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Separation of Cl-Containing Chlorophylls by Column Chromatography

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Using Toyopearl and cyclohexane : cyclohexanol solvent, four Cl-containing Chls were separated from ^{36}Cl-labeled cells of the blue-green, *Plectonema boryanum*. In normally grown cells, all four Cl-containing chlorophylls amounted to less than 1/2,000 of the total Chl and about 1/50 of P700, values much lower than previously reported contents of Chl RC I, and varied from alga to alga. The level of Cl-containing Chl was markedly enhanced when the cells were poisoned with methyl viologen. These results suggests that these Cl-containing Chls are not related to the reaction center of PS I.

Key words: Chlorophyll RC I — Cl-containing chlorophyll — ^{36}Cl incorporation into chlorophyll — Cyanobacteria — *Plectonema boryanum* — Toyopearl column chromatography.

Chl RC I, a Cl-containing derivative of Chl *a* (Scheer et al. 1986, Dörnemann and Senger 1986), was first isolated from a mutant of *Scenedesmus* and postulated as the key pigment in the reaction center of PS I (Dörnemann and Senger 1981, 1982). Recently, the same pigment was isolated from blue-greens (Katoh et al. 1985), but the separation of this pigment on column chromatograms of Sepharose gel was not always clear and the isolation required thin-layer chromatography at the final step. In the present study, the column chromatography of Toyopearl was improved so as to enable separation of minor Chl derivatives from bulk Chl; using blue-green cells labeled with radioactive chlorine (^{36}Cl), the determination of Chl RC I was attempted. Experiments were conducted using freshwater blue-greens because these can grow in media containing extremely low Cl concentrations [for instance, in C medium of Kratz and Myers (1955), $MnCl_2$ in microelement solution (A_5) is the sole source of Cl], which facilitated the efficient labeling of cells with ^{36}Cl. This study revealed that the contents of Cl-containing Chl in normally cultured algal cells were considerably lower than those of P700 in all the algal species tested.

Materials and Methods

Algal culture—Four strains of blue-greens, *Plectonema boryanum, Anabaena variabilis* (M3), *Anacystis nidulans* and *Fremyella diplosiphon*, were obtained from the Institute of Applied Microbiology, University of Tokyo; *Anabaena cylindrica* and *Tolypothrix tenuis* were obtained from National Institute of Basic Biology; and an R2 strain of *Synechococcus* was donated by Dr. R. Haselkorn (University of Chicago, Chicago, U.S.A.). Algae were propagated axenically in BG-11 medium (Allen 1968) and, to deplete the Cl level, cultured in a Cl-free medium which was a modification of BG-11 with $CaCl_2$ and $MnCl_2$ replaced by equivalent molarities of $Ca(NO_3)_2$ and $MnSO_4$, respectively, but due to reagent impurities the medium still contained 0.048–0.065 μM Cl. When the growth rates of the algae became discernibly slow after changes in Cl-free medium

every 4 days, the cells were labeled with ^{36}Cl. In Cl-free BG-11 medium supplemented with ^{36}Cl (4.27 μM, 514 mCi/mole), the cells were allowed to grow to the stationary phase (4 to 5 days) to propagate the cell mass to 600 to 1,350 times that of the inoculum.

Chl extraction—Cells, collected, washed and suspended in 50 mM Tris-HCl (pH 7.4), were disrupted by being passed through an ice-cold French press and immediately mixed with 4 volumes of cold acetone (−20°C), followed by centrifugaton at 10,000×*g* for 10 min. The pellets were then extracted twice with warm methanol (45°C). Acetone and methanol extracts were mixed with petroleum ether, and the upper green layer of petroleum ether was collected and washed repeatedly with 50% (v/v) aqueous acetone to remove water-soluble materials such as Cl ion. Then the petroleum ether layer was collected, evaporated, dissolved in a small volume of ethanol and reevaporated to remove all traces of water.

P700-enriched particles—Freshly prepared spinach chloroplasts were washed three times with ice-cold solution contaning HEPES (20 mM, pH 7.4) and NaCl (10 mM), frozen to −196°C with liquid nitrogen and lyophilized in vacuo. Removal of light-harvesting and PS-II pigments with diethyl ether exraction, leaving the pigments of PS-I reaction center, was performed according to Ikegami (1976). Lyophilized chloroplasts were extracted twice with water-containing diethyl ether [mixture of water-saturated and dehydrated diethyl ether at the ratio of 4 : 1 (v/v)] at 0°C. The Chl content of the resultant pellets was 1/43 that of the original preparation and their Chl *a* : *b* ratio was 15.7. These were used as P700-enriched particles.

Column chromatogram—Toyopearl HW-55F (Toyo Soda, Tokyo) was washed with distilled water and then acetone repeatedly to remove all traces of water. It was then washed with cyclohexane : acetone (1 : 1, v/v), with cyclohexane : aceton (3 : 1, v/v), twice with pure cyclohexane and then allowed to equilibrate with dehydrated pure cyclohexane for more than five days, until the gel had shrunk to less than two-thirds of its original volume. Into a glasstube with sintered glass at the bottom (internal diameter: 2 cm) filled with pure cyclohexane 10 cm deep, the HW-55F suspension was poured slowly to a thickness of 5 to 7 cm. The column bed was washed with 10 volumes (170 ml) of pure cyclohexane. The adsorption properties of the shrunken HW-55F differ from those of the original gel, and sufficient equilibration with pure cyclohexane is important to obtain a sharp and reproducible separation of pigments.

Eluates of column chromatogram were collected every 30 drops (ca 1.32 ml) with a fraction collector. Each fraction was examined for the absorbance spectrum with a Hitachi spectrophotometer 200-10, and then treated as follows to bring to the ^{36}Cl measurements with a scintillation counter. The samples were first dried in scintillator vials in an oven (110°C); then few drops of 16.5% H_2O_2 containing 20 mM Na_2CO_3 were added and warmed in an oven to digest the pigments. This was repeated until the samples became colorless, after which they were heated to 160°C to remove all traces of H_2O_2 and brought to the measurement of radioactivities. In the control experiments with measured amounts of ^{36}Cl, the loss of Cl in the course of H_2O_2 digestion were less than 2.0%. The addition of Na_2CO_3 was useful for the retention of Cl which might be emitted by acids produced by oxidation.

Amounts of Chl *a* and *b* were determined according to Mackinney (1941). P700 was measured by ferricyanide-minus-ascorbate difference absorption at 700 nm using a Hitachi 356 spectrophotometer. The oxidation of the thylakoid suspension (25 to 30 μM Chl in 20 mM Tris-HCl, pH 7.4) by $K_3Fe(CN)_6$ (0.8 mM) was monitored against the reference containing 10 mM Na-isoascorbate. The difference absorption coefficient at 700 nm was assumed to be 64 $mM^{-1} \cdot cm^{-1}$ according to Hiyama and Ke (1972).

Results

The dried preparation of pigments isolated from ^{36}Cl-labeled cells of *Plectonema boryanum* was

dissolved in 1.0 ml of pure cyclohexane and, after filtration through a glass filter to remove insoluble materials, applied to a Toyopearl HW-55F column which had been equilibrated with the same solvent. In the experiment (Fig. 1), a relatively large amount of the sample was charged because the specific degradation rate of ^{36}Cl is very low (due to its long half life of 3×10^5 years). Using pure cyclohexane, β-carotene was separated from the column first; immediately after it was removed, the solvent was changed to a mixture of cyclohexane : cyclohexanol (200 : 1, v/v), which eluted a gray band of pheophytin a. After 25 ml of 120 : 1 mixture had been allowed to flow, the solvent was changed to 80 : 1, at which time a dense green band at the middle of the column started to migrate and separate into a faster migrating, faint band of Chl *a*′ and a slower, predominant band of Chl *a*. After Chl *a*′ was eluted out, the solvent was changed to a 40 : 1 mixture to facilitate the removal of Chl *a*, after which two bands remained on the column, one a pale green band at the middle and the other a red band of myxoxanthin at the top. The former was then eluted by cyclohexane : cyclohexanol (20 : 1, v/v), and the latter with cyclohexane : acetone (1 : 1, v/v). The change of solvent for the last step had to be preceded by a small volume of cyclohexane : acetone (5 : 1, v/v) to avoid an abrupt change of gel properties. A faint green band of chlorophyllide *a* remaining at the top of the column was removed with a sizable quantity of cyclohexane : acetone (1 : 1, v/v). Although the mixture of methanol : acetone should have released it efficiently, the Toyopearl column showed a massive expansion of the bed volume with methanol, resulting in cessation of the solvent flow.

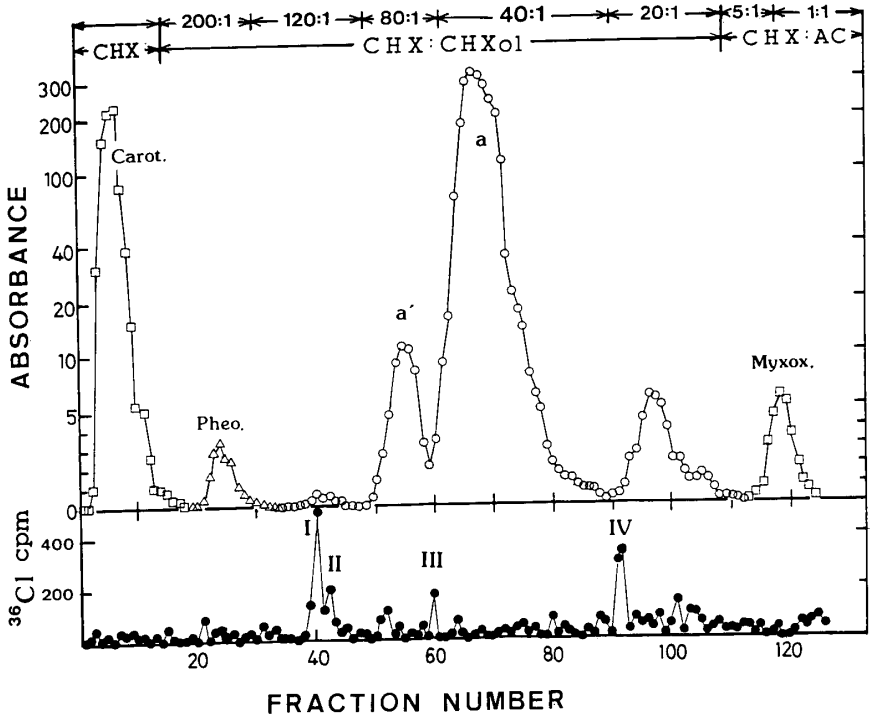

Fig. 1 Elution pattern of Toyopearl HW-55F column chromatography of pigments extracted from *Plectonema boryanum* cells. A chromatogram of a sample containing 56.2 mg of Chl *a* was developed first with 20 ml of pure cyclohexane (CHX), then stepwise with 20 ml of a 200 : 1, 25 ml of a 120 : 1, 40 ml of an 80 : 1, 20 ml of a 20 : 1 cyclohexane : cyclohexanol mixture (v/v), and finally with 10 ml of cyclohexane : acetone (5 : 1) and 25 ml of cyclohexane : acetone (1 : 1). In the upper part, absorbance larger than 5.0 was plotted on a logarithmic scale on the ordinate : squares, absorbance at 484 nm due to β-carotene (fractions 3–18) and to myxoxanthin (112–127); triangles, absorbance at 671 nm due to pheophytin *a* (21–29); circles, absorbance at 664 nm due to Chl *a*′ (50–59) and *a* (60–88). Lower part: rdioactivity of ^{36}Cl.

Measurement of ^{36}Cl radioactivity revealed four Cl-containing components (Fig. 1, bottom), none of which coincided with the bands of separated pigments. The first and second (ClChl-I and -II) were eluted with cyclohexane : cyclohexanol (120 : 1), the third (ClChl-III) between Chl *a'* and Chl *a*, and the fourth (ClChl-IV) after the large band of Chl *a*. Among these, ClChl-I was predominant, but even this was visually indiscernible unless a sizable quantity of pigments was charged. Of the four Cl-containing Chls, the first two (ClChl-I and -II) showed identical absorption spectra with the maximum at 665 nm, coinciding with that of Chl RC I.

The quantities of these ClChls, calculated on the basis of the absorbance of Cl-containing fractions, were about 1/5,400 of the total Chl for ClChl-I and less than this figure for the other ClChls. The radioactivity of 588 cpm in the ClChl-I fraction (Fig. 1, fraction no. 40) should correspond to 4.54 μatoms Cl after the corrections for isotopic dilution, and the absorbance of ClChl-I in the same fraction corresponded to 4.65 μmoles of Chl *a*, if the molar absorption coefficient at the λmax is assumed to be the same. This suggests that one ClChl-I molecule has one atom of Cl. The Cl contents in the other ClChl fractions were smaller than this value, probably because they were not completely free from bulk Chl *a*.

Fig. 2 shows the elution pattern of acid-treated pigments in the same column chromatography. Most part of Chl *a* and *a'* has been transformed into pheophytin *a*, and myxoxanthin was completely degraded with this treatment. Most part of Cl-containing Chls were lost, and a peak of ^{36}Cl radioactivity appeared immediately prior to pheophytin *a* instead, indicating that the Cl-containing components had been converted into pheophytin-like pigment compounds (ClPheo). The pheophytinized products of the four separated ClChls were all eluted

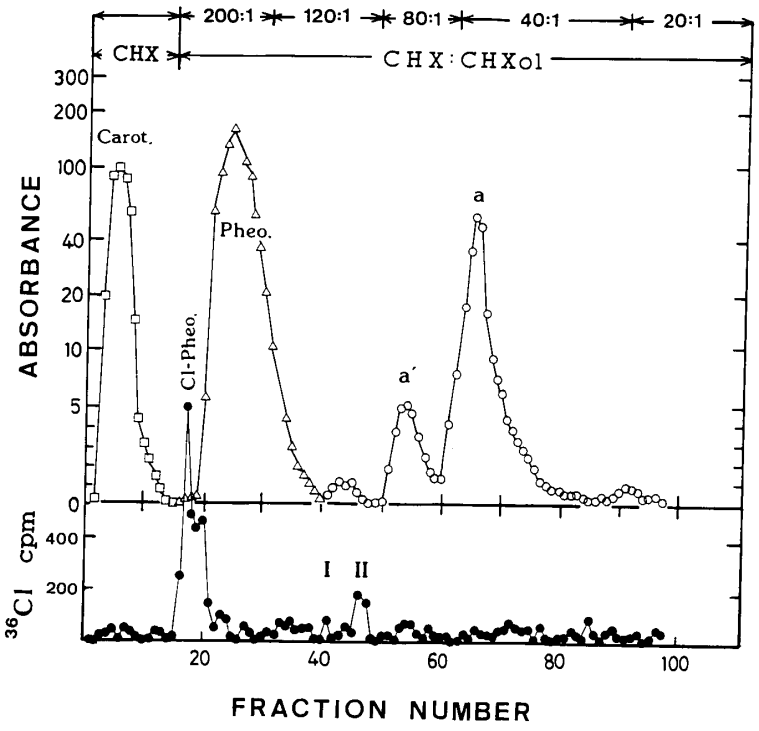

Fig. 2 Elution pattern of Toyopearl HW-55F column chromatography of acid-treated pigments of *Plectonema boryanum*. The extracted pigments (containing 69.6 mg Chl *a*) were dissolved in 2.0 ml of 80% aqueous acetone, to which 0.5 ml of acetic acid was added and allowed to react for 30 min at room temperature. Treated pigments were transferred to petroleum ether, washed repeatedly with distilled water, evaporated and dissolved in 0.5 ml of pure cyclohexane, and applied to the column chromatography. For the conditions of development, the scale of the ordinate, and symbols, see the legend to Fig. 1.

Table 1 Contents of Cl-containing Chls and P700 in various blue-greens (per 10^4 Chl)

	ClChl[a]	ClChl-I[a]	P700
Fremyella diplosiphon	1.09	—	68.9
Plectonema boryanum	4.41	1.89 (1.85)[b]	84.0
Anabaena variabilis (M3)	0.29	0.16 (0.18)[b]	78.2
Anacystis nidulans	3.55	—	91.4
Synechococcus R2	1.40	0.49 (0.82)[b]	100.7
Tolypothrix tenuis	4.97	—	71.7
Anabaena cylindrica	0.66	0.49 (0.58)[b]	73.5

[a] Estimated from the ^{36}Cl radioactivity in chlorophyll fraction.
[b] Values in parentheses estimated from the absorbance of ClChl-I fraction.

at the same position as ClPheo and no distinctions in their absorption spectra were detected.

Using the same arrangements of the column chromatography, the ratios of the ClChl to the total Chl and to P700 were measured for seven freshwater species of blue-greens (Table 1). The amounts of Cl-containing Chls were less than 1/2,000 those of total Chl and less than 1/20 those of P700 and varied from alga to alga, in contrast to the rather stationary values of P700. The number of Cl atoms per molecule in the collected fractions free from the contamination of bulk Chl *a*, calculated with the corrections for isotopic dilution on the basis of $^{36}Cl/Cl$ of culture media,

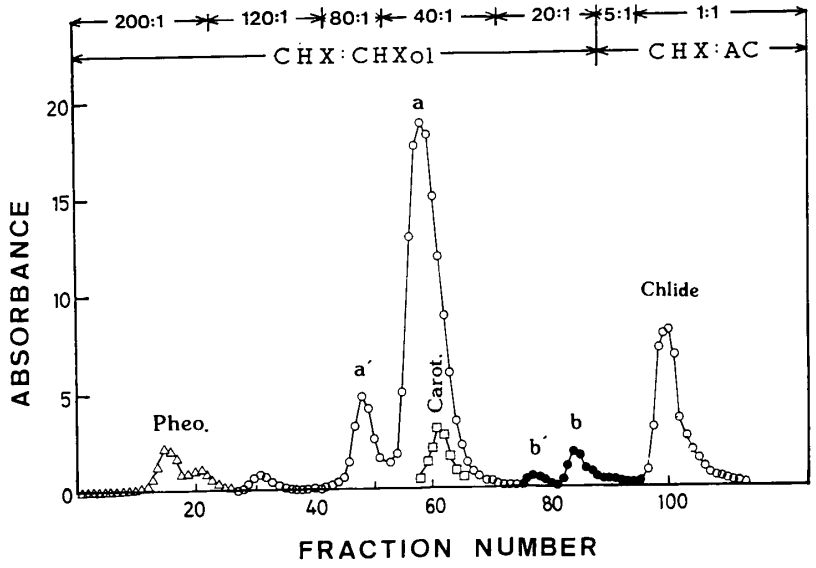

Fig. 3 Elution pattern of Toyopearl HW-55F column chromatography of the pigments from P700-enriched particles of spinach chloroplasts. Lyophilized spinach chloroplasts (23 mg Chl) were extracted twice with water-containing diethyl ether (80% saturation) at 0°C, and the resultant pellet was dried and then homogenated with warm methanol (45°C) to extract pigments. The conditions of development were the same as in Fig. 1, except that the initial development with pure cyclohexane was omitted because no yellow band of β-carotene was seen when pigments were charged on the column. Triangles, absorbance at 671 nm due to pheophytin *a*; open circles, absorbance at 664 nm due to Chl *a'* (fractions 43–51), to Chl *a* (52–72) and to chlorophyllide *a* (97–111); closed circles, absorbance at 644 nm due to Chl *b'* (77–81) and to Chl *b* (83–95); squares, absorbance at 484 nm due to violaxanthin (59–64).

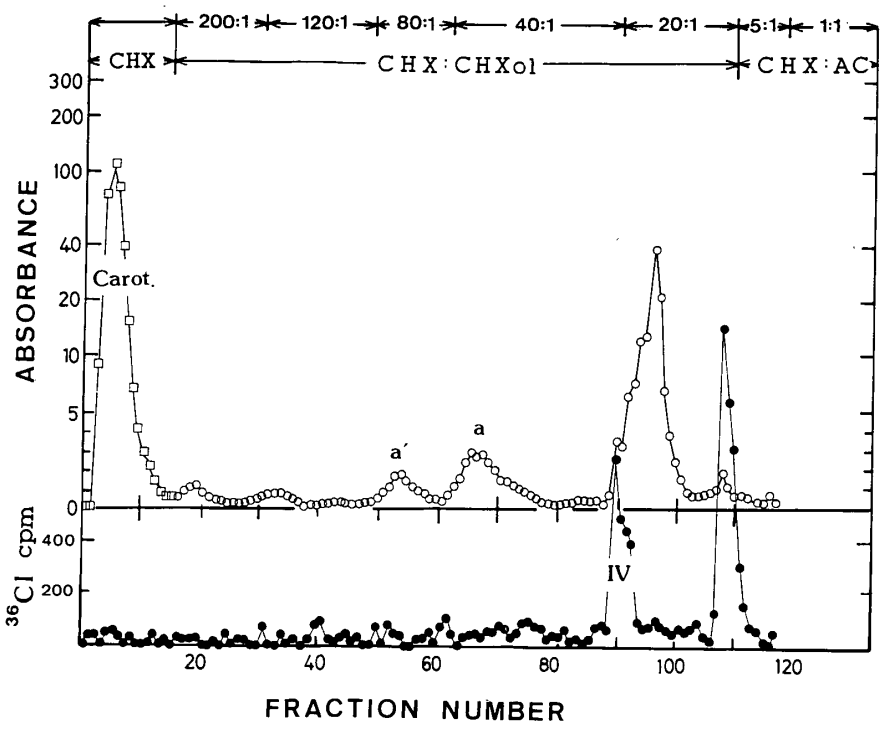

Fig. 4 Elution pattern of the pigments of methyl viologen-poisoned *Plectonema boryanum* cells with Toyopearl HW-55F column chromatography. To middle logarithmic phase cells in ^{36}Cl-supplemented medium, 50 μM (final concentration) of methyl viologen was added, and after 6 h illumination the cells were harvested. For the conditions of development the scale of the ordinate, and the symbols, see the legend to Fig. 1.

were in the range between 0.55 and 0.94.

Fig. 3 shows the elution pattern of the pigments from P700-enriched particles from spinach chloroplasts. As previously reported by Ikegami and Katoh (1975), the Chl of the PS-I reaction center was less readily extracted than other Chl forms by the treatment with aqueous diethyl ether. In diethyl ether-extracted chloroplasts, the contents of Chl *b* and carotenoids were decreased extensively, while the Chl eluted at the position of ClChl-I (or ClChl-II), with an absorption maximum at 665 nm (fraction no. 29 to 34 in Fig. 3), increased to the extent of 1/214 of the total Chl, which, however, was still much smaller than the content of P700 in this preparation (1/17.7).

The incorporation of Cl into Chl was markedly enhanced when the cells were poisoned with methyl viologen (MV). After a 6-h incubation with 50 μM MV in the light, ^{36}Cl was recovered in the fraction of ClChl-IV at a level 15 times higher than in the untreated cells and in the chlorophyllide fraction to the same extent, but no Cl incorporation was seen in the ClChl-I and -II fractions (Fig. 4). The ClChl-IV isolated from MV-poisoned cells showed an absorption maximum at 672 nm, differing from the other ClChls, but the ratio of Cl atom to Chl was found to be close to unity (0.89). No ^{36}Cl incorporation into Chl was observed when the cells were incubated in the dark, indicating that some light reaction, like the Mehler reaction, may have been responsible to the chlorination of Chl. An O_2^- radical is probably involved in the process like chloroperoxidase.

An attempt to chlorinate purified Chl *a* enzymatically using chloroperoxidase of *Caldariomyces imago* (Sigma, U.S.A.) yielded only very little of a chlorinated form of Chl *a*, but produced a sizable quantity of chlorinated pheophytin, which was eluted at the same position as ClPheo. This was probably due to the low pH (4.3) of the reaction medium, adjusted to facilitate the

reaction of this enzyme, the optimum pH of which is 2.2. Of the chlorinated products of Chl recovered, the greatest quantity was for ClChl-I and no radioactivity was obsrved at the position of ClChl-III or -IV.

The separation of pigments with the solvent system of cyclohexane : cyclohexanol mixtures as described above was obtained with cyclohexane : isopropanol mixtures of the same ratio as well. We used the cyclohexanol mixtures because they afford smaller changes in gel volume than the latter, though the cyclohexanol mixtures are more viscous and less easily removed by evaporation than the latter. When the flow rate slowed down, it could be readily resumed by applying an appropriate air pressure to the column without sacrificing the separation performance.

Rechromatography of the separated Chls in the same column showed that no conversion of Chl *a* into other forms, including conversion to Chl *a'*, or to pheophytin, or vice versa, had occured.

Discussion

Using Toyopearl chromatograms and cyclohexane : cyclohexanol mixtures, photosynthetic pigments, including phenophytin *a*, *b*, Chl *a*, *a'*, *b*, *b'*, one Cl-containing pheophytin *a* (ClPheo) and four types of Cl-containing Chls (ClChl-I, -II, -III and -IV) could be separated. A method to separate Chl *a* and *b* was developed by Omata and Murata (1980, 1983) using DEAE-Sepharose CL-6B and Sepharose CL-6B and hexane-isopropanol mixtures. These agarose gels, however, show extensive shrinkage of bed volume with an accompanying change of chromatographic properties when the content of the polar solvent is lowered to the range in which Chl *a* is adsorbed to the gel [in the case of isopropanol : hexane, lower than 1/40 (v/v)], making it difficult to separate those pigments which are eluted prior to Chl *a*. Toyopearl is more stable in this respect. It also shrinks in nonpolar solvents, such as pure hexane or cyclohexane, and swells in polar solvents especially in the presence of methanol, but the physical conditions of gel bed are less affected by the type of solvent used. With this system, most the acetone-extracted photosynthetic pigments can be chromatographically separated without preliminary purification with DEAE-agarose chromatogram, which causes epimerizationof Chl *a* to Chl *a'* (Omata and Murata 1980).

The extremely low level of ClChls in the cells was rather unexpected. The total amounts of ClChls was about 1/2,000 that of the total Chl *a* and the most predominant ClChl-I was 1/50 or less than that of P700. Separation of such a small quantity of pigment is feasible only with the use of radioactive Cl or with chromatography of the highest sharpness such as HPLC. The observed value differs from the Chl RC I content which had been reported to be of the same level as P700 in variety of algal species (Dörnemann and Senger 1981, 1982, Katoh et al. 1985). The scarcity of Cl contents in Chl cannot be interpreted as being due to incomplete incorporation of radioactive Cl because the amount of ClChls, calculated from the absorbance of the Cl-containing fraction, gave almost the same values. In the case of ClChl-I, the ratio of Cl atom : Chl was calculated to be close to unity if the absorption coefficient was assumed to be identical to that of Chl *a*.

The fact that, in *Anabaena variabilis* cells, the content of ClChls is less than 1/280 the content of P700 (Table 1) is not compatible with previous results with the same alga (Katoh et al. 1985). The chromatographic performance of Sepharose CL-6B gel, used in the previous work, was much less stable than that of Toyopearl in highly polar solvents, so that the final determinations of Chls were often made using another chromatogram with thin-layer plates. The higher values for Chl RC I in the previous report would therefore be accounted for either by incomplete chromatographic separation or by a secondary transformation of Chl in the course of thin-layer chromatography. Judging from their relative amounts in this alga, no Cl-containing Chl should have been visibly detected as a separate band on a CL-6B column chromatography, so the collected fraction should probably had not been separated enough from Chl *a'*, which is eluted very close to

ClChl-I and -II. The possibilities that Chl *a* undergoes chlorination on occasion of cell disruption and that the chlorinated derivative is oxidatively converted into Chl RC I in the course of thin layer chromatography in the presence of O_2 have recently been suggested by T. Watanabe in Tokyo University (personal communication).

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