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Some Factors Affecting the Hill Reaction Activity in Cotton Chloroplasts[1]

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ABSTRACT

A method of plant culture was developed for growing large leaves of glandless cotton on single stems. Chloroplasts isolated from these leaves actively reduced ferricyanide when assayed for the Hill reaction. Hill reaction activity increased 133% when the 0.5 M sucrose isolation medium was replaced with 10% (w/v) polyethylene glycol, both buffered at pH 7.6. The presence of 2 or 5% (w/v) bovine serum albumin in the sucrose buffer did not increase Hill activity. Ferricyanide reduction in the dark occurred in all assays, and the possibility of gossypol as the reductant is discussed. Half-life of the chloroplasts stored in 10% glycerol at −23 C was 23 days. The ammonium ion at 0.01 M enhanced Hill reaction activity up to 171%. Leaves containing chloroplasts with the highest Hill reaction activity were found near the 8th node below the apex. Leaf water potentials less than −28 bars reduced the activity about 50%. Daylight conditions during the winter months in the greenhouse reduced the activity about 30%.

The studies of subcellular particles in cotton are few because of difficulties in isolating active samples. Endogenous inhibitors apparently reduce the activity of these particles and directly interfere with the assays. Recent reports show that when adsorptive agents are added to the isolation medium, it was possible to isolate particles with higher activities. Mitochondria with active enzyme (7) and electron transport systems (3) have been isolated from cotton hypocotyls when bovine serum albumin was added. Active enzymes have been isolated from cotton roots, leaves, and cotyledons in the presence of an insoluble form of polyvinylpyrrollidone (H. B. Lane, B. Ward, personal communication). In this study, active chloroplasts were isolated from cotton leaves with the aid of polyethylene glycol. Some characteristics of these chloroplasts are presented in this paper.

MATERIALS AND METHODS

Plant Materials. Cotton plants (*Gossypium hirsutum* L.) of the glandless strain M-8 (6) were greenhouse-grown in a sand-vermiculite-peat (1:1:1) mixture in 10-inch clay pots. Dolomitic limestone was initially added to maintain a soil pH of about 6.5. Automatic irrigation with a nutrient solution kept the soil near field capacity. Controlled greenhouse temperatures were 30 ± 2 C during the day and 19 ± 2 C at night.

A serial set of primary leaves on each plant was obtained by removing all axillary growth. Each leaf was tagged on the day it was 1 to 2 cm wide. Leaves nearly the same chronological age were selected for the experiments. Potted plants were preconditioned for 24 hr in reduced light intensities (about 800 ft-c), primarily to reduce the size of the starch grains in the chloroplasts. Large grains usually ruptured the chloroplasts during the isolation procedures. Ten hours before analysis, the plant shoots and leaves were drenched with warm water and enclosed in a polyethylene bag in order to increase the leaf water potential to a maximum value (−3 to −5 bars). Just prior to chloroplast isolation, leaves were excised and placed in shaved ice. Water potentials of individual leaves as indicated were measured in a pressure chamber similar to that of Waring and Cleary (15).

Chloroplast Isolation. Two-gram samples of deveined leaves were blended four times at high speed at 1 C, each time 4 sec On and 6 sec Off, in 40 ml of isolation medium consisting of 10% (w/v) polyethylene glycol and 0.1 M potassium phosphate buffer adjusted to a pH of 7.6. The homogenate was squeezed through nylon parachute cloth with a mean hole size of 43μ × 75μ. Extracts were centrifuged at 8000*g* for 3 min. After two washes with 25 ml of the isolation medium, the chloroplasts were stored for short periods in the isolation medium at 1 C. For longer periods the chloroplasts were stored in 10% glycerol at −23 C.

Assay for Hill Reaction. Ferricyanide reduction by illuminated cotton chloroplasts was assayed potentiometrically in a medium containing 0.5 M sucrose, 0.02 M KCl, 0.03 M $MgCl_2$, and 0.02 M tris buffer at pH 7.4. Each assay totaling 6 ml contained initially 0.9 μmole of $K_3Fe(CN)_6$, 0.1 μmole of $K_4Fe(CN)_6 \cdot 3H_2O$, and chloroplasts containing 0.1 mg of chlorophyll. The latter was determined according to the method of Arnon (1) at 625 nm. Reaction cell temperatures were 25 ± 0.5 C in a water bath which also served as a heat absorber for the light source. About 3800 ft-c (measured by a Weston illumination meter model 756[2]) of incandescent light was obtained at the reaction cell, an intensity that was about 4 times the saturation level of the chloroplasts in these experiments.

Platinum and calomel electrodes were placed in the reaction cell along with a bubbling tube for agitation. The potentials were measured with a microvoltmeter having an input resistance of 200 megohms. A typical strip chart recording of the potentials for a single assay (Fig. 1) included 2 min of the initial dark ferricyanide reduction by endogenous components (curve AB), plus 2 min of reduction by the illuminated chloroplasts (curve

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[2] Carbowax 6000 was used in these experiments. The mention of brand names does not imply United States Department of Agriculture approval to the exclusion of other brands.

BC), followed by 2 min of additional dark reduction (curve CD). A value for the endogenous reduction occurring during illumination was deducted graphically from the total reduction on a drafting machine by transposing curve CD to match an extension of AB and form the curve ABEF. The reduction rate was then determined directly from the chart with a standard curve of the ferri- to ferrocyanide concentration ratio (right scale, Fig. 1) or from the equations given by Spikes *et al.* (12) with uncorrected standard potentials (E_c^0). Less than 2.5% difference was found between these two treatments within the ratios used. The rates of ferricyanide reduction by illuminated chloroplasts were constant within the ferri- to ferrocyanide ratios of 4 and 0.25 (12).

RESULTS AND DISCUSSION

Isolation of Chloroplasts. Cotton chloroplasts isolated in a sucrose-phosphate medium exhibited low rates of ferricyanide reduction when assayed for the Hill reaction (Table I). The findings are similar to reports of low oxidative activity in mitochondria which were isolated from cotton hypocotyls in a sucrose medium (3, 7). These authors assumed that an endogenous inhibitor was present, possibly gossypol, occurring primarily in the pigment glands of the cotton hypocotyls. They found that the presence of bovine serum albumin in the isolation medium doubled O_2 uptake in a glanded cotton variety and tripled the activity of the mitochondria in a glandless variety (3). Table I shows that there was little or no increase in Hill reaction activity when 2 or 5% (w/v) BSA[3] was added to the sucrose medium.

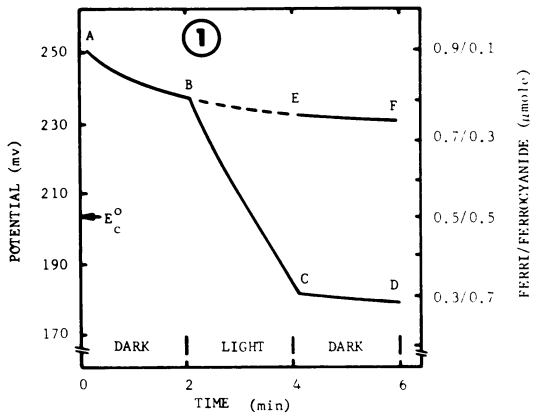

FIG. 1. A strip chart recording showing the potential decline of the platinum electrode (curve ABCD) during an assay of ferricyanide reduction to ferrocyanide by cotton chloroplasts. Curve ABEF is typical of ferricyanide reduction without illumination. See "Materials and Methods" for an explanation.

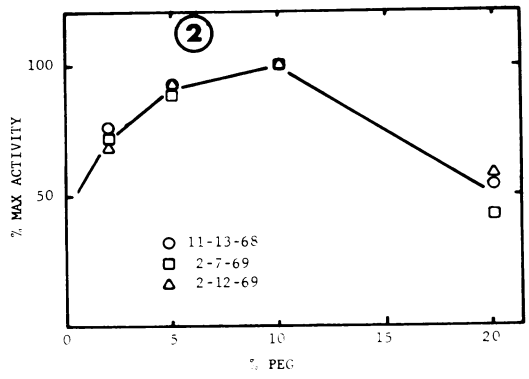

FIG. 2. Hill reaction activity in cotton chloroplasts isolated in different concentrations of PEG (Table I) buffered at pH 7.5 with 0.1 M potassium phosphate.

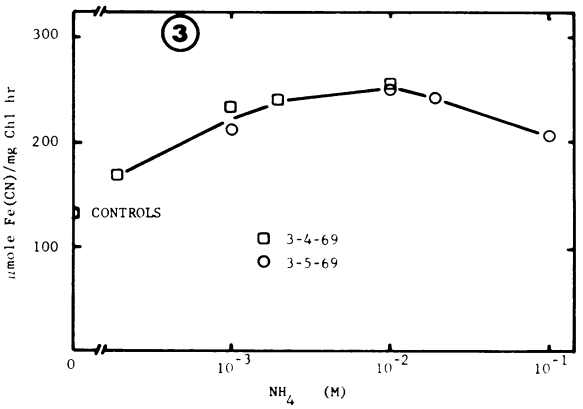

FIG. 3. The effect of the ammonium ion at different concentrations on Hill reaction activity in cotton chloroplasts. The $(NH_4)_2SO_4$ was dissolved in the assay medium prior to adding the chloroplasts.

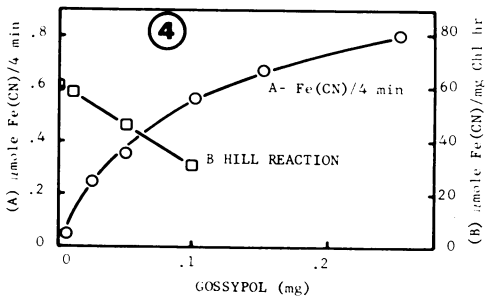

FIG. 4. A: The direct reduction of ferricyanide by gossypol during 4 min in the standard assay medium. B: The effect of gossypol on the Hill reaction activity of cotton chloroplasts. The gossypol dissolved in ethyl alcohol was added immediately before the assays were made.

PEG alone enhanced activity in cotton chloroplasts more than in combination with sucrose (Table I). Figure 2 shows the effects of various PEG concentrations in the isolation medium on the Hill reaction activity. The curve rises to a maximum at 10% PEG and then decreases at 20%. The activity at the 2 and 5% levels remained low, with or without 0.5 M sucrose. When the chloroplasts were uncoupled during the assay with 0.01 M NH_4^+, PEG-isolated chloroplasts showed a 42% increase in activity over the sucrose controls (Table I).

When EDTA was added to the basic isolation medium, the reductive activity of the chloroplasts increased with higher concentrations of EDTA (Table I). These results suggest that the chloroplsts prepared in EDTA were weakly uncoupled. However, in the same chloroplasts the effect of the ammonium ion decreased as the isolation EDTA was increased.

In several other plant species, Hill reaction activity had been preserved by adding PEG to the isolation medium at concentrations ranging from 0.6% in maize isolations (8) to 30% in isolations of sumac, maple, oak, locust, and others (2). The inactivating agents, which were assumed to be tannins, were adsorbed during the isolation by the PEG and not by the chloroplasts. At the same time, the chloroplasts were protected by a coating of precipitated cytoplasmic protein when PEG was at the 30% level (2), and possibly at lower concentrations (8). Tannins have been observed in healthy cotton leaves (A. A. Bell,

[3] Abbreviations: BSA: bovine serum albumin; PEG: polyethylene glycol.

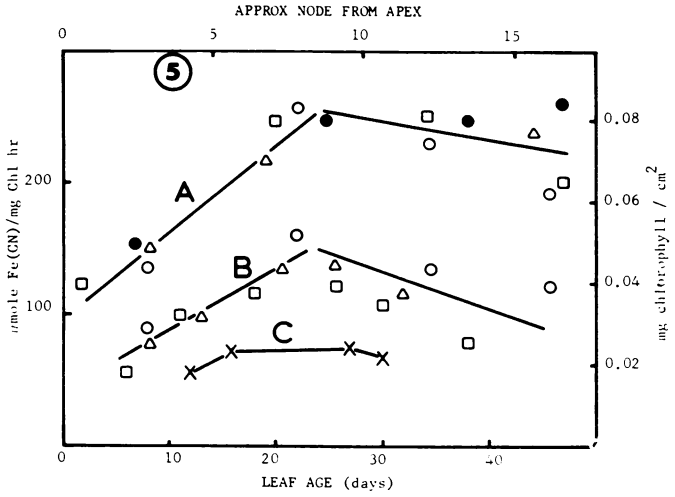

FIG. 5. Effect of leaf age in cotton plants on chlorophyll content of the leaves (A), Hill reaction activity in chloroplasts from turgid leaves (B), and activity in similar but wilted leaves (C). Like symbols indicate data from a serial set of leaves from the same plant grown as described in "Materials and Methods."

Table I. *Effects of Different Isolation Media on the Hill Reaction in Cotton Chloroplasts*

Chloroplasts were isolated from 2-g leaf samples in the media listed below, each buffered with 0.1 M potassium phosphate at pH 7.5. The chloroplasts were then washed twice, taken up in the same medium, and assayed. (Average molecular weight of PEG, 6000). The results are the mean values of three replicates.

Isolation Medium	Ferricyanide Reduction	
	$-NH_4^+$	$+NH_4^{+1}$
	μmoles $Fe(CN)_6$/mg chlorophyll·hr	
Sucrose (15 bar)	45	46
Sucrose (15 bar) + BSA (2%)	46	52
Sucrose (15 bar) + BSA (5%)	44	43
PEG (10%)	108	257
PEG (10%) + sucrose (15 bar)	87	...
PEG (10%) + EDTA		
0.001 M	121	264
0.01 M	135	251
0.1 M	141	252

[1] Chloroplasts were uncoupled with 0.005 M $(NH_4)_2SO_4$ in the assay.

personal communication), which may account for the inhibition of the sucrose-isolated chloroplasts. In the succeeding experiments, 10% PEG was selected for the isolation medium and was used in these experiments as furnished by the manufacturer without further purification. Preliminary trials with PEG that was passed through a cation exchanger showed additional gains in chloroplast activity.

Osmotic potential in the isolation medium is usually lowered by using 0.5 M (−14.5 bars) sucrose, thus preventing osmotic rupture of chloroplasts. This was not prevented in cotton chloroplasts by using 0.5 M sucrose, yet when the sucrose was replaced with 10% PEG, no rupture occurred. The osmotic potential of 10% PEG in 0.1 M phosphate buffer was measured and was found to have a value of −8 bars. Apparently, the cytoplasmic precipitation by the PEG afforded protection to the chloroplast membranes (2) even at a less than favorable osmotic potential. Under the light microscope, they appeared whole and opaque and were assumed to have retained their membranes (14). Starch grains were seldom released, during isolation with 10% PEG.

Table II shows the Hill reaction activity after consecutive washes of the chloroplast residue from the initial homogenate. Two washes with the PEG isolation medium adequately removed both the inhibitors and the dark reductants, resulting in maximum activity. The loss of activity after further washes was probably caused by elution of essential components of the Hill reaction.

Assay. Colorimeter assays of ferricyanide at 420 nm were unreliable where PEG was carried over and produced turbidity in the final assay medium. Similar amounts of PEG had no apparent effect on the potentiometric measurements of ferricyanide. The single small chloroplast sample (0.1 mg of chlorophyll) required by the potentiometric method for assaying both the dark and light reduction was found advantageous. When homogeneous leaf samples were isolated and assayed for the Hill reaction, the coefficient of variation was usually less than 7%.

Uncoupling with the ammonium ion (4) maximized activity at a concentration of 0.01 M (Fig. 3) for cotton chloroplasts. At higher concentrations the ammonium ion apparently inhibited the Hill reaction. A 200% increase of Hill reaction activity was reported (4) for spinach chloroplasts uncoupled by the ammonium ion. A similar uncoupling in cotton increased activity up to 171%. Endogenous agents in the cotton leaves released by homogenization may have inhibited the uncoupling potential, resulting in less enhancement. PEG itself has been suggested as an uncoupler of the Hill reaction (8), but evidently the uncoupling action of PEG is not complete in cotton. When EDTA was added to the assay medium containing chloroplasts that were prepared without EDTA, no apparent trend in activity was found for increasing amounts of EDTA up to 10^{-3} M.

A pH of 7.4 ± 0.2 in the isolation medium resulted in maximum Hill reaction activity. Leaf material usually lowered the isolation buffer 0.2 pH unit during homogenization. The assay pH optima averaged 0.3 unit higher than the isolation pH. Uncoupling with the ammonium ion did not influence the pH optima.

Table II. *Effect of Washing Chloroplasts on the Hill Reaction and Dark Reduction of Ferricyanide in Cotton*

Cotton chloroplasts were isolated from a homogenous 4-g sample in a 10% PEG buffer at pH 7.5 as further described in "Materials and Methods." After each centrifugation, the residues were taken up in decreasing amounts of wash medium to maintain final uniform chlorophyll concentrations; 3-ml aliquots were then taken, and wash medium was added to give uniform chloroplast density. Chlorophyll was determined and assays made. The mean values of three replicates are presented.

Wash No.	Photoreduction		Dark Reduction
	$-NH_4^+$	$+NH_4^{+1}$	
	μmoles $Fe(CN)_6$/mg chlorophyll·hr		*μmoles $Fe(CN)_6$/mg chlorophyll·4 min*
1	82	111	32
2	114	143	19
3	98	138	6
4	86	142	6
5	84	147	6

[1] Chloroplasts were uncoupled with 0.005 M $(NH_4)_2SO_4$ in the assay.

Table III. *Hill Reaction Activity in Cotton Chloroplasts from Turgid and Wilted Leaves*

Turgid and wilted leaves of similar chronological age were selected from potted plants having similar leaf development rates. Wilted leaves were from plants without irrigation for about 3 days. An individual isolation and assay for each leaf was made at pH 7.7.

	Date of Assay (1968)					
	7/65	7/29	7/30	8/1	8/7	12/12
	μmole $Fe(CN)_6$/mg chlorophyll·hr					
Turgid leaves (−4 bars)	168	153[1]	101[1]	100[1]	96	98
Wilted leaves (−28 bars)	86	69[1]	57[1]	58[1]	65	52
Decline, %	49	55	44	42	32	37
No. samples per value	2	4	5	5	3	2

[1] Data averaged for young and old leaves.

Storage. Freezing isolated chloroplasts generally prolongs their activity and usefulness (16). Cotton chloroplasts held at −23 C in 10% glycerol containing 0.03 M $MgCl_2$ and 0.02 M KCl exhibited a half-life of 23 days. In 10% PEG or 1 M sucrose the half-life was about 3 days, while an intermediate half-life of 9 days was obtained with 2% BSA. During storage in the above media the activity of the chloroplasts declined rapidly at first until a steady rate of decline followed. When enhanced by ammonium, the activity was lost very quickly, resulting in shorter half-lives than before, with the exception of chloroplasts in BSA. Apparently the BSA stabilized the system which ammonium uncoupled. The half-life of chloroplasts stored in PEG at 0 C was about 65 hr.

Effects of Gossypol. The curve of fericyanide reduction in the dark in Figure 1 (line ABEF) is typical of all the samples of freshly isolated cotton chloroplasts. Even boiled samples resulted in similar curves, suggesting the presence of a stable endogenous reductant. This component was partially removed from the leaf homogenate by consecutive washings, as shown in Table II. Although the endogenous dark reductant was not identified, gossypol was considered a possibility. Gossypol is known to complex with proteins (5), and during chloroplast isolation, it may be bound to the proteins which are precipitated on the chloroplasts by the action of PEG (2). High levels of bound gossypol were detected in the isolated chloroplasts by using Smith's analytic method for gossypol in cotton leaves (11).

Free gossypol, *i.e.*, the readily extracted and possibly the most reactive form, was measured in the chloroplasts. The results were 0.03 to 0.05 mg of gossypol per 0.1 mg of chlorophyll. Chlorophyll was estimated at 11.8% of the chloroplast dry weight; thus, free gossypol in the chloroplast isolations may vary from 3.5 to 5.9% of the chloroplast dry weight.

Gossypol itself reduces ferricyanide in the dark, and the rate is not influenced by light. Figure 4A shows the amount of ferricyanide reduced in 4 min for different concentrations of 99% pure gossypol. From these data and from the measurements of the dark reduction by fresh chloroplasts (0.1 to 0.3 μmole of ferricyanide per 0.1 mg of chlorophyll), a range of free gossypol from 0.01 to 0.03 mg/0.1 mg of chlorophyll was estimated in isolated chloroplasts. This estimation is near the values of free gossypol from direct analyses that were discussed above. The direct effect of gossypol on the Hill reaction is shown in Figure 4B. Gossypol apparently inhibited the Hill reaction as a linear function of its concentration.

Effects of Leaf Age. Each cotton plant was obtained with a single series of large leaves when grown according to the methods described. Uniform rates of leaf initiation and development usually occurred above the 5th node. Figure 5B shows the effect of leaf development and stem position on Hill reaction activity of the isolated chloroplasts. The leaves selected were fully turgid, having a water potential of −4 to −5 bars. Activity increased as leaves expanded up to 23 ± 3 days of age. Leaves of this age were generally at the 8th node below the apex. After 23 days, the activity decreased at a slower rate, and with a greater variation between plants that were analyzed.

Figure 5A shows the relation of leaf chlorophyll content to leaf age or stem position. During the first 23 days of leaf development, the chlorophyll content per unit leaf area increased to a maximum, after which there was a slow decline. These results agree in part with the work of Treharne, Cooper, and Taylor (13), who noted a similar pattern in the chlorophyll content of orchard grass. Woolhouse (17) has reported a different trend in *Perilla*, in which chlorophyll content increased after the leaves were fully expanded, but decreased rapidly before leaf abscission. Additional cotton data (not shown) indicated that the leaf area increased according to the S-curve growth pattern. On the 23rd day, leaf expansion was about 90% complete, and the rate of leaf expansion was about 0.9% of the maximum rate, the latter occurring on the 11th day.

These data for cotton show that leaf development is almost completed at the time that maximum chlorophyll content of the leaf and maximum activity in the chloroplasts are reached. This suggests that the net photosynthetic rates might be found to peak at the same leaf age.

Effects of Water Stress. Chloroplasts from cotton leaves under a water stress were usually less active. Table III shows the results of six experiments in which wilting decreased the Hill reaction activity of the chloroplasts between 32 and 55%. These plants were grown in a moist environment for 40 days, after which selected turgid leaves were analyzed for the Hill reaction. Following this, the plants with the remaining leaves were held for 3 days without water, and the resulting wilted leaves were analyzed. The water potential in the turgid and wilted leaves was about −4 and −28 bars, respectively. Figure 5C also shows that chloroplasts from leaves of a wilted plant exhibit the same pattern of activity as do chloroplasts from turgid leaves (Fig. 5B), yet the range of activity is lower. Preliminary experiments have indicated that a fraction of the original activity returned when the wilted leaves were rehydrated.

In swiss chard, Nir and Poljakoff-Mayber (9) measured 32 and 85% lower Hill reaction activity in wilted leaves that lost 29 and 61% water content of the leaves. Santarius (10) reported that the loss of leaf water of fodders beets suppressed the activity in the chloroplasts, but not completely until more than 85% of the turgid leaf water was lost. The above results indicate that

Table IV. *Effect of Seasons on Leaf Initiation Rate and the Hill Reaction in Cotton Grown in a Greenhouse at Raleigh, North Carolina*

Leaf initiation rates were determined from tagged leaves as described in "Materials and Methods." The standard technique was used for isolating and assaying the chloroplasts. Each value of leaf initiation rate and Hill reaction activity is the mean value of five or more samples.

Date	Leaf Initiation Rate	Activity	Day Length
mo/yr	*leaves/day*	*μmoles $Fe(CN)_6$/mg chlorophyll·hr*	*approx. hr*
7/68	0.34	127	14.4
11/68	0.29	104	10.8
1/69	0.25	67	9.8
3/69	0.38	120	11.4

leaf water potential should be high and uniform between leaves under investigation for Hill reaction activity.

Seasonal Differences. Seasonal differences were observed in the activity of chloroplasts isolated from greenhouse-grown cotton. The results from assays of several experiments performed throughout the autumn, winter, and spring seasons are shown in Table IV. During the winter months, activities were about 30% below those of the other months. Slow plant growth, as indicated by leaf initiation rate, corresponds to the periods of low activity. Coincidental short day lengths during leaf development apparently influenced the activity and the growth rate, although the total integrated illumination, which is lowest in the winter months, could have also affected the results.

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