DEMONSTRATION OF THREE CLASSES OF SPINACH CHLOROPLASTS BY COUNTER-CURRENT DISTRIBUTION

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1. Introduction

Conventional preparations of "whole" chloroplasts are very heterogeneous. They consist of particles which are more or less damaged by the isolation procedure.

Two main classes have been distinguished by light and electron microscopy [1,2,3,4]. One class (class I) has an opaque appearance and no visible grana by phase contrast microscopy. These are probably fairly intact chloroplasts surrounded by an outer membrane and containing stromal material. The other class (class II) has lost its outer membrane and also much of the stromal material. In phase contrast microscopy these chloroplasts appear dark and usually contain visible grana. Most preparations reported in the literature as "whole" chloroplasts are mixtures of these two classes. Separation of the two types was first accomplished by Albertsson and Baltscheffsky [5] using counter-current distribution and by Leech [6] using density gradient centrifugation.

In this communication we have demonstrated that chloroplasts from spinach can be resolved into at least three classes. Two of these resemble the intact chloroplasts and are denoted as class IA and IB.

2. Materials and methods

Dextran was obtained from Pharmacia, Uppsala, Sweden, as "Dextran 500" batch No. 4024.

Polyethylene glycol (PEG) was obtained from Union Carbide, New York as "Carbowax, polyethylene glycol 4000".

2.1. *Chloroplast preparation*

All operations were carried out in cold room at +2°C Spinach was grown in artifical light at +15°C with 16 hr light (5000 Lux) and 8 hr darkness. Leaves were harvested after a 7–8 hr dark period, cut into pieces by a pair of scissors and then homogenized with three volumes of 0.05 M tris HCl pH 7.8, and 0.4 M sucrose, in a chilled knife blendor. The knives were rotated for 3–4 periods of 2 sec each. The homogenate was filtered through 4–8 layers of cheese cloth. The filtrate (150 ml) was first centrifuged for 1 min at 80–100 *g* in 4 centrifuge tubes of 50 ml capacity. The supernatant was then centrifuged for 10 min at 300 *g*. The pellet was resuspended in the same volume of medium as used for homogenization (or occasionally in 0.4 M sucrose only) and washed three times in it (i.e. centrifuged and resuspended three times). The last resuspension was done in about 3 ml of 0.4 M sucrose.

2.2. *Counter-current distribution*

Phase system (A) containing 6.3% (w/w) dextran and 6.3% (w/w) PEG 4000 was prepared by mixing 63 g of 20% (w/w) dextran, 31.5 g of 40% (w/w) PEG 4000, 50 ml of 30% (w/w) sucrose, 5 ml 0.2 M K phosphate buffer, pH 7.8 (ratio 1 KH_2PO_4 to 9 K_2HPO_4) and making up to a weight of 175 g with water. 25 ml 0.4 M sucrose was then added. The whole mixture was shaken at +2°C and allowed to separate in a funnel. The two phases were collected and stored separately.

Chloroplast sample system was prepared as follows: 3.78 g of 20% (w/w) dextran, 1.89 g of 40% (w/w) PEG 4000, 3 ml of 30% (w/v) sucrose, 0.30 ml of 0.2

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M K phosphate buffer, pH 7.8, were mixed and made up to 10.5 g with H_2O. 1.5 ml of the chloroplast suspension in 0.4 M sucrose was then added. Except for the presence of chloroplasts, this mixture is now identical to phase system A. The volume ratio top/bottom is 6.6/4.6. For counter-current distribution a volume ratio of 1:1 was desired. Therefore 2 ml of the bottom phase from phase system A was added to the chloroplast sample system. The whole mixture was shaken and used for loading the CCD apparatus.

Some batches of dextran give rise to slightly different distributions of chloroplasts in the phase systems. One can compensate for this by altering the ionic composition and/or polymer concentration [10]. Thus, since Na^+ always gives rise to a higher partition than K^+, batch variations may be alleviated by altering the Na^+/K^+ ratio in the pH 7.8 phosphate buffer.

An automatic thin-layer counter-current distribution apparatus [7] with 120 chambers (marketed by IRD, Bromma, Sweden) was used. The bottom phase chamber has a capacity of 0.7 ml. Since the chloroplasts partition between the upper phase and the interface, the method of liquid-interface counter-current distribution [8,9] was employed. Each of the chambers 10–119 were charged with 0.6 ml bottom phase and 0.6 ml top phase. Chambers 0–9 were charged with 1.2 ml of the chloroplast phase system mixture. The settling time was 8 min and the shaking time was 30 sec. The temperature was about +2°C. After 120 transfers the fractions were collected and diluted three-fold with 0.4 M sucrose, containing 0.05 M tris HCl, pH 7.8 and mixed in order to break the phase system. Absorbance of the diluted fractions was measured with a Zeiss PMQ II spectrophotometer.

3. Results

The results of a counter-current distribution experiment with chloroplasts from cotyledons is shown in fig. 1. Three peaks are obtained. The chloroplasts from the different peaks were studied with a phase contrast

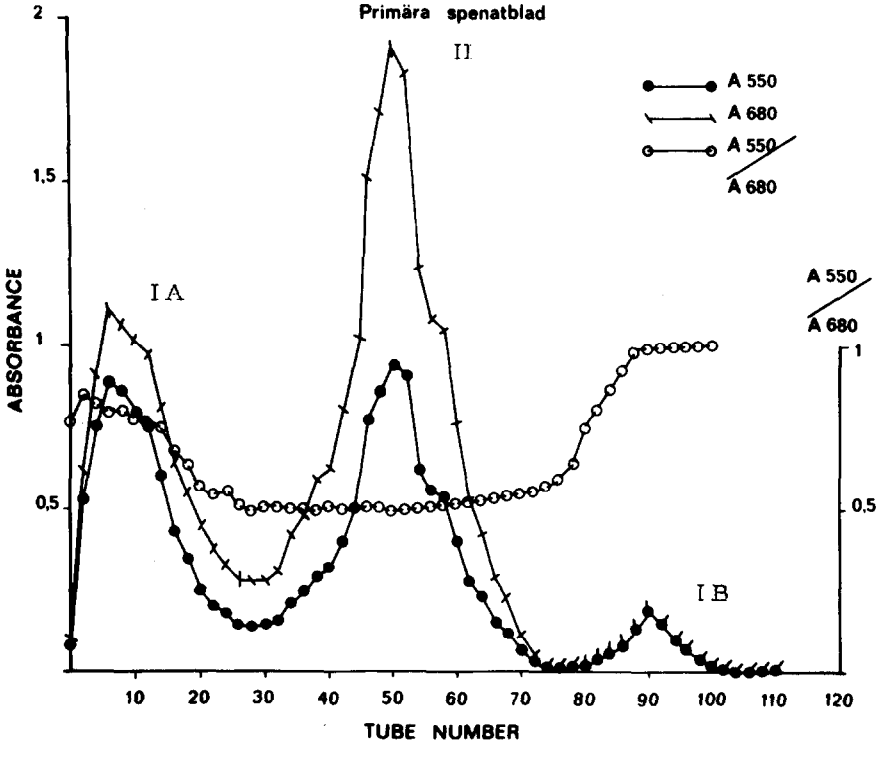

Fig. 1. Counter-current distribution of chloroplasts from spinach cotyledons. Peaks IA and IB represent intact chloroplasts (class I) while peak II represents stripped chloroplasts (class II).

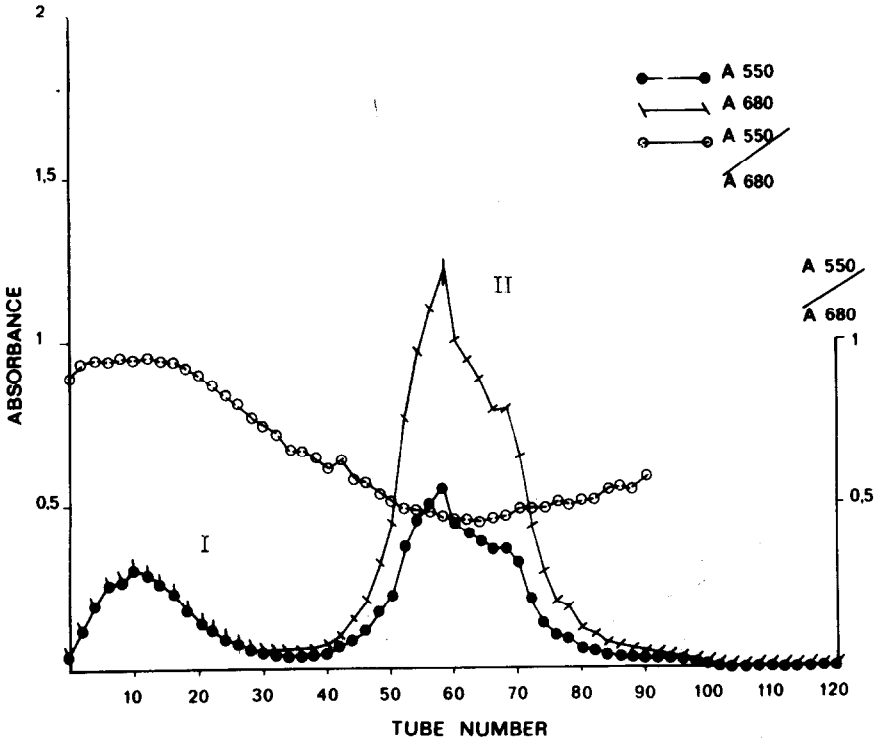

Fig. 2. The same as fig. 1 but with normal leaves.

microscope. The middle peak consisted almost entirely of class II chloroplasts i.e. chloroplasts with dark appearance and visible grana. The material found under this peak is very heterogeneous and consists of chloroplasts which are swollen and damaged in varying degrees. The two other peaks contained both class I chloroplasts. The right-hand peak (IB) contained apparently 90–100% of class I chloroplasts while the left-hand peak (IA) contained about 80% of class I, the rest being of ckass II. In the left-hand peak the % intact chloroplasts decreases with tube number.

In fig. 1 the absorbance of the different fractions at two wavelengths, 550 nm and 680 nm, and also the ratio between the two has been plotted. As can be seen, the ratio between the absorbance at 550 nm and 680 nm is different for different peaks. It is highest for the right-hand peak and lowest for the peak in the centre. As will be shown in a separate publication [10] there is a linear relationship between the absorbance ratio A_{550}/A_{680} and the % content of chloroplasts of class I in a chloroplast suspension.

Fig. 2 shows a similar experiment with chloroplasts from normal leaves. In this case only two peaks are obtained. The right-hand peak contains class II chloroplasts and the left-hand peak class I chloroplasts. However, in tubes no. 80–90 between 10–30% of class I chloroplasts could be observed in the microscope. Also the ratio A_{550}/A_{680} increased in this region. Thus there is a small but significant population of intact chloroplasts in normal leaves with the same distribution behaviour as class IB chloroplasts of cotyledons. See also fig. 1b, tube No. 41–43, in reference [5].

4. Discussion

Particles are separated by counter-current distribution, mainly according to their surface properties [8,9]. A large difference in surface properties between class I and class II chloroplasts is expected since the latter have more or less lost their outer membrane.

From fig. 1 it appears that the difference in surface properties between class IA and class IB is even greater than between class I and class II. Since class IA and IB both contain "intact" chloroplasts, both presumably with the outer membrane, it must be concluded that the outer membranes of class IA and IB chloroplasts are different. To be certain of such a conclusion, however, one must await result of an examination of the two types (IA,IB) of chloroplasts by electron microscopy and chemical analysis.

There might be several reasons for the presence of two or even more types of chloroplasts in a leaf. One is that there are distinct stages during the development or growth cycle of the chloroplasts. Another is that two genetically different strains of chloroplasts grow side by side in a leaf either in the same type of cells or in different cells for example in the mesophyll and parenchymal vascular bundle sheath cells [11,12]. At present the possibility that the two types of chloroplasts, IA and IB, are products of the isolation procedure cannot be excluded. However, we consider this possibility unlikely since the presence of class IB is essentially restricted to cotyledons.

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