SHORT COMMUNICATIONS

BBA 43266

A spectrophotometric method for the quantitative estimation of intact (Class I) chloroplasts

Most preparations of spinach chloroplasts contain two main classes which can be distinguished by phase-contrast microscopy and electron microscopy[1–5]. One of these (Class I) has an opaque appearance with a halo and the grana are not visible. These chloroplasts are fairly intact, bounded by their outer membrane and contain much of the stromal material. The other class appears darker in phase-contrast microscopy and has usually distinct visible grana and the chloroplasts have lost part or all of the outer membrane and also much of the stroma. Separation of the two classes has been accomplished by counter-current distribution[6,7] and density gradient centrifugation[5]. The estimation of the relative ratio of Class I to Class II chloroplasts is important for judging the quality of a preparation and to correlate this with its biochemical activities. Microscopic counting is tedious and time-consuming and a simpler and quicker method is therefore desirable. RIDLEY AND LEECH[8] examined the possibility of using the size distribution recorded by a Coulter counter but found that it could not be used quantitatively for determination of the two classes. ROBINSON

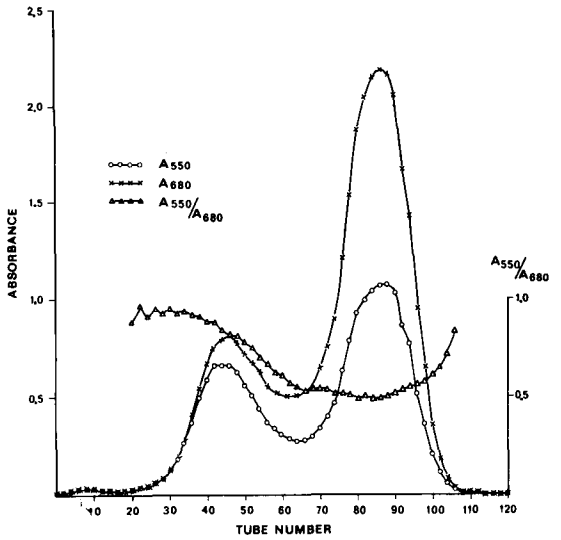

Fig. 1. Counter-current distribution of spinach chloroplasts from normal leaves. The peak to the left contains Class I chloroplasts and the peak to the right Class II chloroplasts. The phase system in each fraction which contains 6.4 % (w/w) dextran, 6.4 % (w/w) polyethylene glycol, 9.2 % (w/w) sucrose and 0.005 M sodium phosphate (pH 7.8) was diluted 3–4 times with a solution containing 0.4 M sucrose and 0.05 M Tris–HCl, pH 7.8, before the absorbance was measured.

AND STOCKING[9] indicated the use of oxygen evolution, determined by an oxygraph, for the estimation of the percent intact chloroplasts. In the following communication a simple, yet accurate, spectrophotometric method is presented which is particularly suitable when many samples are to be characterized. It was observed in counter-current distribution experiments with chloroplasts[7] that the absorbance characteristics (*e.g.* the absorbance ratio $A_{550\ nm}/A_{680\ nm}$) of the two classes were different. Such a difference can be utilized for the determination of the percent of Class I chloroplasts.

Fig. 1 depicts a counter-current distribution curve of spinach chloroplasts prepared by differential centrifugation in a manner previously described[7]. Microscopic inspection showed that the left peak contained mainly Class I and the right peak mainly Class II chloroplasts. In between the two peaks is a mixture of the two classes with a decreasing proportion of Class I chloroplasts with increasing tube number.

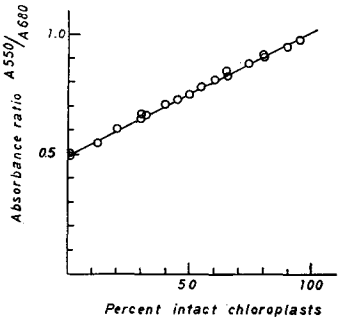

Fig. 2. Absorbance ratio *versus* percent intact (Class I) chloroplasts. The samples were taken from different extraction tubes from four different counter-current distributions of spinach chloroplasts like that in Fig. 1.

The absorbance at 550 nm and 680 nm of the different fractions was also measured with a Zeiss PMQ II spectrophotometer and their ratio plotted in Fig. 1. As can be seen, Class I chloroplasts have a higher $A_{550\ nm}/A_{680\ nm}$ ratio than Class II chloroplasts.

The number of Class I and Class II chloroplasts from different tubes were counted under the microscope. If the percent of Class I chloroplasts is plotted against the absorbance ratio $A_{550\ nm}/A_{680\ nm}$, a linear plot is obtained (Fig. 2), and hence such an absorbance ratio can be used for the quantitative determination of Class I chloroplasts after a standard curve like that in Fig. 2 has been determined.

We believe that the difference in absorbance ratio between the two classes of chloroplasts is due to the following: when the outer membrane of an intact Class I chloroplast is stripped off, much material leaks out but practically all the chlorophyll remains bound to the thylakoid membrane system of the Class II chloroplasts so formed. As a result the refractive index of Class II chloroplasts is much smaller than that of Class I chloroplasts (compare their appearance under the phase-contrast microscope). Therefore the absorbance at 550 nm, which is to a great extent due to light scattering, is smaller per chloroplast for Class II than for Class I. The absorbance at 680 nm, however, is not affected to the same degree by a transformation from Class I to Class II chloroplasts, since it is mainly due to chlorophyll which is present in about the same amount in both classes. This explanation is supported by the following experimental results: (1) When a suspension of Class I chloroplasts is allowed

to stand at room temperature and the chloroplasts transform into Class II chloroplasts, as judged with the microscope, the $A_{550\,nm}/A_{680\,nm}$ ratio changes from 1.0 to a value close to 0.5. (2) The absorbance values depend on which spectrophotometer is used. Depending on the arrangement of the apparatus, including the distance between photocell and cell holder, different quantities of scattered light are recorded.

Since light scattering contributes to the absorbance and is essential for the spectrophotometric difference between the two chloroplast types, the same spectrophotometer which is used for determining the standard curve, where microscope counts are plotted against absorbance ratio, must be used throughout a series of experiments. Also, the same suspension medium should be used for making the standard curve as for the determinations and the chloroplasts should be stable in the medium. It is important that the concentration of chloroplasts be in a range such that a linear relationship between absorbance and concentration pertains. This range is different for different spectrophotometers and wavelengths. In the case reported, the absorbance at 550 nm was linear to concentration up to an absorbance of about 0.8. At 680 nm the corresponding value is much higher. No gross contamination with particles other than chloroplasts is allowable in the suspension since such particles contribute to light scattering. When these requirements are fullfilled the method is rapid and simple and is particularly useful in fractionation experiments in which many fractions have to be analyzed.

Department of Biochemistry,
University of Umeå,
Umeå (Sweden)

BJÖRN KARLSTAM
PER-ÅKE ALBERTSSON

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