BBA 45904

PROPERTIES OF *ANABAENA VARIABILIS* CELLS GROWN IN THE PRESENCE OF DIPHENYLAMINE*

TERUO OGAWA, LEO P. VERNON AND HARRY Y. YAMAMOTO**

C. F. Kettering Research Laboratory, Yellow Springs, Ohio (U.S.A.)

(Received October 13th, 1969)

SUMMARY

Anabaena variabilis cells have been cultivated in the presence of diphenylamine (12 mg/l) which inhibits the biosynthesis of β-carotene, echinenone and zeaxanthin. The content of chlorophyll *a* is also reduced by diphenylamine. The biosynthesis of myxoxanthophyll is, however, stimulated by this reagent.

The membrane fragments prepared from Anabaena cells grown in the presence of diphenylamine have the activities of both Photosystem 1 ($NADP^+$ reduction with DCIP–ascorbate as electron donor) and Photosystem 2 (DCIP reduction with 1,5-diphenylcarbazide as electron donor).

The fluorescence spectra of these cells at 77°K show peaks at 696 and 731 nm and a shoulder around 687 nm. The fluorescence intensity at 687 and 696 nm is higher in these cells than in normal-Anabaena cells.

INTRODUCTION

Inhibition of carotenoid synthesis of *Mycobacterium phlei* by diphenylamine was first reported by TURIAN[1]. In the early investigations of GOODWIN AND OSMAN[2,3], diphenylamine was used as an inhibitor of carotenoid synthesis of *Rhodospirillum rubrum*. Strongly carotenoid-deficient cells of *Chromatium* were also obtained later by using diphenylamine[4–6]. These studies have shown that a certain concentration of diphenylamine inhibits carotenoid synthesis by these photosynthetic bacteria as well as for non-photosynthetic bacteria.

Recent studies in this laboratory have shown that the removal of carotenoid is important for the isolation of a fragment enriched in the reaction center chlorophyll, P700, from plants[7–9]. In the course of these studies we endeavored to cultivate carotenoid-deficient cells of *Anabaena variabilis* using diphenylamine, which inhibited the synthesis of carotenoids in this alga. However, we observed that the synthesis of myxoxanthophyll was stimulated by this reagent.

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

* Contribution No. 367 from the Charles F. Kettering Research Laboratory.

** Present address: Department of Food Science and Technology, University of Hawaii, Honolulu, Hawaii.

In the present paper we will describe pigment composition of Anabaena cells grown in the presence of diphenylamine in comparison with that of cells grown in the normal medium. The properties of these cells and their membrane fragments will also be described.

EXPERIMENTAL

Anabaena variabilis cells were grown in modified Detmer's medium[10] in the absence and presence of diphenylamine (12 mg/l), under 150 foot-candles of light from fluorescent lamps. After 9 days of culture, the cells were harvested and membrane fragments were isolated from the cells by the sonication procedure described earlier[11]. The cells grown in the absence and presence of diphenylamine will be described as normal-Anabaena cells and diphenylamine-Anabaena cells, respectively.

Carotenoids were separated by one-dimensional thin-layer chromatography on Micro-Cel C (Johns–Manville Co.), using hexane containing 5% acetone as the developer. Since the β-carotene band from diphenylamine-Anabaena contains precursors of carotenoids, β-carotene was purified by rechromatography on a thin layer of MgO and Hyflo Super Cel (1:2, w/w) using hexane as the developer. The following molar extinction coefficients were used for the determination of the carotenoid contents: $1.35 \cdot 10^5$, $1.21 \cdot 10^5$, $1.34 \cdot 10^5$ and $1.35 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ for β-carotene, echinenone, zeaxanthin and myxoxanthophyll in methanol, respectively. An extinction coefficient of $6.58 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 666 nm was used for the determination of chlorophyll *a* in methanol[12]. Protein was determined by the method of LOWRY *et al.*[13].

Absorption and fluorescence spectra were measured according to the procedure described previously[7,11].

RESULTS

Absorption spectra

The absorption spectra of cell suspensions and membrane fragments of diphenylamine- and normal-Anabaena are shown in Fig. 1. The absorption spectrum of diphenylamine-Anabaena cells (Curve A) shows a peak at 485 nm and shoulder around 520 nm due to carotenoids absorption, whereas the absorption spectrum of normal-Anabaena cells (Curve B) shows the carotenoid absorption around 490 nm as a shoulder, suggesting a difference in the carotenoid composition in these two types of cells. Phycocyanin has an absorbance peak at 628 nm in normal-Anabaena cells and at 624 nm in diphenylamine-Anabaena cells. As seen from Curve B, the absorbance of chlorophyll *a* at the peak position (679 nm) in normal-Anabaena cells is approximately the same as the absorbance at 628 nm; however, the absorption spectrum of diphenylamine-Anabaena cells (Curve A) shows great reduction of chlorophyll *a* absorbance, which appears only as a shoulder around 675 nm. The difference in carotenoid absorption was also observed in the absorption spectra of the membrane fragments prepared from diphenylamine-Anabaena cells (Curve C) and normal-Anabaena cells (Curve D). As seen from Curves C and D, the peak position of the red band of chlorophyll *a* is located at 680 and 678 nm for the membrane fragments of normal- and diphenylamine-Anabaena, respectively, and the Soret band is located at 438 nm for the membrane fragments of both types. These two

absorption spectra also show that the carotenoid/chlorophyll ratio is higher in the membrane fragment of diphenylamine-Anabaena than in that of normal-Anabaena.

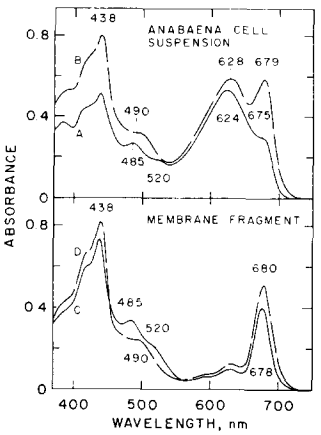

Fig. 1. Absorption spectra of the Anabaena cell suspensions and membrane fragments of diphenylamine- and normal-Anabaena. Curve A, diphenylamine-Anabaena cells; Curve B, normal-Anabaena cells; Curve C, diphenylamine-Anabaena membrane fragments; Curve D, normal-Anabaena membrane fragments. Both the Anabaena cells and membrane fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5).

Composition

The differences between these two types of cells are clearly demonstrated in their pigment composition, which is shown in Table I. As seen from this table, the chlorophyll content in diphenylamine-Anabaena cells is approximately half that of normal-Anabaena cells. Diphenylamine-Anabaena cells do not contain echinenone and zeaxanthin and the content of β-carotene is greatly reduced in these cells. However, the content of myxoxanthophyll in diphenylamine-Anabaena cells is twice as much as that in normal-Anabaena cells.

The ratio of total carotenoid to chlorophyll *a* was higher in diphenylamine-Anabaena cells than in normal-Anabaena cells. The membrane fragments prepared

TABLE I

COMPOSITION OF ANABAENA CELLS

*Component**	*Diphenylamine-Anabaena*	*Normal-Anabaena*
Chlorophyll *a*	11.8	26.0
β-Carotene	0.8	2.7
Echinenone	0	2.8
Zeaxanthin	0	0.5
Myxoxanthophyll	4.5	2.2
Total carotenoids/chlorophyll *a*	0.47	0.33
Protein**	1.3	0.6
P700***	0.8	0.8

* Pigments in nmoles per mg freeze-dried cells.
** Protein of the membrane fragments in mg per 100 nmoles chlorophyll *a*.
*** P700 in membrane fragments in moles per 100 moles chlorophyll *a*.

from these cells have been analyzed for their protein content. The results, shown in Table I, show that more protein was found in the membrane fragment of diphenylamine-Anabaena than that of normal-Anabaena.

The P700 content in diphenylamine-Anabaena membrane fragment was the same as that in normal-Anabaena membrane fragment in spite of the great reduction of chlorophyll content in diphenylamine-Anabaena cells. This suggests that the reduction of chlorophyll content in diphenylamine-Anabaena cells is due to a decrease in the number of chlorophyll units which contain P700 in the ratio of 0.8 mole per 100 total chlorophyll *a* moles.

Photochemical activities

The activities for $NADP^+$ and DCIP reduction were measured with the membrane fragments prepared from two types of Anabaena cells. The results are shown in Table II. Both the diphenylamine- and normal-Anabaena membrane fragments showed $NADP^+$ photoreduction activity with ascorbate–DCIP used as the electron donor system, and higher activity was obtained with the diphenylamine-Anabaena membrane fragment than with normal-Anabaena membrane fragment. None of these membrane fragments showed DCIP reduction activity with water as electron donor (Hill reaction). However, when 1,5-diphenylcarbazide was added as electron donor to Photosystem 2 (ref. 14), these membrane fragments reduced DCIP upon illumination. The rate of DCIP reduction was higher with the diphenylamine-Anabaena membrane fragment than with the normal-Anabaena membrane fragment.

TABLE II

PHOTOCHEMICAL ACTIVITIES OF ANABAENA MEMBRANE FRAGMENTS

*Reaction**	*Membrane fragments*	
	Diphenylamine-Anabaena	*Normal-Anabaena*
Photosystem 1 activity		
NADP (with ascorbate + DCIP)**	150	109
Photosystem 2 activity		
DCIP reduction (Hill reaction)	0	0
DCIP reduction (with 1,5-diphenylcarbazide as donor)***	35	25

* All reaction activities are expressed in μmoles/mg chlorophyll per h.

** The reaction mixture contained: 0.2 mM DCIP, 2 mM sodium ascorbate, 0.4 mM $NADP^+$, 6 mM $MgCl_2$, 15 μM chlorophyll, and saturating amount of crude enzymes prepared from *A. variabilis*. Reactions were run anaerobically under illumination with red light (Corning filter CS 2-58) at $1.5 \cdot 10^5$ erg·cm^{-2}·sec^{-1}.

*** Assayed under the condition described by VERNON AND SHAW[14].

Fluorescence measurements

In Fig. 2 the 77°K fluorescence spectra resulting from excitation at 437 and 520 nm are given for the diphenylamine- and normal-Anabaena cells. Both types of cells showed peaks at 696 and 731 nm as well as a shoulder around 687 nm and, when excited at 520 nm, showed a peak at 664 nm due to phycocyanin fluorescence.

In the 77°K fluorescence spectra excited at 437 nm, the fluorescence intensity at 731 nm was about the same for both types of cells in spite of the great difference in their carotenoid composition. However, the fluorescence intensity of the diphenylamine-Anabaena cells at 687 and 696 nm was approximately twice as much as that of the normal-Anabaena cells. The higher fluorescence intensity of the diphenylamine-Anabaena cells at 687 and 696 nm is also demonstrated in the fluorescence spectra excited at 520 nm, where the short wavelength fluorescence is intensified.

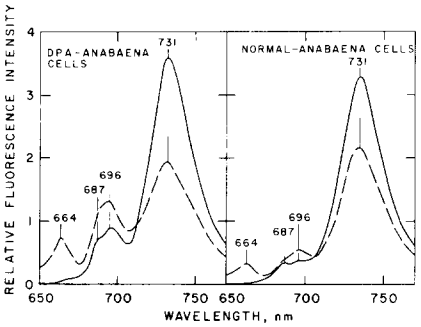

Fig. 2. Fluorescence spectra of diphenylamine- and normal-Anabaena cells measured at 77°K and resulting from incident light at 437 nm (———) and 520 nm (— — —). Each sample has the same chlorophyll concentration.

DISCUSSION

The inhibition of carotenoid synthesis in photosynthetic bacteria by diphenylamine has been well documented[2–6], and the present investigation shows that a similar inhibition is observed with a blue-green alga. In the present case, however, the myxoxanthophyll synthesis is not inhibited, but is stimulated by this reagent. The stimulation of myxoxanthophyll synthesis may be explained if a common precursor of all the carotenoids is assumed. The inhibition of the enzyme system for the synthesis of β-carotene, echinenone and zeaxanthin may force the common precursor to synthesize more myxoxanthophyll.

Diphenylamine reduces growth and carotenoid synthesis of *Euglena gracilis* equally; about 50% of carotenoid synthesis is inhibited[15]. Similar data were also obtained by preliminary experiment with *Chlorella vulgaris*. This might mean that the permeability of chloroplast membrane in these algae to diphenylamine is lower than that of chromatophore membrane in photosynthetic bacteria. Success to inhibit carotenoid synthesis of *A. variabilis* in this study might suggest that the permeability of the membrane of this alga to diphenylamine is rather close to that of photosynthetic bacteria.

The changes of bacteriochlorophyll absorption bands caused by the inhibition of carotenoid synthesis have been reported by BERGERON AND FULLER[5]. The decrease of chlorophyll content in diphenylamine-Anabaena cells is due to either the inhibition of chlorophyll synthesis by diphenylamine, or the failure of some chlorophyll to attach on the membrane when certain carotenoids are missing.

Activity measurements suggest that sonication damages the reaction between H_2O and Photosystem 2, since the addition of an electron donor (1,5-diphenylcarbazide) for Photosystem 2 restores the activity for DCIP reduction.

The fluorescence bands at 685 and 696 nm observed at 77°K are emitted from chlorophyll *a* in Photosystem 2 of chloroplasts and the band at 715–735 nm originates from chlorophyll *a* in Photosystem 1 (refs. 16, 17). The fluorescence data presented in this paper suggest that either the yield of fluorescence of Photosystem 2 is affected by the change of carotenoids composition while that of Photosystem 1 is not affected, or alternatively that the amount of those chlorophyll *a* molecules which have the emission band at 687 and 696 nm at 77°K is increased.

ACKNOWLEDGMENTS

This investigation was supported in part by Research Grant GB-8434 from the National Science Foundation.

The authors express their appreciation to Dr. B. C. Mayne for the use of the fluorometer in this study.

REFERENCES

1 G. TURIAN, *Helv. Chim. Acta*, 33 (1950) 1988.
2 T. W. GOODWIN AND H. G. OSMAN, *Biochem. J.*, 53 (1953) 541.
3 T. W. GOODWIN AND H. G. OSMAN, *Biochem. J.*, 56 (1954) 222.
4 G. COHEN-BAZIRE AND R. STANIER, *Nature*, 181 (1958) 250.
5 J. A. BERGERON AND R. C. FULLER, *Nature*, 184 (1959) 1340.
6 E. C. WASSINK AND G. H. M. KRONENBERG, *Nature*, 194 (1962) 4828.
7 T. OGAWA AND L. P. VERNON, *Biochim. Biophys. Acta*, 180 (1969) 334.
8 L. P. VERNON, H. Y. YAMAMOTO AND T. OGAWA, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 911.
9 H. Y. YAMAMOTO AND L. P. VERNON, *Biochemistry*, 8 (1969) 4131.
10 A. WATANABE, *J. Gen. Appl. Microbiol.*, 6 (1960) 283.
11 T. OGAWA, L. P. VERNON AND H. H. MOLLENHAUER, *Biochim. Biophys. Acta*, 172 (1969) 216.
12 T. OGAWA AND K. SHIBATA, *Photochem. Photobiol.*, 4 (1965) 193.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND A. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 L. P. VERNON AND E. R. SHAW, *Biochem. Biophys. Res. Commun.*, 36 (1969) 878.
15 J. W. GOODWIN AND M. JAMIKORN, *J. Protozool.*, 1 (1954) 216.
16 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 88 (1964) 304.
17 N. MURATA, M. NISHIMURA AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 126 (1966) 234.