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ABSORPTION AND FLUORESCENCE SPECTRA OF SPINACH CHLOROPLAST FRACTIONS OBTAINED BY SOLVENT EXTRACTION

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SUMMARY

Separation of previously postulated chlorophyll components *in vivo* was attempted by fractional extraction of chloroplasts with acetone and methanol of various concentration. Dilute solvents were found to extract more or, less intact chlorophyll–carrier complexes; more concentrated solvents dissociate these complexes, first in the extracts, then also in the residual chloroplast material. The absorption and the fluorescence spectra of the extracts and the residues further suggest a partial separation of two types of complexes, with the "long-wave" component, chlorophyll *a* 683, extracted ahead of the "short-wave" component, chlorophyll *a* 668. Particularly revealing are the fluorescence spectra at −196°; it seems that at this temperature all chlorophyll *a* complexes become fluorescent (while only one fluoresces strongly at room temperature). The relative intensities of the fluorescence maxima at 685 nm, 696–700 nm, and 735 nm change with solvent concentration, suggesting partial separation of the complexes.

INTRODUCTION

The absorption bands of chlorophyll *a in vivo* can be resolved into two major components[1,2]. If these correspond to two different pigment-bearing complexes, one may be able to separate them by fractionation. They could be different aggregates of chlorophyll molecules (*cf.* JACOBS *et al.*[3], BRODY AND BRODY[4], and LOVE AND BANNISTER[5]), or chlorophyll complexes with different carrier molecules. (Chlorophyll bands are known to shift with the polarity of the solvent, from 660 nm for chlorophyll *a* in hexane to 672 nm for the same pigment in carbon disulfide[6].)

To separate the different chlorophyll *a* complexes, a stepwise extraction of chloroplasts with aqueous methanol and acetone of growing concentration was attempted. (A similar procedure was used by THOMAS AND VAN DER WAL[7].) The results are described below. Similar results, obtained with chloroplast fragments solubilized by digitonin and then fractionated by centrifugation, has been reported in another paper[8].

* A portion of this paper is a part of a Ph.D. thesis (in Biophysics) submitted by C. N. Cederstrand to the University of Illinois, 1965.

METHODS

Separation by differential solvent extraction

The chloroplast suspensions were obtained from spinach (*Spinacia oleracea*), as described previously[2].

Aliquots of 0.5 ml of these suspensions were transferred, immediately after preparation, to two series of test tubes. One contained 5-ml portions of aqueous acetone (0 to 100 % acetone, increasing in increments of 10 %); the other, similar methanol–water mixtures. After shaking, the solutions were kept, as far as possible, in the dark at about 0°. After 10 min, the samples were centrifuged for 10 min at 0° at 12000 × *g*. The supernatant liquids were decanted and the pellets re-suspended in the Tris–sucrose buffer (pH 7). Both the supernatants and the suspensions were kept in the cold and in the dark until needed. Since chlorophyll decomposes gradually when removed from the chloroplasts, spectral measurements were made as soon as possible. The absorption spectra were measured on a Bausch and Lomb recording spectrophotometer (Spectronic 505); its reflectance attachment was modified to serve as an integrating sphere.

Fluorescence measurements

The spectrofluorometer used was described by GOVINDJEE and co-workers[9,10]. The fluorescence curves were corrected for the response of the photomultiplier, for the transmission of the scanning monochromator and any filters used, as well as for variations in photon energy. In dilution experiments, when the fluorescence of two samples had to be compared, a correction was needed also for differences in fractional absorption. We used liquid samples of 2.0 ml; the corresponding optical path length was 0.1 mm. Usually, only 5 % of the incident light were absorbed in the absorption maximum. The situation was less clear in the case of frozen samples, because of increased scattering. However, earlier experiments on chloroplast suspensions[10] had suggested that as long as absorption in the molten state does not exceed 5%, absorption in the frozen state is not high enough to cause distortion of the fluorescence spectrum by re-absorption.

RESULTS

Absorption spectra

Representative absorption spectra of chloroplasts re-suspended after partial extraction with acetone of different concentrations, are given in Fig. 1. The band heights (apparently suggesting that more chlorophyll is extracted by 20% and 60 % than by 50 % acetone!) will be examined later. What we note here, is that as the solvent concentration is increased, the red absorption maximum is shifted, and the chlorophyll *b* shoulder at 650 nm disappears. In 50–60 % acetone, a new chlorophyll species, absorbing at 740 nm, is formed, as previously reported by GOVINDJEE[11] and by AGHION, PORCILE AND LIPPINCOTT[12]; no further investigation of this species (which may be microcrystalline) was undertaken in this study.

Fig. 2 shows the position of the peak of the main red absorption band of re-suspended chloroplasts after extraction with acetone or methanol of different concentrations, up to 80 %. (Beyond this point, so little pigment remains in the chloro-

plasts that absorption measurements on them become unreliable.) The maxima were measured to ± 0.5 nm. We note the constant position of the absorption maximum of residual chlorophyll up to approx. 26 % acetone or 38 % methanol. It may be significant that these concentrations correspond to similar dielectric properties (dielectric constants of 67 and 64, respectively[13]).

The absorption spectra of extracts form a similar series. As shown in Fig. 2, right, below 10 % acetone or 25 % methanol, the peaks remain in the same position as in intact chloroplasts; they then begin to shift until they reach limiting positions >55 % acetone and >65 % methanol.

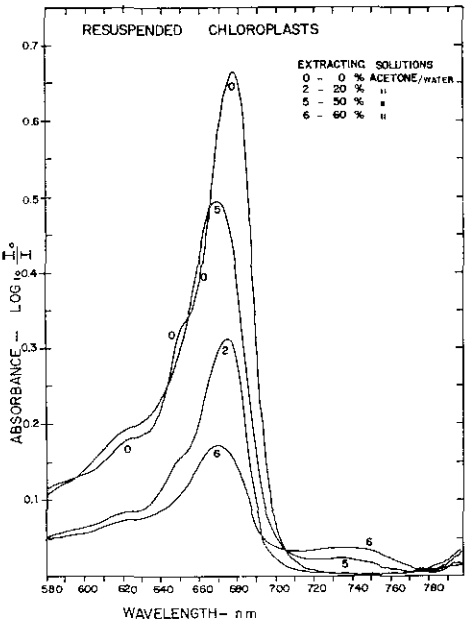

Fig. 1. Absorption spectra of re-suspended spinach chloroplasts after extraction with acetone solutions of different strengths.

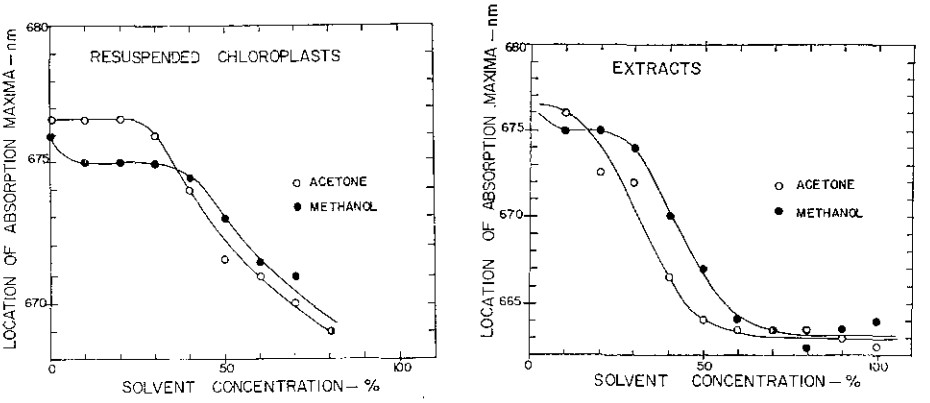

Fig. 2. Left: Position of the chlorophyll *a* absorption maxima in re-suspended spinach chloroplasts after extraction with solvents of different concentrations. Right: Position of the chlorophyll *a* absorption band in spinach chloroplast extracts, made with solvents of different concentrations.

The half-width of the absorption band, which can be considered as evidence of its simple or complex structure[2] is 22–24 nm in dilute solvents (Fig. 3). It increases with the concentration of the solvent and reaches a maximum of 33 nm in 30 % acetone or 40 % methanol; this broadening must be due to superposition of two bands, one belonging to extracted complexes and one to molecularly dispersed chlorophyll. The half-width of the band in low-concentration extracts is somewhat uncertain because of weak absorption, but is definitely narrower than in the spectrum of the original chloroplasts (29 nm, after subtraction of the chlorophyll *b* band). This agrees with the hypothesis that chlorophyll *a in vivo* consists of two different components, with band peaks at 668 and 683 nm (*cf.* ref. 2); and that one of them is extracted preferentially by dilute solvents.

The spectroscopically estimated amounts of chlorophyll in different extracts are plotted in Fig. 4 (left). There is a sudden increase in extracted chlorophyll above 60 % acetone or 75 % methanol. It coincides with complete dissociation of extracted chlorophyll from its carrier (*cf.* Fig. 2, right).

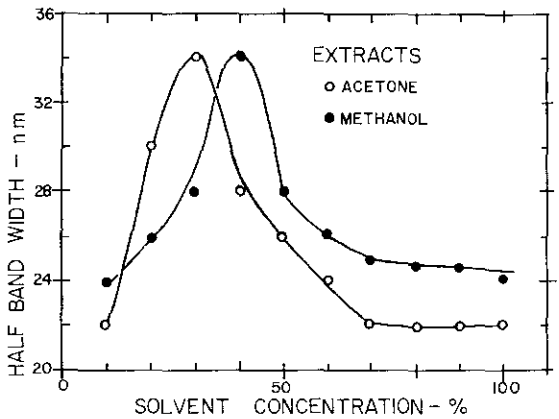

Fig. 3. Half-bandwidth of the red chlorophyll *a* absorption band in extracts from spinach chloroplasts as function of solvent concentration.

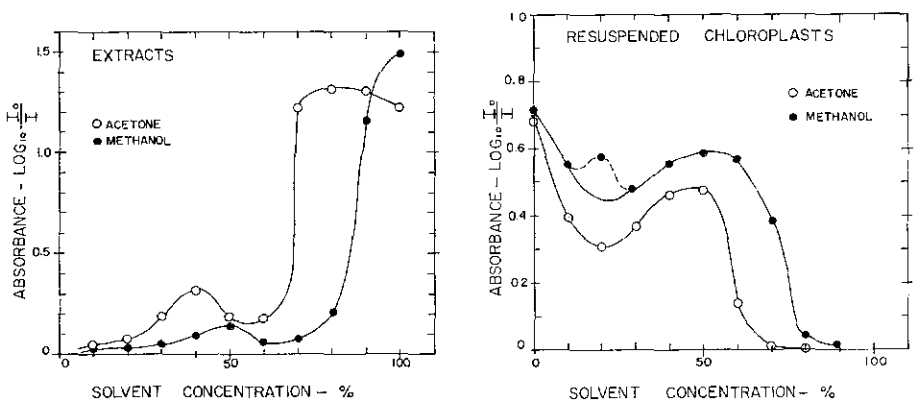

Fig. 4. Left: Height of the chlorophyll *a* absorption band as function of solvent concentration in extracts from spinach chloroplasts. Right: Height of the chlorophyll *a* absorption band in extracted and re-suspended spinach chloroplasts as function of solvent concentration.

In Fig. 4 we note the previously mentioned minimum of the amount of pigment extracted in 50 % acetone (or 60 % methanol). This is the region at which the broad 740 nm band (chlorophyll microcrystals?) appears in the spectrum of resuspended chloroplasts (*cf.* Fig. 1), and is probably associated with it; instead of dissolving chlorophyll, these solvents cause some of it to crystallize within the chloroplast.

In the 15–50% range, the absorbance of resuspended chloroplasts (Fig. 4, right) rises with solvent concentration. This seems paradoxical because up to 40 % acetone or 50% methanol, the amount of chlorophyll in the extract grows too (Fig. 4, left). A plausible explanation is that with the break-up of the residual pigment complexes in the chloroplasts, the mutual shading of pigment molecules (the "sieve effect") is decreased, and absorption becomes stronger despite the loss of pigment to the extracts. (This shading explains why the integrals of the absorption band are lower *in vivo* than in solution.)

In the absence of sieve effect, and if the intrinsic band strengths were the same *in vivo* as *in vitro*, the sum of the band integrals of the extract and the residue should be constant. Experimentally, however, this sum varies by as much of a factor of two or even three (Fig. 5). In general, it increases with increasing extraction, as expected, because of diminishing sieve effect. (Perhaps, a change in extinction coefficient is also brought about by the different refractive index, the dielectric constant and the polarizability of the different solvent concentrations.) This rise is interrupted, and a dip occurs in 60% acetone and, less pronouncedly, in 70% methanol. This is. most likely due to the formation of the 740 nm complex (chlorophyll microcrystals?). When the band integrals are extended to include the 740 nm region, the decrease at 60% is markedly reduced.

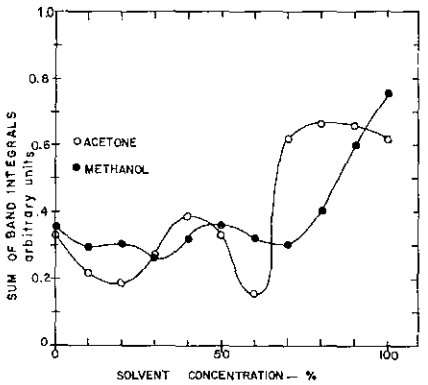

Fig. 5. Sums of the red absorption band integrals of re-suspended spinach chloroplasts and of solvent extracts, as function of solvent concentration.

Fluorescence spectra

The fluorescence spectra of the extracts and of the re-suspended chloroplasts were measured at 20° and at −196°. The location of the fluorescence peaks, like that of the absorption peaks, cannot be measured with a precision greater than ±0.5 nm. Fig. 6 shows fluorescence spectra after extraction with 30 % acetone, at room temperature, obtained by excitation at 420 nm. The main bands lie at 675 nm in the extract, and at 685 nm in the residue; they are followed, at the longer wavelengths, by vibrational bands at 735–740 nm. When the locations of the main fluore-

scence peaks are plotted as function of solvent concentration, the curves shown in Fig. 7 are obtained. The shift in the position of the fluorescence peaks in re-suspended chloroplasts (left) occurs, as expected, at the same concentrations as that of the absorption peaks (Fig. 2, left). The fluorescence maxima in the extracts (Fig. 7, right), offer a less easily understood picture. While the absorption peak of the extract remains for a while in the position characteristic of the intact chloroplasts (Fig. 2, right), the fluorescence peak displacement begins right away; in methanol, it ends at 60 %, at the same concentration where the displacement of the absorption peak ends, but in acetone, it continues up to 100 % acetone.

Relative fluorescence yields are shown in Fig. 8. At the lowest solvent concentration the fluorescence yield in the extracts (Fig. 8, right) is about one-tenth of that observed at the highest concentration. This confirms that the initially extracted material is a natural chlorophyll–carrier complex, which is known to fluoresce with a yield of about 3 % (at least, when excited in the blue band)[14], while in solutions, the yield is of the order of 30 % (independently of the wavelength of the exciting light). The fluorescence yield of the re-suspended chloroplasts (Fig. 8, left) merely

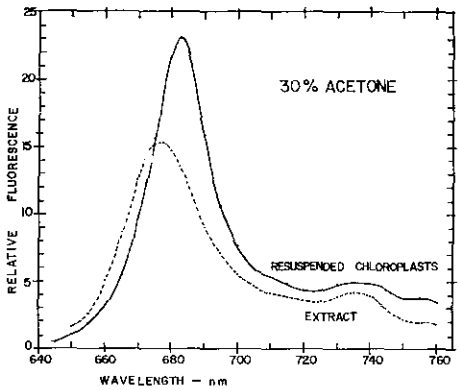

Fig. 6. Fluorescence spectra, at room temperature, of re-suspended spinach chloroplasts after extraction with 30 % acetone, and of the extract.

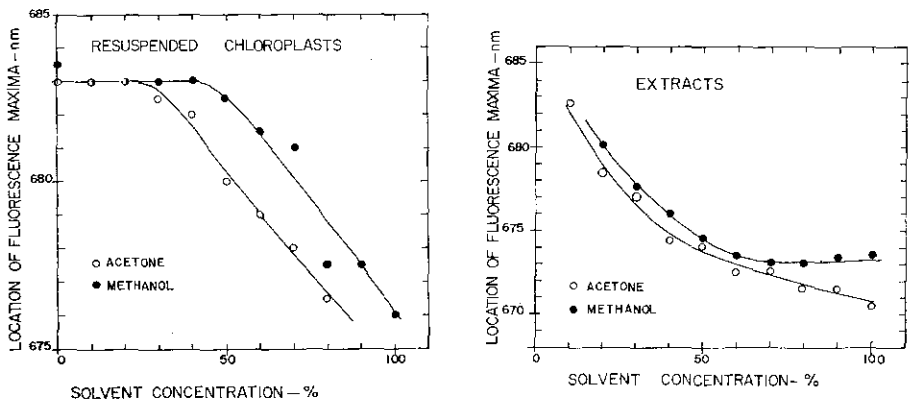

Fig. 7. Left: Position of chlorophyll *a* fluorescence maxima at room temperature, in re-suspended spinach chloroplasts, as function of solvent concentration. Right: Position of chlorophyll fluorescence maxima in extracts from spinach chloroplasts obtained by solvents of different concentrations.

doubles after treatment with concentrated solvent. This suggests continued attachment of chlorophyll to a carrier. The minimum yields, found after treatment with 50 % acetone and 60 % methanol, must be due to the formation of the chlorophyll form (crystals?) giving rise to the 740-nm absorption band (Fig. 1).

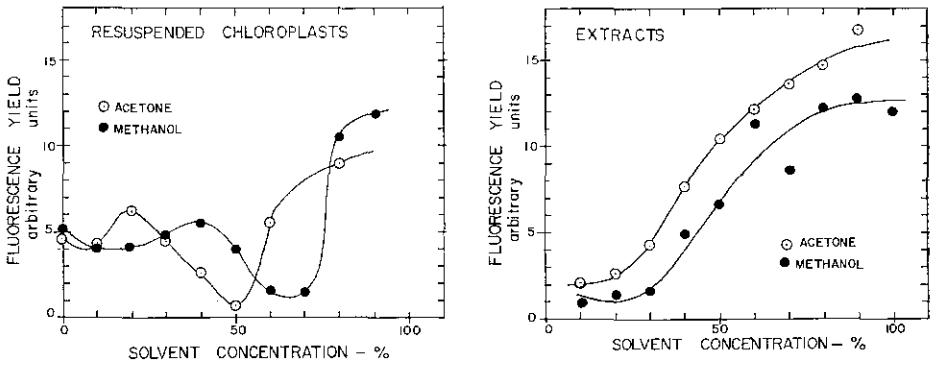

Fig. 8. Left: Fluorescence yield (quanta emitted/quanta absorbed) of re-suspended spinach chloroplasts after extraction with solvents of different concentrations. Right: Fluorescence yield (quanta emitted/quanta absorbed) of extracts obtained from spinach chloroplasts by means of solvents of different concentrations.

Pigments which, at room temperature, fluoresce only weakly, often become strongly fluorescent in liquid N_2 when energy losses by internal conversion and quenching are reduced. 2.0-ml samples were frozen on the bottom window of the Dewar flask, and covered with liquid N_2 to a depth of 5 cm. An increase in total intensity of fluorescence and in the complexity of its spectrum was observed. (Results relating to the intensity of fluorescence must be, however, treated with caution because of the uncertain optical path in the frozen samples.)

At solvent concentrations above 50 %, the chloroplast extracts had, at −196°, simple fluorescence spectra, resembling the room temperature spectra shown in Fig. 6; but at the lower solvent concentrations (less than 40 %), where the survival of a chlorophyll–carrier complex had to be postulated, the fluorescence spectrum at −196° proved much more complex. We noted previously that methanol is less effective than acetone in removing chlorophyll from its carrier; we note that the low-temperature fluorescence spectra of dilute-acetone extracts (Curves 1 and 2 in Fig. 9) are quite similar to those of intact chloroplasts (Curve 0 in Fig. 10). Three strong components appear in them, with sometimes an indication of a fourth band at the longer waves.

The fluorescence bands with the longest wavelengths ($\geqslant$ 696 nm) are relatively stronger at the lowest methanol concentrations (10 %); the band with the shorter wavelength (685 nm) appears strongest in the region of 30–40 % (Fig. 9). Above 50 % methanol, the 674-nm band, characteristic of molecular solutions of chlorophyll *a* in methanol, emerges as by far the strongest (Fig. 9; see also Fig. 10, right). The dilute-acetone extracts show, in addition to the 674-nm band, only a broad fluorescence peak in the neighborhood of 710 nm. This peak disappears when the acetone concentration increases above 35 % (*cf.* Fig. 10, left). (The dilute-acetone curves are somewhat similar to those obtained in 30 % methanol.)

The low-temperature fluorescence spectra of chloroplasts before extraction, or of chloroplasts re-suspended after extraction, have three peaks (Fig. 11). The bands are separated by as much as 30 nm; because of this, there is little apparent shifting of the maxima when the relative amplitudes of the components vary. In the fluorescence spectra of original chloroplast material (Curve 0), two strong components and one weak one appears. The long-wave band (at 735 nm) predominates over the middle

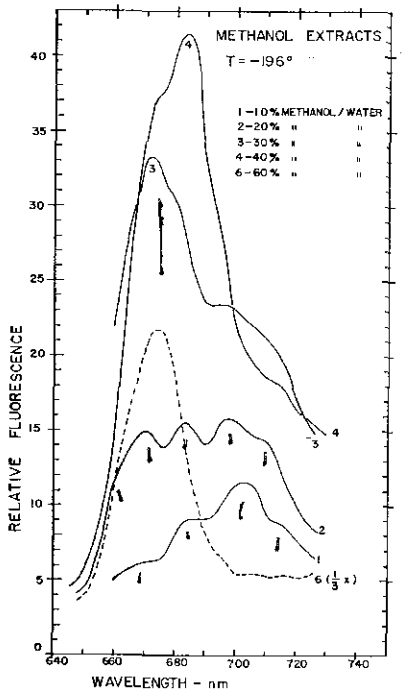

Fig. 9. Fluorescence spectra of methanol extracts from spinach chloroplasts at −196°, for different solvent concentrations.

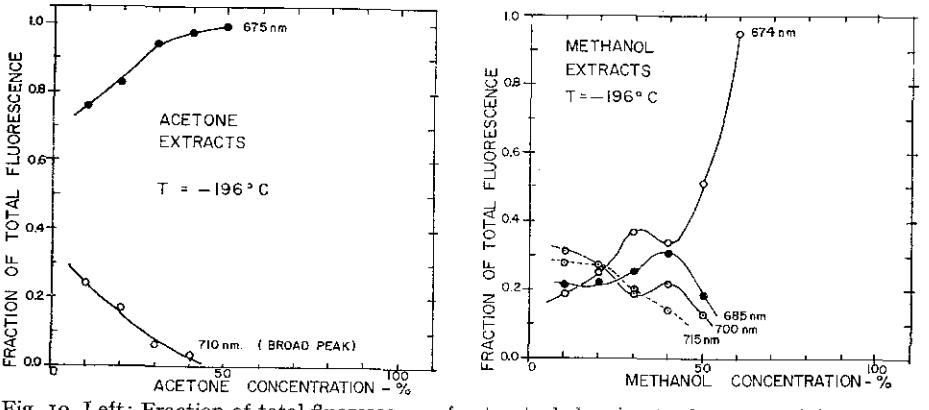

Fig. 10. Left: Fraction of total fluorescence of extracts, belonging to the 710- and 675-nm bands, respectively (calculated by band integration) as function of acetone concentration. Right: Contribution of the four fluorescence bands in extracts to total fluorescence as function of methanol concentration (calculated from the relative heights of the several peaks).

one (696 nm); the short-wave band (680–685 nm) is still weaker. The band at 735 nm decays very rapidly with increasing solvent extraction; it is essentially gone when a 50% solvent concentration is reached. The 696-nm band, on the other hand, is not appreciably weakened up to 50% methanol; it, too, disappears after extraction with more concentrated solvent. The 680–685-nm band is relatively weak in chloroplast residues from extraction with dilute methanol; but as more concentrated solvent is used, it becomes predominant, in the residue as well as in the extract.

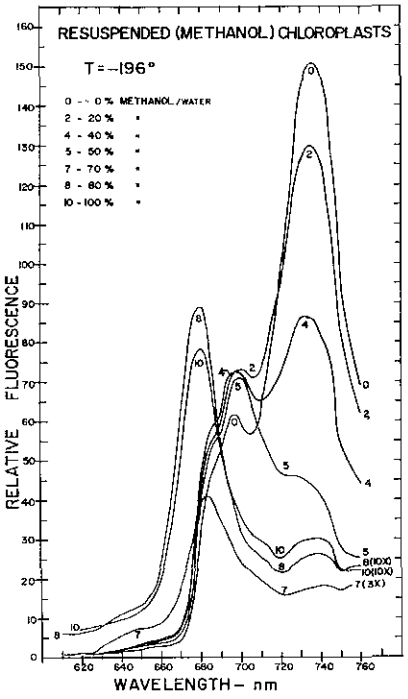

Fig. 11. Fluorescence spectra of re-suspended chloroplasts at —196° after extraction with methanol of different concentrations. Curves are on the same scale; except that scales for Curves 7, 8 and 10 are as indicated in the lower right corner.

In Fig. 12 (left), the contribution of the three components (735, 696 and 685 nm) to the total fluorescence of re-suspended chloroplasts at —196°, is plotted as function of methanol concentration. Up to 20% methanol concentration, the re-suspended chloroplasts show almost no change in the emission spectrum. Between 20 and 50%, the fractional contribution of the 735-nm band decreases sharply, whereas that of the 696-nm band increases to a maximum. It appears that methanol of about 50% extracts preferentially chlorophyll *a* complexes fluorescing at 735 nm, and leaves in the chloroplast predominantly complexes which fluoresce at about 700 nm. In the 50–60% range, the latter complexes also disappear from the chloroplasts; the fluorescence of the residual pigments (main band at 680 nm) now suggests either persistence of the original 685-nm band, or formation of a molecular chlorophyll *a* solution in a medium of high refractive index (a lipoid) with a fluorescence band in about the same position.

Upon still further increase in solvent concentration (>60% methanol), all

chlorophyll–carrier bonds are broken in the extract, and only the 685-nm band remains. This band is due to the traces of chlorophyll left in the chloroplasts.

The results obtained in acetone (Fig. 12, right) are similar, even if less sharp.

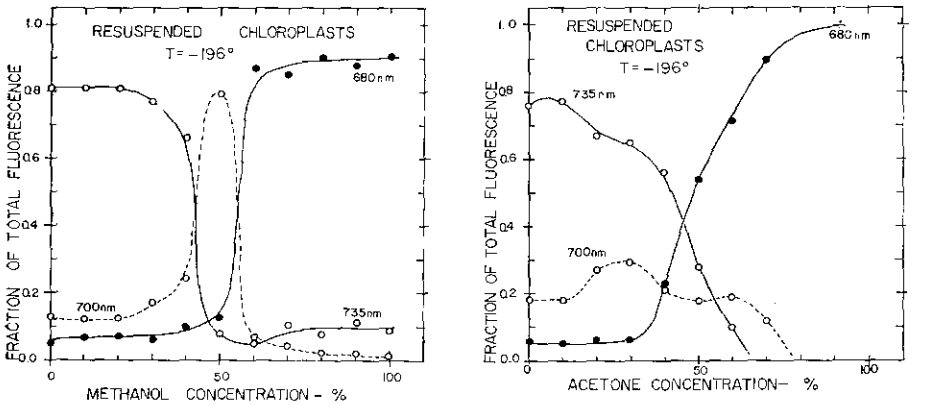

Fig. 12. Left: Fraction of total fluorescence belonging to each component as function of methanol concentration (estimated from the heights of the fluorescence bands). Right: Fraction of total fluorescence belonging to each component as function of acetone concentration (estimated from the heights of the fluorescence bands).

DISCUSSION

The similarities between chlorophyll *a in vivo* and in extracts made with dilute solvents, which include the positions of the absorption maxima (676 and 675 nm, *cf.* left and right in Fig. 2), the position of the fluorescence maxima (685 and 683 nm, *cf.* left and right in Fig. 7) and in the fluorescence yields (*cf.* left and right in Fig. 8) suggest that such solvents extract essentially intact chlorophyll–carrier complexes from the chloroplasts, but with apparent preference for one of the two main complexes present *in vivo*. THOMAS AND VAN DER WAL[7], too, have concluded that unchanged chlorophyll–carrier complexes are present in extracts obtained with dilute acetone from chloroplasts of *Aspidistra elatior*.

Absorption and fluorescence spectra at room temperature give indications of the presence *in vivo* of two (or more) different chlorophyll *a*-bearing complexes; much stronger evidence for this complexity is found in low-temperature fluorescence spectra.

Since the samples could be melted and refrozen many times, and continue to display the same complex spectrum in the frozen, and simple structure in the molten state, the additional components observed at −196° do not arise from permanently ruptured pigment complexes. Three fluorescence bands appear in untreated chloroplasts at −196° (see refs. 4, 10, 15–19). This triplet structure is very clear also in the low-temperature fluorescence spectrum of re-suspended chloroplasts (Fig. 10). Upon extraction, the "long-wave" complex (735–740 nm) disappears at the lowest solvent concentrations, and the "short-wave" complex (695 nm) disappears next. The 685-nm band remains to the last; it is not certain whether it is the same band that had dominated the spectrum *in vivo* at room temperature, or a new band due to the residual chlorophyll in the chloroplasts having been removed from its original complexes and dissolved in a lipoid. If we assume that two "pigment systems" are present

in chloroplasts, the short-wavelength chlorophyll *a* fluorescence (680–685 nm), observed in re-suspended chloroplasts (Fig. 10) at −196° and in dilute extracts (Fig. 9) may be attributed to the bulk of chlorophyll *a* in System II. The half-bandwidth of this fluorescence band of chlorophyll *a* dissolved in acetone is 22 nm. The presence, in the extract, of molecularly dispersed chlorophyll, with a fluorescence band at slightly shorter wavelengths, could account for the slight broadening of the band.

The fluorescence spectra of the extracts at −196° (Fig. 9) display four bands, which overlap so strongly as to make determination of the band-width of the 685-nm component impossible. The appearance of new bands at the longer wavelengths suggests that the main chlorophyll *a* component in System I, which seems to be only weakly fluorescent or non-fluorescent at room temperatures, becomes fluorescent at low temperatures. Life-time measurements of BRODY AND RABINOWITCH[20] and TOMITA AND RABINOWITCH[21], have led to the hypothesis that a large part of chlorophyll *a* *in vivo* is non-fluorescent at room temperature.

The extract band at 670–675 nm (Fig. 9) must be attributed to molecularly dissolved chlorophyll *a*. As expected, this band grows in intensity as the solvent concentration increases.

The origin of the two long-wavelength fluorescence bands, which appear at −196° (in digitonin-solubilized chloroplast fractions[8], as well as in chloroplast extracts in dilute solvents, and in re-suspended chloroplasts after such extraction), at 696 and 740 nm, is of great interest. One may think at first that one of them, at least, could be due to chlorophyll *a* 683 nm (the bulk of chlorophyll *a* in System I, which is practically non-fluorescent at room temperature). GOVINDJEE[15] suggested, however, that these bands are more likely to originate in energy "traps" which had been postulated in both pigment systems (the 740-nm band in the "trap" in System I (KOK's[16] P700) and the 698-nm band, in the hypothetical "trap" in System II (with an absorption band somewhere near 680 nm)). At room temperature, the fluorescence from the two "traps" is very faint because of its quenching by the initial photochemical steps of photosynthesis; at −196°, the excitation energy is still conducted efficiently to the traps, but freezing of all chemical activity makes this energy available for fluorescence. A low-temperature effect on fluorescence in the bulk of chlorophyll is much less plausible, since there the competing process is not a chemical reaction, but resonance energy transfer, which is unlikely to be strongly affected by temperature.

Extraction experiments (Fig. 10) suggest that chlorophyll *a* 668 is more readily extracted by dilute organic solvents than chlorophyll *a* 683. This could account for the narrowness of the absorption band in the most dilute extract (half-width 22–24 nm, see Fig. 3), compared to intact chloroplasts (half-bandwidth 28 nm).

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