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INCREASED CONTENT OF CYTOCHROMES 554 AND 562 IN *ANABAENA VARIABILIS* CELLS GROWN IN THE PRESENCE OF DIPHENYLAMINE*

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SUMMARY

Of the four major cytochromes (cytochromes 550, 554, 557 and 562) present in *Anabaena variabilis* cells, two *c* type cytochromes (cytochromes 550 and 554) were removed from the membrane when cells were sonicated and cytochromes 557 and 562 (which are probably *b* type cytochromes) remained tightly bound to the membrane. Cytochrome 554 was obtained in the reduced state while the others were in the oxidized form.

Cytochromes 554 and 562 were present in markedly higher amounts in Anabaena cells grown in the presence of diphenylamine, whereas cytochromes 550 and 557 were present at about the same concentrations in both types of cells. The contents of P700 and chlorophyll *a* in normal cells were twice as high as those in the diphenylamine-grown cells (expressing concentrations on the basis of cell weight of freeze-dried cells). The data on cytochrome and P700 composition of the membrane of the two cell types indicate that at least the cytochromes 554 and 562 are inserted into the membrane system independently of the chlorophyll and other cytochromes. Photooxidation of cytochrome 557 was observed with membrane fragments from diphenylamine-grown cells. The kinetics of the absorbance changes observed in the blue region were biphasic, and relatively slow changes were observed following the initial fast P700 photooxidation. The slow changes may be related to an unidentified component of the membrane fragments.

INTRODUCTION

Diphenylamine has been used as an inhibitor of carotenoid synthesis in photosynthetic bacteria[1,2]. A recent study in this laboratory has shown that *Anabaena variabilis* cells grown in the presence of diphenylamine differ from normally grown cells in their carotenoid composition; echinenone and zeaxanthin are absent and the content of β-carotene is reduced in the diphenylamine-Anabaena cells, whereas myxoxanthophyll content is increased[3]. The membrane fragments prepared from

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such cells have higher activities for cyclic photophosphorylation and NADP+ photoreduction than do normal-Anabaena membrane fragments[3,4].

In the present paper we compare the cytochrome composition of diphenylamine cells with that of normal cells. Of the four major cytochromes present in blue green algae, two *c* type cytochromes (cytochromes 549 and 554) have been purified by HOLTON AND MYERS[5,6]. Cytochromes 557 and 562, which are possibily *b* type cytochromes, are tightly bound to the membrane. The results presented below show that the contents of cytochromes 554 and 562 are markedly higher in diphenylamine cells than in normal cells, whereas the concentrations of the other cytochromes do not change. Illumination of membrane fragments causes the oxidation of cytochrome 557 and produces an absorbance change having slower kinetics than P700 or cytochrome oxidation kinetics.

EXPERIMENTAL

Preparation of samples

Anabaena variabilis cells were grown in the presence or absence or diphenylamine according to the procedure reported previously[3]. The procedure for the preparation of membrane fragments from these cells was the same as before[7]. The supernates obtained after centrifugation of the sonicated cells and washing of the membrane fragments were combined and used for the determination of soluble cytochromes.

Determination of cytochromes

Reduced-*minus*-oxidized spectra were measured at 20°C or 77°K with a Cary 14 recording spectrophotometer modified for low temperature spectra measurements, using the 0–0.05 slide wire. Cytochromes on the membrane fragments were determined without prior extraction of chlorophyll. After adjusting the base line with the same sample in the sample and reference cuvettes, solid ascorbate, dithionite or ferricyanide was added in excess to the sample or reference cuvettes. The cytochrome determinations in the supernates were carried out after removal of the majority of the phycocyanines by ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatants at 0° and the mixture was shaken and centrifuged for 20 min at 20000 × *g*. Table I shows the effect of ammonium sulfate concentrations on the cytochrome content of the supernates after centrifugation. The

TABLE I

EFFECT OF AMMONIUM SULFATE CONCENTRATIONS ON CYTOCHROME CONTENT IN THE SUPERNATES

% *Saturation with* $(NH_4)_2SO_4$	*Normal-Anabaena*		*Diphenylamine-Anabaena*	
	Chlorophyll a / Cytochrome 550	*Chlorophyll a / Cytochrome 554*	*Chlorophyll a / Cytochrome 550*	*Chlorophyll a / Cytochrome 554*
30	604	—	274	224
40	694	2000	320	204
45	614	2120	288	204
50	1500	2000	434	199
60	Trace cytochrome 550	3100	Trace cytochrome 550	318

cytochrome contents are expressed as the ratios of chlorophyll *a* to each cytochrome. As seen from this table, approximately constant ratios of chlorophyll *a* to cytochrome were obtained for cytochromes 550 and 554 for normal- and diphenylamine-Anabaena when 30–45 % saturation of ammonium sulfate was used. More than 85 % of the phycocyanine was removed with 30 % saturation of ammonium sulfate; however, the spectral change due to residual phycocyanine was still too great to obtain exact difference spectra for the cytochromes when the cytochrome contents were low, as was the case for cytochrome 554 of normal-Anabaena. Although all the phycocyanine was precipitated with 60 % saturation of ammonium sulfate, most of the cytochrome 550 and some of the 554 were also precipitated; even 50 % saturation of ammonium sulfate removed some cytochrome 550. All the cytochromes 554 and 550 remained in the supernates when 45 % saturation or lower concentrations of ammonium sulfate were used, as was done during these experiments.

Determination of chlorophyll a

Chlorophyll *a* was determined after extraction by methanol using an extinction coefficient of $6.58 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 666 nm (ref. 8).

Measurements of light-induced absorbance changes

The light-induced absorbance changes were measured in the flash spectrophotometer described by KE *et al.*[9]. The intensity of the actinic light (red light, Corning CS-2-58) was $2.5 \cdot 10^5$ $erg \cdot cm^{-2} \cdot sec^{-1}$.

RESULTS

Cytochromes of diphenylamine- and normal-Anabaena

Reduced-*minus*-oxidized difference spectra at 20° of the membrane fragments and supernates obtained from sonicated cells of diphenylamine-Anabaena are shown in Fig. 1. The ascorbate-reduced or dithionite-reduced *minus* untreated spectra of the membrane fragments were identical with ascorbate-reduced or dithionite-reduced *minus* ferricyanide-oxidized (A–F, or D–F) spectra of Fig. 1A, which indicates that all the cytochromes on the membrane fragments were in the oxidized state. The ascorbate-reduced *minus* ferricyanide-oxidized spectrum of the supernate (A–F in Fig. 1B) was the same as the untreated *minus* ferricyanide-oxidized spectra and the dithionite-reduced *minus* ascorbate-reduced spectra (D–A) was identical with the dithionite-reduced *minus* untreated spectra which shows that one of the cytochromes (554) in the supernate was in the reduced state and the other cytochrome (550) with a lower redox potential was in the oxidized state.

Fig. 2 shows the ascorbate-reduced *minus* ferricyanide-oxidized spectra of the membrane fragments and supernates of diphenylamine-Anabaena at 22°C and 77°K. Similar data were also obtained for the membrane fragments and supernatants of normal-Anabaena. The (A–F) spectra of the supernates have a peak at 554 nm at 20°C and two peaks at 552 and 547 nm at 77°K, which are in good agreement with those of cytochrome *f* reported by HILL AND BONNER[10] and BOARDMAN AND ANDERSON[11]. The (A–F) spectra of the membrane fragments have a peak at 557 nm at 20°C and at 555 nm at 77°K; no peaks were observed around 548 nm, which indicates that no cytochrome *f* was bound to the membrane fragments. Therefore

the major cytochrome on the membrane fragments which is reduced by ascorbate has a peak at 557 nm at 20°C and at 555 nm at 77°K. This cytochrome, which will be described as cytochrome 557 in this paper, might have the same function as cytochrome 559 in higher plants and is possibly a *b* type cytochrome.

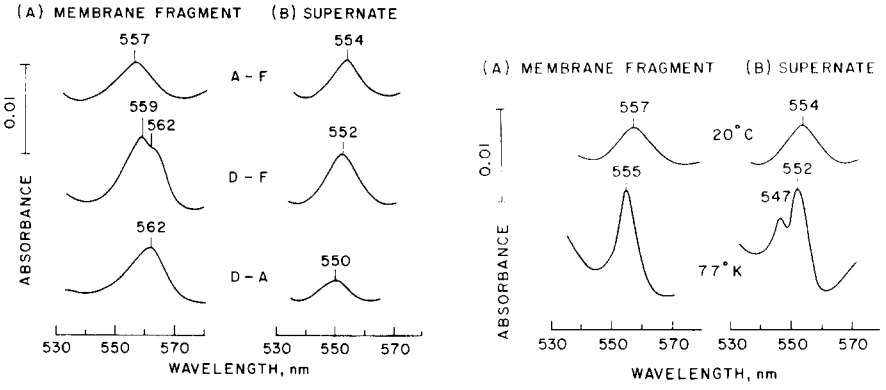

Fig. 1. Reduced-*minus*-oxidized difference spectra of diphenylamine-Anabaena membrane fragments (A) and supernates (B) at 20°: (A–F), ascorbate-reduced *minus* ferricyanide-oxidized; (D–F), dithionite-reduced *minus* ferricyanide-oxidized; (D–A), dithionite-reduced *minus* ascorbate-reduced. Chlorophyll *a* concentration, 29.8 μg/ml. Absorbance path length, 10 mm.

Fig. 2. Ascorbate-reduced *minus* ferricyanide-oxidized spectra of diphenyl-Anabaena membrane fragments (A) and supernate (B) at 20°C and 77°K. Chlorophyll *a* concentration, 0.1 mg/ml. Absorbance path length, 3 mm.

Dithionite reduces all the cytochromes on the membrane fragments and in the supernates. The (D–A) spectra show the difference spectra of those cytochromes which are not reduced by ascorbate. The (D–A) spectra of the membrane fragments have a peak at 562 nm at 20°, which agrees with the maximum of reduced-*minus*-oxidized spectra of cytochrome b_6 in spinach chloroplasts[11]. This cytochrome will be designated as cytochrome 562 in this paper. The (D–A) spectra of the supernate show a peak at 550 nm, which is very close to the peak position of cytochrome 549 isolated from *Anacystis nidulans* by HOLTON AND MYERS[5,6]. Of the three *c* type cytochromes (cytochromes 549, 552, and 554) in this alga, cytochromes 549 and 554 are major components and only a small amount of cytochrome 552 is present. Assuming that the content of cytochrome 552 is also very small in Anabaena, it is considered that the (D–A) spectra of the supernatant show reduced-*minus*-oxidized spectra of cytochrome 549 which will be described as cytochrome 550 in this paper according to the peak position of the (D–A) spectra of the supernate.

Fig. 3 shows reduced-*minus*-oxidized difference spectra at 20° of the membrane fragments and supernates obtained from sonicated cells of normal-Anabaena. These difference spectra show the same cytochromes as those of Fig. 1 for diphenylamine-Anabaena, with cytochromes 557 and 562 on the membrane fragments and cytochrome 550 in the supernate in the oxidized state and cytochrome 554 in the supernate in the reduced state. The magitudes of the absorbance differences of normal-Anabaena were smaller than those of diphenylamine-Anabaena, indicating less cytochromes in normal-Anabaena on a chlorophyll basis. There were also some differences in the shapes of the (D–F) spectra between normal- and diphenylamine-Anabaena. The

(D–F) spectrum of the diphenylamine-Anabaena membrane fragments showed a shoulder at 562 nm, whereas that of the normal-Anabaena membrane fragments did not. The (D–F) spectra of normal-Anabaena supernate had a peak at 551 nm, which is 1 or 2 nm shorter than the peak position of the (D–F) spectra of diphenylamine-Anabaena supernate. These data suggest there are differences in the molar proportions of the cytochromes between these two types of cells.

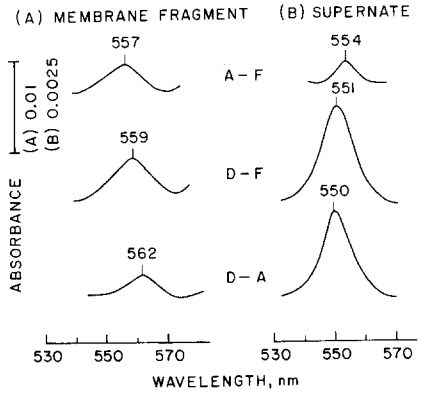

Fig. 3. Reduced-*minus*-oyidized difference spectra of normal-Anabaena membrane fragments (A) and supernate (B) at 20°. (A–F), (D–F), (D–A) have the same meaning as described in Fig. 1. Chlorophyll *a* concentration, 43.5 μg/ml. Absorbance path length, 10 mm.

The contents of cytochromes and P700

Cytochromes 557 and 562 were determined from the (A–F) and (D–A) spectra, respectively, of the membrane fragments at 20° using a difference molar extinction coefficient of $2.0 \cdot 10^4$ for both cytochrome 557 ($\varepsilon_{M,\,557\text{ nm}}-\varepsilon_{M,\,575\text{ nm}}$) and cytochrome 562 ($\varepsilon_{M,\,562\text{ nm}}-\varepsilon_{M,\,575\text{ nm}}$)[12]. No correction was made for the contribution of cytochrome 554, since no detectable cytochrome 554 was bound to the membrane fragments, as shown in Fig. 2. The (A–F) and (D–A) spectra of the supernate were used to determine the contents of cytochromes 554 and 550, respectively. A difference molar extinction coefficient of $2.5 \cdot 10^4$ was used for both cytochrome 554 ($\varepsilon_{M,\,554\text{ nm}}-\varepsilon_{M,\,570\text{ nm}}$)[12] and for cytochrome 550 ($\varepsilon_{M,\,550\text{ nm}}-\varepsilon_{M,\,570\text{ nm}}$).

The ratio of chlorophyll *a* to P700 is the ratio of the chlorophyll *a* absorbance at 678 nm to the absorbance difference at 705 nm of the dithionite-reduced *minus* ferricyanide-oxidized spectra.

The molar ratios of chlorophyll *a* to cytochrome and P700 are summarized in Table II, which gives average values from three preparations. The cytochrome composition of normal-Anabaena is different from that of higher plants. The ratio of chlorophyll to cytochrome 554 (cytochrome *f*) in normal-Anabaena was 1890, which is much higher than that in higher plants[11–13]. The molar proportions of the cytochromes 554, 557 and 562 in normal-Anabaena are as follows: cytochrome 554: cytochrome 557:cytochrome 562 = 1:7:4. These proportions are quite different from those in spinach chloroplasts (cytochrome *f*:cytochrome 559:cytochrome b_6 = 1:2:2, ref. 11). The ratio of chlorophyll *a* to cytochrome 562 in normal-Anabaena was 450, which agrees fairly well with the ratio obtained for the blue green alga, *Phormidium luridum*[14]. In diphenylamine-Anabaena the ratios of chlorophyll *a*

TABLE II

MOLAR RATIOS OF CHLOROPHYLL *a* TO CYTOCHROME AND P700 IN ANABAENA CELLS

	No.	*Chlorophyll a* / *Cytochrome 550*	*Chlorophyll a* / *Cytochrome 554*	*Chlorophyll a* / *Cytochrome 557*	*Chlorophyll a* / *Cytochrome 562*	*Chlorophyll a* / *P700*
Normal-Anabaena	1	755	2020	289	433	110
	2	694	1990	248	484	93
	3	602	1690	270	433	91
	Av.	684	1890	269	450	98
Diphenylamine-Anabaena	1	263	118	129	106	96
	2	288	204	125	90	100
	3	313	176	136	103	115
	Av.	288	166	130	100	104

TABLE III

CYTOCHROME AND P700 CONTENTS OF ANABAENA CELLS

	pmoles cytochrome and P700 per mg freeze-dried cells				
	Cytochrome 550	*Cytochrome 554*	*Cytochrome 557*	*Cytochrome 562*	*P700*
Normal-Anabaena	39	14	97	58	266
Ratio	2.8	1	6.9	4.1	19
Diphenylamine-Anabaena	41	71	91	118	114
Ratio	0.6	1	1.3	1.7	1.7

to cytochromes were lower than in normal-Anabaena, and the following molar proportions were obtained for diphenylamine-Anabaena: cytochrome 554 : cytochrome 557 : cytochrome 562 = 1 : 1.3 : 1.7. The ratio of chlorophyll *a* to P700 was approximately the same for both types of cells.

Table III shows the cytochrome and P700 contents per mg freeze-dried cells, which were calculated from the ratios of chlorophyll *a* to cytochrome and P700 in Table II using the known values of chlorophyll *a* contents per mg freeze-dried cells[3]. On the basis of mg freeze-dried cells the contents of cytochromes 550 and 557 were approximately the same for both types of cells; however, the contents of cytochromes 554 and 562 were markedly higher in diphenylamine-cells than in normal-cells. The content of P700 in diphenylamine-cells was one half of that in normal-cells.

Light-induced absorbance changes of Anabaena membrane fragments

Fig. 4 shows the kinetics of the light-induced absorbance changes of Anabaena membrane fragments which were obtained using 2-sec illumination periods with red light. As shown by the decrease of absorbance at 556 nm, cytochrome 557 on the diphenylamine-Anabaena membrane fragments was oxidized upon illumination,

whereas normal membrane fragments did not show an absorbance decrease due to photooxidation of cytochrome 557.

The rapid changes of absorbance at 410, 425, and 435 nm in Fig. 4 are due to oxidation of P700 and also, in the case of diphenylamine-Anabaena membrane fragments, cytochrome 557. In addition to these rapid changes, absorbance changes which have slower kinetics were observed between 390 and 570 nm. At 410 nm the kinetics were apparently biphasic (the rapid decrease and slow increase of absorbances) and no rapid absorbance changes were observed at 445 nm. These slow absorbance changes disappeared when the membrane fragments were illuminated in the presence of ferricyanide, which indicates that these absorbance changes are due to either the oxidation of an unknown compound or a spectral shift of pigments (carotenoids, etc.) caused by environmental changes of the pigments relating to an oxidation of P700 or cytochromes.

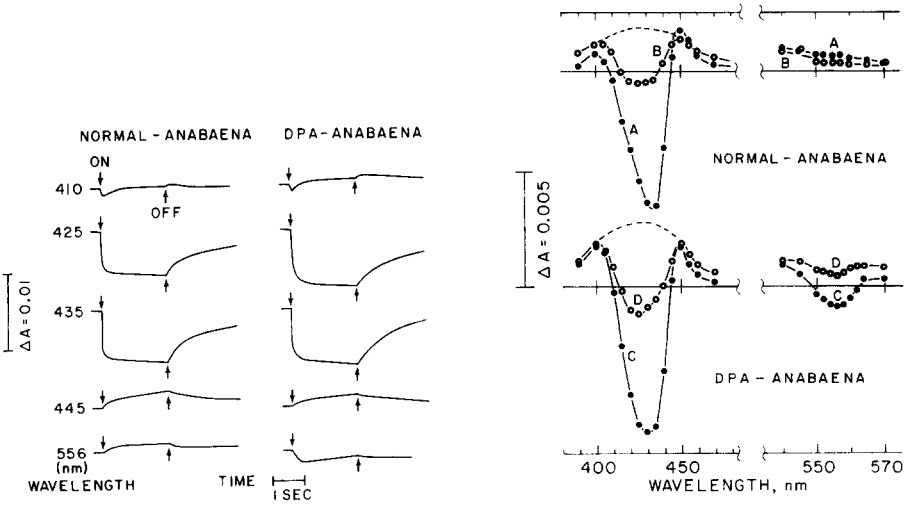

Fig. 4. Light-induced absorbance changes of normal- and diphenylamine(DPA)-Anabaena membrane fragments. The membrane fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5) containing 1 mM ascorbate and 0.1 μM 2,6-dichlorophenol indophenol. The chlorophyll *a* content of each sample was 15.4 μg/ml. For the measurement of absorbance changes at 556 nm a three times higher concentration of chlorophyll *a* was used.

Fig. 5. Light-*minus*-dark difference spectra of normal- and diphenylamine(DPA)-membrane fragments. Difference spectra of the absorbance changes which occurred in 150 msec and between 150 msec and 2 sec are shown by closed and open circles, respectively. The dashed line represents the completion of the assumed absorbance difference spectrum of the component responsible for the slow responses in this region. Experimental conditions and chlorophyll *a* concentrations were the same as described in Fig. 4, except that the chlorophyll *a* concentration for the measurement of the difference spectra between 540 and 570 nm was 46.2 μg/ml.

Difference spectra obtained by plotting the rapid and slow absorbance changes are shown in Fig. 5, where absorbance changes which occurred before and after 150 msec are shown, respectively, for normal and diphenylamine-Anabaena membrane fragments. The difference spectrum of the rapid absorbance changes of diphenylamine-membrane fragments (Curve C) showed a minimum around 556 nm due to oxidation of cytochrome 557, whereas that of normal membrane fragments (Curve A)

did not show any such peak. Curve A also showed maxima at 400 and 450 nm and a minimum at 435 nm which are related to the oxidation of P700. Some of the spectral changes with slow kinetics are also involved in Curve A. Curve C showed a minimum of 430 nm which is 5 nm shorter than that of Curve A. The shift of the minimum is due to the oxidation of cytochrome 557 in diphenylamine-membrane fragments, which causes a decrease in absorbance around 425 nm.

The difference spectrum of the slow absorbance changes showed maxima at 450 and 400 nm and a minimum at 425 nm for both normal and diphenylamine membrane fragments (Curve B and C, respectively). The slow absorption changes were also observed between 470 and 570 nm but the magnitude of the difference spectrum in these wavelengths regions was much smaller than between 390 and 470 nm and peaks were not apparent between 470 and 570 nm. As shown by the minimum around 556 nm in Curve D, some of the spectral changes of cytochrome 557 were involved in Curve D.

The most simple interpretation of the kinetic changes is that they represent three compounds: P700, cytochrome 557 (in Curves C and D) and another unknown compound which exhibits the slow kinetics. Considering Curves A and B, which are not complicated by changes related to cytochrome oxidation, it appears there is not a complete separation of P700 changes and those of the unknown component. Thus, the maximum at 450 nm in Curve A would be a combination of changes of P700 and the unknown component, while the maximum at 400 nm in Curve A would be almost entirely due to the slower acting component. Also, the changes occurring between 150 msec and 2 sec, shown in Curve B, would include some residual slow oxidation of P700, which produces the minimum at 425–430 nm in this curve. This residual P700 change would counter the positive changes in this region due to the slower acting component, whose change in absorbance is indicated by the dashed line connecting the two maxima of Curve B.

DISCUSSION

In the previous paper, we have demonstrated the inhibition of carotenoid synthesis and stimulation of myxoxyanthophyll synthesis in a blue green alga, *Anabaena variabilis*[3]. It is shown in the present investigations that the amounts of cytochromes 554 and 562 are markedly increased when Anabaena cells are grown in the presence of diphenylamine. Recently it has been observed that the rate of cyclic photophosphorylation in diphenylamine-membrane fragments is also markedly higher than that observed with normal-membrane fragments, which was explained in terms of an increased cytochrome content in the photosynthetic apparatus[4]. The data on the cytochrome composition presented in this paper strongly support the concept that the high cyclic phosphorylation activity of the diphenylamine-membrane fragments is due to high cytochrome contents and suggest the participation of cytochrome 562 and perhaps cytochrome 554 in cyclic electron flow. However, removal of a large portion of cytochrome 554 apparently does not effect cyclic phosphorylation to a large extent[15].

The molar ratio of chlorophyll to cytochrome *f* in the chloroplasts of higher plants is about 400 (refs. 11–13), which is approximately the same as the chlorophyll to P700 ratio[15]. In normal-Anabaena cells, however, the ratio of chlorophyll to

cytochrome 554 was much higher than the ratio of chlorophyll to P700 (the ratio of P700/cytochrome 554 = 19), which suggest that cytochrome 554 is not the primary electron donor to P700 in this alga. BIGGINS has shown that in *Phormidium luridum* one fourth of the cytochrome 554 is bound to the membrane and the ratio of cytochrome b_6 to the bound form of cytochrome 554 is about 4 (ref. 14), whereas in Anabaena cells we were unable to detect the bound form of cytochrome 554. The ratio of chlorophyll to total cytochrome 554 in Phormidium is calculated to be 500, which is approximately the same ratio as has been observed in higher plants. Therefore, the low content of cytochrome 554 observed in normal-Anabaena seems to be unusual among the blue green algae.

It is not obvious at present why the oxidation of cytochrome 557 was observed only with the diphenylamine-Anabaena membrane fragment. One possibility is that some electron carrier between cytochrome 557 and P700 is solubilized and removed from the normal membrane fragment. Since diphenylamine membrane fragments contain no or a very small amount of cytochrome 554, it seems unlikely that oxidation of cytochrome 557 by P700 is mediated by cytochrome 554. The higher ratio of P700: cytochrome 557 in the diphenylamine-Anabaena membrane fragments may be sufficient to explain the photooxidation of cytochrome 557 in this case.

The vast differences observed for the cytochrome composition in the photosynthetic membrane system of the two Anabaena cell types is of interest in regard to the mechanism of membrane assembly. DE PETROCELLIS *et al.*[16] have reported on the composition of thylakoid membranes during greening of a mutant strain of *Chlamydomonas reinhardi*, and conclude that there is no set chemical composition which applies to that photosynthetic membrane, but rather that the composition varies during the greening process. Such data are in accord with the concept that there is a multistep process in the formation of membranes, and there is considerable flexability in the interaction of these steps. Our data are certainly in agreement with this concept. The ways in which the presence of diphenylamine influences the formation of the membrane system of *Anabaena variabilis* are not known at present, but such a relationship opens the way for further study on this important process.

The low concentration of cytochrome 554, the algal counterpart of cytochrome *f* in higher plants, deserves some comment. In higher plants cytochrome *f* is present in chloroplasts at about the same molar concentration as P700, whereas in normal *Anabaena variabilis* the concentration of this cytochrome is very low in relation to P700. This has significance in terms of the possible role of this cytochrome as a primary electron donor to the oxidized P700. It appears that this is not possible in this alga, since there are not sufficient cytochrome 554 molecules to serve each photocenter of Photosystem I.

Preliminary experiments showed that the redox potential of the slow absorbance changes is the same or very close to that of P700 (+ 450 mV), which suggests that these absorbance changes are due to a spectral shift of some pigment(s) caused by environmental changes of the pigment(s) related to the oxidation of P700. It is unlikely that these slow absorbance changes are due to spectral changes of carotenoids, since both normal and diphenylamine membrane fragments showed slow absorbance changes with approximately the same magnitudes in spite of great differences in carotenoid composition between these two types of membrane fragments[3]. Further study is necessary on these slow absorbance changes.

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