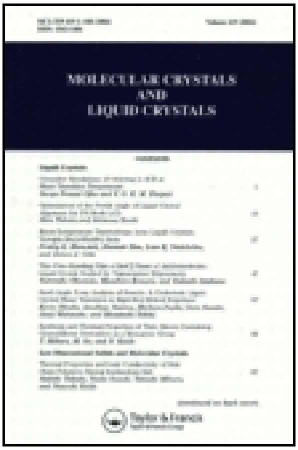

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Crystals of Chlorophyll-A: Optical Properties and Spectrum in the Visible Region

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Crystals of Chlorophyll-A: Optical Properties and Spectrum in the Visible Region

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The preparation of crystals of chlorophyll-*a* suitable for single crystal work is described. The results of an optical and microspectrophotometric study are presented. The crystal face investigated is not appreciably dichroic, and the spectrum in the region 13 000–25 000 cm^{-1} shows a moderate blue shift in respect of that in solution, tentatively interpreted on the basis of intermolecular interactions, where the water molecules may play an important role. A new weak shoulder appears at 21 800 cm^{-1}. The relative *f*-values of the vibronic components of the crystal spectrum have been measured.

INTRODUCTION

Since the beginning of the century the preparation of chlorophyll crystals has been attempted by several investigators.[1] However, doubts have been cast on the reliability of the results. They lack often reproducibility, because of the complex chemical behaviour of the pigment, of the difficulties encountered in its purification and storage, and of its lability to atmospheric agents.

In 1954 Jacobs, Watter and Holt described three methods for the preparation of "gram batches" of pure crystalline chlorophylls.[2] These authors showed that the microcrystals obtained (a few μm across) present sharp diffraction patterns. As a further proof of crystallinity, they pointed out the substantial red shift (about 1100 cm^{-1}) from the solution spectrum, of the peak at lower frequency in the electronic spectrum of microcrystals dispersed in heavy paraffin. Stoll and Wiedmann criticized the report by Jacobs, and by carefully planned experiments, gave a full proof that the chlorophylls can be crystallized.[3] From the photographic documentation presented, the crystals appear to be aggregates of thin prismatic needles. In view of the situation, we describe here the preparation of crystals of chlorophyll-*a* suitable for single crystal work, and the results of a study on the crystal plates by optical and microspectrophotometric techniques, made with the following aims: (i) to show that the crystals are those of the pure pigment, and compare their absorption spectrum with that of the solution; (ii) to derive structural information from an optical analysis in polarized light, and (iii) to discuss, from a qualitative point of view, the absorption spectrum of chlorophyll-*a* in the solid state.

After a brief description of the method which, with some modifications, was found to be the most efficient for the preparation of the pigment with a high degree of purity, we discuss at some length the results of the optical and microspectrophotometric investigation.

EXPERIMENTAL

Preparation of chlorophyll-*a*

The pigment was obtained from the leaves of *spinacia oleracea*. The method proposed by Anderson and Calvin,[4] suitably modified, was used to obtain pure chlorophyll-*a*. Every coloured pigment, including carotenoids and chlorophylls, was extracted with anhydrous acetone added with $MgCO_3$, and the liquid phase separated at low temperature with a centrifuge. The acetonic solution is directly fed to a column filled with powdered polyethylene (Fertene UG 1800, MI 1,2; Montedison). After balancing with acetone: water (60:40) the column is eluted with the same mixture (65:35). Three bands form, of which the slowest splits during elution in two: the band of chlorophyll-*a*, pea-green, and that of chlorophyll-*b*, blue-green. The final pass through icing sugar is superfluous if only the central part of the zone containing chlorophyll-*a* is collected.

The purity of the pigment in solution throughout the various steps of the preparation was checked by recording the electronic spectra with Unicam PM 800, and Perkin–Elmer 402 spectrophotometers, the infrared spectrum with a 257 Perkin-Elmer instrument, and by thin layer chromatography.[5]

Figure 1 (dotted line) shows the absorption spectrum in the visible region of a solution in acetone:water (80:20) of chlorophyll-*a*, collected from the poly-

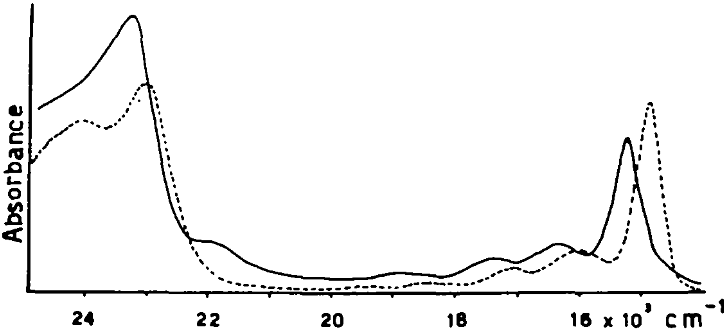


FIGURE 1 Absorption spectrum of chlorophyll-*a* in the range 14 000-25 000 cm^{-1}. (*a*) solution of acetone-water (80:20); (*b*) crystal. Intensities in arbitrary units.

ethylene column. The stock solution of chlorophyll-*a* in petroleum ether (bp 50-70°) was kept in the dark at 0°. In these conditions it can be stored for several weeks without alteration, as shown by the constancy of the electronic and infrared spectra. The purity of the pigment was judged by the ratios of the optical densities between minima and maxima in the electronic spectra, which were found to be comparable to those of the most pure preparations reported in the literature.[1]

Crystals of chlorophyll-*a*

According to Jacobs, Vatter and Holt[2], traces of water are essential for obtaining the pigment in the crystalline state. This observation was kept in mind when testing several crystallization techniques. All attempts to obtain crystals of chlorophyll-*a* by evaporation of benzene, petroleum ether, *n*-hexane and cyclohexane solutions yielded waxy aggregates. Negative results were also obtained when evaporating the solutions on ice, and lyophilizing the residue. Satisfactory results were achieved with the following procedure. A petroleum ether solution of the pure pigment is reduced to small volume by evaporation under reduced pressure, and then added slowly to water. A further slowly evaporation under reduced pressure follows. An accurately clean glass plate is slided along the liquid surface, and immediately covered by a second glass. The sandwich is slightly pressed, and placed in a dark vessel under reduced pressure until the solvent has evaporated. When the nucleation centres are formed, the islands of the concentrated solution of chlorophyll enrich progressively the crystals which reach the maximum size in a few days. Typical observed dimensions are 500 μm in length, while the thickness was estimated to range from 10 to 20μm. The crystals mounted on glass fluoresce. However, it is difficult to discriminate between light re-emission from the crystal and that from the surrounding droplets of

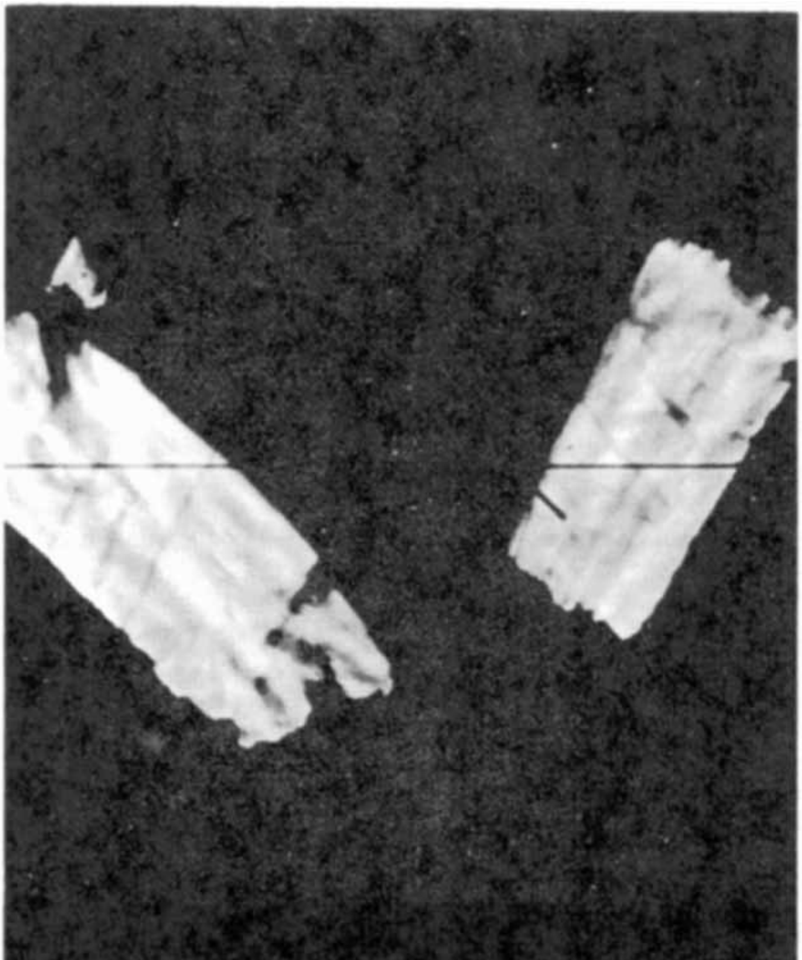
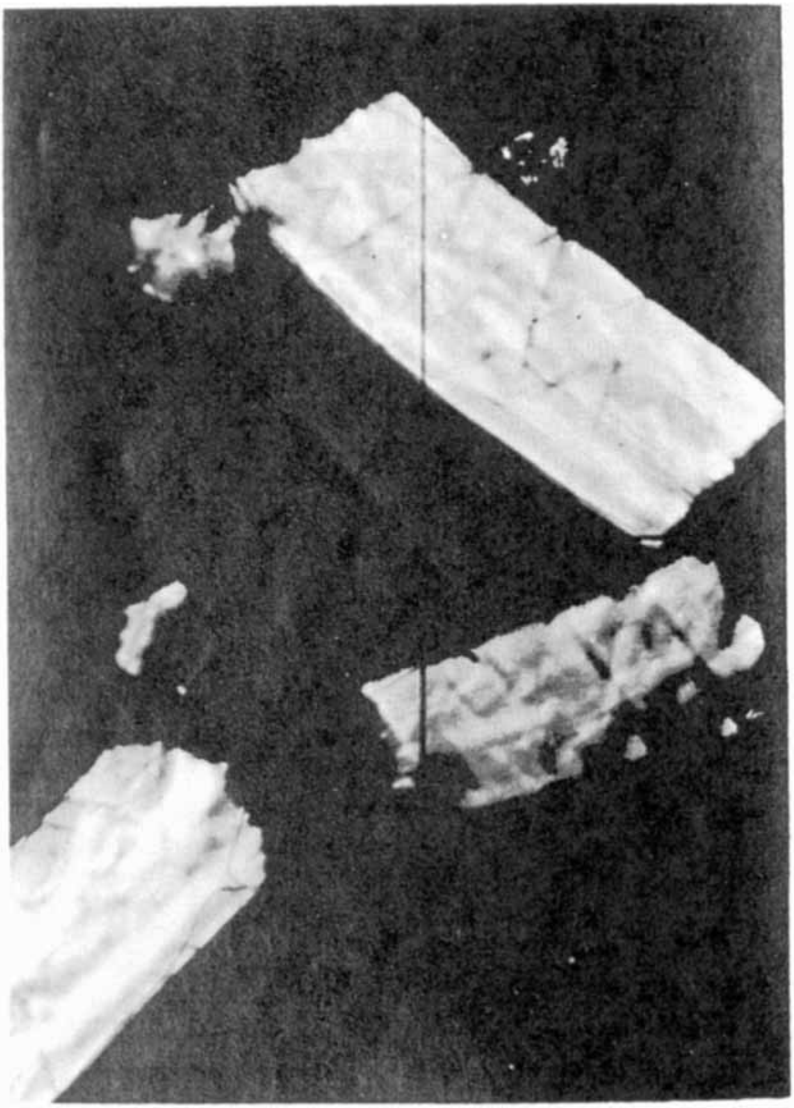


PLATE 1*a*, *b* Crystals of chlorophyll-*a* in parallel light between crossed polarizers, oriented in two positions differing 45°.



concentrated solution. The crystals could never be detached from the mount without being strongly damaged.

In order to check the purity of the crystals, and to make sure that the pigment is not degraded during the various steps of the preparation, several samples were dissolved in small amounts of petroleum ether. The solution was analyzed by cellulose thin-layer chromatography, and by recording the electronic spectra. The results were compared with those obtained from the stock solution. The perfect agreement between the two sets of results showed conclusively that the crystals are indeed made of pure chlorophyll-*a*.

The slides containing the crystals were observed in parallel and convergent light with a polarizing microscope Polmi A Jena. Microscopic observations were also made at low temperature with a liquid nitrogen cryostat.

Spectroscopic part

Spectra of the crystal in the region 13 300-25 000 cm^{-1} were recorded with a Cary 11 spectrophotometer, suitably modified. The sample beam is polarized with a Polaroid HNP'B filter, after being reflected by a 45° mirror along the microscope axis. The emerging beam is collected by one of the photomultipliers of the instrument. The reference beam was suitably diaphragmed with a filter made of polarizers to compensate for the light losses of the other beam. An air-coolod Osram 800 W Iodine lamp was used as a source of visible radiation. The glass slides containing the crystals were oriented on the rotating table of microscope, and homogeneous regions of the crystal were selected. Several crystal sections were investigated, and for each, the spectra along the two directions of extinction were recorded repeatedly from lower to higher frequencies, starting from 13 300 cm^{-1}. Although the experimental conditions were rather extreme because of the small crystal sections, consistent results were obtained in all runs.

RESULTS AND DISCUSSION

Optical properties and morpholgy of crystals of chlorophyll-*a*

The crystals of chlorophyll-*a* are thin (of the order of 10μm, spanning presumably many hundreds of molecules), of a ribbon-like form and of yellow-green colour, that is maintained also between crossed polarizers (see Plates 1*a*, *b*). The ribbons appear to have a slightly crumpled surface, probably connected to friction on the glass encountered during the growth of the crystal, or to slight surface inhomogeneities. The folding occurs mainly as well defined angles from the crystal edges. Plates 1*b* shows the same crystal of Plate 1*a* rotated 45° between crossed polarizers. The extinction is approximately parallel to the long axis of the crystal, and almost complete for the regions presenting the maximum of transmission in Plate 1*a*. The uniformity of the crystal plates at 45° from the extinction position in an indication that the plates are satisfactorily homogeneous in thickness. Optical observations between crossed polarizers, carried out while slowly cooling repeatedly the crystal down to 77K, did not evidence any morphological change. Provided that the constraints of the glass mount did not false the observations, one may conclude that the crystal habit investigated is energetically favoured in respect of the amorphous state even at room temperature.

Under convergent light, crystals of chlorophyll-*a* exhibit conoscopic patterns similar to those observed for a section of a biaxial crystal normal to one of the bisectrices of the optical indicatrix (Plate 2). No evidence was found of the traces of the optical axes, an indication that the crystal section contains probably the obtuse bisectrix.

It is rather difficult to obtain crystallographic information from observations in polarized light of chlorophyll crystals because of the concurrence of several factors, such as birefringence, strong absorption in the visible region, and, possibly, optical activity due to the several centres of asymmetry present in the molecule. The parallel extinction indicates that the structure is substantially oriented, and that a degree of internal order exists, also if the lattice is presumably of low symmetry.

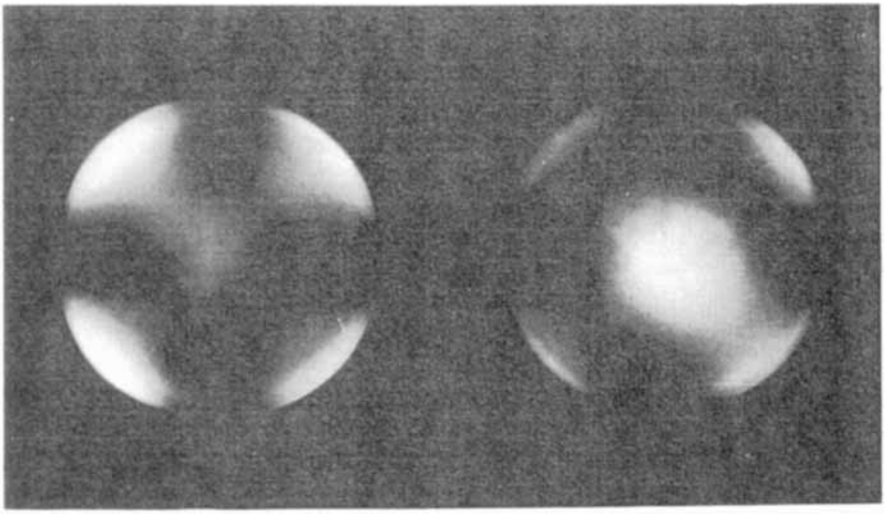


PLATE 2 Conoscopic patterns of a chlorophyll-*a* crystal section (1600x).

Absorption spectra of the chlorophyll-a crystal in the visible region

The absorption contour in the range 14 000–25 000 cm^{-1} of the chlorophyll-*a* crystal is shown in Figure 1 (full line). The upper limit is dictated by the transmission of the microscope, while intensities are given in arbitrary units, because the exact thickness of the crystal plate could not be measured. Since the spectrum was not found to be appreciably dichroic, only one contour is given.

The spectra of chlorophyll-*a* in the two phases, solid and solution, present a strong resemblance to each other, and differ only in some minor respects: (i) there is a generalized blue shift of the absorption contour for the condensed phase; (ii) the intensity ratio for the maxima at 23 280 cm^{-1}, and at 15 250 cm^{-1} differs significantly in the two spectra: (iii) in the crystal spectrum a weak shoulder is found in the neighbourhood of 21 800 cm^{-1}, and (iv) the overall absorption intensity, roughly estimated, is much larger in the solution than in the solid.

We did not find trace of absorption maxima in the neighbourhood of 13 500 cm^{-1} in the crystal spectrum, reported by previous authors,[2, 6–8] and considered to be a proof of crystallinity. A red shift is in fact usually observed when going from the solution to an ordered phase, but there are no theoretical reasons to exclude the occurrence of blue-shifts.[9] In the above spectral region, only the exponentially decaying tail-end of the first intense peak was observed by us. The present results do not strictly contradict previous findings, since the transition that causes the absorption at 13 500 cm^{-1} could be polarized perpendicularly to the crystal face here investigated, and thus go undetected. However, this would imply a rather unlikely regular packing of the porphyrin planes in the crystal lattice.

The main feature of the crystal spectrum, i.e. the lack of dichroism in the whole spectral range, prevents one to obtain information on the relative polarization of the absorption systems. It agrees with the low degree of order hypothesized for the crystal lattice, that will cause the light vector to meet molecules with different orientation.

As far as the generalized blue shift is concerned, this is rarely observed for molecular crystals. Barring the possibility of an anomalously large red shift of the solution spectrum in respect of that of the free molecule, a plausible explanation that can be put forward is that the blue shift is connected to a relative stabilization of the ground state (about 350 cm^{-1} in respect of the solution), due to specific interactions, whose nature can at present be only subject of speculation. Two hypotheses, that are not mutually exclusive, can be advanced. The first is that water molecules, that seem to play an important role in the formation of chlorophyll-*a* crystals, are specifically bonded to the chromophore [10] in such a way that a stabilization of the ground state above the first few excited ones occurs. Approximatively one Kcal is not unreasonable amount of relative stabilization energy to be expected when bonds with water molecules are involved. The second hypotesis contemplates specific interactions between molecules of chlorophyll, possibly mediated by the same water molecules. If strongly bonded dimers, or higher polymers, are the units of which the lattice is built, one can envisage situations similar to those discussed, for instance, by Kasha, Rawls and Ashraf El-Bayoumi[11] for cases where intermolecular overlap is small, where the mutual orientation of molecules in the polymer is such that the allowed transitions are shifted to the blue in respect of the free molecule. This presupposes that for both the main absorption systems in chlorophyll-*a* the transition moments are similarly oriented. In any case, the implications are that the local aggregates of chlorophyll molecules, bonded by strong intermolecular forces, are organized in a lowly ordered molecular crystal with weak inter-dimer (or polymer) forces. After all, the tendency of molecules of chlorophyll to form aggregates in solution or in *vivo* is well known.

In order to analyze the spectral data in more details, the absorption contours in solution and of the crystal have been simulated in terms of Gaussian and Lorentzian functions respectively, the different choice being dictated by the

TABLE I

Results of a deconvolution of the spectra in solution (acetone : water, 80 : 20) and of a crystal of chlorophyll-*a*. Frequency of the maxima in cm^{-1}. Values in parentheses indicate postulated components. The *f*-values refer to the most intense peak of the system taken as unity

Solution spectrum[a]		Crystal spectrum		
ν	*f*-ratio	ν	*f*-ratio	Assignment
14880	1.00	15250	1.00	0–0
(15400)	0.08			
16000	0.33	16360	0.28	0–0+1100
16500	0.07	16780	0.07	0–0+1500
17100	0.19	17400	0.21	0–0+2×1100
		(18150)	0.04	0–0+2×1500
18400	0.07	18900	0.12	0–0+2×1100+1500
		19700	0.04	0–0+3×1500
19800	0.03	20480	0.03	0–0+2×1500+2×1100
22400	0.14	21800	0.11	0–0
23000	1.00	23280	1.00	0–0
23600	0.35	23800	0.23	0–0+520
24000	0.66	(24230)	0.19	0–0+950
24600	0.45	24660	0.46	0–0+1400

[a] The upper limit was taken as for the crystal spectrum

criterion of the best fitting. The results are collected in Table I. The *f*-values of the vibronic components are given with reference to the most intense band of the system, assumed as unity. The most relevant feature is a change in the intensity ratio of the two strongest bands usually indicated with Q and B. It increases from 1.7 of the solution to 2.7 in the crystal spectrum. This feature, as well as the higher intensity of the solution spectrum, are at present of little value, because the third component of the crystal spectrum could drastically change the situation. The half-widths of the vibronic components of solution and crystal spectra are comparable (300-500 cm^{-1}), and show a tendency to increase towards higher frequencies.

As far as the new shoulder observed in the crystal spectrum at 21 800 cm^{-1} is concerned, we can at present only speculate a connection with solvatation centres in the lattice. Other alternatives such as a transition shifted to the red (perhaps the other B component), or a triplet state excitation enhanced by the crystal field cannot be neglected.

In Table 1 we offer tentative analysis for the crystal spectrum based on three systems, with origin at 15 250, 21 800, and 23 280 cm^{-1}. Only for the lower frequency system a vibrational analysis can be attempted. We have based it on one excited state vibrational frequency, 1 100 cm^{-1}, and possibly on a second of 1 500 cm^{-1}. In accord with theoretical considerations, it is customary to interpret the lower frequency absorption system in chlorophylls, and derivatives in term of two origins Q_x and Q_y, and of some vibronic components.[12, 13] However, we feel that on one hand the theoretical treatments applied to complex systems such as those under scrutiny have not yet reached a degree of accuracy adequate to represent a reliable guide for the spectral assignment, and on the other the experimental data should be systematized in the simplest way, according to Occam's prescription. The vibrational frequencies postulated in Table I for the excited state can be correlated to C–N and/or C=C stretchings, and to a ring deformation, according to the recent assignments proposed for the infrared spectra of metallo-porphyrins, and metallo-porphyns.[14]

Acknowledgement

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