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SPECTRAL ABSORPTION PROPERTIES OF ORDINARY AND FULLY DEUTERIATED CHLOROPHYLLS *a* AND *b*

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SUMMARY

The spectral absorption properties of fully deuteriated chlorophylls *a* and *b*, ordinary chlorophylls *a* and *b*, and methyl chlorophyllides *a* and *b*, isolated in a state of high purity, have been compared. Substitution of phytyl by methyl did not affect the wavelengths of the absorption maxima or the ratio of the absorption at the principal maxima. Deuteriation reduced the absorption in proportion to the molecular weight of the deuteriated pigments. Deuteriation also shifted the absorption maxima to slightly shorter wavelengths, an effect attributable primarily to the 6 deuterium atoms attached to carbon atoms in the conjugated system.

INTRODUCTION

The isolation of fully deuteriated chlorophylls[1] has prompted an investigation of the effect of deuteriation upon the spectral absorption properties. To this end, the ordinary chlorophylls and the fully deuteriated chlorophylls were isolated by similar methods, and the wavelengths of the absorption maxima, the specific absorption coefficients, the molecular absorption coefficients, and the ratios of the absorption at the "blue" and the "red" absorption maxima were compared. Further confirmation of the spectral absorption properties of the ordinary chlorophylls was obtained by examination of the methyl chlorophyllides, which were easily isolated in a state of high purity.

Many measurements of the absorption spectra of the ordinary chlorophylls have been reported. MACKINNEY[2], ZSCHEILE AND COMAR[3], and ZSCHEILE, COMAR AND MACKINNEY[4] have reported coefficients based upon the weight of purified chlorophylls *a* and *b* dissolved in diethyl ether. SMITH AND BENITEZ[5] and FALK[6] have reported coefficients of impure preparations, the quantity of the chlorophyll being calculated from the magnesium content. Some of these values are compared in Table I. Spectral absorption curves have also been reported for solutions in various solvents such as methanol[7-9], acetone[10], and dioxane[11].

The ratios of the absorption at the "blue" maximum to that at the "red", frequently employed as a criterion of purity[1-15], have usually been in satisfactory agreement[1-10]. Recently, however, a much lower ratio for chlorophyll *a* in diethyl ether has been reported by ANDERSON AND CALVIN[12] (compare Table I).

Molecular spectral properties of the chlorophyllides *a* and *b* (ref. 14), like those of the esterified hydroxy carotenoids[16], are independent of the molecular weight of the colorless groups forming the esters. The highest of several discrepant values for the molecular absorption coefficients of ethyl chlorophyllide *a* in diethyl ether, reported by HOLT AND JACOBS[14], were but 1–1.7 % lower than those reported for chlorophyll *a* by ZSCHEILE AND COMAR[3]. The highest values for the *b* ester were 1.8–5 % higher than those for the chlorophyll *b*. The ratio of the absorption, "blue" to "red", was 1.33 for ethyl chlorophyllide *a* compared to 1.32 for chlorophyll *a* (ref. 3) and 3.06 for ethyl chlorophyllide *b* compared to 2.82 for chlorophyll b (ref. 3).

Because of the favorable solubility relations and because of the greater amount of comparable data, most of our determinations of the absorption properties were made with solutions in diethyl ether. Moreover, as shown by our current investigations, soon to be reported, the chlorophylls in this solvent are dispersed in monomolecular form, not as dimeric or polymeric species as reported for some other solvents[17,18]. For comparison with our earlier work[7–9], spectral maxima and absorption ratios for methanol solutions were also determined.

PROCEDURES

The ordinary chlorophylls were isolated from fresh spinach leaves (200-g portions) and from a few other sources[15]. The deuteriated chlorophylls were obtained principally from *Scenedesmus obliquus* and from *Chlorella vulgaris* grown in heavy water (usually approx. 100-g portions of the centrifuged cells[1,13]).

Before extraction, the plant material was scalded with boiling water for about 1 min. As described already, the pigments were usually extracted with methanol–petroleum ether (b.p. 20–40°) or occasionally with acetone or acetone–petroleum ether[1,15].

After extraction, the pigments were transferred to petroleum ether by addition of an excess of water or salt solution. The separated petroleum ether layer was then washed with water and, with the aid of suction, drawn into two columns of powdered sugar (8 × 35 cm, formed by pressing the dry sugar into the tubes). The adsorbed pigments were washed with a little petroleum ether and then with petroleum ether plus 0.5 % *n*-propanol. In some preparations, colloidal chlorophyll and colorless waxy material were adsorbed at the top of the columns. As these materials sometimes reduced the percolation rate, the uppermost 2–3 cm of the sugar were worked loose with a spatula in order to facilitate the filtration and the dissolution of the chlorophyll by the petroleum ether–propanol. By this washing procedure, the carotenes, much fatty material, and traces of pheophytin *a* and chlorophyll *a* were carried into the percolate ahead of the chlorophyll *a*. Then the chlorophyll *a* was carried into the percolate ahead of most of the lutein plus zeaxanthin[1,15]. At this stage, the chlorophyll *b* zone, which was near the middle of the column, was removed and packed into another tube, where the chlorophyll *b* was eluted with petroleum ether plus about 4 % ethanol. The chlorophylls in the respective percolates could usually be induced to separate by washing the petroleum ether with water followed by cooling with dry ice. They could always be extracted by shaking the petroleum ether repeatedly with water[1]. The pigments were collected by centrifugation and dried in vacuum. At this stage of purification, however, various impurities accompanied the chloro-

phylls. The yields were about 0.15 g for ordinary chlorophyll *a*, 0.05 g for ordinary chlorophyll *b*, 1.25 g for deuterio-chlorophyll *a*, and 0.06 g for deuterio-chlorophyll *b*.

For further purification, about 0.15 g of chlorophyll *a*, 0.25 g of deuterio-chlorophyll *a* or 0.1 g of the *b*-pigments were dissolved in about 25 ml of diethyl ether (free of ethanol) and diluted with 75 ml of petroleum ether. Each of these solutions was adsorbed in a separate sugar column (8 × 35 cm). If some of the chlorophyll had separated, the solid pigment was permitted to collect on top of the powdered sugar where it later dissolved when the columns were washed with petroleum ether–propanol, or fresh diethyl ether (free of ethanol) was added to the petroleum ether to bring the chlorophyll into solution. The columns with the chlorophyll *a* were washed with petroleum ether plus 0.5 % *n*-propanol, those with the chlorophyll *b* with petroleum ether plus 1 or 1.5 % *n*-propanol. After extensive washing, the main portions of the chlorophyll zones were removed from the tubes, and the pigments were eluted with petroleum ether–ethanol. In order to remove colorless impurities eluted from the sugar[1,19], the petroleum ether elutriates were extracted successively with 100-ml portions of aqueous methanol (50, 60, 70, 80 and 90 % methanol for chlorophyll *a* and 50, 60, 70 and 80 % methanol for chlorophyll *b*) and then with water. At this stage, the chlorophylls usually separated from the petroleum ether. After refrigeration of the solutions with solid CO_2, the separated chlorophylls were collected by decantation plus centrifugation and dried in vacuum, first at 20°, finally at 100° for 1 h at 0.001 mm Hg. The preparations were then preserved in evacuated and sealed ampoules. There were obtained about 0.07 g of chlorophyll *a*, 0.15 g of deuterio-chlorophyll *a*, 0.04 g each of chlorophyll *b* and deuterio-chlorophyll *b*.

In some preparations, the use of methanol was avoided entirely. Extractions were made with acetone, and the chlorophyll solutions were extracted with aqueous acetone. In a few preparations, the individual chlorophylls, after the first adsorption, were readsorbed in sugar columns and washed with benzene. This adsorption from benzene aided the separation of certain yellow pigments, but these pigments were also removed by the purification procedure described above. The washing with benzene did not serve for the separation of chlorophylls *a* and *b*, and it also stimulated the photochemical conversion of the chlorophylls into red-brown pigments, which trailed the green zones in the columns. These altered chlorophylls were removed by readsorption on sugar followed by washing with petroleum ether–propanol.

In spite of the dehydration in vacuum, the solid chlorophylls sometimes retained traces of water. This was indicated by exchange reactions after dissolving the chlorophyll in CCl_4 (ref. 20) and also by measurement of the infrared spectra[1].

A few preparations of ordinary chlorophyll *a*, purified by readsorption, were dissolved in benzene which was evaporated under reduced pressure. The residue was then dissolved in petroleum ether (b.p. 20–40°) and crystallized by chilling with CO_2. Well-formed crystals separated, but when centrifuged down at room temperature, these crystals sintered together to form a solid mass, which was dried in vacuum at room temperature, then at 100°.

To eliminate the possibility of changes induced by drying, chlorophyll *a* was also separated from leaf extracts and purified by repeated adsorption of powdered sugar followed by extraction of the elutriate with aqueous methanol and with water. The pigment was transferred to ether, and the absorption maxima and the absorption ratios were determined.

The methyl chlorophyllides were prepared by the enzymic methanolysis of the chlorophylls in the leaves of cocklebur (*Xanthium pennsylvanicum*). The chlorophyll *a* plus *b* esters were extracted with methanol–diethyl ether–petroleum ether and separated by chromatography on sugar with benzene plus about 1 % *n*-propanol as wash liquid. After further purification by chromatography, each ester was recrystallized from acetone by the addition of petroleum ether.

For measurement of the absorption coefficients, 1–2-mg samples of each chlorophyll, purified by readsorption, were weighed roughly on an analytical balance. The samples, in small weighing bottles, were allowed to equilibrate in the humidified microbalance room and weighed to the nearest 2 μg. They were dissolved in pure, dry diethyl ether (200 ml at 20°), and the concentration corrected to 25°. The spectral absorption curves were determined at 25° in a constant-temperature room using a Cary 14R recording spectrophotometer calibrated for wavelength and absorption values.

RESULTS

Qualitatively, the spectral absorption properties of the ordinary chlorophylls in ether (Tables I–III) were close to those reported by ZSCHEILE AND COMAR[3], by ZSCHEILE, COMAR, AND MACKINNEY[4], by SMITH AND BENITEZ[5], and by FALK[6]. These curves, with ether, were not affected by the methods employed for purification of the chlorophyll. But they varied when other solvents were present in the ether. With U.S.P. ether containing about 3.5 % ethanol as preservative, the absorption maxima for chlorophyll *a* occurred at slightly longer wavelengths, 430 and 661.5 mμ, and the "blue" to "red" ratio was reduced to 1.26.

The qualitative curves for chlorophylls *a* and *b* in methanol confirmed those reported before[7–9]. As shown by Tables II and III, the absorption maxima and the absorption ratios of the ordinary chlorophylls and their methyl esters are virtually identical, both in methanol and in diethyl ether. Neither chlorophyll *a* nor the methyl chlorophyllide *a* in diethyl ether exhibited an absorption ratio so low as that reported by ANDERSON AND CALVIN[12].

With respect to the absorption ratios and the wavelengths of the absorption maxima, the properties of the deuterio-chlorophylls were identical with those reported before[1,13].

The quantitative values for the absorption coefficients, reported in Table I, are the highest that were obtained. Most of the readsorbed *a* preparations (both ordinary and deuterio *a*) yielded values within 2.5 % of those reported. The values for the *b* preparations were all within 1 % of those reported. The ratios of the maximum absorption in the "blue" to that in the "red" were within $\pm$1 % for all preparations.

For comparison of the light absorption by ordinary chlorophylls with that by deuterio-chlorophylls, the molecular absorption coefficients are also recorded in Table I. As supported by infrared absorption[1,13] and nuclear magnetic resonance[21], the molecular absorption coefficients of the deuteriated chlorophylls were calculated for completely deuteriated pigments.

Deuterium has two effects upon the spectral characteristics of the chlorophylls. It reduces the specific absorption coefficients in proportion to its higher atomic weight. On a molecular basis, the absorption coefficients of the deuterio-chlorophylls and the ordinary chlorophylls are equal (Table I). As with the deuterio-carotenoids[13],

TABLE I

SPECTRAL PROPERTIES OF ORDINARY CHLOROPHYLLS (Chl *a*, Chl *b*) AND OF DEUTERIO-CHLOROPHYLLS (D-Chl *a*, D-Chl *b*) WITH VALUES REPORTED BY ZSCHEILE AND COMAR (Z, C), SMITH AND BENITEZ (S, B), AND ANDERSON AND CALVIN (A, C)

Solvent, diethyl ether. Temperature, 25°.

Properties	*Chl a*	*D-Chl a*	*Chl b*	*D-Chl b*
λ_{max}, red (found)	660.5	659.0	642.0	640.5
λ_{max}, red (Z, C)	660.0	—	642.5	—
λ_{max}, red (S, B)	662.0	—	644.0	—
Abs. coef., red (found)	96.6	88.6	61.8	57.9
Abs. coef., red (Z, C)	102.1	—	56.8	—
Abs. coef., red (S, B)	100.9	—	62.0	—
Mol. abs. coef., red (found)	86 300	85 600	56 100	56 700
λ_{max}, blue (found)	428.5	428.0	452.5	451.0
λ_{max}, blue (Z, C)	429.0	—	453.0	—
λ_{max}, blue (S, B)	430.0	—	455.0	—
Abs. coef., blue (found)	125.1	116.1	175.3	165.7
Abs. coef., blue (Z, C)	135.0	—	171.0	—
Abs. coef., blue (S, B)	131.5	—	174.8	—
Mol. abs., blue (found)	111 700	112 200	159 100	162 000
Ratio abs., blue/red (found)	1.30	1.31	2.84	2.86
Ratio abs., blue/red (Z, C)	1.32	—	2.82	—
Ratio abs., blue/red (S, B)	1.30	—	2.82	—
Ratio abs., blue/red (A, C)	1.19	—	—	—

TABLE II

SPECTRAL PROPERTIES OF ORDINARY CHLOROPHYLL *a* (Chl *a*) AND OF METHYL CHLOROPHYLLIDE *a* (Me chlide *a*)

Solvent	λ_{max} (mμ)		*Ratio blue/red*	
	Chl a	*Me chlide a*	*Chl a*	*Me chlide a*
Ether	428.5 660.5	428.0 659.0	1.30	1.29
Methanol	432.5 665.0	433.0 665.5	0.99	1.03

TABLE III

SPECTRAL PROPERTIES OF ORDINARY CHLOROPHYLL *b* (Chl *b*) AND OF METHYL CHLOROPHYLLIDE *b* (Me chlide *b*)

Solvent	λ_{max} (mμ)		*Ratio blue/red*	
	Chl b	*Me chlide b*	*Chl b*	*Me chlide b*
Ether	452.5 642.0	451.0 641.5	2.84	2.84
Methanol	470.0 652.5	470.0 652.5	2.80	2.79

the deuterium also shifts the absorption maxima of the chlorophylls to slightly shorter wavelengths[1,13] (Table I). By analogy with the chlorophyllides and the carotenoids[1,14,16,18], this shift results primarily from the 6 deuterium atoms coupled with the carbon atoms in the conjugated system, not from the other 66 deuterium atoms, 3 deuterium atoms on saturated-ring carbon atoms, 21 deuterium atoms in the methyl, ethyl, and propionyl groups, and 42 deuterium atoms in the methyl and phytyl ester groups.

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