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HYDROPHOBIC AFFINITY PARTITION OF SPINACH CHLOROPLASTS IN AQUEOUS TWO-PHASE SYSTEMS

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SUMMARY

The surface properties of spinach chloroplasts, both of intact chloroplasts with surrounding envelope and broken chloroplasts consisting of the inner lamellar system, have been studied by partitioning them between two aqueous phases, especially using counter-current distribution technique. The two-phase system consists of poly(ethyleneglycol), dextran and water. The two polymers are enriched in opposite phases and by binding deoxycholate or palmitate to one of the polymers the affinity of chloroplasts for the corresponding phase is strongly enhanced. The partition of the two classes of chloroplasts, however, is not affected to the same degree and the affinity of the chloroplast envelope for deoxycholate and palmitate is stronger than that of the lamellar system. This has been correlated to the chemical composition of the two types of membranes. By studying the effect of salts on the partition it has been found that the lamellar system bears a larger number of negative charges as compared to the envelope of the intact chloroplast.

INTRODUCTION

Partition in aqueous dextran-poly(ethyleneglycol) two-phase systems is in many cases a useful technique for separation and study of biological substances [1]. A substance partitions between the two liquid phases according to its surface properties such as electrical charge and hydrophilic-hydrophobic character [2]. If electrolytes are included in the two-phase system an unequal distribution of cations and anions across the interface creates a potential difference between the phases. This interfacial potential depends on the salt used and steers the partition of other charged substances present in deficit as compared with the salt [1–3]. Certain salts eliminate the interfacial potential and the partition of a substance is, in this case, independent of its net charge.

Another approach to steer the partition and to increase the selectivity of a phase system is to bind specific groups covalently to the phase polymers. Such "affinity partition" has been recently demonstrated [4–6]. Poly(ethyleneglycol) esterified with

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palmitic acid has been used to selectively extract serum albumin from plasma [6] and also to study binding of the palmitoyl group to different proteins [7]. The same polymer esterified with different fatty acids or deoxycholate was used to study the hydrophobic surface properties of red blood cells [8].

In the present work we used this method to study the hydrophobic affinity of chloroplast membranes. Hydrophobic groups were attached covalently to either poly(ethyleneglycol) or dextran and their effect on partition of two types of chloroplasts, class I and II, was studied. Since class I chloroplasts retain their envelopes while class II chloroplasts have lost their envelopes and expose the thylakoid lamellar system the hydrophobic affinity of the two membrane systems could be compared.

MATERIALS AND METHODS

Dextran 500, batch No. 5996, molecular weight M_r = 500 000, was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden. Poly(ethyleneglycol), molecular weights M_r = 3000–3700 and 6000–7500 respectively, was obtained from Union Carbide, New York, USA as Carbowax 4000 and 6000.

Poly(ethyleneglycol)-deoxycholate was synthesized from 100 g Carbowax 6000 which was dissolved in 600 ml toluene. 100 ml toluene was distilled off to remove traces of water. 40 g deoxycholic acid (Sigma Chemical Co., St. Louis) and 50 g dicyclohexylcarbodiimide were dissolved in the poly(ethyleneglycol)-solution which was kept at 40 °C for 5 days. After filtration, the polymer was allowed to precipitate at 3 °C and the mucous product was collected by centrifugation in the cold. It was recrystallized twice from 250 ml absolute alcohol and precipitated in the cold. The yield was very low, 15–25 per cent, and the amount of poly(ethyleneglycol)-bound deoxycholate varied from time to time in an unexplainable manner. The degree of substitution was determined by comparing the optical rotation of poly(ethyleneglycol)-deoxycholate in water solution with a standard solution of sodium deoxycholate. The poly(ethyleneglycol)-deoxycholate used in this work was found to contain 0.026 mmol deoxycholate groups per g, corresponding to a degree of substitution of 8 per cent.

Dextran-deoxycholate. 100 g dextran was suspended in 300 ml dry acetone and after 1 h the polymer was collected by suction filtration. It was then suspended in 300 ml toluene. 12 g deoxycholic acid and 30 g dicyclohexylcarbodiimide were dissolved and the mixture was stirred mechanically at room temperature over night. The dextran was recovered by suction filtration and washed with 500 ml toluene and then with 500 ml absolute alcohol followed by 500 ml acetone. After drying at room temperature the product was used without further purification. The amount of bound deoxycholate has not been determined.

Poly(ethyleneglycol)-palmitate was prepared by esterifying poly(ethyleneglycol) (Carbowax 6000) with palmitoyl chloride as described previously [6].

Chloroplast preparation

Chloroplasts from spinach were prepared essentially according to ref. 9. The preparation consisted of 70–95 per cent class I chloroplasts and the remaining part class II chloroplasts and was suspended in 0.3 M sucrose before partition or countercurrent distribution experiments.

Partition

Single partitions were performed in test tubes with 2.0 g phase systems of the compositions given in Table I. Batch systems were prepared by mixing stock solutions of dextran (20 %), poly(ethyleneglycol) 4000 (40 %), buffer (0.2 M), sucrose (30 %) and the appropriate salt. An aliquot of 1.80 g was taken from this batch system immediately after mixing. 0.10 g of a solution containing poly(ethyleneglycol)-deoxycholate and poly(ethyleneglycol) 6000 (for systems A and B, Table I) or dextran-deoxycholate and dextran (for system C, Table I) was added to give the desired concentration of polymer-ester.

After temperature equilibration in coldroom, 2–4 °C, 0.10 ml chloroplasts suspension in 0.3 M sucrose was added. The system was brought to equilibrium by inverting the tube 50 times and was left to separate for 30–45 min in cold. Samples, 750 μl from top phase and 500 μl from bottom phase, were withdrawn with constriction pipettes and diluted with 1.50 ml 0.3 M sucrose/50 mM potassium phosphate buffer (pH 7.8). The same volume of this solution was added to the residue of the phase system (left in the test tube). The absorbance of the diluted samples and the diluted residue was measured at 680 nm against a corresponding blank without chloroplasts. The absorbance at 550 nm was also measured in some cases. The measurements were done with a Zeiss PMQ II spectrophotometer.

The total quantity of chloroplasts added to each phase system was calculated by summing up the absorbance values obtained for top phase sample, bottom phase sample and residue with consideration taken into the volumes. The quantity was expressed as absorbance units at 680 nm and was in the range 1.5 to 3.0 units, corresponding to 20 to 40 μg chlorophyll. The amount of material in top phase and bottom phase was calculated from the absorbance at 680 nm of the diluted samples taking the volumes of the phases into account and is expressed as percentage of the total amount of chloroplasts in the phase system.

Counter-current distribution

An automatic thin-layer counter-current distribution apparatus described by Albertsson [10] was used. The composition of the phase systems used in counter-current distributions are listed in Table II. They were prepared from stock solutions as described by Karlstam and Albertsson [11] and by Larsson et al. [12] with minor modifications.

All counter-current distributions were performed at 2–4 °C using partition cell blocks with 120 chambers. Chambers numbered 0–5 were loaded with the sample system containing chloroplasts. The remaining chambers (numbered 6–119) were loaded with equal volumes (0.55–0.70 ml depending on the block used) of top and bottom phase of the counter-current distribution system. The interface and bottom phase were kept stationary, while the upper phase was mobile. This was ensured by taking the volumes of the bottom phases 10 per cent smaller than the volumes of the bottom-plate cavities. 120 transfers were made, with a settling time of 8 min and a shaking time of 30 s. After the run, the content of each chamber was diluted with 1.50 ml 0.3 M sucrose/50 mM potassium phosphate buffer (pH 7.8), in order to obtain one-phase systems, and the fractions were collected in test tubes. The absorbance at 680 nm and 550 nm was measured on every second or third tube against a similarly diluted blank system.

RESULTS AND DISCUSSION

In a dextran-poly(ethyleneglycol) two-phase system chloroplasts are distributed between the upper phase, the lower phase and the interface. The partition is determined by the surface properties of the membranes exposed to the surroundings [1]. Since most salts give rise to an electrical interfacial potential between the phases [2, 3], the distribution of the chloroplasts depends on the number of charged groups on their surfaces. At pH 7.8, the value used for all the partition experiments, the chloroplast membranes are negatively charged (isoelectric point 3.7–4.2 for class I and 4.6–4.9 for class II chloroplasts. Westrin, H. and Albertsson, P.-Å., unpublished results). By including a salt such as NaCl which makes the top phase negative compared to the bottom phase [3], the chloroplasts should have affinity preferentially for the bottom phase. Na_2SO_4 on the other hand gives a phase system with an interfacial potential close to zero [3]. In the latter system the distribution of the chloroplasts should be determined solely by properties other than the charge of the particles, e.g. hydrophobic-hydrophilic character of that part of the membranes exposed to the surroundings. When poly(ethyleneglycol) or dextran is partly esterified with a hydrophobic acid, the distribution of chloroplasts is also determined by the affinity of the membranes for this polymer-bound group.

Fig. 1 shows the partition of a chloroplast preparation in phase system A

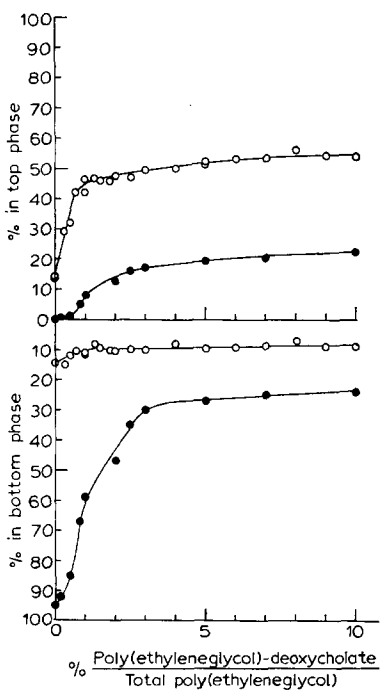

Fig. 1. Distribution of chloroplasts as function of the concentration of poly(ethyleneglycol)-deoxycholate. System A, containing Na_2SO_4, having an interfacial potential close to zero (open circles) and system B, containing NaCl, having a negative top phase (filled circles). For composition of the phase systems see Table I.

TABLE I

COMPOSITION OF SYSTEMS FOR SINGLE PARTITION

	Phase system		
	A	B	D
Total dextran 500 (%(w/w))	6.3	6.3	6.1
Poly(ethyleneglycol), type Carbowax 4000 (%(w/w))	5.67	5.67	6.1
Poly(ethyleneglycol), type Carbowax 6000, including substituted poly-(ethyleneglycol) (%(w/w))	0.63	0.63	0
Poly(ethyleneglycol)-deoxycholate/Total poly(ethyleneglycol) (%)	0–10	0–10	0
Dextran-deoxycholate/Total dextran (%)	0	0	0–10
Na_2SO_4 (mmol/kg)	10	0	0
NaCl (mmol/kg)	0	10	0
Potassium phosphate buffer (pH 7.8) (mmol/kg)	1	1	0
Sodium phosphate buffer (pH 7.8) (mmol/kg)	0	0	1
Sucrose (mol/kg)	0.3	0.3	0.3

(with Na_2SO_4) and phase system B (with NaCl), described in Table I, as a function of the poly(ethyleneglycol)-deoxycholate concentration. This modified polymer is concentrated in the top phase. In line with the assumption above, less material is found in the top phase and more in the bottom phase when the system contains NaCl (filled circles) compared to when the system contains Na_2SO_4 (open circles). Addition of poly(ethyleneglycol)-deoxycholate increases the affinity of the chloroplasts for the top phase. When the concentration of esterified deoxycholic acid is increased the distribution changes drastically. In system A the amount of chloroplasts in the top phase increases from 15 to 55 per cent in the interval tested. Since the concentration in bottom phase is not affected, the material must come almost exclusively from the interface in the poly(ethyleneglycol)-deoxycholate-free system. In system B the amount in the top phase increases less, from 0 to 22 per cent. The main change observed in this system is the decrease of material in the bottom phase, from 95 to 24 per cent and the transfer therefore is mainly to the interface.

The accumulation of material on the interface is a common phenomenon when particles are partitioned in aqueous two-phase systems. It is most predominant when the affinity of the particles is the same for the two phases and the theoretical background for this has been treated by Albertsson [1]. When the chloroplasts are partitioned in system A (Table I) without ester-bound deoxycholic acid, each phase contains around 15 per cent of the chloroplasts and consequently the main part of the particles, 70 per cent, is found at the interface. By including poly(ethyleneglycol)-deoxycholate in the phase system the affinity of the chloroplasts for the top phase is enhanced due to hydrophobic interactions. The chloroplasts are therefore partly extracted by the top phase from the interface.

In system B (Table I) on the other hand the affinity for bottom phase is enhanced by the interfacial potential generated by NaCl and the chloroplasts are found almost exclusively in the bottom phase when the system contains no poly(ethyleneglycol)-deoxycholate. When this poly(ethyleneglycol)-ester is included in the system

in increasing amount, the affinity of the chloroplasts for bottom phase is gradually decreased. At poly(ethyleneglycol)-deoxycholate content corresponding to 10 per cent of the total poly(ethyleneglycol), the affinity of the chloroplasts for the two phases is equal and the particles therefore collect mainly at the interface. This means that the affinity of the chloroplasts for the top phase, due to the added deoxycholate groups, balance the effect of the interfacial potential working in the opposite direction.

The ratio of the absorbances at 550 nm and at 680 nm (A_{550}/A_{680}) has earlier been shown to be a measure of the proportion of intact (class I) and broken (class II) chloroplasts [13]. A high ratio, close to 1.0 is obtained for class I chloroplasts and a low ratio around 0.5 to 0.6 indicates a class II population. In experiments like those shown in Fig. 1 the ratio A_{550}/A_{680} was always higher for samples from the top phases than from the corresponding bottom phases when poly(ethyleneglycol)-deoxycholate was present. The ratio was 0.1 to 0.2 units higher for top phases in systems B and 0.05 to 0.1 units higher for top phases is systems A compared to the bottom phases. In systems without poly(ethyleneglycol)-deoxycholate the ratio was equal or reversed for the two phases. In system B with poly(ethyleneglycol)-deoxycholate there could for example be 90 per cent intact chloroplasts in the top phase sample and 60 per cent intact chloroplasts in the bottom phase sample from the same test-tube. For system A the values were around 90 and 75 per cent respectively and the remaining part was class II chloroplasts as judged from phase contrast microscopy. These differences indicate that the envelope has higher affinity for the deoxycholate group as compared to the lamellae.

Since single tube experiments only measure the over all partition of the total population of chloroplasts we chose counter-current distribution to compare in more detail the behaviour of the different classes of chloroplasts. We used the liquid-interface counter-current distribution technique [1] where the bottom phase and the interface were kept stationary while the upper phase was moving. Thus, chloroplasts to the right in the counter-current distribution diagram have more affinity for the upper, poly(ethyleneglycol) rich phase while chloroplasts to the left have more affinity to the interface and bottom phase. It is known from earlier work with counter-current distribution [11, 12] that class I and II chloroplasts give rise to separate peaks and the effect on their position in the diagram by addition of different poly(ethyleneglycol)-derivatives therefore gives a direct indication of the affinity of each of the two chloroplast classes for the added derivative.

A phase system in which 2 per cent of the poly(ethyleneglycol) was replaced by poly(ethyleneglycol)-deoxycholate was chosen for counter-current distributions of the chloroplast preparation. The result of a distriubution in system B (Table II) is shown in Fig. 2a. From the ratio A_{550}/A_{680} it can be seen that chloroplasts to the left, which have been almost immobile during the run, belong to the class II population while class I chloroplasts are found in the middle of the counter-current distribution train. This has also been confirmed by phase contrast microscopy and electron micrographs. The distribution pattern should be compared to that shown in Fig. 2c. This diagram shows a counter-current distribution of chloroplasts in phase system C, Table II, and corresponds to the diagrams described elsewhere [11, 12]. In this diagram, class I chloroplasts are distributed to the left and class II to the middle. The relative location of the peaks containing class I and class II chloroplasts respectively are consequently shifted in system B (Fig. 2a).

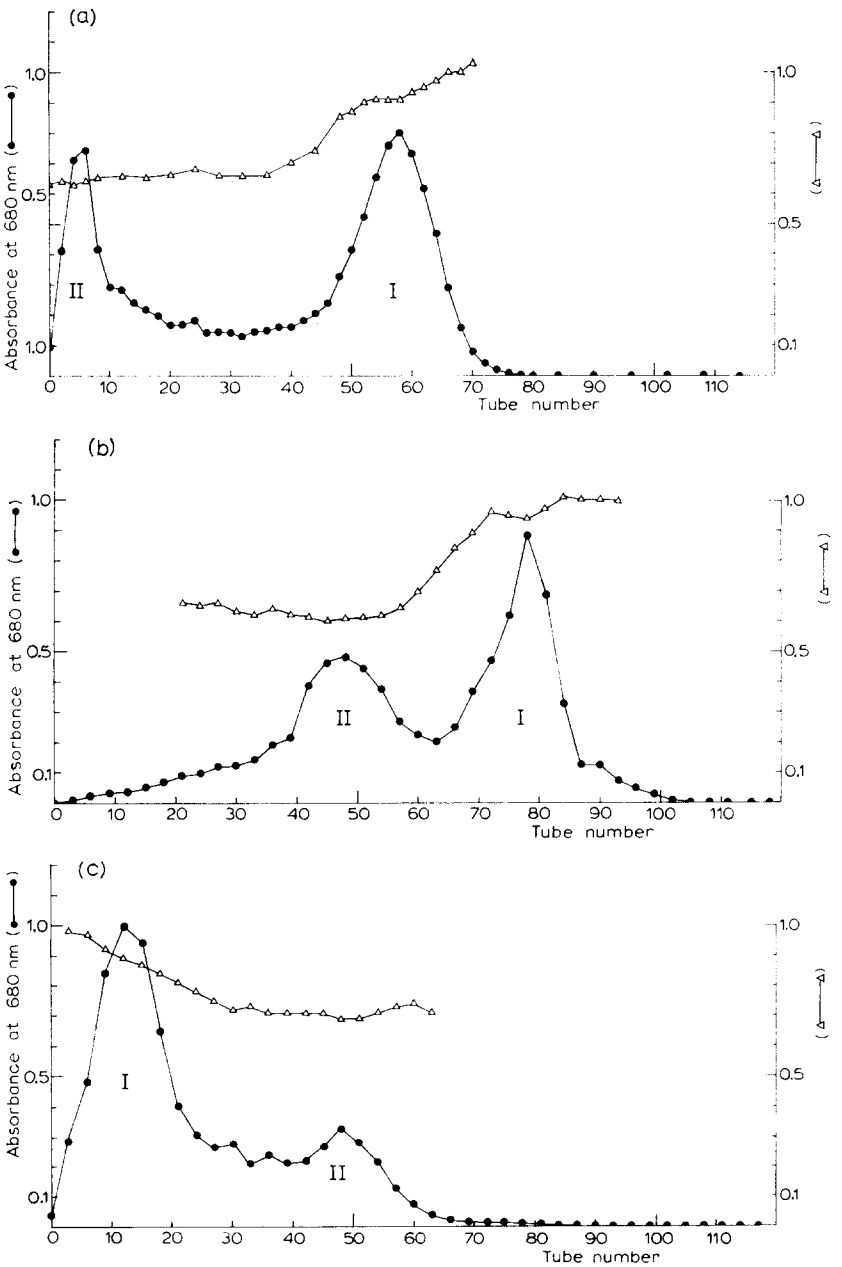

Fig. 2. Counter-current distribution of chloroplasts in (a) system B (containing poly(ethyleneglycol)-deoxycholate and NaCl, Table II) (b) system A (containing poly(ethyleneglycol)-deoxycholate and Na_2SO_4, Table II) (c) system C (containing no poly(ethyleneglycol)-ester and no salt except for buffer, Table II). Two distinct peaks are obtained in all cases. ●–●, absorbance at 680 nm; △–△, the ratio of absorbances at 550 and 680 nm ($A_{550/680}$). A high ratio indicates class I chloroplasts and a low one class II chloroplasts. The peak consisting of class I chloroplasts is designated as I and that of class II chloroplasts as II.

TABLE II

COMPOSITION OF PHASE SYSTEMS USED FOR COUNTER-CURRENT DISTRIBUTION

	Phase system				
	A	B	C	D1	D2
Total dextran 500 (%(w/w))	6.3	6.3	6.3	6.1	6.1
Poly(ethyleneglycol), type Carbowax 4000 (% (w/w))	6.17	6.17	6.3	6.1	6.1
Poly(ethyleneglycol)-deoxycholate, M_r = 6000–7500 (%(w/w))	0.13	0.13	0	0	0
Poly(ethyleneglycol)-deoxycholate/Total poly-(ethyleneglycol) (%)	2	2	0	0	0
Dextran-deoxycholate/Total dextran (%)	0	0	0	0	2
Na_2SO_4 (mmol/kg)	10	0	0	0	0
NaCl (mmol/kg)	0	10	0	0	0
Potassium phosphate buffer (pH 7.8) (mmol/kg)	1	1	5	0	0
Sodium phosphate buffer (pH 7.8) (mmol/kg)	0	0	0	5	5
Sucrose (mol/kg)	0.22	0.22	0.22	0.22	0.22

A reversal of the location of the peaks, similar to Fig. 2a, is also obtained when NaCl is exchanged for Na_2SO_4 (system A, Table II) but the resolution of the two peaks is not so good, Fig. 2b. The class I chloroplasts have a peak maximum at tube number 78 and class II at the number 48 in system A, Fig. 2b, compared to tube numbers 58 and 6 respectively in system B, Fig. 2a. Since system A has an interfacial potential close to zero the two types of chloroplasts distribute solely according to the ratio between hydrophobic and hydrophilic sites on their membranes exposed outwards. When a system with the same composition as system A but without poly-(ethyleneglycol)-deoxycholate is used for counter-current distribution both types of chloroplasts have low affinity for the top phase and the maximum values of the overlapping peaks are found in tube number 12 for class I and in tube number 18 for class II. This shows that the separation of class I and class II chloroplasts in system A (Table II), Fig. 2b, is exclusively due to different affinity for the deoxycholate groups. Again it has been shown that the class I chloroplasts have higher affinity for the deoxycholate group.

When the counter-current distribution is carried out with system B, Fig. 2a, the interfacial potential (negative top phase and positive bottom phase) lowers the affinity of the chloroplasts for the top phase and neither of the two chloroplast classes are transferred as far to the right in the diagram as with system A (with interfacial potential close to zero). The shift in the interfacial potential when going from system A to system B changes the distribution in such a way that the peak for class II chloroplasts is shifted 42 tube numbers to the left while the peak for class I chloroplasts is shifted only 20 tubes. This greater shift for class II chloroplasts shows that this type of chloroplasts bears a higher number of negative charges on their exposed surface than do class I chloroplasts.

The same effect as for poly(ethyleneglycol)-deoxycholate, but less pronounced, has been obtained with poly(ethyleneglycol) esterified with palmitic acid. Poly(ethyleneglycol)-palmitate also reverses the order of the two peaks but the resolution is decreased. Smaller amounts of poly(ethyleneglycol)-palmitate than of poly(ethylene-

glycol)-deoxycholate are needed for corresponding effects in counter-current distributions (0.003 per cent poly(ethyleneglycol)-palmitate compared to 2 per cent poly(ethyleneglycol)-deoxycholate of total poly(ethyleneglycol)). This difference in the amount of the substituted polymer required is also in agreement with partition studies of red blood cells [8]. However, above 0.01 per cent poly(ethyleneglycol)-palmitate (of total poly(ethyleneglycol) in single partition tests the chloroplast membranes, both envelope and lamellae, are broken. As judged from electron microscopy the lamellae are broken into large fragments (about 1 μm long) and the envelope forms vesicles. The same effect has not been seen for poly(ethyleneglycol)-deoxycholate up to 60 per cent of the substituted polymer.

The effect of deoxycholate bound to the dextran has also been tested. Since dextran-deoxycholate should pull the chloroplasts down into the bottom phase, it is desirable to start with a phase system in which the chloroplasts distribute exclusively to the top phase. Such a phase system is obtained by reducing the polymeric concentrations to 6.1 per cent of each polymer, exchanging potassium phosphate buffer for sodium phosphate buffer and excluding other salts [1, 11], system D in Table I. As can be seen in Fig. 3 the amount of chloroplasts in the top phase decreases strongly with increasing concentration of dextran-bound deoxycholate. The amount of material in the bottom phase increases simultaneously, but less pronounced, and the material is thus to a great extent accumulated on the interface.

Counter-current distribution of chloroplasts in a system without dextran-deoxycholate (system D1, Table I) is shown in Fig. 4a. The introduction of deoxycho-

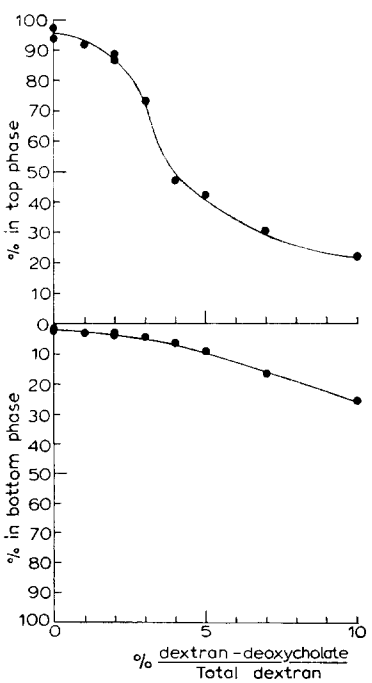

Fig. 3. Distribution of chloroplasts as function of dextran-deoxycholate concentration. System D, Table I.

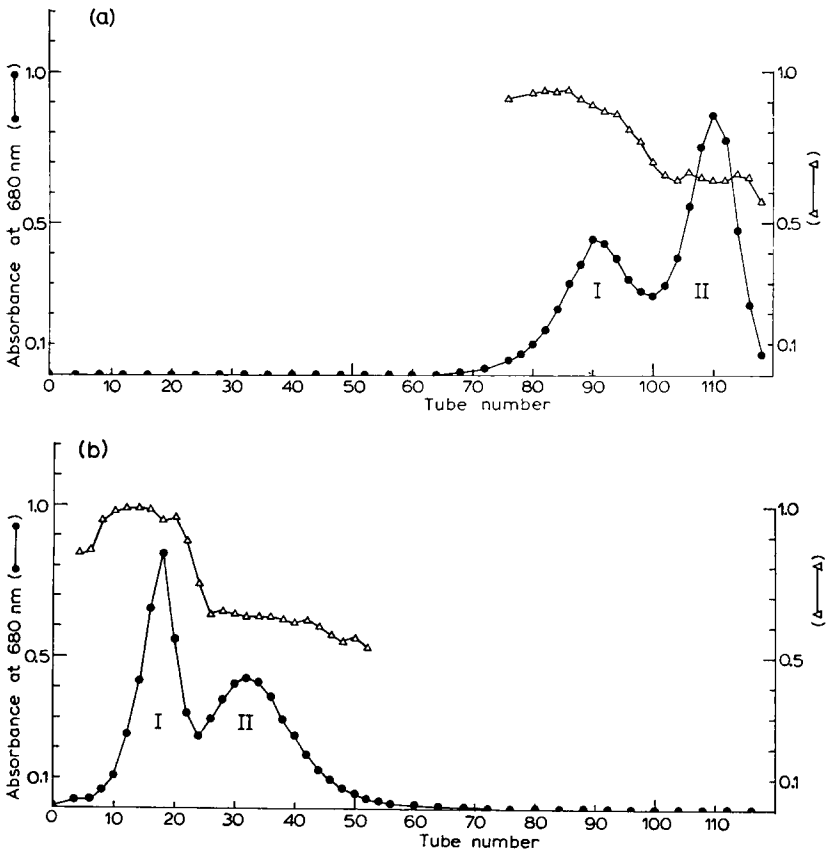

Fig. 4. Counter-current distribution of chloroplasts in (a) system D1 (Table II) (b) system D2 containing dextran-deoxycholate (Table II). Class II chloroplasts (designated II) in the right and class I chloroplasts (designated I) in the left peak. ●–●, absorbance at 680 nm; and △–△, A_{550}/A_{680}.

late groups on dextran (system D2, Table II) drastically diminishes the mobility of the chloroplasts along the counter-current distribution train (Fig. 4b). However, the two classes of chloroplasts are now affected to the same extent. From the corresponding experiments with poly(ethyleneglycol)-deoxycholate one would have predicted a larger effect on class I than on class II chloroplasts. One explanation for this apparently equal affinity of the two types of chloroplasts for deoxycholate bound to dextran could be the larger molecular weight of the dextran as compared to the poly(ethyleneglycol). The binding of one dextran-deoxycholate molecule to a binding site on the chloroplast could hinder the binding of other dextran-deoxycholate molecules to binding sites in the neighbourhood of the first one. Therefore the effect of dextran-deoxycholate on the partition should be the same for all chloroplasts assuming the surface density of binding sites exceeds a certain (relatively low) value.

The results presented have shown the existence of an interaction between the chloroplast membrane and the hydrophobic groups investigated. This affinity for poly-(ethyleneglycol)-deoxycholate and poly(ethyleneglycol)-palmitate is more pronounced

for the envelope membranes than for the inner lamellar membranes. The difference in interaction can be explained by differences in lipid composition [14, 15]. The envelope, but not the lamellae, contains sterols, steryl glycosides, steryl esters and acetylated steryl glycosides [14]. Deoxycholate has a structure resembling these steroid derivatives and might therefore bind stronger to envelope membranes than to membranes lacking these lipids. Another difference in the lipid composition is the higher proportion of saturated fatty acids in galactolipids and phospholipids of the envelope [15]. This may influence the affinity of the palmitoyl group for the envelope and for the lamellar membranes. The protein composition of the two types of membranes is also different [16, 17]. The envelope membrane proteins are few and have a higher molecular weight than lamellar proteins.

Whether the polymer-bound hydrophobic groups interact with the lipids of the membrane or with the membrane proteins cannot be determined from these experiments. The binding of the hydrophobic group to the lipids would mean that the polymer-bound group dip into the lipid bilayer and interact with the hydrophobic parts of the lipids. If, on the other hand, the proteins determine the affinity for the tested hydrophobic groups this should demand more specific binding sites for both deoxycholate and palmitate groups on the envelope membrane proteins than on the lamellar membrane proteins.

The presented results show that the partition in aqueous polymeric two-phase systems containing a small amount of polymer-bound hydrophobic groups are more specific for certain membrane types than for other. This technique can thus be useful in characterization and separation of membranes and membrane fragments differing in surface properties.

ACKNOWLEDGEMENT

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