Phytochemistry, 1965, Vol. 4, pp. 725 to 730. Pergamon Press Ltd. Printed in England

CHLOROPHYLL *c* IN SOME MEMBERS OF THE CHRYSOPHYCEAE

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(*Received* 6 *February* 1965)

Abstract—A number of algae belonging to the Chrysophyceae were examined for the presence of chlorophyll *c* by three methods. It was found in *Chrysochromulina chiton*, *Chrysochromulina* sp. (Plymouth No. 200), *Chrysochromulina* sp. (Plymouth No. 137), *Isochrysis galbana*, *Prymnesium parvum* and *Dicrateria* sp. (Plymouth No. 133). (These algae have recently been placed in a new Class, the Haptophyceae.) The chlorophyll *c* content was generally in the range 20–40 per cent of total chlorophylls, chlorophyll *a* being the only other chlorophyll present. Their chlorophyllide content in culture was negligible. Chlorophyll *c* could not be demonstrated in heterotrophically grown *Ochromonas danica*. This organism showed an acetone-soluble pigment whose absorption spectrum resembled pheophytin *a*. A comparison of the three methods of assay gave results which were in poor agreement (sometimes varying over a twofold scale using the same culture). The trichromatic method gave results with two green algae and *Ochromonas danica* which indicated the presence of a small amount of chlorophyll *c*, whereas none was found by the other methods. It seems advisable to carry out chlorophyll *c* assays by more than one method, and even then only approximate results are obtained.

INTRODUCTION

THERE have been conflicting reports on the presence or absence of chlorophyll *c* in the Chrysophyceae. Allen, French and Brown[1] have suggested that chlorophyll *c* was present in *Prymnesium parvum* Carter on the basis of a derivative absorption spectrum. They detected only chlorophyll *a* in *Ochromonos danica* and *Ochromonas malhamensis*. Jeffrey[2] has shown that *Isochrysis galbana* and *Sphaleromantis* sp. contained chlorophyll *c* to the extent of about 28 per cent and 22 per cent respectively of total chlorophylls. Parsons[3] has shown that *Monochrysis lutheri* and *Syracosphaera carterae* contained about 5 per cent and 19 per cent of total chlorophylls as chlorophyll *c*. On the other hand Dales[4] claimed that chlorophyll *c* was not present in the eight species of Chrysophyceae that he examined and Gibbs[5] stated that *Ochromonas danica* contained only chlorophyll *a*.

The present work springs from an interest in *Prymnesium parvum* Carter. Ratios of optical densities of 665 mμ/635 mμ in methanolic extract, and 663 mμ/626 mμ in ethereal extract, were equal to 3·35 and 4·92 respectively, which indicated the possible presence of chlorophyll *c*. The results reported are a study on the chlorophyll *c* content of this organism and other members of the Chrysophyceae.

RESULTS

Table 1 shows the percentage of the various chlorophylls present in a number of organisms, as determined by the trichromatic method of Parsons and Strickland.[6] Chlorophyll *c* was

[1] M. B. ALLEN, C. S. FRENCH and J. S. BROWN, In *Comparative Biochemistry of Photoreactive Systems* (Edited by M. B. ALLEN), p. 36, Academic Press, New York (1960).
[2] S. W. JEFFREY, *Biochem. J.* **86**, 313 (1963).
[3] T. R. PARSONS, *J. Fisheries Research Board Can.*, **18**, 1017 (1961).
[4] R. P. DALES, *J. Marine Biol. Assoc. United Kingdom*, **39**, 693 (1960).
[5] S. P. GIBBS, *J. Cell. Biol.* **15**, 343 (1962).
[6] T. R. PARSONS and J. D. H. STRICKLAND, *J. Marine Research*, **21**, 155 (1963).

determined by two additional methods, the results of which, expressed as percentages of the trichromatic value, are also shown in Table 1.

Typical absorption curves for the 90% aqueous acetone extract of chlorophyll *c* obtained by the method of Parsons[7] are shown in Fig. 1, before and after hydrochloric acid treatment. The absorption maxima for the untreated extract being 450–452 mμ, 585 mμ and 632 mμ. The latter two peaks were found to be similar in magnitude (585 mμ slightly greater than 632 mμ) and about 10 per cent of the 450 mμ peak in all cases, with the exception of *Isochrysis galbana*. In this organism the 585 mμ and 632 mμ peaks averaged 9·8 per cent and 17·2 per cent respectively of the 450 mμ value.

TABLE 1. THE CHLOROPHYLLS OF VARIOUS ALGAE

Method of estimation	A % total chlorophyll			B % A value	C % A value
Chlorophyll	*a*	*b*	*c*	*c*	*c*
Organism					
Chrysochromulina chiton	58·7	nil	41·3	145	53
Chrysochromulina sp.	50	6·9	43·1	133	69
(Plymouth 200)	59·4	nil	40·6	181	68
Chrysochromulina sp.	59·2	2·5	38·3	167	64
(Plymouth 137)	61·4	nil	38·6	192	72
Isochrysis galbana	76·4	nil	23·6	91	76
	64·0	nil	36·0	74·6	68
Ochromonas danica	92·5	nil	7·5	nil	—
	89·0	nil	11·0	nil	—
Prymnesium parvum	68·7	nil	31·3	132	50
Dicrateria sp.	50·6	3·3	46·1	145	63·5
(Plymouth 133)	54·0	1·6	44·4	154	—
Dunaliella primolecta	67·5	25·3	7·2	nil	—
Pedinomonas minor	64·7	21·3	14·0	nil	—
Halidrys siliquosa	72·1	nil	27·9	80·5	53

(Method A: Parsons and Strickland[6]; Method B: Parsons[7]; Method C: from the optical density of the acetone extract of Parsons[7] at 630 mμ using the calibration curve of Parsons[7] after correction for the absorption at 750 mμ.)

A noticeable difference compared with the results of Parsons[7] was found after hydrochloric acid treatment. There was a drop in the magnitude of the absorption maximum at 430 mμ of 18–35 per cent as compared with that at 450 mμ. The pheophytin peak at 430 mμ was stable with time for at least 30 min after addition of hydrochloric acid. The concentration of hydrochloric acid in the acetone extract did not influence the magnitude of the peak at 430 mμ over the range 0·0066–0·0375 N. However, at the lower concentrations the rate of conversion to pheophytin was slow, taking about 10 min at 0·0066 N. At the concentration recommended by Parson[7] it was practically instantaneous.

The absorption curves of chlorophyll *c* from *Prymnesium parvum* in 90% acetone, isolated by chromatography, resembled those shown in Fig. 1 (except that the drop in the major peak after hydrochloric acid treatment was only 11 per cent.). The phase test was positive, the results resembling those of Granick,[8] and Strain and Manning.[9] The chromatography

[7] T. R. PARSONS, *J. Marine Research*, **21**, 164 (1963).
[8] S. GRANICK, *J. Biol. Chem.* **179**, 505 (1949).
[9] H. STRAIN and W. M. MANNING, *J. Biol. Chem.* **144**, 625 (1942).

also confirmed the presence of chlorophyll *a* and the absence of chlorophyll *b* in this organism.

In none of the cultures examined was there any significant level of chlorophyllides. The acetone extract of *Ochromonas danica* obtained by the method of Parsons[7] was, however, yellow-green, but did not appear to contain chlorophyll *c*. The absorption curves of this extract showed major peaks at 414 mμ and 665 mμ, which were hardly affected by hydrochloric acid treatment. The latter peak was about 46 per cent of the former. The curve resembled that of pheophytin *a*.

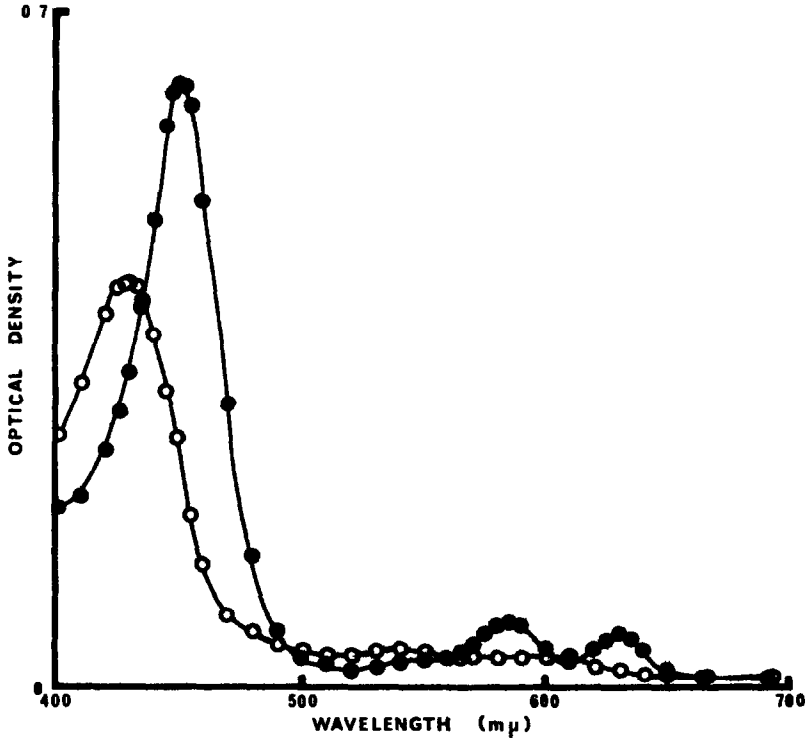

FIG. 1. ABSORPTION SPECTRA OF AQUEOUS ACETONE EXTRACTS OF *Dicrateria* SP. (PLYMOUTH NO. 133) OBTAINED BY THE METHOD OF PARSONS.[7]

(●, aqueous acetone extract; ○, aqueous acetone extract after hydrochloric acid treatment.)

DISCUSSION

The results of the absorption curves on 90% acetone extracts obtained by the method of Parsons[7] showed clearly that all the Chrysophyceae (after Fritsch[10]) examined, except *Ochromonas danica*, contained chlorophyll *c*; as did the brown alga, *Halidrys siliquosa*. Agreement between the three methods of estimation used was very poor. In particular the two green algae and *Ochromonas danica* showed the presence of chlorophyll *c* by the trichromatic method, whereas in fact there was almost certainly none present. The trichromatic method did not show the presence of chlorophyll *b* in any of the Chrysophyceae or in

[10] F. E. FRITSCH, *The Structure and Reproduction of the Algae*, Vol. 1, p. 507, Cambridge University Press (1948).

Halidrys siliquosa. The method of Parsons,[7] whilst permitting confirmation of the presence of chlorophyll *c* by the absorption curves of the acetone extract, generally gave results which were higher than those obtained by the trichromatic method. To some extent this may reflect the diminished absorption peak at 430 mμ as compared with the results described in the original method, in which no diminution on change of maxima from 450 mμ to 430 mμ was observed. If a rough correction was made for this in calculating the results (by assuming that the percentage underestimate at 450 mμ after hydrochloric acid treatment was the same as the percentage drop in magnitude on alteration of the 450 mμ peak to 430 mμ with hydrochloric acid) the chlorophyll *c* values fell within ± 30 per cent of the trichromatic ones. The chlorophyll *c* content, calculated from the absorption maximum at 630 mμ, was determined because Jeffrey[2] had shown that the stability of the extinction at the red end of the spectrum was greater than that at the blue, and had recommended that it always be used in chlorophyll *c* assays. Because the extinction was much lower in this region the sensitivity was correspondingly much lower than at 450 mμ. The method gave results which were 50–75 per cent of the trichromatic values.

As can be seen from the results the simple methods available for the determination of chlorophyll *c* give only approximate results; values sometimes varying over a two-fold range. The method of Parsons[7] was, however, particularly valuable for confirming the presence of chlorophyll *c* by means of absorption spectra. The reason for the differing results after hydrochloric acid treatment is not known, but possibly may reflect slight differences in the physical conditions of assay, particularly since the percentage drop in absorption maximum is not the same in the various algae, and the peak in the blue region is known to be somewhat unstable (Jeffrey[2]).

The lowest value obtained with the chlorophyll *c* containing algae was about 17 per cent of total chlorophylls, and the highest 55 per cent. The majority of results fell in the range 20–40 per cent of total chlorophylls. No evidence was obtained for the presence of bacteriochlorophyll in any of the bacterized cultures and there was no difference between the hexane-extracted acetone extracts of chlorophyll *c* of the axenic and bacterized cultures. It thus appeared that the presence of bacteria in some of the cultures could be neglected.

The presence of a pigment resembling pheophytin *a* in *Ochromonas danica* is unusual. If one assumes that the pigment was pheophytin *a*, it was found to constitute about 20 per cent of the total chlorophyll pigment. The cells had been harvested by centrifugation and then immediately extracted with 90% acetone. There was presumably little, if any, breakage of cells before addition to the acetone. It is to be noted that the culture medium for this organism has a pH of 5. Virtually none of this would be retained in the deposit mixed with the acetone. It thus appears unlikely that the pheophytin was an artefact of preparation. It could be that some intracellular compartments of the cell have a sufficiently low pH to effect conversion of chlorophyll *a* to the pheophytin.

The classification of the algae is at present in a state of flux and the recent reclassification of Parke and Dixon[11] has placed the majority of the organisms (all except Ochromonas, and the green and brown algae) here described, into the new Class Haptophyceae. It is noteworthy that chlorophyll *c* was demonstrated in all these organisms except Ochromonas, which still remains in the Chrysophyceae (as do Sphaleromantis and Monochrysis, although their classification is not certain). It is possible that although no chlorophyll *c* was demonstrated in *Ochromonas danica*, this might be due to its heterotrophic mode of growth. Parsons[3] found

[11] M. PARKE and P. S. DIXON, *J. Marine Biol. Assoc. United Kingdom*, **44**, 499 (1964).

that *Monochrysis lutheri* contained only about 5 per cent of its total chlorophyll as chlorophyll *c*. This proportion was increased by growth in a nutrient-deficient medium at high light intensity and lower temperature.

EXPERIMENTAL

Cultures

Chrysochromulina chiton (Plymouth No. 146), *Chrysochromulina* sp. (Plymouth No. 200), *Chrysochromulina* sp. (Plymouth No. 137), *Isochrysis galbana* (Plymouth I), *Dicrateria* sp. (Plymouth No. 133), *Dunaliella primolecta* (Plymouth No. 81) were grown in Erdschreiber medium at 14° with the regimen of 16 hr light (200 lumen per sq. ft) and 8 hr darkness per day. The cultures were unialgal, but not bacteria-free, and were originally provided by Dr. Mary Parke of the Marine Laboratory, Plymouth. *Ochromonas danica* (axenic) was provided by the Culture Collection of Algae and Protozoa, Cambridge, and grown in a medium containing KH_2PO_4 30 mg; $MgSO_4 \cdot 7H_2O$ 100 mg; glucose 1 g; $NaNO_3$ 200 mg; glutamic acid 100 mg thiamine HCl 1 mg; biotin 10 μg; vitamin B_{12} 1 μg; soil extract 40 ml; distilled water to 1 l., pH 5, sterilized by autoclaving. *Pedinomonas minor* was provided by Dr. Hans Ettl as a unialgal, bacteria-containing culture. This was grown in a medium containing K_2HPO_4 1 mg; $MgSO_4 \cdot 7H_2O$ 2·05 mg; $FeCl_3 \cdot 6H_2O$ 0·168 mg; KNO_3 10 mg; soil extract 2 ml; distilled water to 100 ml; sterilized by autoclaving (Ettl—personal communication). The culture had been rendered axenic by a dilution technique. *Ochromonas danica* and *Pedinomonas minor* were grown under the physical conditions described above. *Prymnesium parvum* (axenic, Israeli strain) was grown as described in Ricketts.[12] *Halidrys siliquosa* was a gift from Dr. Leonard Evans of the Department of Botany, Leeds.

Chlorophylls

Cultures were normally harvested by centrifugation at 1000 *g*/10 min after about one month of growth. The deposits were then pooled and centrifuged in a smaller tube at 1000 *g*/5 min. The supernatants were decanted and drained to remove as much liquid as possible and immediately treated with 90% aqueous acetone in the dark. (The *Halidrys siliquosa* was cut into small pieces and homogenized in 90% acetone before centrifuging). After about 5 min the suspension was centrifuged and the supernatant decanted for determination of the chlorophylls by the method of Parsons and Strickland.[6] This involved measurement of optical densities at 630, 645 and 665 mμ and then calculation of the chlorophyll concentrations using simultaneous equations. The residue was always practically colourless after this procedure. After determination in this way an aliquot of the 90% acetone extract was taken for determination of chlorophyll *c* by the method of Parsons.[7] The method involved extraction of the aqueous 90% acetone extract with hexane under conditions which led to removal of the carotenoids and chlorophylls *a* and *b*. The chlorophyll *c* content was then determined by the decrease in optical density of the acetone extract at 450 mμ after treatment with hydrochloric acid. The organisms were also examined for the presence of chlorophyllides by the method of Parsons.[7] This was done by comparing the optical density of the hexane extract at 665 mμ with that of the original aqueous 90% acetone extract, allowance being made for the greater dilution in the former and the different absorptions in the two solvents. Differences between the two values indicated the presence of chlorophyllides.

[12] T. R. Ricketts, *J. Roy. Microscop. Soc.* **83**, 459 (1964).

The pigments of 90% acetone extracts of axenic *Prymnesium parvum* were transferred to ether by the method of Allen, Goodwin and Phagpolngarm.[13] The extract was evaporated in a stream of air (1–2 min) and chromatographed in the dark on 1·5 × 16 cm icing sugar columns (British Sugar Corporation) after dissolving the residue in petroleum spirit, b.p. 40–60°, and a few drops of ether. The column was eluted with (*a*) petroleum spirit, 5 ml, (*b*) 0·5% *n*-propanol in petroleum spirit, 5 ml; (*c*) 0·5% demethylaniline in petroleum spirit, 5 ml; (*d*) 0·5% *n*-propanol in petroleum spirit, 15 ml; and finally with (*e*) 2% *n*-propanol in petroleum spirit (After Strain, Manning and Hardin[14].) This procedure removed chlorophyll *a* and the carotenoids. Chlorophyll *c* was retained towards the top of the column as a broad light yellow-green band which was then eluted with ether containing 10% methanol. The eluate was freed of methanol by washing with water, and then diluted with ether before determining its absorption spectrum.

Acknowledgements—I would like to thank the Department of Scientific and Industrial Research for financial support.

[13] M. B. ALLEN, T. W. GOODWIN and S. PHAGPOLNGARM, *J. Gen. Microbiol.* **23**, 93 (1960).
[14] H. H. STRAIN, W. M. MANNING and G. HARDIN, *Biol. Bull. Marine Biol. Lab.* **86**, 169 (1944).