Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 112, No. 2, November, 1965
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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS **112**, 355–360 (1965)

Enhancement by Carotenoids of Nicotinamide Adenine Dinucleotide Phosphate Photoreduction in Isolated Chloroplasts

I. Isolation and Purification of Active Fractions[1]

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Received June 7, 1965

A carotenoid fraction has been isolated from green plant tissues which enhances NADP photoreduction by isolated chloroplasts by 200–350%. It also enhances the Hill reaction with NADP as electron acceptor by 40–60%. None of the known carotenoids tested showed this activity, and it is ascribed to an as yet uncharacterized mono- or dihydroxy carotenoid probably identical with the carotenoid in the protein-carotene complex isolated from chloroplasts.

The possible role of the various carotenoids found in green tissue in photosynthesis has been repeatedly investigated (1–3). To date, however, no specific function in photosynthesis has been assigned to any of these carotenoids except for the possible protection of chlorophyll from photooxidation (2). In a recent publication, Bamji and Krinsky (4) established that in contrast to their previous suggestion (3), the de-epoxidation of anthrazanthine to zeaxanthine was not a light-driven reaction occurring under anaerobic conditions.

One of us (JSK) has recently reported the isolation of a protein-carotene complex from spinach chloroplasts, which was a required cofactor for the photoreduction of ferricyanide and cytochromes by a soluble protein-chlorophyll complex (5, 6). We have subsequently attempted to determine whether this carotenoid has a function in the photosynthetic reactions of isolated chloroplasts. This communication describes the isolation and purification from crude protein-carotene complex of carotenoid fractions which strongly enhance the photoreduction of NADP by isolated chloroplasts. It also describes the direct isolation of these carotenoid fractions from chloroplasts and from the supernatant solution obtained during chloroplast isolation.

MATERIALS AND METHODS

Chloroplasts were isolated by the method of Jagendorf and Avron (7) and disrupted by suspension in 2×10^{-3} *M* $MgCl_2$. The protein-carotene complex was isolated as previously described (8).

Photoreduction of NADP was measured as described by Vernon and Zaugg (9); reduced dichlorophenol-indophenol was used as an electron donor. The reaction mixture, in a final volume of 3 ml, contained: 0.03 μmole of 2,6-dichlorophenol-

[1] Contribution 2001 from the N.C. State Agricultural Experiment Station. Supported in part by Public Health Service grant GM-09710 from the National Institute of Health, Division of General Medical Sciences.

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TABLE I

EFFECT OF THE VARIOUS CAROTENOIDS ISOLATED FROM A CRUDE PROTEIN-CAROTENE COMPLEX PREPARATION, ON NADP PHOTOREDUCTION BY ISOLATED CHLOROPLASTS WITH REDUCED DPIP AS ELECTRON DONOR

Fraction	mμmole carotenoid	Units activity[b]	Units/μmole carotenoid
I[a]	137	None	—
II	147	168	1145
1	52	Inhibits	—
2	22	5.5	250
3	16	Inhibits	—
4	46	11	241
5	20	26	1300
6	7.4	Inhibits	—
III	1124	1490	1325
1	42	Inhibits	—
2	462	157	340
3	405	644	1592
4	64	90	1410
5	68	82	1410
6	97	Inhibits	—
7	56	97	1730

[a] The Roman numerals refer to fractions obtained by partition on a silica-methanol column; the Arabic numerals to subfractions developed on a starch column. For details, see text.

[b] Defined as enhancement of NADP photoreduction by chloroplasts of 100 μmole/mg chlorophyll/hour over control value.

indophenol (DPIP); 0.5 μmole of NADP, 0.03 μmole of (*p*-chlorophenyl)-1,1,dimethylurea (CMU); 1.2 μmoles of $MgCl_2$; 0.5 μmole of ADP; 10 μmole of phosphate; 60 μmoles of tris buffer, pH 8.0; 15 μmoles of ascorbate; twice the saturating amount of spinach ferredoxin; and 20 μg of chloroplast-chlorophyll. For measurement of the Hill reaction, the same reaction mixture was used, but the CMU, DPIP, and ascorbate were omitted. Samples were illuminated for 2 minutes with 7500 lux of incandescent light passed through a 5-cm water filter and a red acetate (600–780 mμ) filter, and the activity was calculated from the increase in optical density at 340 mμ. The activity was also tested at 30,000 lux, both with and without the carotenoids, but the activity was the same as with the lower light intensity.

The carotenoids were added to the reaction mixture in a minimum volume of ethanol, the final concentration of which was <2%. Controls run concomitantly showed this concentration of ethanol to have no measurable effect on chloroplast activity. The activity of the carotenoids was determined by their enhancement of NADP photoreduction, a unit of activity representing an enhancement of 100 μmoles of NADP reduced per milligram chlorophyll per hour over control rates. Active fractions were tested at a number of concentrations, and the specific activity was calculated from the concentration giving optimal effect.

Isolation of carotenoids from crude protein-carotene complex. The crude complex in petroleum ether was warmed to 30°C and mixed with ¼ volume of methanol. The methanol was separated, mixed with two volumes of water, and extracted with one volume of diethyl ether which was then added to the petroleum ether phase. The combined solvent phase was mixed with ¼ volume of methanol saturated with KOH and left to stand, with repeated shaking, for 1 hour. The alkaline methanol was removed, mixed with water, and extracted with diethyl ether which was combined with the solvent phase. The combined solvent phase was washed with water, dried under vacuum, dissolved in a minimum volume of *n*-hexane, and partitioned on a silica-methanol column (10). The hydrocarbons were eluted with petroleum ether (Fraction I), the monohydroxy-carotenoids with ethyl ether-petroleum ether (1:1) (Fraction II), and the polyhydroxy-carotenoids with methanol (Fraction III). The total amount of carotenoids in the various fractions was estimated from the optical density at 445 mμ using the extinction coefficient for β-carotene (132 liters/mmole). The fractions were transferred to ethanol and their effect on NADP photoreduction by isolated chloroplasts was determined. The active fractions (II and III) were transferred to petroleum ether and chromatographed on a starch column. They were developed, and the faster moving bands were eluted with 2% diethyl ether in *n*-hexane for fraction II and 4% diethyl ether in *n*-hexane for fraction III. Slower moving bands were extruded from the column and eluted from the starch with diethyl ether-hexane (1:1). All fractions were transferred to ethanol and the total amount and activity in NADP photoreduction was determined.

Isolation of carotenoids from chloroplasts. Chloroplasts in aqueous suspension were mixed with an equal volume of methanol and 2 gm of Hy-flo Supercel per 100 ml, and then filtered to dryness. The filtrate was discarded and the solid mat on the filter was extracted with acetone-petroleum ether (1:1) until the extracts were colorless. The combined extracts were washed with water in a separatory funnel to remove the acetone and then saponified and chromatographed as described above.

RESULTS

I. Carotenoids Isolated from the Protein-Carotene Complex

Most fractions tested had no effect or inhibited NADP photoreduction, but a number of fractions greatly enhanced NADP photoreduction at low concentrations (Table I). At higher concentrations even these fractions inhibited NADP reduction (Fig. 1a).

The inhibition at higher concentration decreased with increased purification and with the most active fractions inhibition occurred only at concentrations 3–4 times higher than those that saturated photoreduction. The partly purified active carotenoids were highly unstable, losing as much as 50% of their activity overnight, even when kept under nitrogen. This could account for the discrepancies in total activity of the components isolated from Fractions II and III. Upon further purification the active fractions became more stable. With the most active fractions, enhancement of 200–350% was obtained.

Hilt reactions with NADP as electron acceptor. The Hill reaction with NADP was far more sensitive to inhibition by added carotenoids than the photoreduction. The addition of active Fraction III, while greatly stimulating photoreduction, inhibited the Hill reaction (Table II, No. 2 vs. Nos. 6 and 7). The carotenoids inhibited oxygen evolution, and in their presence reduced DPIP served as electron donor for NADP reduction (No. 6). In the presence of CMU, all NADP reduced could be accounted for by the ascorbate oxidized (Nos. 4 and 5), while little ascorbate was oxidized in the absence of CMU and carotenoid (No. 3). When some of the most active fractions obtained from the starch columns were rechromatographed on starch, a fraction was obtained which enhanced the Hill reaction by 30–65% and photoreduction by 250% (Fig. 1b). In most cases, the maximal specific activity was nearly the same for both reactions, which could mean that some other component becomes rate-limiting. In contrast to the photoreduction, the enhancement of the Hill activity was poorly reproducible from one batch of chloroplasts to the other.

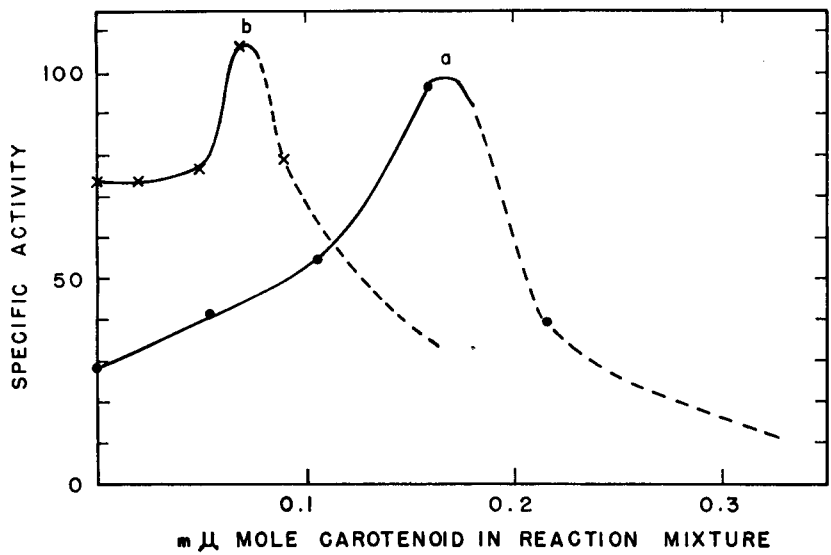

FIG. 1. Enhancement by a carotenoid of NADP photoreduction and Hill reaction by spinach chloroplasts: (a) NADP photoreduction; (b) Hill reaction with NADP as acceptor. The inhibition at higher concentration (broken lines) decreased with increased purification of the carotenoid. Specific activity represents μmole NADP reduced/mg chlorophyll/hour. For details, see text.

TABLE II

CAROTENOID INHIBITION OF THE HILL REACTION AND STIMULATION OF PHOTOREDUCTION[a]

	Treatment	NADP red. (μmole)	Ascorbate ox. (μmole)	O_2 evolved (calculated) (μmole)
1.	Control	0.102	—	0.051
2.	+ carotenoid (1:10)*	0.034	—	0.017
3.	+ red. DPIP	0.118	0.018	0.050
4.	+ red. DPIP + CMU	0.046	0.054	0.000
5.	+ red. DPIP + carotenoid (1:10) + CMU	0.064	0.073	0.000
6.	+ red. DPIP + carotenoid (1:10)	0.112	0.063	0.025
7.	+ red. DPIP + carotenoid (1:7)	0.081	0.081	0.000

[a] 20 μg chlorophyll, 2 minutes light, 3-ml volume. Reaction mixture as described in text.
[b] Molar ratio of chlorophyll to carotenoid

II. Carotenoids Isolated from Chloroplasts and Supernatant Fluid

The total activity found in carotenoids isolated from chloroplasts was low, and the large amount of contaminating carotenoids made the separation and purification of the active fractions very difficult. In light of the enhancement of NADP reduction obtained with the active fractions, the possibility that chloroplasts lose their carotenoids during isolation was investigated. The supernatant solution obtained from chloroplast isolation and washing was cleared by centrifugation at 35,000 *g*, the pellet was discarded, and the carotenoids were isolated from the clear supernatant solution and chromatographed as described above. Both Fractions II and III contained highly active components, and the total activity was much higher than that obtained from the chloroplasts (Table III). The most active fraction obtained had an absorption spectrum closely resembling that of the purified protein-carotenoid complex (5) and did not correspond to any of the known carotenoids. We have been able to isolate these active carotenoids also from lettuce and soybean chloroplasts, supernatant solutions, and—to a limited extent—from mustard seeds. We tested a number of known carotenoids (α-carotene, β-carotene, Lutein, Zeaxanthine, violaxanthine, neoxanthine), obtained by chromatography on MgO for enhancement of NADP photoreduction, but none showed any activity.

Attempts were made to find conditions for chloroplast isolation, under which the chloroplasts will lose all their active carotenoids. Chloroplasts were isolated in media of different pH, then assayed for NADP photoreduction at pH 8.0 with and without the addition of an active carotenoid fraction (Fig. 2). While chloroplasts which were isolated between pH 5.5 and 9.5 showed a marked enhancement of NADP photoreduction by the addition of carotenoids, those isolated at pH 10–10.5 showed both the highest specific activity and smallest effect

TABLE III

EFFECT OF THE VARIOUS CAROTENOIDS ISOLATED FROM SUPERNATANT SOLUTIONS OBTAINED FROM CHLOROPLAST ISOLATION, ON NADP PHOTOREDUCTION BY ISOLATED CHLOROPLASTS[a]

Fraction	mμmole carotenoid	Units activity	Units/μmole
I	13,300	None	—
II	4590	1120	245
1	27	78	2900
2	854	83	97
3	59	16	271
4	1883	Inhibits	—
5	773	73	95
6	68	None	—
III	7350	None	—
1	50	480	9600
2	190	75	395
3	43	None	—
4	1780	Inhibits	—
5	1960	Trace	—
6	789	Trace	—
7	1190	Trace	—

[a] For details, see Table I and text.

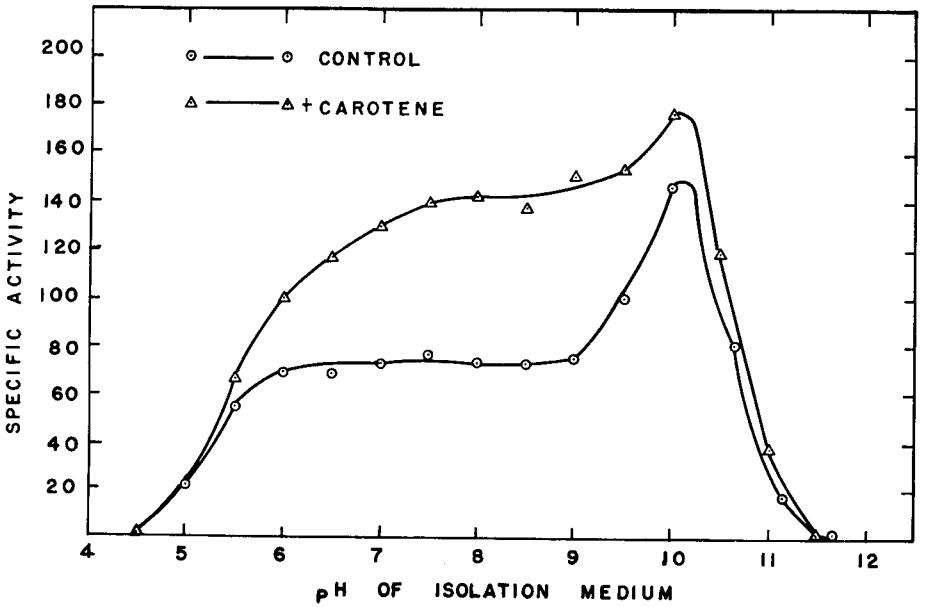

FIG. 2. Effect of pH of isolation on chloroplast NADP photoreduction, and its enhancement by an active carotenoid fraction. All assays were carried out at pH 8.0. Specific activity represents μmole NADP reduced/mg chlorophyll/hour.

of added carotenoids. In agreement with this, chloroplasts isolated at pH 8.0 lost most of their active carotenoids, while those isolated at pH 10.5 retained about half the total activity (Table IV).

The active carotenoids did not serve as a substitute for any of the additives to the reaction mixture. The requirement for ferredoxin increased with increased activity of the carotenoid fractions, and decreasing the concentration of dichlorophenol-indophenol below 10^{-5} *M* lowered the specific activity of the control and greatly reduced the effect of added carotenoids. When 10^{-3} *M* methyl ammonium chloride, an uncoupler of photophorylation, was added to the reaction mixture instead of ADP and phosphate, the rate of NADP photoreduction was not altered, and the enhancement effect of the carotenoids was the same as in the presence of ADP and phosphate.

TABLE IV

EFFECT OF pH OF ISOLATION ON THE RETENTION OF ACTIVE CAROTENOIDS BY CHLOROPLASTS[a]

pH	Units activity[b]	
	Chloroplasts	Supernatant solution
8.0	1000	5550
10.5	3520	3750

[a] Data represent summary of activities in the various subfractions obtained from a starch column. For details, see text.

[b] For definition, see Table I.

DISCUSSION

With the procedures described here we were able to isolate a number of carotenoids containing at least one hydroxy group and which have a strongly enhancing effect on the NADP photoreduction and Hill reaction of isolated chloroplasts. Attempts are currently being made to determine the structural entity common to these fractions and responsible for their activity. The highly active fractions were more unstable, even when stored under N_2 at $-20°$, than any of the known carotenoids in photosynthetic tissue, especially those fractions isolated from the chloroplast supernatant solution. This fact accounts at least in part for the loss of total activity by chromatography, which could not be recovered by treatment

with reducing agents or by recombining all the separated fractions. The loss of total activity upon chromatography was much greater than the loss of total carotenoids, the latter being mostly due to crystals of lutein which precipitated from Fraction III and were removed by filtration. The crystals were always totally inactive.

It is considered possible that the activity in Fraction II may be a partially esterified product of Fraction III. The saponification procedures used were not sufficiently rigorous to assure complete hydrolysis of all esters. Zeaxanthine dipalmitate retains a measurable amount of the mono- and diesters after saponification under conditions similar to those used here (A. Lawrence Curl, personal communication).

Chloroplasts and photosynthetic tissue contain a large number of different carotenoids, some of which, such as β-carotene and lutein, are present in very large amounts (11). The components described here are present, in contrast, in very small amounts, which makes their purification difficult. With fractions of highest specific activity, a ratio of chlorophyll to carotenoid at optimal concentration of 120:1 was obtained (Fig. 1). Assuming an essentially pure fraction, this would indicate one molecule of carotenoid per reaction center (12).

The active carotenoids did not substitute for any of the components of the reaction mixture, but from their marked effect on NADP photoreduction as compared with the Hill reaction, they appear to function in light reaction I. In agreement with this, we could never get an enhancement of DPIP or ferricyanide reduction with the active carotenoids (13). From the fact that the enhancement was obtained in red light (600–750 mμ) just as well as in white light, it follows that these carotenoids do not function as accessory pigments in the photochemical reaction. Their possible role in photoreduction is currently under investigation.

ACKNOWLEDGMENT

We gratefully thank Mrs. Thea Schostag for her able and untiring technical assistance.

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