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POSSIBLE INFLUENCE OF THE RATE OF SPECIMEN COOLING ON THE DETERMINATION OF ENERGY DISTRIBUTION IN PHOTOSYNTHESIS BY FLUORESCENCE EMISSION AT 77 K

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Summary

The rate of cooling to 77 K appears to be a determining factor in obtaining valid low temperature emission spectra of photosynthetic organisms. Evidence is shown that the usual method of cooling the algae or chloroplasts in suspension leads to artefacts in the spectra and considerable discrepancies in quantitative determinations.

The fluorescence spectra of photosynthetic organisms, when taken at the temperature of liquid nitrogen, provide a frequently used criterion for the evaluation of the underlying pigment systems. Thus, fluorescence emission spectra at 77 K have been used to assign fragmented thylakoids into those particle fractions containing both pigment systems and those possessing predominantly Photosystem I (see the review by Goedheer, [1]). Another application of the method is the determination of energy distribution between the various photosystems prominent in current research [2, 3].

In this communication, attention is drawn to the rate of cooling, one of the various parameters influencing the measured spectra. If the freezing process is too slow, biological membranes are considerably damaged by crystallization and osmotic processes connected with their structural water [4]. Since the microenvironment of the pigment complexes within the thylakoid membrane is the main parameter determining the obtained fluorescence spectra, while absorption characteristics are much less affected, the importance of a membrane preparation undamaged during cooling is evident [5]. To avoid distortions and artefacts in the spectra, the cooling rate of the specimen should be at least around 10^4 degrees/s [6, 7].

It was noticed that the photosynthetic organisms and organelles for many of the published spectra were frozen as an aqueous suspension, normally in cuvettes of 2 mm width. Water, however, can be assumed to act as an

insulator under these circumstances, since it possesses a relatively low thermal conductivity (0.001340 cal/s per cm^2 at 273 K). In order to cool down to the temperature of liquid nitrogen, the heat of the suspended organelles has to be removed across the thermal barrier of the surrounding medium and, in many cases, that of the plexiglas covers of the sample mount as well. A cooling rate much slower than the 10^4 K/s required, with ensuing damage to the pigment-containing membrane, can be expected.

A demonstration of this point is given in Fig. 1, which shows the effect of

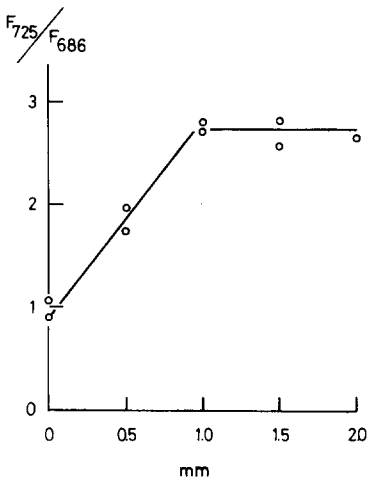

Fig. 1. Ratio of long wavelength (Photosystem I) to short wavelength (Photosystem II) emission, taken at liquid N_2 temperature, as a function of the spacing of the cuvette used. *Chlorella* autospores from fully synchronized cultures were used in the determination. The distance between the two plexiglas window panes of the cuvette was altered by the use of brass spacers of appropriate thickness. In the reference (at 0 mm spacing) the algae were adsorbed to a double layer of cheesecloth mounted on two interlocking polypropylene rings. Chlorophyll concentration/cm^2 exposed surface area was about equal in all instances, approx. 2.1 μg. The probes were cooled down by immersion in liquid N_2. Excitation at 595 nm. See ref. 8 for further details.

increasing the rate of cooling by reducing the thickness of the aqueous suspension, on the relation of the Photosystem I (F_{725}) to II emission (F_{686})in *Chlorella* . The lowest ratio, indicating a relatively intact membrane, is obtained if the sample is adsorbed on cheesecloth and thus in direct contact with the surrounding liquid nitrogen. The use of melting nitrogen with such samples (in order to avoid the Leidenfrost phenomenon) did not significantly alter the ratio obtained with the cheesecloth method. Fully synchronized autospores of *Chlorella* have been used in the experiment shown to avoid any side effects which might be introduced by damaged chloroplasts. Similar results, however, were obtained with spinach plastids as well (see column 1 in Table I).

The cooling rate constitutes an important factor in quantitative measurements. An example of this is the calculation of the fraction of absorbed light funneled into Photosystem II (β) and Photosystem I (α) of chloroplasts, reported by Butler and Kitajima [3]. In Table I these calculations have been repeated with sets of experimental data obtained from the same chloroplast preparation. The only difference in the various determinations was the freezing rate, altered by increasing the average thickness of the aqueous layer between chloroplast and liquid N_2. Care was taken that the chlorophyll

TABLE I

THE LIGHT DISTRIBUTION BETWEEN THE PHOTOSYSTEMS, CALCULATED ACCORDING TO BUTLER AND KITAJIMA [3], IN SAMPLES OF THE SAME CHLOROPLAST PREPARATION USING DIFFERENT TYPES OF SPECIMEN PROBES

Spinach chloroplasts, prepared in 0.4 M sucrose/2 mg/ml ascorbate/1 mg/ml bovine serum albumin/0.1 M Tricine, pH 7.8, washed and stored at 0° C in the dark in 0.4 M sucrose/0.1 M Tricine, pH 7.8. For the measurements, the suspension (1.2 mg chlorophyll/ml) was diluted, using this medium, to a concentration of 0.6 μg chlorophyll/cm^2 illuminated area. Excitation with blue light between 450—500 nm. The emission peaks appeared at 687 nm (F_{685}), 695 nm (F_{695}) and 738 nm (F_{732}). The signals at 687 and 738 nm were used in the calculations after appropriate corrections for photomultiplier sensitivity and instrument deviation. The values are averages from 10 (cheesecloth adsorbed) to 4 (suspension frozen in cuvettes) independent determinations. Individual measurements differed no more than 10% from each other.

Probe	Addition	F_{732}/F_{685}	$(F_O/F_V)_{685}$	α	β	$\alpha(-Mg)/\alpha(+Mg)$	$\beta(-Mg)/\beta(+Mg)$
Cheesecloth mounting	—	2.13	4.1	0.135	0.865	1.42	0.955
	5 mM Mg^{2+}	1.09	4.8	0.095	0.905		
1-mm cuvette spacing	—	2.67	1.6	0.337	0.663	1.47	0.858
	5 mM Mg^{2+}	1.51	1.9	0.228	0.772		
2-mm cuvette spacing	—	2.72	1.2	0.380	0.620	1.36	0.864
	5 mM Mg^{2+}	1.61	1.1	0.283	0.717		

content was identical and low enough in all probes to avoid concentration artefacts.

As can be seen from the data, not only the ratio F_{732}/F_{685} was considerably influenced, but the value of α, the fraction of harvested light channeled into Photosystem I ($\alpha + \beta = 1$) as well. This result clearly shows that an experimental determination of energy distribution using the spectral properties at liquid N_2 temperature remains doubtful as long as the artefacts of freezing are not properly considered. It is, however, noteworthy that the ratio of α in the absence and presence of Mg^{2+}, a measure of the Mg effect first described by Murata [2], is similar in all circumstances.

In summary, the data suggest that all quantitative interpretations based on spectral data obtained with aqueous suspension have to be viewed with serious reservations.

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