of activity by including BSA in the washing medium agrees in substance with the report of Price and Thimann (15). In the one instance tried, a BSA wash did not show the decided stimulation when the blending volume was reduced to a 1:3 rather than 1:5 ratio.

Table VI shows some characteristics of the succinoxidase system from etiolated cotton seedling hypocotyls prepared by blending 30 seconds with sucrose-phosphate-2 % BSA, washing once with the same medium, washing again with sucrose-phosphate, and final suspension in sucrose-phosphate. These particle preparations were similar in most respects to other plant preparations, including the rate of utilization of succinate (1, 2, 14, 15, 17). Excluding magnesium from the reaction mixture had perhaps a slight depressing effect but not to the extent reported for mung beans (14) and soybeans (17). The system responded to added cytochrome c, this material causing about 30 % stimulation. There have been reports of cytochrome c stimulation (10, 12, 17) and non-stimulation (2, 4, 18) of plant preparations. The effect of cytochrome c was about the same with or without glutamate in the reaction mixture. Glutamate addition to the reaction mixture caused the greatest response of any of the factors tested. This stimulation by glutamate was probably due to the trapping of oxalacetate by transaminase activity, thus preventing the accumulation of oxalacetate with its inhibitory activity (10). Without glutamate the rates were not only slower but were generally not linear for the 70 minute measurement period. The effect of omitting ATP was

TABLE VII

OXIDATION OF VARIOUS SUBSTRATES BY COTTON PARTICULATE FRACTIONS PREPARED BY BLENDING & WASHING WITH SUCROSE-PHOSPHATE-2 % BSA*

SUBSTRATE	$Qo_2(N)$		SUCCINOXIDASE $Qo_2(N)$**
	WITHOUT DPN	WITH DPN	
Citrate (0.02 M)	20	15	304
	4	0	591
	55(30)***		
Fumarate (0.02 M)	31	123	479
	69	157	591
		79(59)	
Isocitrate (0.02 M)	20	49	304
	7	75	533
		62(101)	
Alpha-ketoglutarate (0.02 M)	94	62	479
	58	52	484
	73(30)		
Malate (0.02 M)	24	110	479
	35	112	484
		89(50)	
"Sparker" malate (0.0017 M)	7	16	304
	42	31	591
	(17)		
Pyruvate (0.02 M) + "Sparker" malate (0.0017 M)	35	44	304
	69	75	591
	(37)		
Pyruvate (0.02 M)	23	20	304
	38	46	591
	48(0)		
Oxalacetate (0.02 M)	0	0	304
	19	0	591
	4(0)		
Glutamate (0.043 M)		16	260
	31	61	533
	18	48	304
	37	87	479
	21	87	591
	0	48	484

* Reaction mixtures same as in table VI; DPN added at 1 mg/flask when shown.
** Succinoxidase activity of same preparations.
*** Figures in parentheses are rates without glutamate in the reaction mixtures; where these coincide with figures not in parentheses, they are direct comparisons on the same preparation.

very slight, agreeing with the report of Biale et al (2) for avocado fruit, Price and Thimann (15) for peas, and Millerd (14) for mung beans, but not the reports of Switzer and Smith (17) for soybeans, and Davies (4) for peas. The presence of DPN had no stimulating effect on succinoxidase activity and even had a small depressing effect. This latter effect was mentioned by Beevers and Walker (1). The lack of response to DPN is to be expected from the general knowledge of a succinoxidase system. Rates were considerably less at pH 6.0 and 8.0, indicating a pH optimum somewhere near neutrality as has been reported for other preparations (12, 14, 17, 18). The system was sensitive to both malonate and cyanide, as expected from the nature of these two inhibitors.

That the particle fraction isolated from cotton seedlings is also capable of oxidizing other substrates is shown in table VII. All the substrates tried were oxidized but at a much slower rate than was succinate, and some were utilized at extremely low rates. Citrate, pyruvate, and oxalacetate comprised this latter group. No evidence was found for increased pyruvate oxidation when the reaction mixture contained a low concentration of malate. This is contrary to the reports of Davies (4), Millerd (14), Switzer and Smith (17), and Beevers and Walker (1), among others, for various plant preparations. Of the substrates tested, fumarate, isocitrate, and malate responded to added DPN, as did glutamate. There was no response of alpha-ketoglutarate to DPN, as some reports have indicated (1, 15, 17). The utilization of glutamate, though slow, agrees with the reports of Switzer and Smith (17) and Freebairn and Remmert (5). Omitting glutamate from the reaction apparently caused some decrease in oxidation rates of the various substrates tested except for isocitrate, in which case there was possibly some increase. The low rates for oxalacetate prohibit drawing conclusions as to the glutamate effect.

The rather slow utilization of the various substrates tested, except for succinate, may be due to the lack of a full complement of necessary cofactors in the present work. It is also possible that even with BSA in the preparative medium the resulting particle fractions were not completely free of the inhibiting material and as such were isolated under sub-optimum conditions.

Summary

Difficulties encountered in attempting to isolate enzymatically-active subcellular particles from etiolated cotton seedling hypocotyls were found to be largely associated with the presence of a toxic substance or substances in the seedlings. This toxic principle apparently inactivated the particles during the isolation procedure and was active against soybean succinoxidase activity.

Inclusion of bovine serum albumin at rather high concentrations in the preparative medium facilitated the isolation of active particles. This material apparently neutralized the inhibiting substance in some manner. Other materials tried and found to be of no value in securing active preparations were cysteine, ethylenediamine tetra-acetic acid, polyvinylpyrrolidone, reduced glutathione, succinate, gelatin, and egg albumin.

Fractions prepared by blending the hypocotyl segments were superior to those prepared by grinding in a mortar.

The resulting particle preparations were similar to other plant preparations with respect to cofactor requirements. Glutamate added to the reaction mixtures markedly increased the rate and stability of oxidation of succinate but not the other substrates. Succinate was oxidized very readily, while citrate, pyruvate, and oxalacetate were oxidized very slowly. Other substrates utilized fairly readily were fumarate, isocitrate, alpha-ketoglutarate, malate, and glutamate. Oxidation of fumarate, isocitrate, malate, and glutamate was stimulated by added diphosphopyridine nucleotide.

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TWO IMPROVED & INEXPENSIVE SYSTEMS FOR MOISTURE STABILIZATION IN SEEDS OR OTHER TISSUES [1, 2]

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Devices to control relative humidity have long been used by plant scientists. Some control systems are rather complex ones with humidistats, radio tubes, pumps, and fans (8). Alterations of seed moisture have been effected by such techniques as pouring known amounts of water over known amounts of seeds, achieving equilibrium at low temperatures in stoppered jars within a few days (2).

Perhaps the most commonly used method to vary seed moisture is storage in closed containers, over salts or solutions of known vapor pressures, for a few days or weeks (e.g., 7). There are several objections to this technique:

I. There is a vapor pressure gradient from the salt or solution surface to the other extremity (usually the top) of the chamber.

II. Unless the sample is completely exposed, as from being in a single layer, there is a vapor pressure gradient from interior to exterior of the sample.

III. The atmosphere within the chamber can be greatly altered whenever the container is opened.

IV. It is difficult or impossible to remove the sample without exposing it to a different atmosphere.

V. Ascertaining moisture changes in the sample is difficult without upsetting the system and/or destroying the sample.

VI. Important effects on seed physiology may result from volatile chemicals used for humidity control (4).

Recently, preradiation moisture treatments have been given to seeds in such a way that some of these objections do not apply: seeds were exposed to a constant flow of air that had first gone through a saturated solution calculated to yield the desired relative humidity at the temperature used (3). To improve on this system and render it more flexible, the following two arrangements were devised.

CONSTRUCTION OF SYSTEMS

PLYWOOD BOXES (see fig 2): Lidless boxes were made of plywood with all joints glued and screwed, and three coats of marine varnish were applied to the interiors. Lids of ¼-inch Lucite were cut and drilled to bolt over rubber gaskets. Open shelves occupying about one-third of the interior volume were devised for use with sample trays of wire screen. With inside dimensions of 17 inches long, 11 inches wide, and 14 inches deep, each box contained about

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