CHLOROFUCINE (CHLOROPHYLL γ), A GREEN PIGMENT OF DIATOMS AND BROWN ALGAE

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Diatoms are the most abundant autotrophic organisms over much of the earth's surface. They and the similarly pigmented brown algae produce prodigious quantities of organic matter. Synthesis of this carbonaceous material, so essential to life in the sea and probably to the formation of petroleum, depends upon absorption of light by pigments in the living algal cells. The number and nature of the green pigments contained in these plants are, however, matters of controversy.

Chlorophyll *a* occurs in all the diatoms and brown algae examined thus far (1–16), but there is no unanimity of opinion regarding the nature and origin of another green substance observed in extracts of these organisms. This second green pigment, originally called chlorofucine (2), later chlorophyllin γ (5, 6), chlorophyll γ (7), and chlorophyll *c* (8), was at first considered a natural constituent of these algae (1–6). Later, from results obtained through the use of an involved analytical method, Willstätter and Page (7) concluded that chlorofucine was a postmortem product. With the exception of Wilschke ((8) p. 355), subsequent workers accepted this conclusion as authoritative even though several of them observed chlorofucine in the extracts of fresh algal material (9–11). Chlorofucine was not observed in land plants (2). It should not be confused with a controversial "chlorophyll *c*" of higher plants (17) which is now believed to have been a mixture of chlorophyll *a* and pheophytin *a* (18).

The occurrence of small amounts of chlorophyll *b* in brown algae was reported by Willstätter and Page (7). There is also one recent report of the occurrence of this pigment in diatoms (16). Chlorophyll *b* has not been detected by other workers who applied various analytical methods to diverse algal species in both these groups (1–6, 8–11, 13, 14). The results of the various pigment investigations are presented briefly in Table I.

In view of the contradictory conclusions just reviewed, it seemed desirable to reexamine the green pigments both of diatoms and of brown algae. Knowledge gained from this reexamination should aid in the interpretation of measurements of photosynthesis in these organisms (14, 15).

TABLE I

Reported Occurrence of Chlorophyll b and Chlorofucine in Various Algae

Bibliographic reference No.	Author	Year	Method	Class of algae*	Chlorophyll *b*	Chlorofucine
1	Stokes	1864		B.	−	+
2	Sorby	1873	Partition; spectral absorption	"	−	+
3	Reinke	1876	" "	" D.?	−	+
4	MacMunn	1885	Spectral absorption of extract	"	?	+
5	Tswett	1905	Partition; spectral absorption	" D.?	−	+
6	"	1906	" "	"	−	+
7	Willstätter, Page	1914	HCl alteration	"	0.05 × *a*	−
8	Wilschke	1914	Fluorescence of extracts	" Y.,D.	−	+
9	Kylin	1927	Partition; capillary adsorption	" D.	−	−(?)
10	Bacharach, Dhéré	1931	Fluorescence of extracts	D.	−	+
11	Dhéré, Fontaine	1931	" " "	B.	−	+
12	" Raffy	1935	" " tissue	"	−	−
13	Seybold, Egle	1938	Chromatographic adsorption	" D.	−	−
14	Montfort	1940	" "	" "	−	−
15	Dutton, Manning	1941	" "	D.	?	−
16	Pace	1941	" "	"	0.1 × *a*	−

*B., brown (Phaeophyceae); D., diatom (Bacillariophyceae); Y., yellow (Chrysophyceae).

EXPERIMENTAL

Plant Material—The pennate diatom *Nitzschia closterium* was grown in pure culture by a procedure similar to one previously described (15), except that "snow-white" fluorescent lamps were used instead of a neon lamp. 1 liter of culture usually yielded 0.5 to 1.0 ml. of centrifuged cells. Brown algae were collected at low tide at Moss Beach, north of Half Moon Bay, California. They were identified by Dr. Gilbert M. Smith of Stanford University. Specimens that were not used immediately were kept indoors in shallow open dishes of sea water at a temperature not higher than 17°.

Methods—Three general methods have been used for analysis of the chlorophylls in extracts of the algae. The principles of the methods are summarized here. Essential details are described in the sections pertaining to preparation of the individual compounds.

By partition of the pigments between immiscible solvents such as petroleum ether and 80 to 90 per cent methanol, it was possible to separate

chlorophyll *a* and carotene from the xanthophylls and chlorofucine. After dilution of the methanol fraction to 50 per cent with water, most of the xanthophylls were removed from the alcohol-soluble chlorofucine by repeated extraction with ether.

Numerous modifications of the chromatographic adsorption method have been tested for the preparation of chlorophylls. In general the best results were obtained with columns of confectioner's powdered sugar which contained 3 per cent corn-starch to prevent caking. Before use, this sugar was dried at 85° for several hours and was then cooled in closed bottles. In packing the columns, successive small portions of sugar were pressed firmly into the adsorption tube (usually 3 × 30 cm.) with a packing plunger slightly smaller than the tube ((19) p. 42). The filtration rate of columns packed with the "C and H" (California and Hawaiian) brand was nearly twice that of columns of the "Sea Island" (Western Sugar Refinery) brand, although both brands were labeled "Grade XXXXXX." Results nearly as satisfactory were obtained with inulin or with some preparations of starch as adsorbents.

A photoelectric spectrophotometer (20) was used for determination of the spectral absorption of pigments in the extracts of plant material, and for the identification of pigments prepared by partition and chromatographic adsorption methods. Most of the results are presented as the so called characteristic absorption curves, the plot of $\log \log(I_0/I)$ *vs.* wavelength (21). For a pure pigment, characteristic absorption curves measured at any concentration are superposable. Variations in the shape of the curves, which are readily observable by superposition, indicate contamination of the pigment with other colored substances. This method of presentation also simplifies the comparison of curves when absolute absorption coefficients are not available. Absorption curves plotted as the $\log (I_0/I)$ are illustrated by small insets in Figs. 1, 2, and 11.

Extraction of pigments from diatoms was incomplete when acetone (absolute or 80 per cent) or ethanol (absolute or 95 per cent) was used as solvent. The pigments were removed rapidly and completely with absolute methanol. Consequently, this solvent was used for determination of the spectral absorption curves of the algal extracts.

Chlorophylls a and b from Sunflower—For standards of comparison it was necessary to obtain spectral absorption curves of chlorophylls *a* and *b* in methanol. These pigments were prepared from sunflower leaves by the partition and chromatographic adsorption procedures employed with algal extracts. About 2 gm. of fresh leaves were extracted rapidly at 20° with 100 ml. of absolute methanol containing about 0.5 per cent redistilled dimethylaniline. (The dimethylaniline served to neutralize plant acids during extraction, to counteract acidity which might otherwise develop

upon the adsorption columns ((19) p. 123), and to minimize pigment oxidation.) Petroleum ether (100 ml.) was added to the extract, followed by about 15 ml. of water. After separation of the methanol layer, the petroleum ether phase was extracted twice with 50 ml. portions of 90 per cent methanol in order to remove the remaining xanthophylls. Residual methanol was removed from the petroleum ether with water, and the green solution was concentrated to a few ml. at reduced pressure and 20°. This concentrated solution was poured onto a column of sugar (3 × 15 or 20 cm.) which was then washed with small portions of petroleum ether containing 0.5 per cent dimethylaniline. As soon as the carotene had been washed

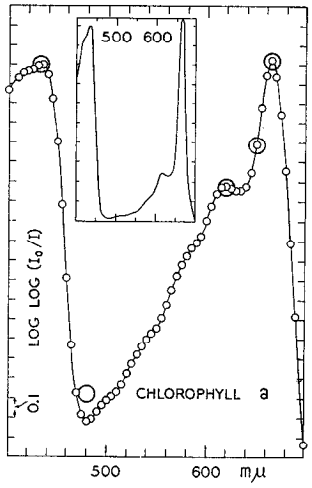

FIG. 1

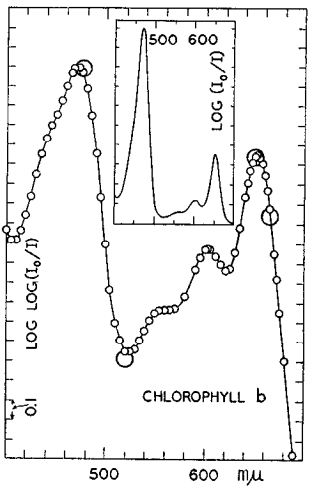

FIG. 2

FIG. 1. Absorption curve of chlorophyll *a*. The inset shows the same data plotted as log (I_0/I). Solvent, methanol. The large circles represent values calculated from Mackinney's results (22).

FIG. 2. Absorption curve of chlorophyll *b*. Solvent, methanol. The large circles are from Mackinney's data (22).

below the chlorophyll, the chromatogram was developed with petroleum ether containing 0.5 per cent dimethylaniline and 0.5 per cent methanol or *n*-propanol. Chlorophyll *a*, which was carried rapidly through the column, was collected separately in the percolate. The yellow-green chlorophyll *b* band moved slowly down the column. This band was removed with a spatula, and the pigment was eluted with freshly distilled ether (U.S.P.). The solution of each chlorophyll was then evaporated nearly to dryness at reduced pressure (20 to 30 mm.). To each residue was added the solvent in which the spectral absorption was to be measured. The remaining ether was removed in a vacuum, and the solutions were then diluted to the

proper concentrations for absorption measurements. The entire preparation required about 1 hour. Consistent spectral absorption curves were usually obtained.

Absorption curves of chlorophylls *a* and *b* in methanol (Figs. 1 and 2) were in satisfactory agreement with values previously determined at a few wave-lengths (22). The absorption by chlorophyll *a* in methanol was quite different from the absorption in ether. The differences were due to solvent effects, rather than to irreversible pigment alteration, because an absorption spectrum of the pigment determined in ether after prior determination in methanol showed the spectral curve typical of ether solutions.

Because of the great lability of the chlorophylls, great care had to be exercised in handling the plant material and also the extracted pigments. If too little alcohol was used for extraction of the pigments, if extraction took too long, or if the chlorophyll remained on the adsorption columns very long, especially in the absence of dimethylaniline and alcohol, additional colored zones were observed on the sugar. In such cases, spectral curves of the recovered chlorophylls exhibited considerable variation in the region between 470 and 600 mμ. If fresh plant material was permitted to stand with dilute alcohol for a day or more, as is often done in the estimation of chlorophyllase activity (23), and if the pigments were then extracted and adsorbed on columns of sugar, as many as fifteen distinct green bands were observed.

Results

Chlorophyll a—Chlorophyll *a* prepared from *Nitzschia closterium* by adsorption agreed in spectral properties, and in adsorption behavior (*cf.* (19) p. 12), with chlorophyll *a* from sunflower leaves (Fig. 3). Moreover, in the red region of the spectrum, where the presence of small amounts of yellow pigment did not interfere, the curve for chlorophyll *a* prepared from *Nitzschia closterium* by partition between petroleum ether and 80 to 90 per cent methanol was in good agreement with curves for preparations obtained by adsorption (Fig. 3). Since most of the green pigment remained in the petroleum ether, the results prove that chlorophyll *a* is the principal green pigment in the organism. Had chlorophyll *b* been present in appreciable quantities, it would have appeared with chlorophyll *a* in the partitioned extract. Incidentally, Fig. 3 indicates that the adsorption procedure described above did not alter the spectral properties of chlorophyll *a*.

Natural Occurrence of Chlorofucine—Fig. 4 shows for the red region of the spectrum a typical absorption curve for a methanol extract of *Nitzschia closterium*. Although the position of the principal absorption maximum and the shape of the curve indicate a large proportion of chlorophyll *a* in the extract, the curve for the extract differs significantly from the chloro-

phyll *a* curve. Similar differences were observed when acetone extracts were examined.

Various methods of extraction, some of which had been employed by Wilschke (8), had little or no effect on the shape of the curve for total pigment absorption, as illustrated in Fig. 5. In one case, diatoms from 1 liter of culture were centrifuged, suspended in 1 ml. of water, placed in a boiling water bath, and diluted with 15 ml. of boiling water. After 1.5 minutes the suspension, which had turned a bright green, was cooled quickly with ice, centrifuged, and the cells extracted with methanol. A similar quantity of the same culture, suspended in 1 ml. of water, was shaken in a current of hydrogen for 10 minutes and then extracted with methanol in the stream

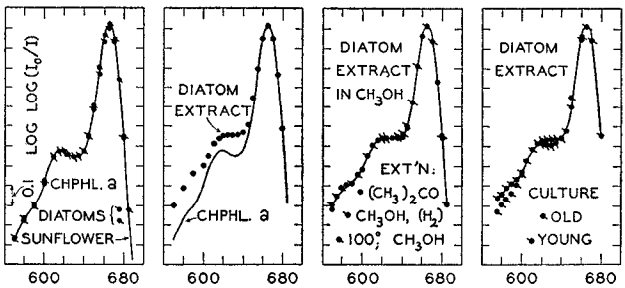

FIG. 3 FIG. 4 FIG. 5 FIG. 6

FIG. 3. Absorption curves of chlorophyll *a* from sunflower leaves and from diatoms by partition (↓) and by adsorption (↑). Solvent, methanol.

FIG. 4. Absorption curves of diatom extract and of chlorophyll *a*. Solvent, methanol.

FIG. 5. Absorption curves of extracts prepared from diatoms by various methods (see the text). Solvent, methanol.

FIG. 6. Absorption curves of extracts prepared from old and young cultures of diatoms. Solvent, methanol.

of hydrogen. A preparation of cells from another culture was extracted with acetone (80 per cent) and the resulting solution then evaporated at reduced pressure and room temperature. The residue obtained in this way was dissolved in methanol and its absorption curve was determined (Fig. 5).

The close agreement between all these absorption curves indicates that the deviation from the characteristic chlorophyll *a* curve is probably due to another pigment, or pigments, normally present in the living cells, rather than to a postmortem product. Furthermore, the spectral curve of methanol extracts of young, rapidly multiplying cultures of diatoms indicated a higher proportion of other pigment than that found in very old, more concentrated cultures (Fig. 6). The spectral absorption curves of methanol

extracts of brown algae (Fig. 7) were similar to those of *Nitzschia* extracts (Figs. 4 and 5). In Fig. 7, the absorption for *Fucus* indicates a smaller proportion of pigments other than chlorophyll *a*.

Fig. 8 shows a calculated curve representing the difference between the curve for diatom extract in Fig. 4 and that for chlorophyll *a*. This calculated curve is slightly influenced by yellow pigment (fucoxanthin) absorption below 610 mμ and is not continued below 570 mμ because of the increasing xanthophyll absorption. Indirect determination of green pigment absorption at shorter wave-lengths (24) was not feasible, because removal

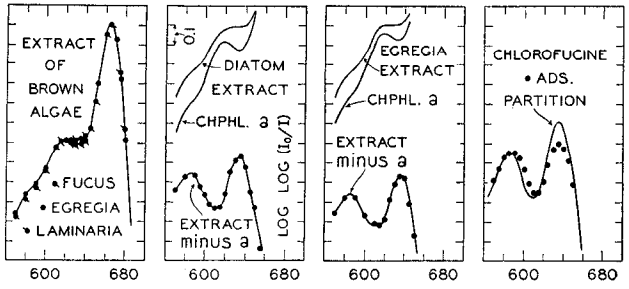

FIG. 7 FIG. 8 FIG. 9 FIG. 10

FIG. 7. Absorption curves of extracts of the brown algae, *Fucus furcatus*, *Egregia menziesii*, and *Laminaria andersonii*. Solvent, methanol.

FIG. 8. Calculated absorption curve representing the difference between absorption by a diatom extract and absorption by chlorophyll *a* in this extract. The lowest curve represents log (log (I_0/I) for extract minus log (I_0/I) for chlorophyll *a*) assuming from Fig. 4 that chlorophyll *a* absorbs 99 per cent of the light at 665 mμ.

FIG. 9. Calculated absorption curve representing the difference between absorption by an extract of *Egregia* and by chlorophyll *a* (*cf.* Fig. 8).

FIG. 10. Absorption curves of chlorofucine prepared by adsorption and by partition (see the text). Solvent, methanol.

of the chlorophylls through saponification resulted in alteration of several algal xanthophylls (7, 25). Fig. 9 shows a calculated curve for the brown alga *Egregia*. The calculated curves obviously do not correspond to chlorophyll *b*, but are in good agreement with the characteristic curve of chlorofucine prepared by partition of methanol extracts of *Nitzschia* (see below, Fig. 11). The agreement is particularly good in view of the fact that the calculation is based on relatively small differences between large absorption values (differences from one-twentieth to one-third of the total absorption of the extracts).

The results summarized in Figs. 4 to 9 lead to the conclusion that chlorofucine, rather than chlorophyll *b*, is the second green pigment of the diatoms and brown algae examined in this investigation.

Absence of Chlorophyll b—Chlorophyll *b*, in amounts up to 1 or 2 per cent of the amount of chlorophyll *a* (Fig. 3), would affect only slightly the spectral absorption of the extracted pigments and thus might escape detection in the original extract (Fig. 4). More sensitive tests for the presence of chlorophyll *b* were therefore employed.

Separate experiments revealed that extremely small quantities of chlorophyll *b* contained in extracts of barley leaves could be detected by adsorption upon columns of sugar (Sea Island). As little as 13 γ of chlorophyll *b* formed a very distinct band on a column 2 × 12 cm., 1.3 γ formed a definite band on a column 1.4 × 12 cm., and 0.03 γ formed a barely perceptible band on a column 0.2 × 5 cm. when petroleum ether with 1 per cent methanol was used to develop the chromatogram. Even when pure chlorophyll *a* was added in great excess to an extract of barley leaves containing 1.3 γ of chlorophyll *b*, the latter formed a definite band in the presence of 2000 times this quantity of chlorophyll *a* (column 1.4 × 12 cm.).

Pure chlorophyll *b* added to extracts of *Nitzschia* was readily recoverable by use of adsorption columns even when the amount of this pigment was less than 0.5 per cent of the amount of chlorophyll *a* present in the extracts. In this case a longer adsorption column (3 × 20 cm.) was necessary, because of the influence of other substances upon the adsorption. As a result of all these experiments, it is evident that chlorophyll *b* either is absent from diatoms and brown algae or is present only in traces.

When mixed with extracts of diatoms and adsorbed, chlorophyll *b* formed a green band below the principal orange band of fucoxanthin *a*. If the columns were then washed with petroleum ether containing 2 to 3 per cent of methanol, both the chlorophyll *b* and fucoxanthin were carried through the adsorbent, leaving chlorofucine behind as a diffuse green band. This comparative test indicates that the strongly adsorbed green pigment observed by Pace (16) under similar conditions was not chlorophyll *b* as he assumed. We believe that it, as well as a similar strongly adsorbed substance observed by Dutton and Manning (15) and occasionally by Montfort (14), was chlorofucine.

Separation and Properties of Chlorofucine—For determination of its spectral absorption properties, chlorofucine was prepared in the following way. Diatoms centrifuged from about 4 liters of culture suspension were extracted with about 80 ml. of absolute methanol containing 0.2 per cent dimethylaniline. The resultant pigment solution was diluted to 200 ml. with methanol, and, after the addition of 37 ml. of water, was extracted with 100 ml. of petroleum ether. As indicated already by Fig. 3, chlorophyll *a* was the only green constituent in the petroleum ether. The methanol layer containing the xanthophylls and chlorofucine was diluted with 160 ml. of water and extracted with 100 ml. of ether (U.S.P.) and then with five 75 ml.

portions of ether. The residual pale green methanol solution was treated with about 40 ml. of ether and a large excess of strong salt solution which caused the chlorofucine to dissolve in the ether. The chlorofucine, recovered by separation and evaporation of the ether, was dissolved in methanol, and its absorption was determined (Fig. 11).

It was difficult to obtain good preparations of chlorofucine from diatoms by this method. If extraction with ether was not carried on long enough, xanthophylls (principally fucoxanthins) contaminated the residual green pigment. If too many extractions were made, all the chlorofucine was re-

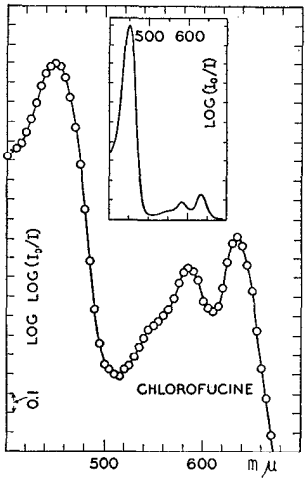

FIG. 11

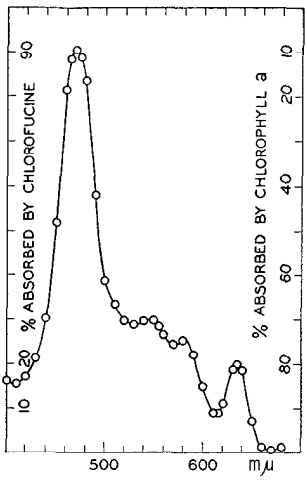

FIG. 12

FIG. 11. Absorption curves of chlorofucine prepared from diatoms by partition of the pigments between 50 per cent methanol and ether. The inset shows the same data plotted as log (I_0/I). Solvent, methanol.

FIG. 12. Relative proportions of light absorbed by chlorofucine and by chlorophyll *a* in a methanol extract of diatoms.

moved. With extracts of brown algae, fewer extractions with ether could be made before all the chlorofucine was removed from the methanol. However, with slight modifications of the method, satisfactory preparations of chlorofucine were also obtained from brown algae. Use of petroleum ether instead of ether for the partition often resulted in formation of emulsions that prevented separation of the two phases. These difficulties in the preparation of chlorofucine by partition probably account for some of the variable results obtained by earlier investigators (7–9).

Chlorofucine either from diatoms or from brown algae was easily prepared by adsorption on columns of sugar. The pigments were extracted

from the organisms with methanol and transferred to ether by partition. The ether solution was concentrated to a very small volume and diluted with petroleum ether. The resultant petroleum ether solution was passed through the adsorption column. (Direct transfer of chlorofucine from methanol to petroleum ether was incomplete, which may further explain some of the variable results obtained by others (8, 9, 13, 14).) When the chromatogram was developed with petroleum ether containing 2 to 4 per cent methanol, chlorofucine formed a pale green, diffuse band in the upper portion of the adsorbent. Below this, fucoxanthins formed orange bands. Other xanthophylls, chlorophyll *a*, and carotene passed rapidly through the column. Chlorofucine eluted from the green band with ether and transferred to methanol exhibited a spectral absorption curve that was significantly different from that of the same pigment prepared by partition (Fig. 10). Pigment obtained from the upper portion of the chlorofucine band on the column exhibited greater spectral variations than that obtained from the lowest portion. These differences were due, apparently, to alteration of the chlorofucine by adsorption upon the sugar rather than to the removal of other pigments. In one experiment, for example, chlorofucine prepared by partition was adsorbed on a column of sugar which was then washed with petroleum ether containing 2 per cent methanol and then with petroleum ether containing 4 per cent methanol. After 2.5 hours, all the pigments on the column were eluted with ether and alcohol, the ether was evaporated, and the spectral curve of the preparation was redetermined. It then showed changes corresponding to those observed with chlorofucine prepared directly by adsorption (Fig. 10).

Chlorofucine more like that prepared by partition was obtained when the pigments extracted from diatoms were adsorbed directly from petroleum ether containing 7.5 per cent *n*-propanol and 2 per cent dimethylaniline. Under these conditions traces of another green pigment similar to chlorofucine were observed above the chlorofucine band.

By means of adsorption, spectral absorption, or partition methods, chlorofucine was observed in all the brown algae that were examined; namely, *Fucus furcatus*, *Hesperophycus harveyanus*, *Pelvetiopsis limitata*, *Nereocystis pyrifera*, *Macrocystis integrifolia*, *Cystoseira osmundacea*, *Pterygophera californica*, *Laminaria andersonii*, *Egregia menziesii*. A more extensive investigation of *Laminaria* revealed chlorofucine both in old and young blades and in the stipes. When extracts of sunflower and of barley leaves were examined by means of the same methods employed with brown algae, chlorofucine was not observed. This result agrees with the early

Chlorofucine exhibits many properties that are similar to those of the common chlorophylls. It is altered by alcoholic KOH, yielding colored products insoluble in ether and petroleum ether and slightly soluble in

water. Chlorofucine obtained in solid form by evaporation of an ether solution is virtually insoluble in petroleum ether but readily soluble in methanol. It is insoluble in water and in dilute aqueous solutions of sodium carbonate and ammonia.

Solutions of chlorofucine are strongly fluorescent (11), although apparently less so than solutions of chlorophyll *a*. When the fluorescent light produced by exposure of an ether solution of the chlorofucine to light from a mercury arc was examined in the photoelectric spectrophotometer, a fluorescence band was observed at 635 mμ, near the absorption band in the red, but no fluorescence was observed at wave-lengths corresponding to the absorption band in the yellow. These results are in agreement with those of Wilschke (8) and of Dhéré and Fontaine (11). There appeared to be a weak, secondary fluorescence maximum in ether at about 690 mμ.

Chlorofucine prepared by adsorption and dissolved in U.S.P. ether had absorption maxima at 627, 579.5, and 446 mμ, in satisfactory agreement with those observed spectroscopically by Tswett (6). For a similar preparation, the maxima in aqueous acetone (80 ml. of acetone to 20 ml. of water) were at 631, 581, and 446 mμ. As with chlorophylls *a* and *b*, the spectral absorption maxima of chlorofucine in both these solvents were more pronounced than those in alcohol solutions.

DISCUSSION

Because diatoms are the principal photosynthetic organisms over some four-fifths of the earth's surface, it appeared of interest to calculate the relative amounts of light absorbed by chlorofucine and chlorophyll *a* in methanol extracts of *Nitzschia closterium*. Results of these calculations are summarized in Fig. 12. It should be noted that light absorption by yellow pigments, which exceeds the absorption by chlorophyll in the blue-green region of the spectrum (15), does not enter into the calculation. Between 455 and 490 mμ the amount of light absorbed by chlorofucine was much greater than that absorbed by chlorophyll *a*. Similar results were obtained for extracts of several species of brown algae, another class that is widely distributed, and of great quantitative importance over large areas (*e.g.*, *Sargassum* in the Sargasso Sea). One is forced to the conclusion that chlorofucine may be an important pigment in the carbon economy of nature.

Demonstration of chlorofucine as a normal constituent of diatoms and brown algae indicates that pigments of the photosynthetic apparatus may be subject to greater variation than had been previously supposed. This leads to the supposition that further variations in the chemical nature of green pigments may be discovered in various plant species and mutants.

In general properties chlorofucine is so similar to chlorophylls *a* and *b* that it should probably be regarded as a chlorophyll type of pigment. Now that the existence of a so called chlorophyll *c* in higher plants has been

questioned (26) and the original claims of discovery have been retracted (18), it may become desirable to change the name chlorofucine to chlorophyll *c*, as first proposed by Wilschke (8).

SUMMARY

In addition to chlorophyll *a*, diatoms and brown algae contain chlorofucine, another green, chlorophyll-like pigment. Neither group of organisms contains detectable quantities of chlorophyll *b*. Chlorofucine is a normal constituent of the cells rather than a postmortem product. Although readily susceptible to chemical alteration, this green pigment has been separated from the other green and yellow pigments, and its spectral absorption properties have been determined. In certain regions of the spectrum, chlorofucine may absorb considerably more light than the chlorophyll *a* contained in the plant cells, an indication that this pigment may play an important rôle in the phenomenon of photosynthesis.

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