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ISOLATION OF DIFFERENT CLASSES OF SPINACH CHLOROPLASTS BY COUNTER-CURRENT DISTRIBUTION A METHODOLOGICAL STUDY

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SUMMARY

1. Various factors such as polymer concentration, ionic composition and pH which determine the partition of chloroplasts from spinach in a dextran–polyethylene glycol–water two-phase system have been investigated.

2. Optimal conditions for the application of counter-current distribution on chloroplasts from spinach are described. Two and sometimes three classes of chloroplasts are obtained.

INTRODUCTION

A biochemical characterization of isolated intact chloroplasts is important for our understanding of the function of this organelle inside the cell. After cell breakage a very heterogeneous population of various cell particles is obtained. This suspension may be purified by differential centrifugation whereby fragments of chloroplasts and most of the other smaller cell organelles are separated from the "whole" chloroplasts. The whole chloroplast preparation thus obtained is still very heterogeneous, consisting of chloroplasts with very different appearance in the phase contrast and electron microscope.

Two main classes can be distinguished. Already in 1954 McCLENDON[1] described two types of isolated chloroplasts which were studied by the phase contrast microscope. JACOBI AND PERNER[2] and KAHN AND VON WETTSTEIN[3] described two types of isolated chloroplasts by electron microscopy. Since then authors have described these two chloroplasts obtained from different plants and through different preparation procedures[4–8]. The two types of chloroplasts are usually called Class I and Class II. Class I chloroplasts have an opaque appearance and their grana are, under certain conditions, not visible with phase contrast microscopy. In the electron microscope these chloroplasts look fairly intact; they are, for example, surrounded by an outer envelope. Class II chloroplasts are those that have lost their outer membrane and also much of their stromal content. In a phase contrast microscope these chloroplasts appear darker as compared to Class I, and the grana are usually visible. The ratio

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between these two types of chloroplasts varies considerably depending on the type of plant from which the material is taken and the method of preparation and media used. In addition there is probably a whole spectrum of chloroplasts in a state intermediate between the two main classes. These two classes were separated from each other by counter-current distribution[9,10] and by density gradient centrifugation[11–13].

Recently we demonstrated[10] that chloroplasts from spinach can be further divided into at least three different populations by counter-current distribution using aqueous polymer two-phase systems. The three classes of chloroplasts differ in their morphology as revealed by electron microscopy, in protein/chlorophyll ratio and also size distribution[14].

The present paper describes the various conditions which must be fullfilled for a successful application of counter-current distribution to spinach chloroplasts.

MATERIALS AND METHODS

Reagents

Dextran trade-marked as "Dextran 500", was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Each batch of the manufacture is given a number and in this work we employed the batch numbers 2660, 3202, 4024 and 8689. All essential statements about these are shown in Table I and are from the booklet accompanying the supply of the products.

Polyethylene glycol was obtained as "Carbowax 4000 and 6000" from Union Carbide, New York, U.S.A. These products have molecular weights ranging from 3000 to 3700 and 6000 to 7500, respectively.

Phosphate buffers were prepared by mixing 0.2 M solutions of the respective sodium or potassium monobasic phosphate ($H_2PO_4^-$) and dibasic phosphate (HPO_4^{2-}) in desired proportions. In most experiments we employed a ratio of 1 part of $H_2PO_4^-$ and 9 parts of HPO_4^{2-} which corresponds to approximately pH 7.8.

Tris–HCl buffers were prepared by making a 0.2 M solution of Tris and adjusting its pH close to 7.8 with HCl. The mixture was then diluted to approx. 0.05 M with respect to Tris before final adjustment to exactly pH 7.8 was made.

All inorganic and organic reagents used were of analytical grade and the water for solutions was distilled once in quartz-glass apparatus.

Plant material

Seeds of spinach (*Spinacia oleracea* L.) were germinated in vermiculite by watering a commercial nutrient solution called Substral (traded by Svenska Substral, AB, Malmö, Sweden) containing all important nutrients required for a satisfactory growth. The plant beds were illuminated with artificial light in a greenhouse. The light was turned on for 16 h per day and the light intensity was about 5000 lx at the top of the mature plants. Total darkness was maintained during the remaining 8 h of the day. The temperature was maintained at 15° and the relative humidity was adjusted to 70 %.

Preparation of chloroplasts by differential centrifugation

Both cotyledons and normal expanded leaves from the same spinach plants were employed to prepare chloroplasts. Leaves from plants of different ages were

used: 2–3 weeks old for cotyledons and 6–8 weeks old for normal leaves. The leaf material was always harvested during a dark period shortly before a new light period to decrease the amount of starch grains in the leaves, as the grains can damage the chloroplasts during the preparation. In most cases the leaves after picking were stored in the cold (2°) in plastic vessels before further use. Before use the midribs were removed and the leaves were cut in smaller pieces to make the homogenization easier. The final procedure was performed in a chilled knife blendor with knives rotating 3–4 times during periods of 2 sec each. The whole operation was carried out in a cold room (2°). As preparation medium, we employed 0.4 M sucrose in either 0.05 M Tris–HCl buffer, pH 7.8, or 0.067–0.15 M sodium phosphate buffer with pH in the range 7.3–7.8. In most of the experiments 50 g of leaf material was processed in a 3-fold volume of medium. The crude homogenate was filtered through 4–8 layers of cheese cloth. The filtrate was centrifuged for 10 min at $300 \times g$ in 50-ml plastic centrifuge tubes in a pre-chilled SS-34 rotor of Sorvall refrigerated centrifuge RC 2-B. Sometimes we began with a first step at 80–100 $\times$ g for 1 min. The 300 $\times$ g sediment was taken as main fraction of chloroplasts for further treatment. The supernatant was discarded and the pellet was resuspended in fresh medium. The sample was washed 3–4 times. Resuspension between each centrifugation was accomplished by sucking the pellet up and down through a pipette with a wide opening. The last washings were often carried out with 0.4 M sucrose only. We found the 300 $\times$ g fraction better than the more conventional 1000 $\times$ g fraction in all respects. It contained a higher percentage of Class I chloroplasts and was also cleaner with respect to the risk of contamination from chloroplast fragments and mitochondria. Therefore we used the washed 300 $\times$ g sediment for examination of different classes of chloroplasts by counter-current distribution. This chloroplast sample was finally suspended in 2–3 ml of 0.4 M sucrose and the amount of material was estimated in terms of total chlorophyll. In a standard preparation between 30–50% of the chloroplasts counted in phase contrast microscope were Class I.

Phase system and partition in single tubes

Concerning the choice of suitable polymers and general technique for work with polymer two-phase systems we refer to refs. 15 and 16. Here we only treat the specific problem of investigating a heterogeneous fraction of chloroplasts using counter-current distribution. Before applying this procedure it is necessary to know the partition coefficient of the test material in the specific phase systems under consideration. The partition is determined by several factors such as the type, the molecular weight and concentration of the polymers, the type of inorganic salts, ionic strength, pH and temperature. The temperature influences the partition mainly indirectly by affecting the composition of the phase system[15]. A coordination of these parameters is necessary to obtain the best possible results.

Sample systems were made from stock solutions of 20% (w/w) dextran 500, 40% (w/w) polyethylene glycol 4000 or 6000, 30% (w/v) sucrose, 0.4 M sucrose and 0.1–0.2 M sodium and potassium salts. The following is an example of the procedure: 1.26 g 20% (w/w) dextran 500, 0.63 g 40% (w/w) polyethylene glycol 4000, 1.00 ml 30% (w/v) sucrose, 0.10 ml 0.2 M potassium phosphate buffer, pH 7.8, and distilled water up to 3.50 g were mixed. Then 0.50 ml of chloroplasts suspended in 0.4 M sucrose was added and the system was mixed again by several inversions. The mixture was

allowed to separate for 30 min at 2°. Then a sample, often 1.00 ml, of the top phase was withdrawn and diluted with buffered sucrose. The dilution factor depended on the amount of material. The latter was determined by absorbance at 680 nm with a Zeiss PMQ II spectrophotometer. All samples must be diluted to the range where the absorbance is a linear function of the concentration of particles. All measurements were performed at absorbances below 2.5.

Knowing the quantity of chloroplasts in the top phase sample and also the total quantity of chloroplasts added to the system, the amount of material in the top phase is calculated and expressed as percentage of the total amount. This is plotted against the parameter changed in each case.

Counter-current distribution

The technique of liquid-interface counter-current distribution[15,16] was used throughout. Three phase systems A, B and C were used. Their composition is given in Table II. System A has 6.3% (w/w) dextran 500 and 6.3% (w/w) polyethylene glycol 4000. System B has 6.4% (w/w) dextran 500 and 6.4% (w/w) polyethylene glycol 4000 and finally System C has 5% (w/w) dextran 500 and 3.5% (w/w) polyethylene glycol 6000. The final concentration of sucrose is 9.2% (w/w) and the concentration of buffer is 5 mM in all systems. The stock systems were prepared at 2° and allowed to separate overnight in a separating funnel. Then the two phases in equilibrium were collected and stored in different vessels.

TABLE I

DATA OF VARIOUS BATCHES OF DEXTRAN 500

Batch No.	*Weight average mol. wt.*	*Number average mol. wt.*	*Intrinsic viscosity number, [η] (dl/g)*
2660	478 000	198 000	0.53
3202	490 000	185 000	0.53
4024	495 000	195 000	0.52
8689	518 000	199 000	0.53

TABLE II

MIXTURES FOR PHASE SYSTEMS A, B AND C USED IN COUNTER-CURRENT DISTRIBUTION

Chemicals involved	*System A*	*System B*	*System C*
20% (w/w) dextran 500, Batch 4024	63.0 g	—	—
20% (w/w) dextran 500, Batch 2660	—	64.0 g	50.0 g
40% (w/w) polyethylene glycol 4000	31.5 g	32.0 g	—
40% (w/w) polyethylene glycol 6000	—	—	17.5 g
30% (w/v) sucrose	50.0 ml	50.0 ml	50.0 ml
0.2 M sodium phosphate buffer, pH 7.8	—	5.0 ml	5.0 ml
0.2 M potassium phosphate buffer, pH 7.8	5.0 ml	—	—
Adding distilled water up to a total weight of 175.0 g			
0.4 M sucrose	25.0 ml	25.0 ml	25.0 ml

TABLE III

MIXTURES FOR PHASE SYSTEMS A_{chl}, B_{chl} AND C_{chl} WITH CHLOROPLAST SAMPLE FOR CHARGING THE COUNTER-CURRENT DISTRIBUTION APPARATUS

Chemicals involved	*System A_{chl}*	*System B_{chl}*	*System C_{chl}*
20% (w/w) dextran 500, Batch 4024	3.78 g	—	—
20% (w/w) dextran 500, Batch 2660	—	3.84 g	3.00 g
40% (w/w) polyethylene glycol 4000	1.89 g	1.92 g	—
40% (w/w) polyethylene glycol 6000	—	—	1.05 g
30% (w/v) sucrose	3.00 ml	3.00 ml	3.00 ml
0.2 M sodium phosphate buffer, pH 7.8	—	0.30 ml	0.30 ml
0.2 M potassium phosphate buffer, pH 7.8	0.30 ml	—	—
Adding distilled water up to a total weight of 10.5 g			
Chloroplast suspended in 0.4 M sucrose	1.50 ml	1.50 ml	1.50 ml
Bottom phase from System A	2.00 ml	—	—
Bottom phase from System B	—	1.50 ml	—

The systems containing chloroplast sample were prepared immediately before charging the counter-current distribution apparatus (see Table III). A volume ratio of 1:1 was chosen for the phases in most experiments. The chloroplasts samples therefore have to be adjusted to this volume ratio. Hence to System A_{chl} and B_{chl} were added 2.00 ml and 1.50 ml, respectively, of bottom phase. This bottom phase was taken from the corresponding large Systems A and B (see Table II). No addition to System C_{chl} was required as the ratio was already approx. 1:1. The chloroplast sample systems were mixed by several inversions before they were added in the counter-current distribution apparatus. The quantity of chloroplast material used in the experiments was 2–10 mg in terms of total chlorophyll.

Counter-current distribution apparatus and charging of sample

The automatic thin-layer counter-current distribution apparatus described by ALBERTSSON[17,18] was used. Two counter-current distribution plates were employed, one with 60 and one with 120 chambers. The capacity of the bottom chamber for these was 1.1 and 0.7 ml, respectively. As the technique of liquid-interface distribution was used, the bottom phase chamber was filled with a volume lower than maximum in order to obtain a stationary interface. This volume was 0.9 ml for Model 60 and 0.6 ml for Model 120.

Experiments with the apparatus containing 60 chambers were performed by loading Chambers 0–2 with 1.8 ml of the chloroplasts sample System (A_{chl}, B_{chl} or C_{chl}) and 3–59 with 0.9 ml of each phase from A, B or C. In a similar manner for Model 120 each of Chambers 10–119 were charged with 0.5 or 0.6 ml each of the top phase and the bottom phase. Chambers 0–9 were filled with 1.0 ml or 1.2 ml of chloroplast sample System (A_{chl}, B_{chl} or C_{chl}).

The shaking time was 30 sec and the settling time was, except when otherwise stated, 8 min. All operations were performed at 2° and the number of transfers was between 60 to 120 depending on the apparatus used. After the last transfer, the fractions were collected in plastic tubes and diluted 3-fold with buffered sucrose to break the two-phase systems. The samples were examined with respect to certain factors described in detail below.

Spectrophotometry and microscopy

Absorbance of diluted samples from counter-current distribution experiments were measured at 550 nm and 680 nm with a Zeiss PMQ II spectrophotometer. The values were plotted against the tube number. The $A_{550\ nm}/A_{680\ nm}$ ratio was also determined and plotted in the same figure[19].

The samples were also investigated in phase contrast microscope. The observations were performed with a Zeiss microscope equipped with "Neufluor" phase contrast objectives and at least 500 chloroplasts were counted in each sample. Two main types were seen and these were the so-called Class I and Class II previously described by several authors. The percentage of Class I chloroplasts could be related to the ratio $A_{550\,nm}/A_{680\,nm}$ on the fractions from counter-current distribution, as described elsewhere[19].

Determination of chlorophyll

Chlorophyll was determined by the method of ARNON[20]. Before adding acetone, dextran is removed by washing the chloroplasts with repeated centrifugations in buffered sucrose (dextran gives precipitations with acetone). Then 1 ml of the suspension was mixed with 4.0 ml of acetone and finally shaken to dissolve the pigments. The sample was protected from light and after at least 10 min the solution was centrifuged in a Sorvall centrifuge RC 2-B in a SM-24 rotor at 6500 rev./min for 20 min. The green supernatant was collected and assayed by measuring the absorbances at 645 and 663 nm. The ratio between chlorophyll *a* and chlorophyll *b* was calculated as by MACKINNEY[21].

RESULTS

Factors influencing partition

Ionic composition

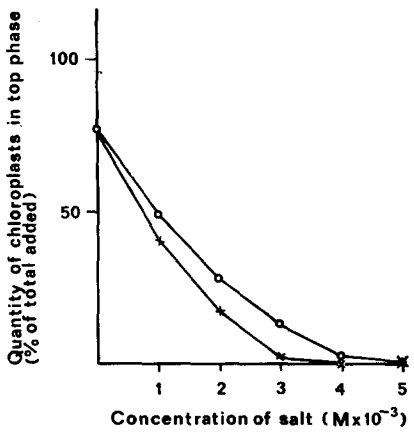
The distribution of particles and macromolecules in a dextran–polyethylene glycol system depends strongly on the ionic composition[15,22,23]. The same holds for chloroplasts. Fig. 1 shows the distribution of spinach chloroplasts in System B. In phosphate buffer alone about 75 % of the chloroplasts remain in the upper phase while the rest of the particles are adsorbed at the interface. When NaCl is included in the phase system, more particles are adsorbed at the interface. KCl has a similar but stronger effect. This behaviour is similar to the behaviour of other particles such as bacteria, algae and red blood cells.

The effect of other ions seem to follow the same rules as hold for other cell particles[15], *i.e.* cations increase the affinity of chloroplasts for the upper phase in the order $Li^+ > Na^+ > K^+$ and the HPO_4^{2-} ion increases the same affinity more than Cl^- or $H_2PO_4^-$.

Thus the kind of ions present have a strong influence. However, the concentration of a given salt is also of importance. Fig. 2 shows the distribution of chloroplasts at different phosphate concentrations. It is seen that more chloroplasts are found in the upper phase with increasing buffer concentrations in the interval 0.001–0.02 M.

Influence of polymer concentration

Increasing the polymer concentration results in a phase system more removed from the critical point. This usually gives rise to an increase in adsorption of particles



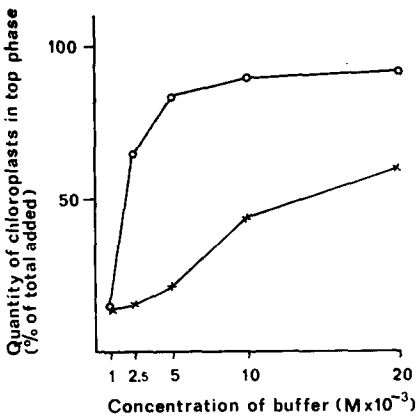


Fig. 1. Partition as a function of salt concentration. The quantity of chloroplasts in the top phase is expressed as percentage of the total quantity of chloroplasts in the system. System B (Table II) with varying concentrations of NaCl and KCl, respectively, was used. ○—○, NaCl; ×—×, KCl.

Fig. 2. Partition as a function of buffer concentration. The quantity of chloroplasts in the top phase is expressed as percentage of the total quantity of chloroplasts in the system. System B (Table II) with varying concentrations of sodium and potassium phosphates, respectively, was used. ○—○, sodium phosphate buffer (1:9) pH 7.8; ×—×, potassium phosphate buffer (1:9) pH 7.8.

at the interface. This is shown in Fig. 3 where the distribution is plotted against the polymer concentration while the ionic composition is constant. Fig. 4 shows similar experiments on the effect of polymer concentration in presence of different phosphates.

Thus, a certain partition of chloroplasts can be achieved either by changing the ions, the salt concentration or the concentration of the polymers.

Different amounts of chloroplasts

If the total amount of chloroplasts added to a system is varied and other factors are kept constant, the quantity of chloroplasts found in the top phase is proportional

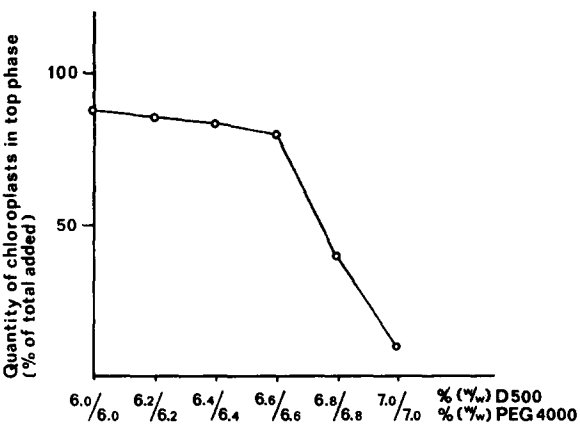


Fig. 3. Partition as a function of polymer concentration. The quantity of chloroplasts in the top phase is expressed as percentage of the total quantity of chloroplasts in the system. Phase systems containing dextran 500 (D500), Batch 2660, polyethylene glycol 4000 (PEG4000) and 5 mM sodium phosphate buffer, pH 7.8, were used.

to the amount of chloroplasts added (Fig. 5). Therefore the distribution ratio between upper phase and interface is concentration independent.

Volume ratio

The effect of volume ratio of the phases is shown in Table IV. In these experiment different amounts of top and bottom phases from a pure System A was added to a 4.0 g of System A_{chl}, containing chloroplasts. The final volume of all systems was 10 ml. As seen, there is a slight tendency for the chloroplasts to favour the interface when the top phase volume is increased.

Counter-current distribution

A number of counter-current distribution experiments on chloroplasts have been carried out in order to study the effect of different factors on the distribution

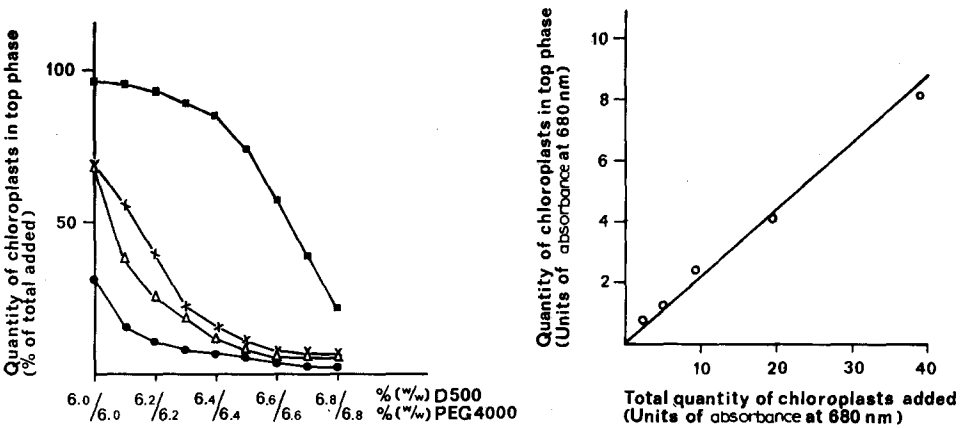


Fig. 4. Partition as a function of polymer concentration. The quantity of chloroplasts in the top phase is expressed as percentage of the total quantity of chloroplasts in the system. Phase systems containing dextran 500 (D500), Batch 8689, polyethylene glycol 4000 (PEG4000) and with four different ionic compositions were used. □, 2.5 mM NaH_2PO_4–7.5 mM Na_2HPO_4; ×, 2.5 mM NaH_2PO_4–7.5 mM K_2HPO_4; △, 2.5 mM KH_2PO_4–7.5 mM K_2HPO_4; ●, 25 mM NaH_2PO_4–75 mM K_2HPO_4.

Fig. 5. Partition as a function of amount of chloroplasts. The quantity of chloroplasts in the top phase was plotted against the total quantity of chloroplasts added. Both quantities are expressed as units of absorbance at 680 nm.

TABLE IV

INFLUENCE OF THE VOLUME RATIO ON THE PARTITION

Phase System A (Table II) was used, and the total volume was fixed at 10 ml.

Volume ratio (top-phase/bottom-phase)	*Percentage of chloroplasts in top-phase*
0.33	36
0.70	38
1.04	39
1.52	31
2.96	24

diagram. The factors studied include time of phase separation, number of transfers, type of phase systems, leaf material and different batches of dextran.

Settling time

The effect of time of phase separation is shown by Fig. 6. Three different settling times, 5, 8 and 12 min respectively, were used. There is a considerable improvement when the settling time is increased from 5 to 8 min but further increase to 12 min does not improve the separation any further. Therefore we chose a settling time of 8 min for subsequent runs.

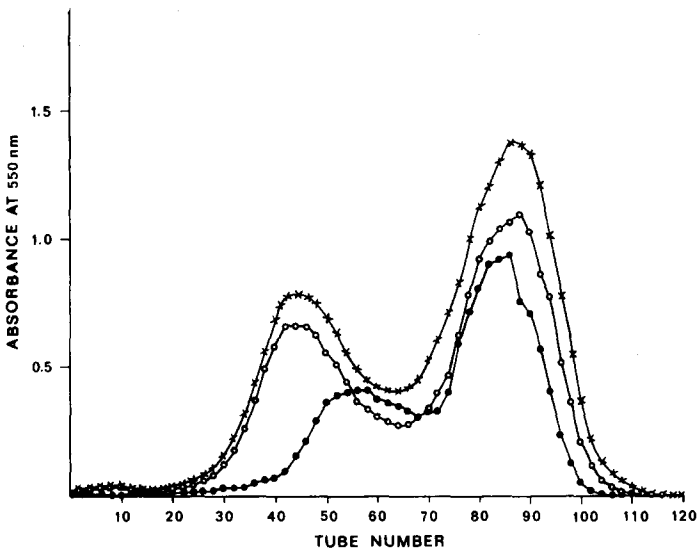


Fig. 6. Effect of settling time on the resolution of different classes of chloroplasts from normal leaves. System B (Table II) was used and 120 transfers were made. ●—●, 5 min; ○—○, 8 min; ×—×, 12 min.

Number of transfers

Fig. 7 shows diagrams obtained from three different runs with the same system but with different number of transfers, 60, 90 and 120, respectively. As seen, the peaks are moved further to the right with increasing number of transfers. The main peak has its maximum position in Tubes number 28, 44 and 58, respectively, that is, the peak moves almost proportionately with the number of transfers as would be expected if the chloroplasts show ideal distribution behaviour. Also the peaks become broader with increasing number of transfers.

Molecular weight of polyethylene glycol

Figs. 8a and 8b show counter-current distribution of chloroplasts in two different dextran–polyethylene glycol phase systems containing polyethylene glycol 4000 and polyethylene glycol 6000, respectively. The temperature and the ionic composition was the same for the two experiments but the polymer concentrations were adjusted in order to get about the same overall distribution between the phases in a single tube experiment. As seen, both systems can resolve the two classes of chloroplasts. Thus a separation is obtained for both molecular weight preparations of

polyethylene glycol provided a suitable distribution is obtained by appropriate polymer concentration.

Leaf material

Chloroplasts give rise to different distribution diagrams depending on whether they are isolated from cotyledons or normal leaves. Figs. 9a and 9b show diagrams after counter-current distribution with 60 transfers of cotyledon and normal leaf chloroplasts, respectively. The following differences can be noted: (1) Chloroplasts from cotyledons give 3 distinct peaks (here named I, II and III), while chloroplasts from normal leaves give only 2 peaks (named I and II). As reported elsewhere I, II and III contain chloroplasts with different structure and chemical composition, and their relative amounts depend on treatment for cell breakage and leaf material used.(2)

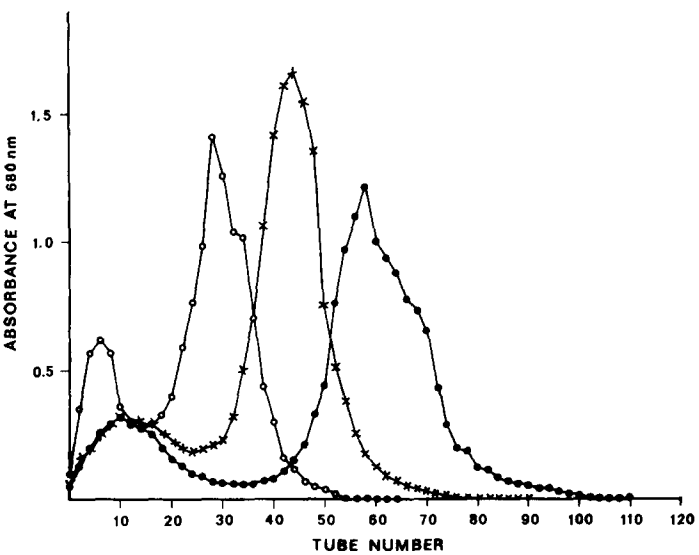


Fig. 7. Effect of number of transfers on the resolution of different classes of chloroplasts from normal leaves. System B (Table II) was used and the settling time was 8 min. ○—○, 60 transfers; ×—×, 90 transfers; ●—●, 120 transfers.

Peak II in the diagrams is not located at the same position; in Fig. 9b it is more to the right. This is a reproducible phenomenon and indicates that the Class II chloroplasts are probably not the same for the two materials. (3) Chloroplasts under Peak I in Fig. 9b is a cleaner preparation, as regards contamination by Class II chloroplasts, than chloroplasts under Peak I in Fig. 9a. This is indicated by the higher $A_{550\,nm}/A_{680\,nm}$ absorbance ratio for Peak I in Fig. 9b.

Dextran batches

The different dextran batches tested gave different partition results with chloroplasts. Fig 10 shows distribution diagrams of three counter-current distribution experiments where three different batches were compared, when other factors were kept constant.

As seen, Batch 2660 gives a resolution of two clear peaks while the others do

not. This is due to a general increase in the partition of the chloroplasts into the top phase by Batches 3202 and 4024. Adjusting the K value by changing the polymer

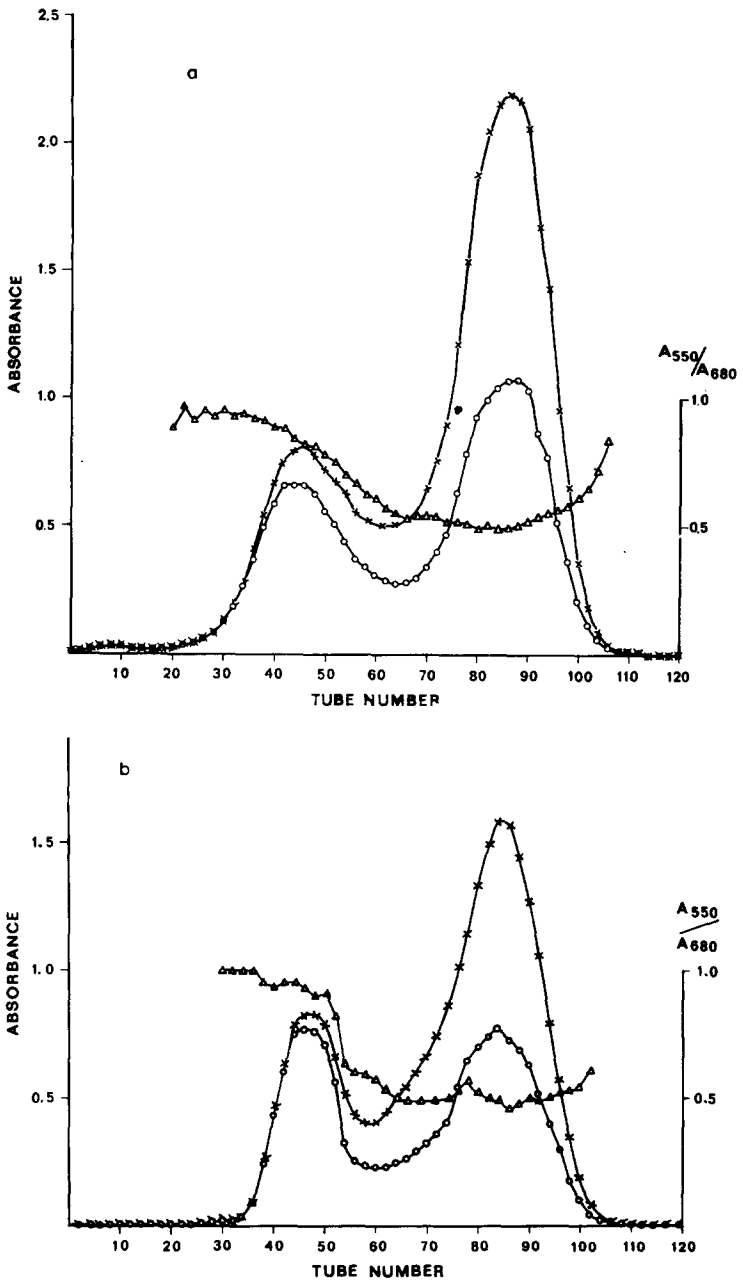


Fig. 8. Counter-current distribution of chloroplasts from normal leaves in two different phase systems containing polyethylene glycol of different molecular weights but giving the same overall partition. Both systems separate chloroplasts into two distinct peaks. In (a) System B (Table II) and in (b) System C (Table II) was used. In both cases we used a settling time of 8 min and 120 transfers. ○—○, $A_{550\,nm}$; ×—×, $A_{680\,nm}$; △—△, $A_{550\,nm}/A_{680\,nm}$.

concentration and ionic composition (from System B to A) to lower values results in an efficient separation of the two classes (Fig. 9b) with Batch 4024. Thus, batch variations can be compensated by changes in phase system composition.

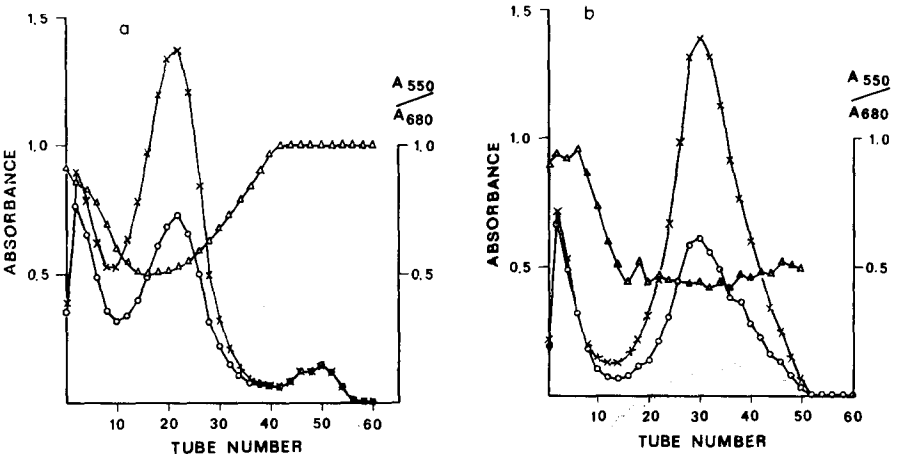


Fig. 9. (a) Counter-current distribution of chloroplasts from cotyledons. Three distinct peaks (I, II and III) were obtained, corresponding to chloroplasts with different morphological and chemical properties. System A (Table II) was used and 60 transfers were made. (b) Counter-current distribution of chloroplasts from normal leaves. Two distinct peaks (I and II) were obtained. Other factors the same as in (a). ○—○, $A_{550\,nm}$; ×—×, $A_{680\,nm}$; △—△, $A_{550\,nm}/A_{680\,nm}$.

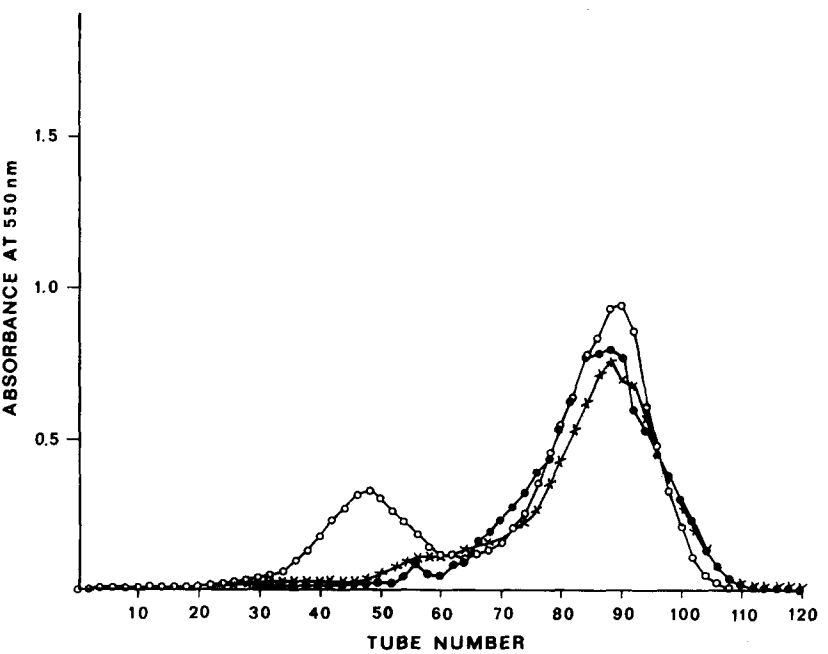


Fig. 10. Effect of dextran batch on the resolution of different classes of chloroplasts from normal leaves. Phase systems of Type B (Table II) were used and 120 transfers were made. ○—○, dextran 500, Batch 2660; ×—×, dextran 500, Batch 3202; ●—●, dextran 500, Batch 4024.

DISCUSSION

The aim of this investigation was to determine the different factors which influence chloroplast partition and to devise a procedure for selection of phase systems suitable for counter-current distribution.

The main factors determining partition are type of polymers, polymer concentration, ionic composition, pH and temperature. Several polymers can be used[15], but for the present work we chose dextran and polyethylene glycol since these are available commercially in large quantities, and have proven to be mild towards various biological activities. Also the behaviour of other cell particles and macromolecules in this phase system has been extensively studied earlier.

The behaviour of chloroplast partition as described in Fig. 1, 3, 4 and 5 are qualitatively similar to the behaviour of cells described earlier[16]. Chloroplasts are, however, extremely sensitive to changes in phase-system composition such as polymer concentration and ionic composition. For counter-current distribution it is desirable to adjust the overall partition of chloroplasts so that part of the material is in the upper phase and the rest at the interface. There are several ways to adjust the partition and these are: changing the polymer molecular weight, polymer concentration, ionic composition or pH. Chloroplasts have, however, certain requirements on the medium for their stability. These include tonicity and pH above 7. Sucrose up to about 9.2 (w/w) was therefore added, and the pH was kept at 7.8. The partition was then adjusted by change in polymer or ionic composition. This procedure is generally recommended, *i.e.* one first selects the pH, tonicity and perhaps also the ionic composition which is most suitable for preservation of the cell organelles of interest, and then one adjusts the partition by change in polymer concentration according to Fig. 3.

The counter-current distribution experiments of Fig. 7 show that the chloroplasts travel along the distribution train as expected for an ideal behaviour, *i.e.* the position of a peak moves proportionally to the number of transfers. An increased separation is therefore obtained with increasing number of transfers. Partition in single tubes of the train also agrees with the tube number.

Several counter-current distributions in various media were carried out before the successful system was found. The results show that the best resolution for a given number of transfers was obtained when the phosphate buffer (in pH range 7.5–8) concentration was kept low, 0.005 M, and a suitable overall K value was adjusted by change in polymer concentration instead of adding NaCl or KCl. We have no explanation why the resolution was not as good when the buffer concentration was higher, for example 0.1 M or KCl was added to adjust the partition. One explanation could be that the chloroplasts are partly separated according to their surface charge and that differences in surface charge are diminished at higher ionic strength. Theoretical considerations and also experiments with cells[15] indicate that surface charge plays a great role in determining partition in the dextran–polyethylene glycol phase system. It is reasonable that the surface charge also influences chloroplast partition and that a resolution of different classes is therefore obtained only in a certain pH range and at certain ionic compositions.

The experiments described here demonstrate that by proper choice of phase system composition, counter-current distribution can be used for analyzing cell organelle suspensions in the same way as has been demonstrated for whole cells[16]. For

the application of this technique to other organelles, certain preliminary work has to be done in order to find a suitable phase system. This can be done along the same lines as described in this paper.

The properties of the chloroplasts under the different peaks have been characterized by phase contrast microscopy, absorbance ratio at 550 and 680 nm and also osmotic swelling and RNA/chlorophyll ratio (unpublished data). They have also been characterized by electron microscopy, size distribution and protein/chlorophyll ratio, and the results are published in a separate paper[14]. The results show that the first peak consists of mainly intact Class I chloroplasts, the second peak of stripped Class II chloroplasts and the third peak of a new type of chloroplast particles (Class III) containing an intact chloroplast surrounded by an extra cytoplasm-like layer with an additional membrane[14]. The properties and possible origin of these chloroplasts are described in a separate paper[14]. Thus, the three classes of chloroplasts differ considerably in their surface properties and this supports the assumption that partition of cell particles is to a great extent determined by the properties of particle surfaces. Counter-current distribution therefore serves as a useful complement to centrifugation methods for separating cell organelles.

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