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A Study of DNA from Chloroplasts Separated by Counter-current Distribution

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Three types of chloroplasts (Peak I, Peak II, and Peak III chloroplasts) isolated by counter-current distribution in aqueous polymer two-phase systems have been studied with respect to DNA content. (The characterization was performed by studying the buoyant density, the melting profile and the amount of DNA of each type). The DNA was found to differ between the three types of chloroplasts.

The counter-current distribution (CCD) of chloroplasts using aqueous polymer two-phase systems has shown that a chloroplast preparation is composed of three types of chloroplasts.[1] These differ with respect to the outer most membrane surrounding the chloroplast. One class has an intact envelope (Peak I), another type has a broken envelope (Peak II) and in the third type the chloroplast envelope is surrounded by a layer of cytoplasm enclosed within a membrane (Peak III). This cytoplasmic layer also contains mitochondria and peroxisomes. It is thus obvious that it is necessary to achieve a good separation of the different types of chloroplasts before a study of the chloroplasts can be carried out.

Chloroplasts have, by many authors,[2-5] been shown to contain DNA. The study was from the beginning concentrated on the characterization of the DNA with respect to buoyant density, melting characteristics, homogeneity, and content of DNA in the chloroplast. The main problem was to obtain clean preparations not contaminated with nuclear DNA, mitochondrial DNA, or DNA from other organisms, such as algae or bacteria.

The aim of this work was to investigate whether the three types of chloroplasts described above differed with respect to content, buoyant density and melting profiles of the DNA contained in them.

MATERIALS AND METHODS

Polymers used were Dextran 500, from Pharmacia Fine Chemicals, Uppsala, Sweden, and poly(ethylene glycol), Carbowax 6000 and Carbowax 4000, from Union Carbide, New York, USA. Cesium chloride was Suprapur, Merck, Darmstadt, Germany. All other chemicals used were of analytical reagent grade. The water used was distilled twice in a quartz apparatus.

Spinach (Viking II, Weibulls, Landskrona, Sweden) was grown in artificial light, with a light intensity of 5000 Lux, alternating with dark periods of 12 h. The temperature during the growth was 18 °C and the relative humidity 70 %.

Preparation of chloroplasts. 500 – 700 g spinach leaves were harvested and washed 3 times with 5 l of newly distilled water at a temperature of 4 °C. The leaves were homogenized 3 × 2 s in 2 l preparation medium (0.05 M potassium phosphate buffer pH 7.8 and 0.4 M sucrose) in a Turmix blendor. The homogenate was filtered through four layers of perlon net (Monodur 31 μ Vereinigte Seidenwebereien AG, Krefeldt, W. Germany). The filtrate was then centrifuged in a Sorvall RC 2B high speed centrifuge for 10 min at 400 *g*. The pellet was resuspended in the preparation medium and centrifuged for 15 min at 400 *g*. This pellet was used in the sample phase system.

Two-phase system. The phase system was composed of 630 g 20 % w/w dextran, 305 g 40 % w/w poly(ethylene glycol) 4000, 500 ml 30 % w/v sucrose, 50 ml 0.2 M potassium phosphate buffer pH 7.8 and water to 2012 g. This system was scaled up by a factor of ten as compared to Ref. 1.

Sample phase system. The chloroplast pellet in a typical experiment weighed about 13 g. It was suspended in 11 g of upper phase of the phase system mentioned above. To this suspension were added 7.31 g 40 % w/v poly(ethylene glycol) 4000, 0.6 g 20 % w/w dextran, 8.5 ml 30 % w/v sucrose and water to 45 g.

Counter-current distribution. The CCD was carried out in an all glass machine (Gallenkamp, England). The capacity of each tube for stationary phase was 40 ml. To get a stationary interphase each chamber except chamber 2 was filled with 35 ml of the lower phase and 45 ml of the upper phase. Chamber 2 was filled with 35 ml of the lower phase and the sample system. Number of transfers were 19, the shaking time 45 s and the settling time 20 min. The temperature during the run was +2 to +4 °C.

Extraction of DNA. The DNA was extracted from chloroplasts using the method described by Rudin and Albertsson.[6] In the present case the volumes of that procedure were scaled down by a factor of six.

Removal of polymers. Poly(ethylene glycol) and lithium phosphate buffer pH 7.8 were added to the upper phase from the extraction procedure to obtain a final concentration of 10 % w/w and 10 mM, respectively. The phase system thus formed separates into an upper phase which is free from dextran and containing all the DNA and a small lower phase. The upper phase was removed and CsCl was added to it to a density of 1.7 g cm^{-3}. In the resulting two-phase system the poly(ethylene glycol) distributed exclusively to the upper phase. The DNA was found in the polymer free CsCl phase. The CsCl was then removed by dialysis.

Ultracentrifugation. The density equilibrium centrifugation experiments were performed in a Spinco analytical ultracentrifuge Model E fitted with a Kel F centerpiece and MSE Centriscan 75 ultracentrifuge using the technique described by Schildkraut, Marmur and Doty.[7] DNA from *Micrococcus lysodeikticus* was used as reference DNA. The calculations were based upon a banding at the density 1.731 g cm^{-3} for the reference DNA. The centrifugations were carried out at 44 000 rpm and 20 °C. After at least 20 h the cell contents were analysed and in the case of Spinco the photographs were analysed in a Beckman Analytrol.

Melting curves. The melting curves were obtained using a Zeiss PMQ II spectrophotometer with termostated cuvette holder. The temperature was raised at the rate of 1 °C/min.

Chlorophyll measurements. Chlorophyll was determined by the method of Arnon.[8]

Calculation of DNA content. The calculation of DNA per chloroplast was referred to the chlorophyll content, one chloroplast containing 1×10^{-6} μg chlorophyll.[9]

RESULTS

The three types of chloroplasts obtained by CCD are classified according to their position in the CCD-diagram: Peak I chloroplasts partition to the left, Peak II chloroplasts partition in the middle and Peak III chloroplasts partition to the right.[1] Fig. 1 shows a CCD-experiment of 19 transfers with chloroplasts as sample. Peak I chloroplasts were collected from tube number 1 to 5, Peak II chloroplasts from tubes number 8 to 10 and Peak III chloroplasts from tubes number 13 to 18. Karlstam and Albertsson showed that the absorbance ratio (A_{550}/A_{680}) could be used to determine percentage of intact chloroplasts.[10] Intact chloroplasts have an absorbance ratio of close to 1. Peak I contains mainly intact chloroplasts. The broken chloroplasts found in Peak I are probably caused by destruction of intact chloroplasts during the end of the CCD.

Each type of chloroplasts collected as above were washed with preparation medium. The

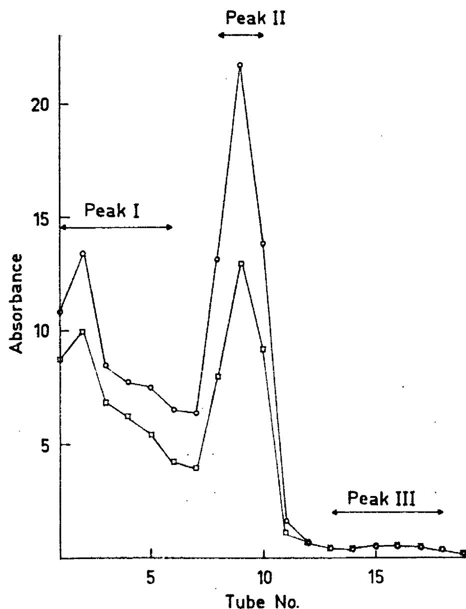

Fig. 1. Chloroplasts from 700 g spinach leaves were distributed in a glass Craig apparatus. Each tube had a capacity of bottom phase of 40 ml. The number of transfers was 19 and the temperature during the experiment 2 °C. Absorbance at 680 nm (O) and absorbance at 550 nm (□).

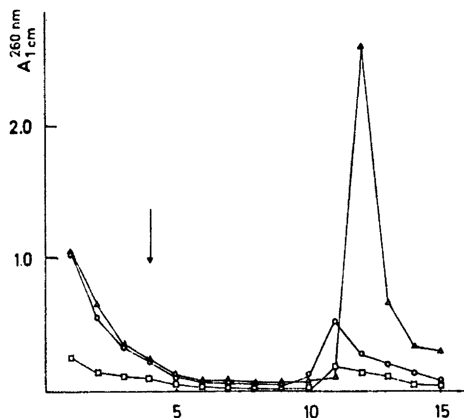

Fig. 2. Extraction of DNA from the three peaks shown in Fig. 1. The extraction profile for Peak I (○), for Peak II (△), and for Peak III (□), respectively. Vertical arrow indicates a shift in phase system to the one in which the DNA is transferred to the upper phase (tube numbers 11 and 12).

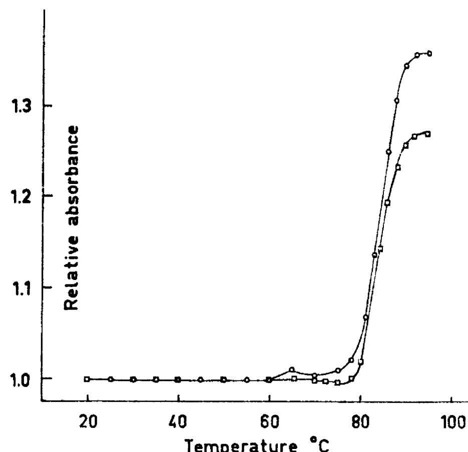

Fig. 3. Melting curves for Peak I DNA (□) and Peak II DNA (○). The concentration of salts was 0.15 M NaCl and 0.015 M sodium citrate.

DNA was then extracted from the chloroplasts in an aqueous polymer two-phase system and the results are shown in Fig. 2. The arrow in the figure indicates a shift of phase system to the one in which the DNA is extracted to the upper phase as the salt concentration diminishes. This usually occurs in tubes number 11 or 12. The DNA used in the subsequent experiments was taken from tube number 11 for Peak I, tube number 12 for Peak II and tube number 11 for Peak III. The number of chloroplasts used in these experiments was based on the chlorophyll content in the three chloroplast pellets obtained after the CCD. The DNA content of the three types of chloroplasts is recorded in Table 2. The value, 9×10^{-14} g DNA per chloroplast for Peak III DNA is somewhat uncertain due to the low amount of DNA obtained in the extraction procedure. It is nevertheless significantly higher than the

Table 1. Buoyant densities of chloroplast DNA from Peak I, Peak II and Peak III chloroplasts.

Preparation	Native DNA	Heat denatured DNA	Alk. denatured DNA
Peak I			
1	1.697	1.714	1.713
2	1.696	1.713	1.713
3	1.696	1.714	1.713
4	1.696		
5	1.696		
Peak II			
1	1.694	1.710	1.710
2	1.695	1.711	1.712
3	1.694	1.710	1.710
4	1.694		
5	1.694		
Peak III			
1	1.697		
2	1.696		
3	1.696		

Table 2.

	DNA per chloroplast ($\times 10^{14}$ g).	Mol per cent (GC) calculated from T_m according to Marmur and Doty[11].	Mol per cent (GC) calculated from buoyant density according to Schildkraut *et al.*[7]
Peak I Chloroplast DNA	3.3	36	37
Peak II Chloroplast DNA	7.2	35	35
Peak III Chloroplast DNA	9	36	37

DNA content in the other fractions for which the values were 3.3×10^{-14} g DNA per chloroplast for Peak I and 7.2×10^{-14} g DNA per chloroplast for Peak II.

Heat denaturation of Peak I and Peak II DNA was performed in a solution containing 0.15 M NaCl and 0.015 M Na-citrate. The results are shown in Fig. 3. A melting point (T_m) of 84 °C and a hyperchromicity of 28 % was obtained for Peak I DNA. For Peak II DNA T_m was the same but the hyperchromicity was 35 %. The hyperchromicity varied between 28 % and 33 % for Peak I DNA and between 30 % and 35 % for Peak II DNA in the melting experiments performed.

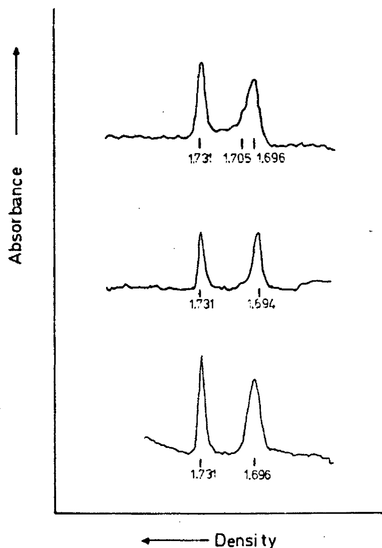

Fig. 4. Densitometer tracings of samples equilibrated in a CsCl density gradient formed by centrifugation at 44 000 rpm. The peak to the left is the standard DNA (*Micrococcus lysodeikticus* DNA). The curve at the top represents Peak III DNA, the curve in the middle Peak II DNA and the curve at the bottom of the diagram Peak I DNA.

The buoyant density analytical patterns for Peak I, Peak II and Peak III DNA are shown in Fig. 4. The three experiments were carried out on the DNA prepared from the three chloroplast types obtained from the same initial chloroplast preparation. In each experiment DNA from *Micrococcus lysodeikticus*, prepared according to Rudin and Albertsson[6] was used as marker. The shoulder at 1.705 for Peak III DNA is probably caused by mitochondrial DNA from the cytoplasm layer surrounding Peak III chloroplasts. Peak I DNA and Peak III DNA:s banded at a density of 1.696 g cm^{-3} while Peak II DNA banded at 1.694 g cm^{-3}. The small peaks between the shoulder and the marker DNA were identified as irregularities in the photograph, due to dust in the lens system. As shown in Table 1 the density values are reproducible, which indicates that there are differences between Peak I DNA and Peak II DNA. Heat denatured and alkaline denatured DNA was run in a CsCl-gradient. Peak I DNA was found to band at 1.713 g cm^{-3} while Peak II DNA banded at 1.711 g cm^{-3}. This is in agreement with the difference found between native Peak I and Peak II DNA.

Table 2 shows the mol per cent Guanosine-Cytosine (G–C) in each type of chloroplast DNA determined from the buoyant density and the melting point, respectively, of the corresponding DNA. The agreement between the two methods is good and shows that the mol per cent (G–C) is 35–37 in the DNA from the three chloroplast fractions.

DISCUSSION

The buoyant density values for spinach nuclear DNA of 1.694 and chloroplast DNA of 1.696 reported by Whitfield and Spencer[5] agree with the values above for Peak II and Peak I DNA, respectively. We therefore conclude that Peak I DNA is chloroplast DNA, Peak II DNA is composed mainly of nuclear DNA, and finally Peak III DNA consists of chloroplast DNA and mitochondrial DNA.

Suyama and Bonner[12] have reported a banding of different mitochondrial DNA from plants at 1.706 g cm^{-3}. Tewari[13] has reported the same buoyant density for mitochondrial DNA. It is therefore reasonable to assume that the shoulder in the buoyant density profile at 1.705 for Peak III DNA in Fig. 4 represents mitochondrial DNA.

Kolodner and Tewari[14] have reported a value of $(0.5-1.5)\times 10^{-14}$ g DNA per chloroplast while Chiba and Sugahara[15] have reported a higher value, $(3.8-6.9)\times 10^{-14}$ g DNA per chloroplast. The lower value may be due to losses of DNA in the extraction procedure. Our value 3.3×10^{-14} g DNA per Peak I chloroplast seems high compared to Tewari's result but agrees fairly well with the results of Chiba and Sugahara. The following reasons can be suggested for the high value 9×10^{-14} g DNA per Peak III chloroplast.

1. Peak III chloroplasts contain less chlorophyll than Peak I chloroplasts.

2. It has been suggested that chloroplasts undergo division.[16] In the mitotic state the chloroplasts contain double amount of DNA. Peak III chloroplasts might be chloroplasts in the mitotic state; this would explain the high amount of DNA.

3. DNA from the mitochondria in Peak III contributes to the DNA content.

A combination of point 3 and one or both of the other is a probable explanation for the higher value of the DNA content in Peak III chloroplasts. The DNA in Peak II chloroplasts is as mentioned earlier probably nuclear DNA. The DNA cannot be losely bound to the membranes because in that case it would have been washed away when the chloroplasts were collected after the CCD. Further the DNA has a high partition coefficient and would have been found to the far right in the CCD-diagram. It is thus probable that the nuclear DNA is rather tightly bound to the membrane system in Peak II chloroplasts. This binding might occur during the cell breakage and the subsequent handling of the leaf extract. DNA obtained from chloroplast preparations containing both intact and broken chloroplasts, would therefore be a mixture of chloroplast and nuclear DNA. It is therefore essential first to isolate intact chloroplasts surrounded by their envelope, before DNA extraction if one wants to claim that the DNA is of chloroplast origin.

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