Spectral Properties of Cell Suspensions

E.I. Rabinowitch, L. Szalay, M. Das, N.R. Murty,
C.N. Cederstrand, and Govindjee
Department of Botany, University of Illinois, Urbana, Illinois 61801

Photosynthesis begins with the absorption of light by plants. To determine correctly the *amount of light* absorbed (not to speak of calculating the true absorption coefficient) is not easy, because strong scattering cannot be avoided. Early measurements of the absorption spectra of leaves and algae, made in transmitted light, have therefore given a very distorted picture of their absorption properties.

An advance came with the use of the integrating sphere, which permits collection of scattered light as well as transmitted light. Its use reduced considerably the difference between the measured absorption spectra of pigments in the living cell and in pigment extracts. Specifically, the apparently much greater absorption *in vivo* in the green and in the far-red part of the spectrum proved to be an illusion caused by scattering (including strong selective scattering in the far-red).

Even after the elimination of scattering effects, however, significant differences remain between the spectra of plant pigments *in vitro* and *in vivo*. These include differences in the positions of the absorption peaks and in the widths and relative heights of the absorption bands. For example, the peak absorbance in the blue (Soret) band *in vivo* appears only slightly higher than that in the red band, whereas it is about 50% higher in solutions of chlorophyll *a* and about 80% higher in those of chlorophyll *b*.

Duysens[1] first pointed out the leveling influence on the absorption spectra of the non-uniform distribution of pigments. The same effect was analyzed by Rabinowitch[2] simply by applying Beer's law twice, first to the pigment solution within a particle and then to a "solution of particles." In a dilute suspension of particles, many light beams pass through the suspension without striking a single particle (sieve effect); this effect decreases as the suspension becomes more concentrated (or as the light path increases), and disappears when each beam has to traverse a large number of particles on its path through the suspension. The sieve effect reduces the absorption throughout the spectrum, but particularly strongly in the absorption peaks.

Comparison of the absorption spectra of cell suspensions with those of pigment extracts does suggest the existence of the sieve effect, but its reliable evaluation is not possible on the basis of this comparison because the extract spectrum itself depends on the solvent, i.e., on the kind of molecules with which the pigment molecules are associated in solution (instead of being associated with each other and with proteins or lipoid molecules as they are *in vivo*).

A more reliable procedure is to compare the absorption spectrum of a cell suspension with that of a sonicate (a suspension of cell fragments obtained by ultrasonic treatment). If sonication is sufficiently thorough, all cells are broken into pieces

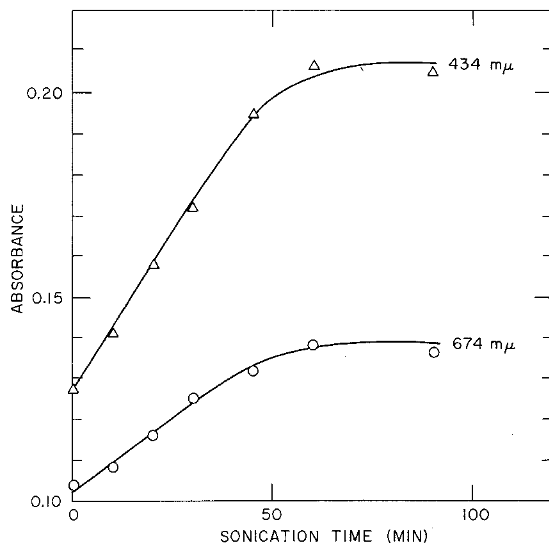


Figure 1. Increase in absorbance at the absorption peaks of chlorophyll upon prolonged sonication.

small enough to eliminate the sieve effect. However, if sonication is carried out in the presence of air, and in an even slightly acidic solution, a large fraction of the pigment undergoes chemical change (probably accelerated by local heating caused by cavitation). To minimize such changes, sonication must be carried out under anaerobic conditions and in a slightly alkaline buffer. (The absence of air precludes photooxidation, while alkalinity minimizes pheophytinization.) With these two precautions, even prolonged sonication does not cause an excessive loss of pigment.

This conclusion is drawn from tests on extracts of the pigments in aqueous methanol; equivalent amounts of whole cells and sonicated cells gave extracts with almost identical absorption curves. The pigment loss upon extraction was only about 10%. A correction can be applied for this loss (with the tentative assumption that the transformation products are colorless). A possible further improvement would be to carry out the sonication at 0° C; but in the experiments described here we were satisfied with the results obtained in alkaline buffer with exclusion of oxygen.

Sonication under these conditions breaks the cells into small pieces and gradually eliminates the sieve effect. Prolonged sonication, therefore, causes an increase in the absorbance of the suspension, as shown in Figure 1 for both the red and the blue maxima. Extending sonication from 1 to 1½ hr neither increased nor decreased the absorption, which confirmed that in 1 hr the sieve effect had disappeared and the sonication did not cause any serious loss of pigment.

Note that R (the ratio of the absorbance values at the peaks of the blue and the red band) is much higher in the sonicate than in the cell suspension: $R = 1.55$ instead of $R = 1.2$ (Figure 2). In the methanol extract from the same cells, $R = 1.6$ (as expected for a mixture of chlorophyll *a* and *b* in the ratio of about 3:1).

These results can be checked by comparison with the solutions of equations derived by statistical treatment of the sieve effect. Duysens' equations for spherical

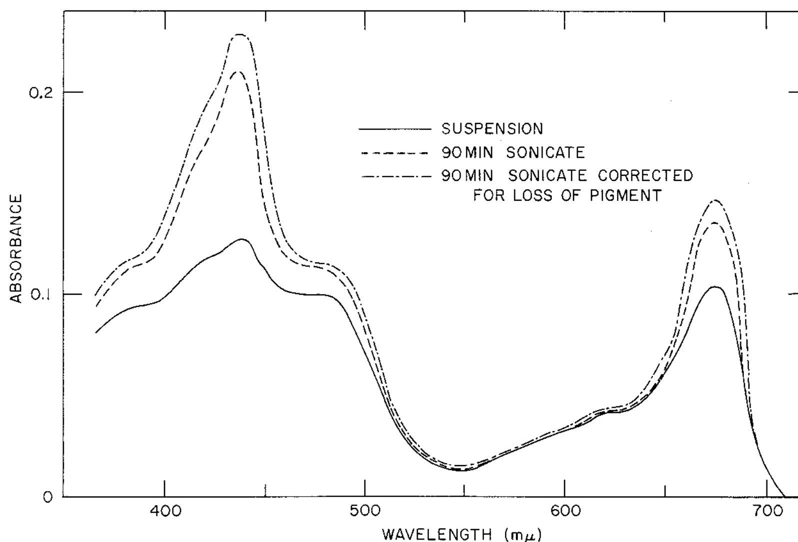
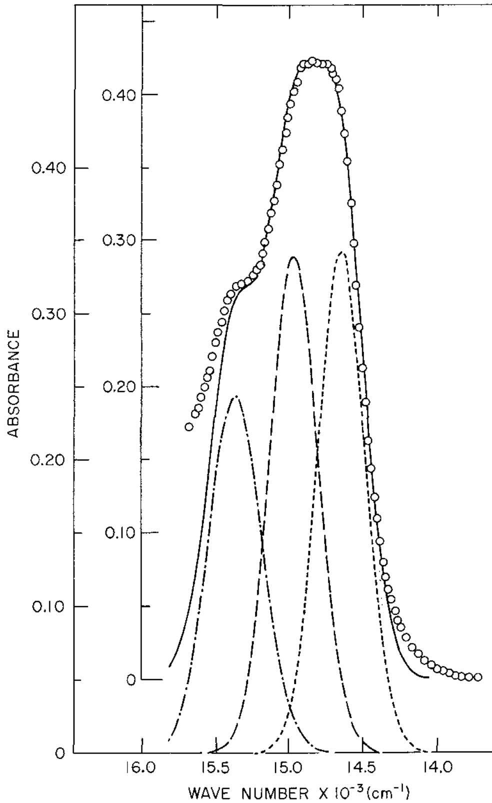


Figure 2. Absorption spectra of a *Chlorella* suspension and of its sonicate.

particles indicate that, from the observed spectrum of the suspension, R should be as high as 1.75 after correction for the sieve effect, whereas the experimental value is only 1.55. However, *Chlorella* chloroplasts are not spheres but bell-shaped, hollow bodies. As a next approximation, the sieve effect was estimated for a suspension of Π-shaped particles. The resulting value, $R = 1.50$, was quite close to the experimental one. Note that R can be calculated without special assumptions concerning the size and absorptivity of the particles.

THE DOUBLET STRUCTURE OF THE RED BAND

The greater width of the red absorption band in green cells (compared with that of chlorophyll *in vitro*) has been discussed by French[3] and by Thomas and Govindjee.[4] Brown and French[5] analyzed this band in *Chlorella* into four "Gaussian" components (one attributed to chlorophyll *b* and three to chlorophyll *a*) by first constructing a derivative spectrum ($dx/d\lambda$ as a function of λ) and then representing it as a sum of derivatives of four Gaussian curves. We made a similar analysis directly on the absorption curve. An important improvement in our instrumentation was the use of an integrating sphere equipped with 12 photocells (on the sides of a dodecahedron), which increased the sensitivity so that precise measurements could be made in the red with a spectral band width of 1 mμ. The instrument and the results are described elsewhere.[6] The main conclusions are illustrated in Figure 3. The red band of chlorophyll in *Chlorella* is well represented by only three Gaussian bands, one attributable to chlorophyll *b* (at 650 mμ) and two attributable to chlorophyll *a* (at 668 and 683 mμ, respectively). A third, weak band at about 700 mμ, suggested by certain observations and considerations, is not indicated by this analysis; but it cannot be excluded, provided its intensity is only of the order of 10% of



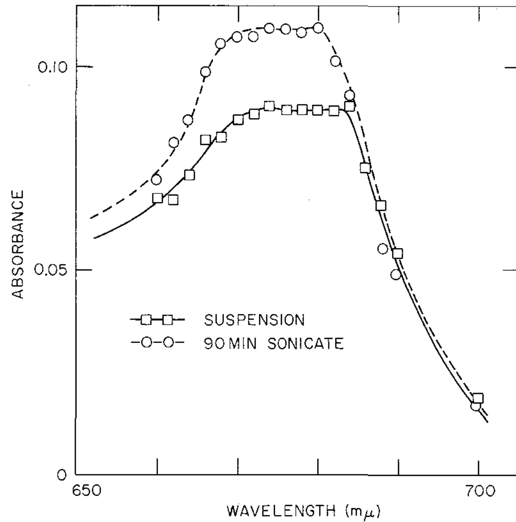


Figure 4. The peaks of the red absorption bands in suspension (solid line) and sonicate (dashed line).

Figure 3. Computer analysis of the red absorption band in a suspension of *Chlorella pyrenoidosa* into one chlorophyll *b* band (650 mμ) and two chlorophyll *a* bands (668 and 683 mμ). Solid line is the sum of the three bands; circles indicate measured absorbances. Deviations at short wavelengths are to be expected because of vibrational sub-bands.

those of the two main components. The two main components are approximately equal in intensity, not only in green algae (*Chlorella*), but also in red algae (*Porphyridium*) and blue-green algae (*Anacystis*), in which (because of the early "red drop" in the action spectra), only one dominant component might have been expected.

Unfortunately, this analysis was carried out before we had estimated the sieve effect. The computer analysis should be repeated on the sonicate. So far, we have determined only the shape of the red peak of the sonicate, and we are satisfied that it confirms the doublet structure found in the intact cell suspension (Figure 4). However, the two components must be somewhat sharper and slightly closer together for the sonicate than has been determined for suspensions.

THE "RED DROP" AND THE DOUBLET STRUCTURE

The well-known "red drop" in the action spectra of photosynthesis and of chlorophyll *a* fluorescence *in vivo* has been used as evidence of the complexity of the red

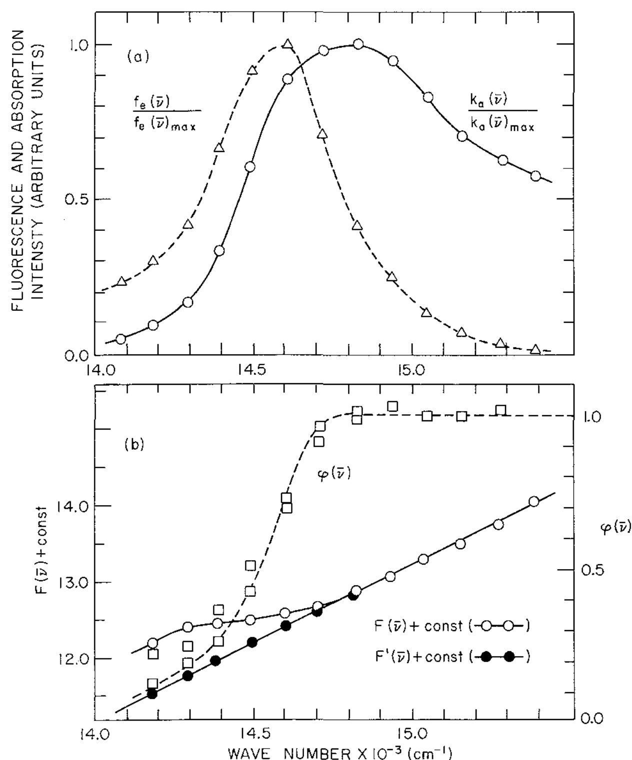


Figure 5. (*a*) Absorption (○) and fluorescence (△) bands of chlorophyll in *Chlorella*. (*b*) □---, Fluorescence yield (φ) of chlorophyll *a*; ○—, $F(\bar{\nu})$ + constant, calculated for φ = constant; ●—, $F'(\bar{\nu})$ + constant, calculated with $\varphi = \varphi(\bar{\nu})$ as shown. Temperature = 311°K.

chlorophyll *a* absorption band *in vivo*. It suggested the presence of a long-wavelength component which is, by itself, relatively inefficient in both photosynthesis and fluorescence. The Emerson effect indicates, however, that this component becomes fully efficient when light of shorter wavelength is supplied simultaneously.

It was natural to try to relate the "red drop" to the doublet structure found in the red band *in vivo*, by suggesting that one of the two components belongs to the inefficient type of chlorophyll *a* and the other to the efficient type. However, this hypothesis is not confirmed by quantitative analysis. A simple consideration suggests that if the long-wavelength component of the doublet were responsible for the "red drop," the halfway point of the drop should lie at about 675 mμ, whereas it actually lies at about 690 mμ.

Figure 5*b* shows the results of an exact determination of the "red drop" in the action spectrum of chlorophyll *a* fluorescence in *Chlorella* (plotted against wave number rather than wavelength). The dashed line is drawn through points representing directly determined relative quantum yield; the squares refer to an indirect determination of the yield, based on comparison of the absorption and fluorescence spectra, according to a theory of Stepanov[7] and Ketskeméty.[8] This analysis is described in detail elsewhere.[9] Of interest here is that application of the Stepanov-

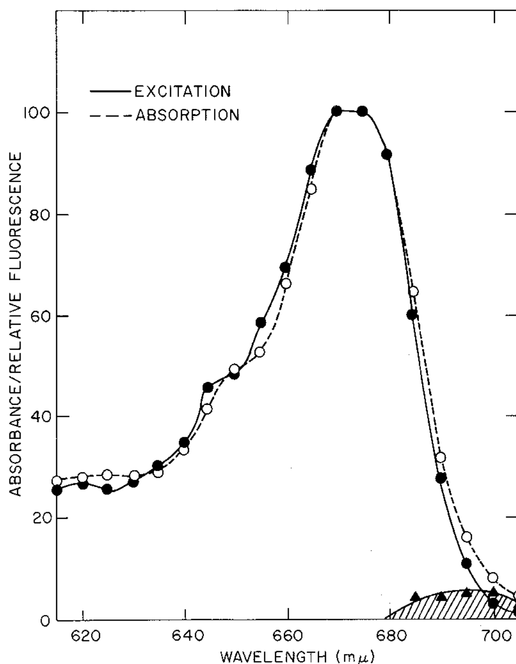


Figure 6. Determination of "inefficient" band component in *Chlorella* from the difference between absorption spectrum and fluorescence excitation spectrum.

Ketskeméty equations* to chlorophyll *a* in *Chlorella* gives the theoretically expected straight line only if the fluorescence yield changes with wave number as shown by the dashed curve in Figure 5*b*. This agreement is more significant as support for the Stepanov-Ketskeméty theory than as confirmation of the experimental quantum yield curve.

Similar data for red and blue-green algae suggest a complex (two-stage?) "red drop" (and a corresponding difficulty in "straightening out" the Stepanov-Ketskeméty curve).

These results indicate that the two main components of the red absorption band do not differ in their contributions to photosynthesis and fluorescence. Calcu-

---

*The Stepanov-Ketskeméty equation which relates the intensities of emission $f_e(\bar{\nu})$ and absorption $k_a(\bar{\nu})$ at the same frequency is given by

$$f_e(\bar{\nu})/k_a(\bar{\nu}) = D(T)\varphi(\bar{\nu})\bar{\nu}^3 \exp(-h\bar{\nu}c/kT)$$

where $\varphi(\bar{\nu})$ is the relative quantum yield of fluorescence and $D(T)$ is a function of temperature alone. In Figure 5*b* the function $F'(\bar{\nu})$ is defined by

$$F'(\bar{\nu}) \equiv 3 \log \bar{\nu} + \log \varphi(\bar{\nu}) - \log f_e(\bar{\nu}) + \log k_a(\bar{\nu}) .$$

From the Stepanov-Ketskeméty relation

$$F'(\bar{\nu}) = h\bar{\nu}c(\log e)/kT - \log D(T) .$$

$F'(\bar{\nu})$ plotted versus $\bar{\nu}$ should yield a straight line, the slope of which depends on T, the vibration temperature of the excited electronic state. $F(\bar{\nu})$ is defined as $F'(\bar{\nu})$ when $\varphi = \text{constant}$.

lation of the absorption band of the "inefficient" chlorophyll *a* component "backwards," from the "red drop" curve, gives the curves shown in Figure 6. The "inefficient" component is much smaller and is located at much longer wavelengths than the long-wavelength component of the doublet in Figure 3. Its intensity is of the order of 10% of that of the main components, and its peak lies at about 700 mμ.

These results show that, even in green cells, the two main red components cannot be identified with the two kinds of chlorophyll *a* postulated to account for the "red drop." In blue-green and red algae, in which the "red drop" already begins at 650 mμ (while the two-component structure seems to be about the same as in green cells), this conclusion is even more obvious.

One possible interpretation is that the two main red band components do not belong to two different forms of chlorophyll *a* at all but result from a doublet splitting of the red band in a system which, although not truly crystalline (as shown by the position of the peak of the red band), may have a kind of pseudocrystalline structure with two pigment molecules to a unit. (Perhaps *in vivo* chlorophyll *a* forms a bimolecular layer, as suggested in some speculative models of the organization of the photosynthetic apparatus.) The resulting band splitting could be similar to that calculated by Hochstrasser and Kasha[10] for crystalline monolayers with two molecules per unit.

That the "inefficient" form of chlorophyll *a* (chlorophyll *a* in system I) may be present in relatively small amounts compared with the "efficient" form (chlorophyll *a* in system II) is easily possible in the "spill-over" model (but not in the "separate package" model) of the two systems.[11]

REFERENCES

1. L.N.M. DUYSENS, Ph.D. thesis, p. 21, Utrecht, The Netherlands, 1952; *Biochim. Biophys. Acta* **19**, 1 (1956).
2. E. RABINOWITCH, *Photosynthesis and Related Processes*, Vol. II, Pt. 2, p. 1863, Interscience, New York, 1956.
3. C.S. FRENCH, in *Light and Life,* p. 447, W.D. McElroy and B. Glass, Editors, Johns Hopkins Press, Baltimore, 1961; *Brookhaven Symp. Biol.* **11**, 65 (1959).
4. J.B. THOMAS, *Biochim. Biophys. Acta* **59**, 202 (1962); J.B. THOMAS AND GOVINDJEE, *Biophys. J.* **1**, 63 (1963).
5. J.S. BROWN AND C.S. FRENCH, *Plant Physiol.* **34**, 305 (1959).
6. C. CEDERSTRAND, E. RABINOWITCH, AND GOVINDJEE, *Biochim. Biophys. Acta,* **120**, 247 (1966).
7. B.I. STEPANOV, *Dokl. Akad. Nauk SSSR* **112**, 839 (1957); *Izv. Akad. Nauk SSSR Ser. Fiz.* **22**, 1367 (1958).
8. I. KETSKEMÉTY, J. DOMBI, AND R. HORVAI, *Acta Physiol. Acad. Sci. Hung.* **12**, 263 (1960); *Ann. Physik* **8**, 342 (1961).
9. L. SZALAY, E. RABINOWITCH, N.R. MURTY, AND GOVINDJEE, *Biophys. J.*, submitted.
10. R.M. HOCHSTRASSER AND M. KASHA, *Photochem. Photobiol.* **3**, 317 (1964).
11. Cf. B. KOK, in *Photosynthetic Mechanisms of Green Plants, Natl. Acad. Sci.–Natl. Res. Council Publ.* **1145**, 45 (1963).