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ON THE CHLOROPHYLLS SEPARATED BY PAPER CHROMATOGRAPHY FROM CHLORELLA EXTRACTS

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SUMMARY

Several chlorophylls were found to be present in low quantities in Chlorella extracts besides standard chlorophyll *a* and *b*. They included chlorophyll *a* and *b* isomers, allomerized chlorophyll, chlorophyllides and a particular protochlorophyll-like pigment. The chromatographic separation and some of the properties of these special chlorophylls are described. Their possible nature is discussed.

INTRODUCTION

In recent years, evidence has accumulated showing that, as predicted by earlier investigators (LUBIMENKO[1]), different chlorophyll forms are present in the green cells[2–6]. It is generally considered that these forms correspond to different modes of attachment of one unique species of chlorophyll molecules (*a* or *b*) to a protein carrier, or to the formation of dimers[6,26].

We intend to present arguments supporting another view, namely that the *in vivo* forms not only differ in their attachment to the protein but also in the structure of the attached chlorophyll molecule itself. In this paper we show that small quantities of several pigments distinct from typical chlorophylls (*a* and *b*) are present in Chlorella extracts. In a second paper, we shall demonstrate that some of the *in vivo* forms correspond to distinct chlorophyll species *in vitro*.

MATERIALS AND METHODS

Chlorella culture and extraction

We used *Chlorella vulgaris* (Pirson strain) cultivated under alternating light (16 h) and dark (8 h) periods. The light was provided by a Philips 50-W fluorescent lamp. MYERS'[7] culture medium was used. Air was continuously bubbled through the algal suspension. Under these conditions a good growth was obtained.

Dark green suspensions were centrifuged and the algae extracted at room temperature (between 20° and 25°) with different solvents (acetone, ether, ethanol and methanol). In some cases, indicated in the text, hot ethanol was used. The ethanol and methanol extracts were first transferred to light petroleum ether (boiling at about 40°) before chromatography. Acetone and ether extracts were chromatographed directly.

Paper chromatography methods

Whatman No. 1 paper was used. The extracts were first subjected to modified CHIBA AND NOGUCHI[8] chromatography (CN. chromatography). The spots were collected in diethyl ether (Merck *pro analysi*) and rechromatographed following SIRONVAL[9] (S. chromatography). The CN. chromatography is a descending one-dimensional chromatography, using toluene as developing solvent. The S. chromatography is also a descending chromatography: the solvent is a mixture of benzene–light petroleum ether–acetone (10:2.5:2, v/v). The atmosphere in the chamber was always carefully saturated with vapours of this mixture before S. chromatography. The chromatograms were run at laboratory temperature (20–25°) in darkness.

Pheophytin

0.2 ml of 0.2 N HCl was added to 2 ml of a given chlorophyll solution in pure diethyl ether. After 1 h at room temperature in the dark, the S. chromatography procedure was used and the pheophytin was eluted in diethyl ether (or in acetone for the preparation of Cu-chlorophylls). The samples were eventually rechromatographed for purification.

Cu-chlorophyll

To 0.5 ml of a pheophytin solution in acetone, 2 ml of a $CuSO_4$ solution were added. The mixture remained 30 sec in a water bath at 100°. The Cu-chlorophyll was extracted from the mixture by 2 ml of light petroleum ether. Purification was achieved by CN. chromatography followed by S. chromatography. The pigment was eluted in diethyl ether and rechromatrographed when necessary.

Allomerized chlorophyll

Allomerized chlorophyll was prepared by the method of WATSON AND LIVINGSTON[10]. $2.7 \cdot 10^{-5}$ M chlorophyll was shaken in air for 3.5 h at room temperature in a $0.7 \cdot 10^{-1}$ M solution of lanthanum chloride in dry methanol. After transfer into petroleum ether, the S. chromatography procedure was used. Different fractions were obtained, one of which corresponded to "Fraction 2" of HOLT AND JACOBS[15,27] (see below).

Spectra

The visible spectra of purified pigments (after suitable rechromatography) were drawn using a Cary Model-15 spectrophotometer. Infrared spectra of some pure pigments were recorded in KBr using a Leitz spectrophotometer by the micromethod of METZNER AND BORNEFELDT[11].

Solvents

The solvents, both for extraction and for chromatography or spectra, were always Merck *pro analysi*. The light petroleum ether boiled at 40°.

RESULTS

Preparation of the different chlorophylls

The CN. chromatography of a total Chlorella extract (just prepared) gave, at the solvent front, the bulk of the carotenoids and pheophytins (probably a mixture).

In the middle of the chromatogram was found a green-bluish zone containing essentially the chlorophylls of type *a* (zone *a*), and below the starting line, a bright-green zone containing essentially the chlorophylls of type *b* (zone *b*). The *a* zone and the *b* zone were eluted separately in diethyl ether. The solutions were evaporated on the starting line of two new chromatograms and were chromatographed (by S. chromatography) for a second time.

The S. chromatography of the *a* pigments separated four zones, numbered 1, 3, 4 and 5 from the solvent front to the starting line. The chromatography of the *b* pigments gave three zones, numbered 1–3 (Fig. 1).

a_1 and b_1 were visible in ordinary light. But, owing to their low quantities, the other pigments were seen only by their red fluorescence under ultraviolet light. After delimitation under ultraviolet light, the different zones were eluted in minimum diethyl ether. The process was repeated several times with new quantities of the total (freshly prepared) extract in order to accumulate more and more of the separated pigments.

After concentration to an extinction of about 1000 in a 1-cm cuvette at 660 mμ in diethyl ether, the different chlorophylls were purified by successive CN. and S. rechromatography. a_1, a_4 and a_5 each gave one single zone in such rechromatography. But a_3 separated into two near, distinct zones. The principal one of these zones contained the bulk of a_3. The other was located below a_3 (in the direction of the solvent front). It was called a_2. The separated a_3 and a_2 pigments were rechromatographed several times.

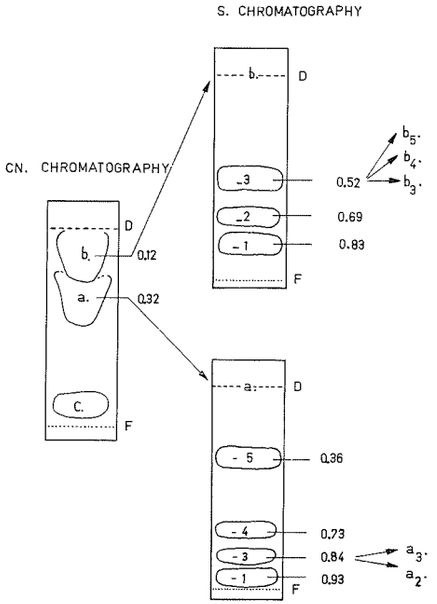

Fig. 1. The schedule used for the separation, by paper chromatography, of the chlorophylls contained in Chlorella extracts, with the approximate R_F values of the spots. CN. chromatography and S. chromatography are, respectively, chromatographic procedures following CHIBA AND NOGUCHI, and SIRONVAL (see METHODS).

The accumulated b_1, b_2 and b_3 pigments were also subjected to successive rechromatography. b_1 and b_2 appeared on rechromatography as single isolated zones, but b_3 divided into three distinct zones, which we called b_3, b_4 and b_5, from the lowest one (in the direction of the solvent front) to the highest one, near the starting line. We have succeeded in purifying b_4 by successive rechromatography. It is a pigment very similar to the *a* pigments.

The whole process is summarized in Fig. 1, where approximate R_F values of the pigments are given.

The absorption spectra of the purified pigments of type a

The absorption spectra of a_1, a_2 and a_3 are shown in Fig. 2 as recorded by a Cary Model-15 spectrophotometer. For the three pigments, the red peak was found at 660–661 mμ, the blue peak being located at 428–429 mμ. The blue:red ratios lay between 1.34 and 1.25. Minor discrepancies in the positions and relative intensities of the red and blue peaks are questionable.

The main differences between the a_1, a_2 and a_3 spectra occurred: (1) in the wavelength region below 429 mμ; (2) in the form of the long-wavelength sides of the blue and red peaks; (3) in the half band width of the red peak.

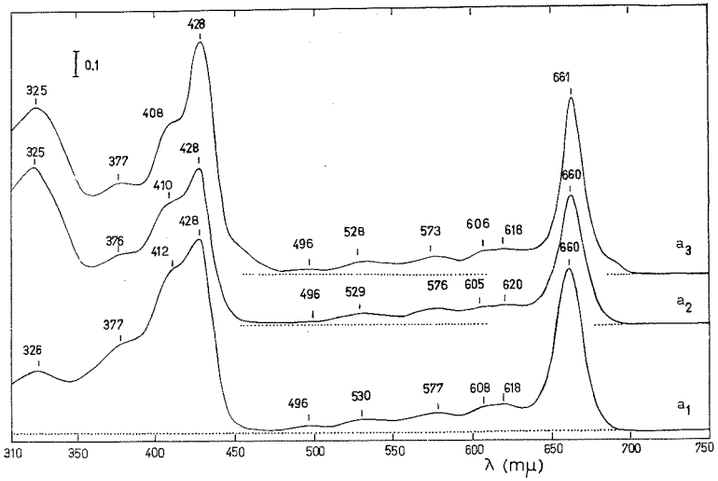

Fig. 2. Absorption spectra of chlorophylls a_1, a_2 and a_3 in diethyl ether.

Table I gives the ratios between the absorbancies at 428 mμ (blue maximum) and the absorbancies at 325 or 410 mμ. It is clear that a_2 and a_3 absorb relatively much more at 325 mμ than does a_1.

The spectrum of a_3 is of particular interest. As seen in Figs. 2 and 3, this pigment absorbed additionally in the 450-, 455- and 690-mμ regions, just at the base of the long-wavelength side of the blue and red peaks. Moreover a_3 had a red half band width significantly narrower than the corresponding widths of a_1 and a_2 (17.5 mμ for a_3 as compared with 19.5 and 19.1 mμ, respectively, for a_1 and a_2). These data, together with the particular chromatographic behaviour of each of the three pigments, support

TABLE I

PRINCIPAL CHARACTERISTICS OF THE SPECTRA OF THE *a* CHLOROPHYLLS (IN DIETHYL ETHER)

Designation of the pigments	*Position of the main absorption bands (mμ)*				*Ratios between the absorptions of the main bands*			MOLISCH *phase test*
	1	*2*	*3*	*4*	*3:4*	*3:2*	*3:1*	
a_1	326	412	428	660	1.25	1.20	3.06	+
a_2	325	410	428	660	1.29	1.28	0.97	+
a_3	325	408	428	661	1.34	1.56	1.40	+
a_4	323	396	417	653	1.82	1.91	1.90	—
a_5	325	410	430	662	1.23	1.49	1.50	+
b_4	326	410	430	663	1.22	1.45	2.58	+

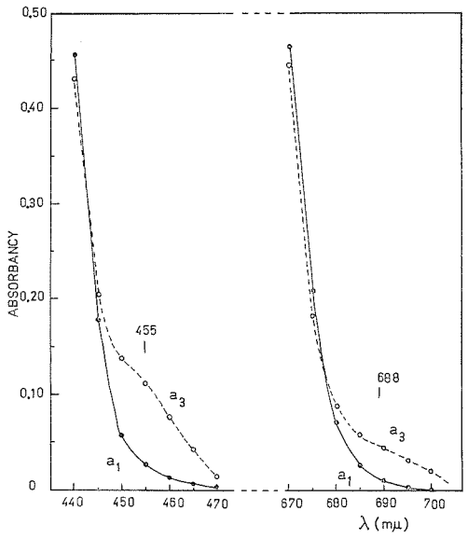

Fig. 3. Details of the absorptions of chlorophylls a_1 and a_3 at the long-wavelength side of the blue and red peaks (in diethyl ether).

the view that the pigments were different. It is interesting that, for the three chlorophylls, the shoulder at about 410 mμ never appeared as a distinct peak, and that the first maximum at the short-wavelength side of the red peak (at about 620 mμ) appeared very flat, tending to divide into two minor bands located at 605–608 and 618–620 mμ (Fig. 4).

The spectrum of a_4 in diethyl ether is shown in Fig. 5. In comparison with the corresponding maxima of a_1, a_2 and a_3 the positions of the red and blue maxima were shifted about 10 mμ to shorter wavelengths (respectively 653 mμ and 417 mμ). The blue:red ratio was high, near 2 (1.81–1.82; Table I). Nothing particular was seen on the long-wavelength side of the red and blue peaks. The first maximum on the short-wavelength side of the red peak (at 602 mμ) was well defined without tendency to

divide into two minor bands (Fig. 4). The shoulder at the short-wavelength side of the blue peak had almost disappeared (a feeble shoulder was located at 396 mμ). The peak at about 325 mμ was clearly visible, as it was in a_2 and a_3 (compare Fig. 5 with Fig. 2). The half band width of the red peak was 18.2 mμ.

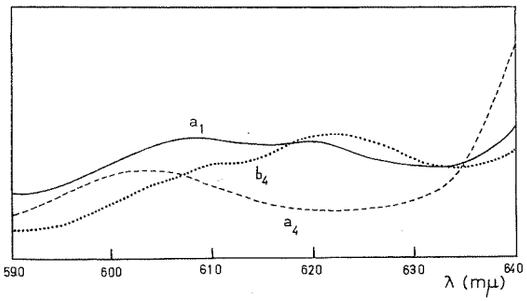

Fig. 4. Details of the absorptions of chlorophylls a_1, a_4 and b_4 in the 620-mμ region. a_2 and a_3 behave like a_1; a_5 like b_4 (in diethyl ether).

The spectra of a_5 and b_4 are given in Fig. 6 (in diethyl ether). They were found to be very similar except at the shorter wavelengths, below 430 mμ, where differences of the type shown between a_1 and a_3 were observed. In comparison with the spectra of a_1, a_2 and a_3, the spectra of a_5 and b_4 were both shifted about 1–2 mμ to longer wavelengths (blue peak at about 430 mμ; red peak at 662 mμ). Fig. 4 shows that in the 620-mμ region the spectra were characterized by the presence of two separated maxima located at 606–607 and 618–621 mμ, as in a_1, a_2 and a_3. However, in a_5 and b_4 the 618–621-mμ maximum was more pronounced than in a_1, a_2 and a_3. Nothing particular was found at the long-wavelength side base of the red and blue peaks (contrary to a_3). The half band width of the red peak was the same for a_5 and b_4: 17.6 mμ, a value very near to that found for a_3.

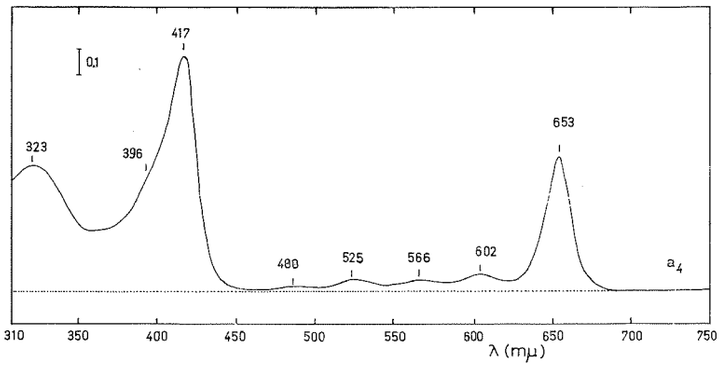

Fig. 5. Absorption spectrum of chlorophyll a_4 in diethyl ether.

The probable nature of the isolated pigments of type a

Among the different isolated pigments, a_1 was the most abundant. It certainly corresponds to standard chlorophyll *a*. The positions of the red and blue peaks in

diethyl ether agree well with the recent data of STRAIN *et al.*[12] and of PERKINS AND ROBERTS[13]. The ratio blue:red (1.25) is of the order of magnitude usually found for chlorophyll *a*. The form of the spectrum below 400 mμ is similar to that found by ZSCHEILE AND COMAR[14].

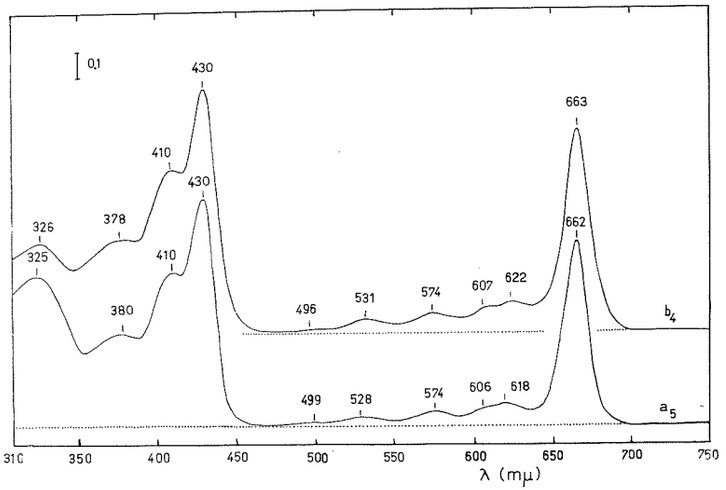

Fig. 6. Absorption spectrum of chlorophylls a_5 and b_4 in diethyl ether.

As shown above, a_2 and a_3 were different from a_1. However, they were certainly not pheophytins and they gave, as did a_1, a positive MOLISCH phase-test. Moreover, the facts (1) that the blue and red peaks were found in diethyl ether at the positions characterizing a_1, and (2) that the blue:red ratio was about 1.30 for both pigments, are good arguments for considering a_2 and a_3 as structurally similar to a_1, *i.e.* as isomers.

Removal of Mg^{2+} gave three distinct pheophytins a_1, a_2 and a_3. The spectra of pheophytins a_1 and a_3 are shown in Fig. 7. Pheophytin a_3 (like pheophytin a_2) absorbed much more than pheophytin a_1 in the region of the 320-mμ peak. Moreover pheophytin a_3 (like chlorophyll a_3) absorbed more than a_1 and a_2 at the base of the long-wavelength side of the blue and red peaks (Fig. 8). Introduction of copper in the molecule gave three distinct Cu-chlorophylls: from chlorophyll a_1, essentially Cu-chlorophyll a_1 was obtained; chlorophyll a_2 gave a mixture of equivalent amounts of Cu-chlorophyll a_1 and a_2 (this was possibly due to imperfections in a_2 isolation); chlorophyll a_3 gave Cu-chlorophyll a_3. The R_F values of the Cu-pigments were 0.90, 0.46 and 0.67, respectively, for Cu-chlorophyll a_1, a_2 and a_3 (S. chromatography). Their absorption spectra were found to be similar; in particular no clear spectral differences could be found between Cu-chlorophylls a_1 and a_3. Fig. 9 gives the spectrum of Cu-chlorophyll a_1 in diethyl ether.

At first sight, the reported facts do not support the view that a_1, a_2 and a_3 are identical with the isomers postulated by FISCHER[25]. The stability of the three forms during the pheophytinisation process and during the transformation into Cu-chlorophylls, indicates rather other structural differences.

The spectrum of a_4 (Fig. 5) was similar to that found by HOLT AND JACOBS[15,27] for one of the "allomerized" ethylchlorophyllides ("Fraction 2" of HOLT AND JACOBS). In fact, a_4 gave a negative MOLISCH phase test, showing that it was oxidized in the

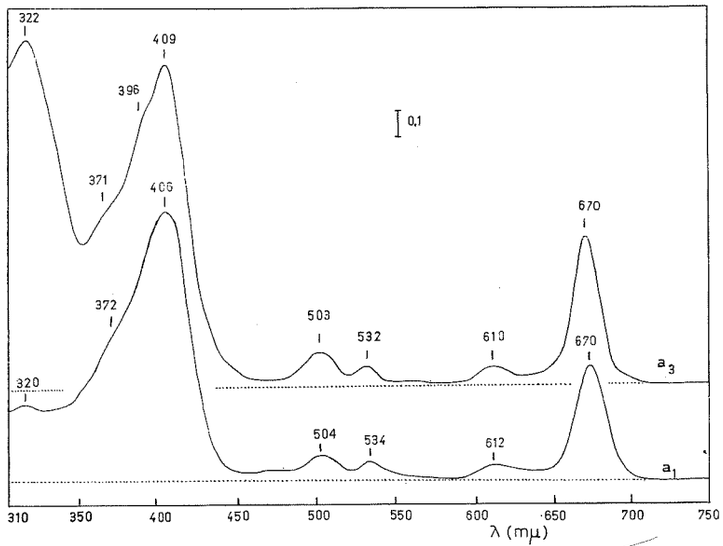

Fig. 7. Absorption spectra of pheophytins a_1 and a_3 in diethyl ether.

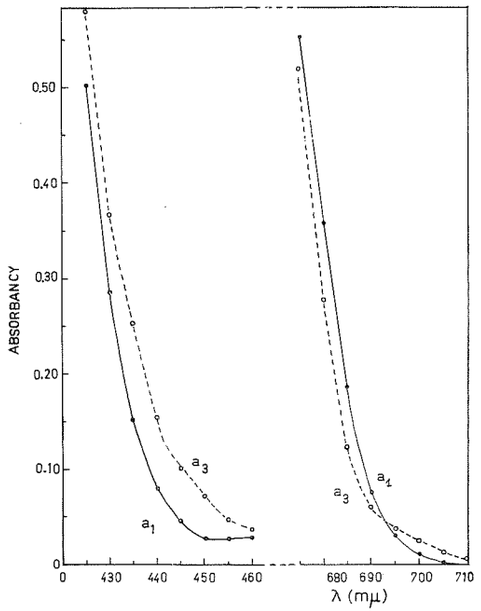

Fig. 8. Details of the absorptions of pheophytins a_1 and a_3 at the long-wavelength side of the blue and red peaks (in diethyl ether).

cyclopentanone ring. When a methanolic solution of a_1 was shaken in air in the presence of $LaCl_3$, different pigments were formed. After extraction in petroleum ether, they were subjected to S. chromatography. Three zones were isolated in this way (the pheophytins not being considered). The most abundant of these zones (zone 1) was found above the pheophytins, the two others being nearer the starting line. After successive rechromatography zone 1 gave a product the spectrum of which was found to be similar to that of "Fraction 2" and of a_4. However, the oxidized a_1 pigment (zone 1) was not identical to the a_4 pigment: cochromatography showed that allomerized a_1 separated from a_4 (it was found near the solvent front while a_4 was found nearer the starting line of the chromatogram).

The oxidation of a methanolic solution of b_4 (in the air, in the presence of $LaCl_3$) gave also several products, none of which was identical to a_4. Oxidation of a_3, a_2 and a_5 was not attempted, owing to the limited quantities available.

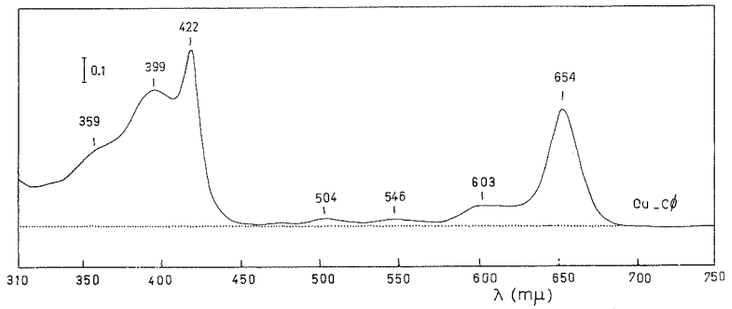

Fig. 9. Absorption spectrum of Cu-chlorophyll a_1 in diethyl ether.

Therefore we know that a_4 was an allomerised chlorophyll a, but we can not yet decide the exact nature of the chlorophyll a involved in oxidation, except that it was not a_1 or b_4.

The action of *Beta vulgaris* chlorophyllase on pure a_1 gave a product the spectrum of which in diethyl ether was near those of a_5 and b_4 (red maximum 663 mμ; blue maximum 429 mμ). However, chlorophyllide a_1 absorbed less than a_5 in the 325-mμ region (it absorbed in this region similarly to b_4). When a diethyl ether solution of a_5 (and of b_4) was shaken with 0.02 N NaOH the pigments accumulated at the interface (this was not the case with a_1 or a_3). Infrared spectrophotometry showed a clear decrease in the intensity of the 3.45-μ and 5.75-μ bands in a_5 in comparison to a_1, while a band appeared at 5.94 μ indicating the absence of phytol and the presence of a free carboxyl group in a_5. However, S. cochromatography of a_5 (or b_4) with chlorophyllide a_1 gave two distinct spots: chlorophyllide a_1 did not migrate, whereas a_5 (or b_4) did. Therefore, if, as indicated by infrared spectrophotometry, a_5 and b_4 were chlorophyllides (*i.e.* chlorophylls devoid of phytol, with a carboxyl group in the propionic side chain), they were different from chlorophyllide a_1; in particular, they were more lipophilic. We would add that a_5 and b_4 were certainly very similar products: S. cochromatography of pure a_5 with pure b_4 gave a single spot. The MOLISCH phase-test was positive for both pigments.

Some preliminary comments on the b pigments

The b_1 pigment was the most abundant. It was essentially chlorophyll b with some chlorophyll a contaminant, the nature of which is unknown.

b_2 was a mixture of two pigments: one was a chlorophyll b, probably distinct from b_1, since it separated from it; the second component was a pigment the spectrum of which was similar to that of a_4. Cochromatography of this component (mixed in b_2) with a_4 gave a single spot.

b_3 was a particular pigment, the spectrum of which is given in Fig. 10. The red maximum in diethyl ether was at 631 mμ, the blue one at 443 mμ. The blue:red ratio was about 4. The b_3 product was probably a mixture, being accompanied by minor substances absorbing in the far-red region: an absorption band was found at 685 mμ and another at about 735 mμ (Fig. 10, above).

b_5 was probably chlorophyllide b with some minor contaminants.

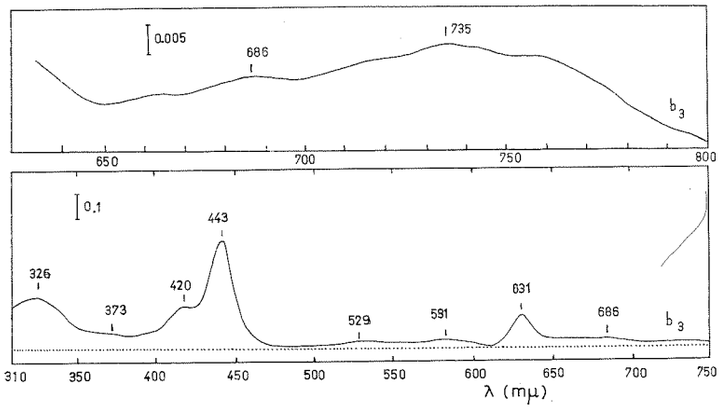

Fig. 10. Absorption spectrum of chlorophyll b_3 in diethyl ether. The spectrum between 640 and 800 mμ is given above as registered with a 10-fold magnification (Cary spectrophotometer).

Artifacts

We have already shown that the rechromatography of purified a_1, a_2 or a_3 by CN. chromatography, followed by S. chromatography, gave only pure a_1 or a_2 or a_3, and nothing else. This proves that the chromatographic procedures in themselves did not transform one of the purified pigments into another.

Moreover, it does not seem that one of the isolated pigments was produced during the time spent in the cold at 1°–2°. Table II shows that the composition of a mixture of the five a pigments (dissolved in diethyl ether) did not vary even after 1 month in the dark at 1°–2°.

Likewise, it does not seem that the nature of the extraction solvent played a determinant role in the composition of extracts from a given Chlorella suspension. Table III shows the proportions of the five pigments separated from comparable ether, acetone, ethanol and methanol extracts. The extractions were performed at laboratory temperature (about 20°) or, in some cases, rapidly in boiling ethanol. The different extraction conditions did not noticeably affect the proportions of the pigments.

Therefore, in our experiments, we failed to demonstrate that one of the

a pigments was artificially produced by the handling procedures. This of course does not prove that they all really exist in the cells.

TABLE II

EFFECT OF TIME SPENT IN THE COLD (1°–2°), IN ETHANOL, ON THE PROPORTIONS OF THE *a* PIGMENTS

The results are given in per cent of the total *a* pigments.

Time in the cold	*Pigments*			
	a_1	a_2+a_3	a_4	a_5
3 days	53	24	9	13
6 days	55	21	10	14
10 days	54	27	7	12
2 weeks	62	21	8	9
1 month	57	21	9	13

TABLE III

EFFECT OF THE SOLVENT USED FOR EXTRACTION ON THE PROPORTION OF THE PIGMENTS FOUND IN THE EXTRACT

The results are given in per cent of the total *a* pigments; one given Chlorella suspension was used for the extractions, which occurred simultaneously.

Solvents	*Pigments*			
	a_1	a_2+a_3	a_4	a_5
Ether	56	22	11	10
Acetone	60	22	6	12
Acetone	56	21	8	14
Ethanol (cold)	53	24	9	13
Ethanol (boiling)	54	27	7	12
Methanol	52	25	10	13

DISCUSSION AND CONCLUSIONS

The a_1 pigment, the most abundant, has been identified above as standard chlorophyll *a*. Differences are, however, found when comparing the spectrum of a_1 with those already published for chlorophyll *a*.

Except for the data of HARRIS AND ZSCHEILE[16], of STRAIN *et al.*[12] and of PERKINS AND ROBERTS[13], the positions published in the literature for the red and blue peaks in pure diethyl ether usually give values shifted slightly to longer wavelengths in comparison with our a_1 values and also with our values for a_2 and a_3.

The chlorophyll a_1 preparation had a relatively low blue:red ratio (1.25). Generally, the blue:red ratios found in the literature (for instance in STRAIN *et al.*[12], PERKINS AND ROBERTS[13], ZSCHEILE AND COMAR[14], SMITH AND BENITEZ[17]), are higher. Only the figures of ANDERSON AND CALVIN[18] and of ARONOFF[19] are lower. PERKINS AND ROBERTS[13] recently showed that the low values were due to the presence of

alcohols, water or other impurities in the solvent. Whether this was the case for a_1 is unknown.

Another peculiarity of the a_1 spectrum was its form in the 410-mμ region. The spectra of the literature generally show a distinct peak at 410 mμ and relatively high 430:410 ratios. The a_1 spectrum had no distinct peak at 410 mμ, and the 430:410 ratio was low. It is also interesting to observe that chlorophyll a_1 (like a_2 and a_3) had no distinct peak at 620 mμ.

However, in spite of these differences, chlorophyll a_1 absorbed below 400 mμ identically with the chlorophyll *a* preparation of ZSCHEILE AND COMAR[14].

As already stated, the a_2 and a_3 pigments are assumed to be isomers of a_1. They probably do not correspond to the isomers postulated by FISCHER and are therefore distinct, one from the other, by some unknown particularity.

It is difficult to decide if a_2 or a_3 corresponds to the *a'* isomer of STRAIN AND MANNING[20,21]. These authors do not give sufficient indications of the *a'* spectrum; *a'* pheophytin was, however, found distinct from normal *a* pheophytin, a result corresponding to our observation that a_3 and a_2 pheophytins are distinct from a_1 pheophytin. Moreover, STRAIN AND MANNING found only two *a* isomers (*a* and *a'*), and we found three. It is true that the separation of a_2 from a_3 was difficult. It is quite possible that this separation was not seen by STRAIN AND MANNING owing to the difficulties in distinguishing minor pigments when adsorbed on a sugar-column. (It is easy to see minor pigments separated on paper by their red fluorescence; but it is not easy to see them when adsorbed on a column.)

The a_3 isomer was the only pigment exhibiting an absorption band at about 690 mμ in diethyl ether—an interesting region because it could be responsible for an absorption *in vivo* around 700 mμ. It does not seem that the 690-mμ band was modified or eliminated by successive rechromatography. But the band was modified in the pheophytinisation process and it did not appear in the a_3 Cu-chlorophyll. As the 450–455-mμ band of a_3 behaved similarly (it was also modified in the pheophytinisation process and also disappeared in the a_3 Cu-chlorophyll), it could be supposed that a_3 was accompanied by a minor pigment, which we were unable to separate chromatographically, except in the Cu-form, which absorbed in the red at 688 and in the blue at 450–455 mμ (in diethyl ether). This point needs to be specially investigated. Recently, after irradiation of a chlorophyll $(a+b)$–protein complex (prepared from *Chenopodium album*) YAKUSHIJI *et al.*[22] found a chlorophyll absorbing in acetone at 690 mμ.

Among the other pigments we have found, reference should be made to b_3. Its red maximum in diethyl ether was located at 631 mμ, the blue maximum being at 443 mμ. Chlorophyll *c* has its red and blue maxima in about the same positions[23]. In fact, the spectrum of b_3 was clearly "protochlorophyll-like". However, b_3 seems different from chlorophyll *c* for two reasons: (1) the blue:red ratio seems to be higher than 10 in chlorophyll *c*, while it was of the order of 4 for b_3; (2) in b_3 a secondary peak appeared at the short-wavelength side of the blue peak at 420 mμ which does not appear in chlorophyll *c*. Final conclusions cannot yet been drawn since b_3 was not isolated in a pure state.

The possible occurrence of pigments accompanying b_3 and absorbing in the far-red region (especially at 685 and 735 mμ in diethyl ether) seems of special significance and requires further attention. In this connection, it is interesting that, in their irradiation

experiments, YAKUSHIJI *et al.*[22] also produced a pigment absorbing at 735 mμ (in acetone).

Further work will tend to elucidate the exact nature of the different chlorophylls found (after appropriate purification). It is, however, clear that Chlorella extracts not only contain chlorophyllide(s) and allomerized chlorophyll, but that chlorophyll *a* (and perhaps chlorophyll *b*) isomers, pigments absorbing in the far-red region, and a special pigment exhibiting a "protochlorophyll-like" spectrum are present in low quantities.

We were unable to show that the additional pigments of the *a* group were formed during the chromatographic procedures or during the time spent in the dark at 1°–2°. If one accepts that the isolated pigments do not exist inside the living cells and that they are not formed during chromatography or during the time spent in the cold, they must be produced during the extraction procedure. In particular, one would be obliged to accept that the acetone extraction of Chlorella cells (at the laboratory temperature of about 20°) produces new molecules distinct from a_1. In fact, STRAIN AND MANNING[20] considered that their *a'* isomer was formed during the extraction at the room temperature. Their conclusion was based on the observation that an extraction at 0° did not yield *a'*, while an elevation of temperature to 100° produced the pigment. We must note that these arguments are not decisive: (1) it is normal that elevation of the temperature accelerates molecular transformations; this does not demonstrate that similar transformations do not occur inside the cells; (2) the absence of appreciable quantities of a given isomer after the extraction at 0° may result from imperfect extraction.

On the other hand, it seems that, if it occurs during the extraction at the room temperature, the production of the different isomers may be considered as relatively easy. We could therefore state with RABINOVITCH[24] (writing about the *a'* chlorophyll of STRAIN AND MANNING) that, "the ease with which the old isomers could be converted into the new ones *in vitro* argues for their presence in the living plants, particularly at the higher temperatures". Indeed, we intend to show in subsequent papers that some of the red absorption bands *in vivo* correspond to different chlorophyll *a* molecules *in vitro*.

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