Biochimica et Biophysica Acta, 501 (1978) 103—111

BBA 47424

PREPARATION OF CHLOROPHYLL *a*, CHLOROPHYLL *b* AND BACTERIOCHLOROPHYLL *a* BY MEANS OF COLUMN CHROMATOGRAPHY WITH DIETHYLAMINOETHYLCELLULOSE

NAOKI SATO and NORIO MURATA

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)

(Received May 10th, 1977)

Summary

Chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a* were prepared by means of column chromatography with Sephadex LH-20 and diethylaminoethylcellulose. This method provides purified preparations of chlorophylls in about 3 h.

To prepare chlorophyll *a*, blue-green or red algae were used as the starting material. Chlorophyll *a* was extracted with 90% aqueous acetone from cells of blue-green algae, *Anabaena variabilis*, *Anacystis nidulans* and *Tolypothrix tenuis*, and with 90% aqueous methanol from thalli of a red alga, *Porphyra yezoensis*. Chlorophyll *a* was collected as precipitates by adding dioxane and water to the extract according to the method of Iriyama et al. [6]. The crude chlorophyll *a* preparation was applied to a Sephadex LH-20 column with chloroform as the eluent and then to a DEAE-cellulose column with a chloroform/methanol mixture (49 : 1, v/v) as the eluent. Analysis with thin layer chromatography revealed that the chlorophyll *a* preparation contained no detectable contaminants.

Bacteriochlorophyll *a* was prepared in a similar manner from purple photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Chromatium vinosum*.

In order to prepare chlorophyll *b*, chloroplasts of spinach leaves were used as the starting material. A mixture of chlorophylls *a* and *b* was obtained in the same way as described for the preparation of chlorophyll *a* from the blue-green algae. To separate chlorophyll *b* from chlorophyll *a*, the mixture was applied to a diethylaminoethylcellulose column which was developed with a hexane/2-propanol mixture (5 : 2, v/v).

Introduction

Column chromatography with powdered sugar has often been used to prepare chlorophylls [1,2]. In this method, however, a large amount of sugar

and a large volume of solvent are consumed, possibly because of a relatively small surface area per unit volume of the powdered sugar. Moreover, the flow rate of eluent is usually very low so that the chromatography process takes a long time. In some cases, cellulose powder [3] has been employed instead of the powdered sugar in which case flow rate increases. Successful separation of chlorophyll *a* and carotenoids in a short time [4] is achieved by using a hydroxypropyl derivative of cross-linked dextran, Sephadex LH-20, which has a large surface area. Solvent fractionation has often been used in combination with the column chromatography [1,5].

A method, different in principle from that mentioned above, has been introduced by Iriyama et al. [6] for a partial purification of chlorophylls. They have found that chlorophylls form precipitates with dioxane in aqueous organic solvents.

In the present study, we employed the dioxane precipitation method and column chromatography with Sephadex LH-20 and DEAE-cellulose in order to prepare chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a*. For an isolation of chlorophyll *a*, blue-green and red algae were the best starting materials, since they contain no chlorophyll *b*.

Methods

Anabaena variabilis, strain M3 and *Anacystis nidulans*, which were obtained from the Algal Collection of the Institute of Applied Microbiology, the University of Tokyo, were grown at 30°C under illumination in Kratz and Myers' C medium [7]. *Tolypothrix tenuis* was grown at 26°C under illumination in the modified Detmer's medium [8] and supplied by a courtesy of Dr. K. Ohki, the Ocean Research Institute, the University of Tokyo. *Porphyra yezoensis* was grown in Yamamoto Nori Research Laboratory and supplied by a courtesy of Dr. S. Araki. Spinach leaves were purchased at a local market. They were disrupted with a Waring blendor for 1 min in a medium containing 0.4 M sucrose and 50 mM phosphate buffer (pH 7.3) and the homogenate was filtered through 8 layers of cheese cloth. Chloroplasts were collected by centrifuging the filtrate at 5000 $\times g$ for 5 min. *Chromatium vinosum* was photoautotrophically grown at 30°C under anaerobic conditions in a medium of Bartsch [9]. *Rhodopseudomonas spheroides* was photoheterotrophically grown in a medium containing 5 g of polypeptone, 1 g of yeast extract and 4 g of sodium lactate per l.

Before use for the chromatography, chloroform was distilled and 0.005 vol. of ethanol was immediately added. All the other solvents were of analytical grade and were used without further purification. DEAE-cellulose (Brown) and Sephadex LH-20 were purchased from Seikagaku Kogyo Co.

The column of Sephadex LH-20 was prepared in a following way. The gel was swollen in methanol overnight and packed in a column (1.7 cm diameter) to a height of 12 cm. A disk of filter paper was put on top of the Sephadex LH-20 column in order to prevent the gel from floating. The column was washed with a chloroform/methanol mixture (2 : 1, v/v), and then with chloroform.

The column of DEAE-cellulose was prepared in a following way. DEAE-

cellulose was washed successively with methanol containing 1 M HCl, methanol, 95% aqueous methanol containing 0.1 M KOH and finally with methanol. The resin was further washed with water containing 1 M HCl, water, water containing 0.5 M KOH and finally with water. Then, the DEAE-cellulose was washed with methanol and dried. It was then immersed overnight in glacial acetic acid and washed with methanol [10]. Approximately 5 ml of a dispersion of DEAE-cellulose in methanol was poured into a column (0.9 cm diameter) and the DEAE-cellulose was manually packed with a glass stick. This operation was repeated until the height of the adsorbent reached approx. 20 cm. The column was washed with methanol, followed by methanol containing increasing concentrations of chloroform, and finally with chloroform. For the separation of chlorophyll *b* from a mixture of chlorophylls *a* and *b*, chloroform in the column was replaced by a hexane/2-propanol mixture (5 : 1, v/v).

Purity of the chlorophyll preparations was examined using thin-layer chromatography. Contamination by carotenoids and degradation products of chlorophylls was checked according to the method of Hager and Meyer-Bertenrath [11]. An adsorbent layer was prepared from a mixture containing 12 g diatomaceous earth (Hyflosupercel), 3 g $CaCO_3$, 3 g silica gel (WAKO-GEL B-O), 0.02 g $Ca(OH)_2$, 0.2 g ascorbic acid disodium salt and 55 ml water. The thin-layer plates were dried at 55°C for 1.5 h. Approx. 30 μl of an acetone solution of chlorophylls was applied to the silica gel plate. After standing for a few minutes, the plate was developed with a petroleum ether/2-propanol mixture (95 : 5, v/v). R_F values of the chlorophylls were as follows: 0.51 (0.63) for chlorophyll *a*, 0.43 (0.48) for chlorophyll *b* and 0.78 (0.67) for bacteriochlorophyll *a*. The values in parentheses are for the decomposition products of the chlorophylls that were formed after standing for a long time. Quinones were analyzed by thin-layer chromatography with silica gel (WAKO GEL B-0) as the adsorbent and a hexane/benzene mixture (15 : 85, v/v) as the developing solvent [12]. Lipids were also examined using thin-layer chromatography with silica gel [13].

Chlorophylls and carotenoids were spectrophotometrically detected either in chloroform or in 80% acetone. Absorption spectra were measured with a Shimadu UV-200 spectrophotometer. Amounts of chlorophyll *a* and chlorophyll *b* in a mixture were determined according to the method of Arnon [14].

Results

Chlorophyll a

All the procedures for chlorophyll preparation were carried out in the cold unless otherwise mentioned. Cells of the blue-green algae, *A. variabilis*, *A. nidulans* and *T. tenuis* (1—2 ml in packed volume) were suspended in 5 ml phosphate buffer (0.2 M, pH 7.0). 45 ml acetone was added to the suspension. After standing for 15 min at room temperature, the suspension was filtered through a Buchner funnel. The residue on the funnel was washed with acetone (approx. 25 ml) and the filtrates were combined. 10 ml dioxane was added and then 15—20 ml distilled water were added drop-wise until green precipitates began to appear. The solution was placed in a freezer at approx. −20°C for 30 min. The green precipitates formed were collected by centrifugation at 5000 × *g* for

5 min. The red-orange supernatant containing xanthophylls and lipids was discarded and the green precipitates were dissolved in 20 ml ethanol. The solution of crude chlorophyll *a* preparation was evaporated to dryness under reduced pressure. When a small amount of water remained, the evaporation procedure was repeated after adding 10 ml ethanol. The crude chlorophyll *a* preparation thus obtained contained carotenoids, lipids, pheophytin *a* and chlorophyllide *a* as contaminants. The crude chlorophyll *a* preparation was dissolved in 1 ml chloroform and was put on the Sephadex LH-20 column and developed with chloroform. Lipids, chlorophyllide *a* and most xanthophylls were adsorbed on top of the column, and chlorophyll *a*, β-carotene and echinenon, which were not tightly adsorbed, were eluted. The green fractions were collected and dried in vacuo. The Sephadex LH-20 column was washed with methanol to remove the adsorbed lipids and pigments, and then the solvent was replaced by chloroform for the next step. The chlorophyll *a* preparation obtained from the Sephadex LH-20 column was dissolved in 0.5 ml chloroform, applied to the DEAE-cellulose column and then the column was developed by a chloroform/methanol mixture (49 : 1, v/v). The elution pattern in the case of *A. variabilis* is shown in Fig. 1. β-Carotene, echinenon and pheophytin *a* were not adsorbed and rapidly eluted out. Chlorophyll *a*, which was eluted later, was completely separated from the carotenoids and pheophytin *a*. The eluate was evaporated to dryness under reduced pressure and chlorophyll *a* thus obtained was dissolved in anhydrous benzene and stored at −20°C. Under this condition, chlorophyll *a* could be stored for a week without forming any detectable degradation products.

Analyses of the chlorophyll *a* preparation in the thin-layer chromatography revealed that the preparation contained neither carotenoids, lipids and quino-

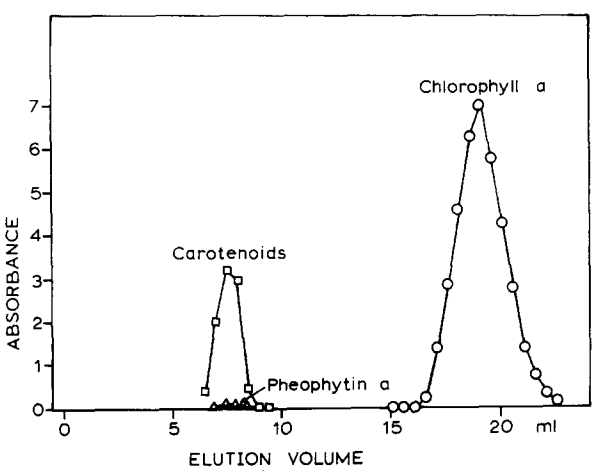

Fig. 1. Chromatogram of crude chlorophyll *a* preparation of *Anabaena variabilis* on a DEAE-cellulose column. The chlorophyll *a* preparation was obtained using the dioxane precipitation method and the chromatography on a Sephadex LH-20 column. 0.5 ml chloroform solution containing 0.5 mg chlorophyll *a* was applied to the DEAE-cellulose column (0.9 × 25 cm) and eluted with a chloroform/methanol mixture (49 : 1, v/v) at a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected. ○, absorbance at 665 nm due to chlorophyll *a*; □, absorbance at 462 nm due to the carotenoids β-carotene and echinenon; △, absorbance at 665 nm due to pheophytin *a*.

nes nor by-products of chlorophyll *a*. An absorption spectrum of a chlorophyll *a* preparation dissolved in diethylether showed no sign of alteration of chlorophyll *a*; the ratio of absorbance of the Soret peak at 429.4 nm to the red peak at 661.2 nm was 1.27—1.28.

To extract chlorophyll *a* efficiently from thalli of the red alga *P. yezoensis*, 90% methanol was used instead of 90% acetone. Chlorophyll *a* was also prepared by the dioxane precipitation method. The same procedure as with the blue-green algae was employed for the purification of chlorophyll *a*. Fig. 2 shows the elution pattern of the DEAE-cellulose column chromatography.

When the crude chlorophyll preparation obtained as the dioxane precipitates was applied directly to the DEAE-cellulose column, an elution pattern similar to that shown in Figs. 1 and 2 was obtained with *A. variabilis*, *T. tenuis* and *P. yezoensis*. Though *A. nidulans* contains zeaxanthin which was eluted at the same elution volume as chlorophyll *a* from the DEAE-cellulose column, this carotenoid was eliminated from the chlorophyll *a* preparation in the Sephadex LH-20 chromatography step. Hence the combination of the chromatography with DEAE-cellulose and Sephadex LH-20 was indispensable to obtain a chlorophyll *a* preparation from *A. nidulans* without carotenoid contamination.

Although the chromatography step with Sephadex LH-20 could be omitted in preparing chlorophyll *a* from *A. variabilis*, *T. tenuis* or *P. yezoensis* the Sephadex LH-20 column was usually used before chromatography with DEAE-cellulose. Some xanthophylls, which could easily be removed from the Sephadex LH-20 column, bound tightly to the DEAE-cellulose so that they were not easily washed out with methanol. In order to reuse the DEAE-cellulose column, the preceding step with the Sephadex LH-20 column could not be omitted.

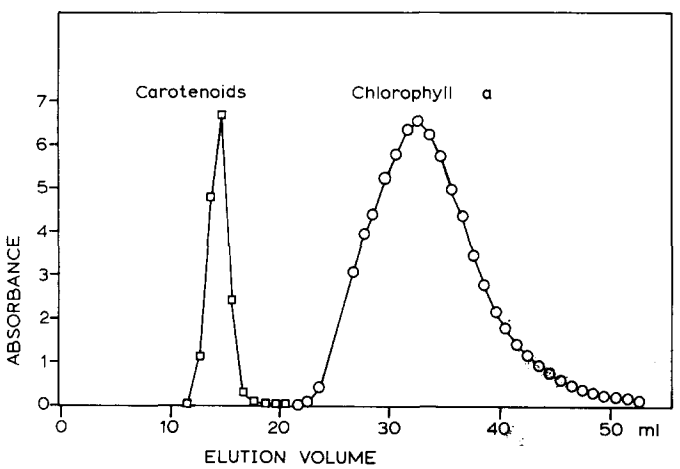

Fig. 2. Chromatogram of a crude chlorophyll *a* preparation from *Porphyra yezoensis* on a DEAE-cellulose columns. The chlorophyll *a* preparation was obtained using the dioxane precipitation method and Sephadex LH-20 column chromatography. 0.3 ml chloroform solution containing 1.0 mg chlorophyll *a* was applied to the DEAE-cellulose column (0.9 cm × 25 cm) and fractions of 1.0 ml were collected. Other conditions were the same as in Fig. 1. ○, absorbance at 665 nm due to chlorophyll *a*; □, absorbance at 462 nm due to carotenoids.

Bacteriochlorophyll a

Cells of *R. spheroides* or *C. vinosum* were first extracted with 90% aqueous acetone at room temperature for 10 min and the suspension was filtered. The orange-colored acetone solution containing most of carotenoids was discarded. The green-colored residue was then extracted with methanol at room temperature for 10 min and the suspension was filtered. 1/7 vol. dioxane and then 1/2 vol. of distilled water was added to the methanol solution. Light green precipitates of bacteriochlorophyll *a* appeared. Bacteriochlorophyll *a* was passed through columns of Sephadex LH-20 and then DEAE-cellulose in the same way as described in the purification of chlorophyll *a*. The elution pattern of the DEAE-cellulose column chromatography is shown in Fig. 3. Carotenoids and bacteriopheophytin *a* were eluted first and bacteriochlorophyll *a* was completely separated from them.

Chlorophyll b

Spinach chloroplasts were used as starting material to prepare chlorophyll *b*. Chlorophylls *a* and *b* were extracted with 90% aqueous acetone and collected in the dioxane precipitation method. The crude chlorophyll preparation was applied to a Sephadex LH-20 column, and then to a DEAE-cellulose column in the same manner as described in the purification of chlorophyll *a*. The elution pattern in the DEAE-cellulose chromatography is shown in Fig. 4. Carotenoids and pheophytins were removed from chlorophylls, while chlorophylls *a* and *b* were not separated from each other. The fractions including chlorophylls *a* and *b* were collected and evaporated to dryness under reduced pressure. The mixture of chlorophylls *a* and *b* was dissolved in a hexane/2-propanol mixture (5 : 1, v/v) and applied to a DEAE-cellulose column which had been equilibrated with the same solvent. The column was developed with a hexane/2-propanol mixture (5 : 2, v/v). The elution pattern as shown in Fig. 5a

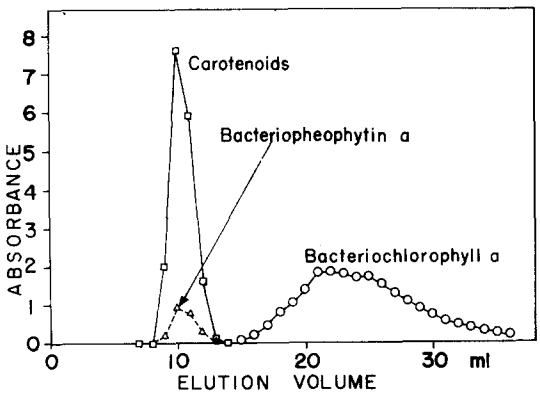

Fig. 3. Chromatogram of crude bacteriochlorophyll *a* preparation of *Rhodopseudomonas spheroides* on a DEAE-cellulose column. The bacteriochlorophyll *a* preparation was obtained using the dioxane precipitation method and Sephadex LH-20 column chromatography. 0.5 ml chloroform solution containing 0.4 mg bacteriochlorophyll *a* was applied to the once-used DEAE-cellulose column (0.9 × 20 cm) and eluted by chloroform at a flow rate of 1.7 ml/min. Fractions of 1.0 ml were collected. ○, absorbance at 779 nm due to bacteriochlorophyll *a*; □, absorbance at 465 nm due to carotenoids; △, absorbance at 756 nm due to bacteriopheophytin *a*.

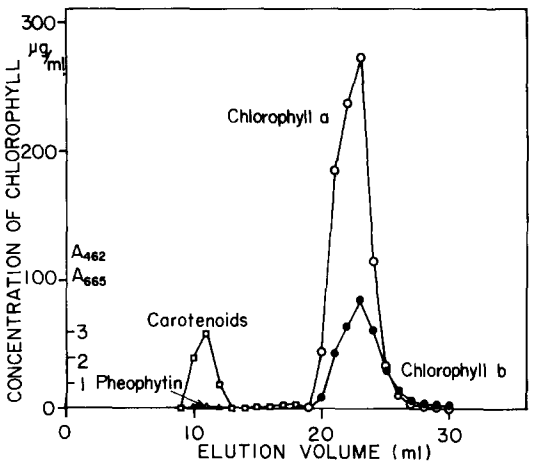

Fig. 4. Chromatogram on a DEAE-cellulose column of a crude preparation of chlorophylls *a* and *b* mixture obtained from spinach chloroplasts. The chlorophyll preparation was obtained using the dioxane precipitation method and Sephadex LH-20 column chromatography. 0.3 ml chloroform solution containing 1.3 mg chlorophyll was applied to the DEAE-cellulose column (0.9 × 18 cm) and eluted by a chloroform/methanol mixture (49 : 1, v/v), and fractions of 1.0 ml were collected. To determine concentrations of chlorophylls *a* and *b*, an aliquot of each fraction was evaporated to dryness and dissolved in 80% aqueous acetone. The chlorophyll concentrations were calculated from the absorbance at 663 and 645 nm according to the method of Arnon [14]. □, absorbance at 462 nm due to carotenoids; △, absorbance at 665 nm due to pheophytins; ○, concentration of chlorophyll *a* (μg/ml); ●, concentration of chlorophyll *b* (μg/ml).

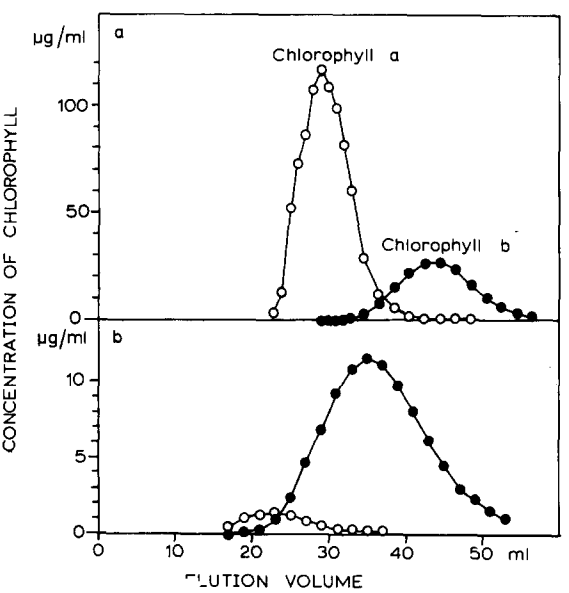

Fig. 5. a. Chromatogram of chlorophyll preparation of spinach chloroplasts on a DEAE-cellulose column. The chlorophyll preparation was obtained using the dioxane precipitation method and chromatography on Sephadex LH-20 and DEAE-cellulose columns. 0.2 ml solution of hexane/2-propanol mixture (5 : 1, v/v) containing 1.0 mg chlorophyll *a* and 0.4 mg chlorophyll *b* was applied to the DEAE-cellulose column (0.9 × 25 cm), and eluted with a hexane/2-propanol mixture (5 : 2, v/v) at a flow rate of 0.7 ml/min. Fractions of 1.0 or 2.0 ml were collected. ○, concentration of chlorophyll *a* (μg/ml); ●, concentration of chlorophyll *b* (μg/ml). b. Chromatogram of the chlorophyll *b*-rich preparation. Fractions of elution volume 37—52 ml in the chromatogram of the upper figure containing 0.22 mg chlorophyll *b* and 0.02 mg chlorophyll *a* were collected and redissolved in a 0.5 ml hexane/2-propanol mixture (5 : 2, v/v). The solution was applied to the DEAE-cellulose column (0.9 × 20 cm) and eluted with a hexane/2-propanol mixture (2 : 1, v/v) at a flow rate of 1.0 ml/min.

indicated that the separation of chlorophylls *a* and *b* was incomplete. Fractions containing a main part of chlorophyll *b* were collected and concentrated by evaporation. The crude chlorophyll *b* preparation was applied to another column of DEAE-cellulose and eluted with a hexane/2-propanol mixture (2 : 1, v/v). The elution pattern of the second chromatography is shown in Fig. 5b. Although the early fractions still contained a small amount of chlorophyll *a*, the fractions of elution volume 35—50 ml contained only chlorophyll *b*. The thin-layer chromatography with silica gel revealed that the chlorophyll *b* preparation thus prepared was not contaminated by chlorophyll *a* or any other pigment. Chlorophyll *a* was also prepared by the rechromatography of fractions in Fig. 5a containing chlorophyll *a* with a hexane/2-propanol mixture (5 : 2, v/v). However, the chlorophyll *a* preparation was contaminated by a very small amount of carotenoid which was not identified. A repeated dioxane precipitation reduced the amount of carotenoid contamination in the chlorophyll *a* preparation. To prepare only chlorophyll *b*, the DEAE-cellulose column chromatography with the hexane/2-propanol mixture as an eluent could be used directly for the crude chlorophyll preparation obtained after chromatography on the Sephadex LH-20 column.

Characteristics of this method

Recovery of chlorophylls in the dioxane precipitation method reached about 90% in either acetone or methanol. In the column chromatography with Sephadex LH-20 and DEAE-cellulose, the chlorophylls were almost completely recovered. The preparation of chlorophyll *a* from the blue-green algae and bacteriochlorophyll *a* from the photosynthetic bacteria in the dioxane precipitation method and the column chromatography with Sephadex LH-20 and DEAE-cellulose took less than 3 h, while the preparation of chlorophyll *b* from spinach chloroplasts took about 4 hours.

The DEAE-cellulose column could be reused to prepare chlorophylls after washing it with methanol, but the elution volume for chlorophylls was reduced after the first use and washing of the column. If the column was washed with glacial acetic acid, the original elution volume was recovered. In a reused column, however, good separation of chlorophylls from the other pigments was achieved by using a chloroform/methanol mixture in a ratio of 100 : 1 or 200 : 1 (v/v) or chloroform on its own as the eluent.

Discussion

In the present study it was found that chlorophylls can be prepared in pure form in a short time using a combination of the dioxane precipitation method and column chromatography with Sephadex LH-20 and DEAE-cellulose. For chlorophyll *a* the blue-green and the red algae are the best starting material since they contain no chlorophyll *b*.

DEAE-cellulose chromatography was the first step in purification of the chlorophylls as DEAE-cellulose adsorbs chlorophylls more tightly than the sugar powder, the cellulose powder and the hydroxypropyl derivative of cross-linked dextran. For the separation of chlorophylls *a* and *b*, a 2-propanol/hexane mixture (0.5 : 99.5, v/v) has been used in the column chromatography

with sugar powder and cellulose powder [1,3]. However, solubility of chlorophylls in this solvent mixture is rather low. In the DEAE-cellulose column the ratio of 2-propanol to hexane can be increased to 2 : 5 as chlorophyll solubility is greatly increased in higher concentrations of 2-propanol, and so markedly reduces the amount of organic solvents required for the separation of chlorophylls *a* and *b* in the column chromatography.

Acknowledgements

The authors thank Dr. K. Ohki, Ocean Research Institute, University of Tokyo, for her kind gift of the culture of *T. tenuis*, and are very grateful to Dr. S. Araki, Yamamoto Nori Research Laboratory, for the supply of the culture of *P. yezoensis*. This study was supported in part by a grant from the Ministry of Education, Japan.

References

1 Strain, H.H. and Svec, W.A. (1966) in The Chlorophylls (Vernon, L.P. and Seely, G.R., eds.), pp. 21—66, Academic Press, New York
2 Tswett, M. (1906) Ber. Deutsch. Bot. Ges. 24, 384—393
3 Strain, H.H., Sherma, J., Benton, F.L. and Katz, J.J. (1965) Biochim. Biophys. Acta 109, 23—32
4 Shimizu, S. (1971) J. Chromatog. 59, 440—443
5 Willstätter, R. and Stoll, A. (1913) Untersuchungen über Chlorophyll, Springer, Berlin
6 Iriyama, K., Ogura, N. and Takamiya, A. (1974) J. Biochem. 76, 901—904
7 Kratz, W.A. and Myers, J. (1955) Am. J. Bot. 42, 282—287
8 Watanabe, A. (1960) J. Gen. Appl. Microbiol. 6, 283—292
9 Bose, S.K. (1963) in Bacterial Photosynthesis (Gest, H., San-Pietro, A. and Vernon, L.P., eds.), p. 507, The Antioch Press, Yellow Springs, Ohio
10 Rouser, G., Kritschevsky, G., Heller, D. and Liever, E. (1963) J. Am. Oil Chem. Soc. 40, 425—454
11 Hager, A. and Meyer-Bertenrath, T. (1966) Planta 69, 198—217
12 Barr, R., Henninger, M.D. and Crane, F.L. (1967) Plant. Physiol. 42, 1246—1254
13 Nichols, B.W., Harris, R.V. and James, A.T. (1965) Biochem. Biophys. Res. Commun. 20, 256—262
14 Arnon, D.I. (1949) Plant. Physiol. 24, 1—15