COUNTER-CURRENT DISTRIBUTION OF SPINACH CHLOROPLASTS IN AN AQUEOUS TWO-PHASE SYSTEM

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Chloroplasts prepared by differential centrifugation appear to be morphologically very heterogenous when analysed with either the light or the electron microscope (Jacobi and Perner, 1961; Kahn and Wettstein, 1961). A conventional "whole"-chloroplast preparation from spinach thus contains a spectrum of particles of different sizes and shapes, some of which resemble very closely those observed in intact cells, while others look more or less swollen, broken or fragmented.

In this communication we will describe how such a chloroplast preparation has been analyzed by counter-current distribution (CCD) in an aqueous polymer two-phase system containing dextran and polyethylene glycol (Albertsson, 1960). Previous studies have demonstrated that various biological particles, including chloroplasts (Albertsson, 1956), can be distributed in polymer two phase systems (Albertsson, 1960); and algal cells, bacteria (Albertsson, 1960, Albertsson and Baird, 1962, Baird et al.,1961) and polio virus (Bengtsson et al., 1962) can be fractionated by CCD in these phase systems in a rather

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specific way and under mild conditions. The surface properties of the particles are the determining factor in this kind of fractionation. Since conventional chloroplast preparations may be lacking physiological function such as cyclic photophosphorylation only because of damage caused by the isolation (Baltscheffsky, 1960) it was considered important to apply this technique on chloroplasts also.

Materials and Methods.

Dextran was obtained from Pharmacia Fine Chemicals, Rochester, Minn., U.S.A. The fraction used has a limiting viscosity number of 48 ml/g and is marketed under the trade name "Dextran 500".

Polyethylene glycol was obtained as "Carbowax 4000" from Carbide and Carbon Chemicals Company, New York, U.S.A.

Na-phosphate buffer, pH 8, 0.2 M, was prepared by mixing Na_2HPO_4 and $NaHPO_4$ in the molar proportions 9:1.

Preparation of chloroplasts. Chloroplasts were prepared according to Arnon et al. (1956) from 50 g batches of spinach leaves which had been obtained at the local market. The medium contained 0.4 M sucrose (Jagendorf and Avron, 1958) and 0.01 M "tris"-buffer and had a pH of 8.0. After one washing the "whole chloroplast" fraction was secured and used in the experiments.

Phase system and partition. A partition experiment was done in the following way. First, a stock polymer mixture (A) containing 30 g 20 % (w/w) dextran, 20 g 30 % (w/w) PEG 4000 and 25 g 30 % (w/v) sucrose was prepared. After vigorous shaking, 3 g of this mixture was weighed into each of a series of tubes. To each of these we then added 0.1 ml phosphate buffer and different amounts of H_2O in order to obtain different polymer concentrations (see Table I) of the final phase system. After mixing, we added 0.5 ml of the chloroplast suspension. The contents were mixed again by inversion and

were allowed to stand for 25 min at 1° C for phase separation. A sample of the top phase was withdrawn, diluted threefold with H_2O and the absorbancy at 680 mu was determined with a Beckman Model B spectrophotometer. The amount of chloroplasts in the upper phase is expressed as per cent of the total amount in the tube. Both determinations are based on absorbancies of samples diluted at least 3 times with water.

Counter-current distribution. An apparatus of the type described by Albertsson (1963) was used. The bottom phase chambers had a capacity of 0.7 ml. Since the chloroplasts partitioned largely between the upper phase and the interface of the particular phase system used, the method of liquid-interface CCD (Albertsson, 1960) was employed.

Thus, only 0.6 ml bottom phase was added to each cell in order to allow the interface to be stationary. The volume of upper phase added to each cell was 0.4 ml. It is essential to add somewhat less top phase than bottom phase in order to suppress the tendency of the lower phase to form droplets in the upper phase. Between 50 and 60 transfers were usually carried out; the settling time was 10 min for the two first transfers and 5 min thereafter. The temperature was 1° C. After the last transfer, 0.5 ml of 0.4 M sucrose in 0.01 M Tris-HCl buffer was added to each cell and mixed with the cell contents. This dilution converts the two-phase system to a one-phase system in which the chloroplasts are suspended. Samples were then withdrawn for absorbancy measurements and microscopic examination.

Microscopy. Samples were fixed for at least 30 min in 2 % formaldehyde in 0.4 M sucrose, 0.01 M Tris-HCl, pH 8, and then inspected with the phase contrast microscope. Several types of chloroplasts could be distinguished. For counting purposes, we distinguished between so-called "intact" chloroplasts, which had a bright appearance with no visible grana; and "broken" chloroplasts, which had a dark appearance and usually contained visible grana. These two types of chloroplasts have been

described by Kahn and Wettstein (1961), and Spencer and Wildman (1962) and the reader is referred to these papers for micrographs. Usually 300-500 particles were counted. The counting is somewhat subjective, but the agreement between two independent observers was usually within 20 per cent.

Results and Discussion

Distributions of chloroplasts in phase systems of different polymer concentration are given in Table I. The chloroplasts show an increasing tendency to collect at the interface as the polymer concentration is increased. This is in agreement with the general observation that phase systems with compositions removed from the critical composition favour adsorption at the interface (Albertsson, 1960).

TABLE I

DISTRIBUTION OF CHLOROPLASTS IN A DEXTRAN-POLYETHYLENE GLYCOL TWO-PHASE SYSTEM.

No.	Mixture A[x] g	Chloroplast[xx] suspension ml	Na-phosphate 0.2 M, pH 8 ml	H_2O ml	Chloroplasts in upper phase, per cent of total
1.	3	0.5	0.1	0.40	80
2.	3	0.5	0.1	0.35	54
3.	3	0.5	0.1	0.30	44
4.	3	0.5	0.1	0.25	31
5.	3	0.5	0.1	0.20	27

x) = 30 g 20 % (w/w) dextran + 20 g 30 % (w/w) polyethylene glycol + + 25 g 30 % (w/w) sucrose.
xx) = in 0.4 M sucrose and 0.01 M Tris-HCl pH 8.

Generally, the selectivity of a phase system increases as the composition is removed from the critical point. On the other hand, maximum separation for a given number of transfers is achieved when the partition is fairly equal between the moving and the stationary layer. Therefore, system No. 3 was selected for CCD. The results of one experiment is shown in Fig. 1. The two distinct peaks of the diagram, (Fig. 1a) indicate two classes of particles. Microscopic examination showed that peak I consisted almost entirely of "intact" chloroplasts, while the second peak (II) consisted largely of "broken" chloroplasts (see Fig. 1b). The small fraction (5-10 %) of particles in peak I which were classified as "broken" chloroplasts did not look like the typical "broken" chloroplasts of peak II. Also, many of the chloroplasts in peak II which were classified as "intact" chloroplasts were somewhat different from the "intact" chloroplasts of peak I. These ambiguities in classification may be due to intermediate forms or the presence of different types of "broken" and "intact" chloroplasts.

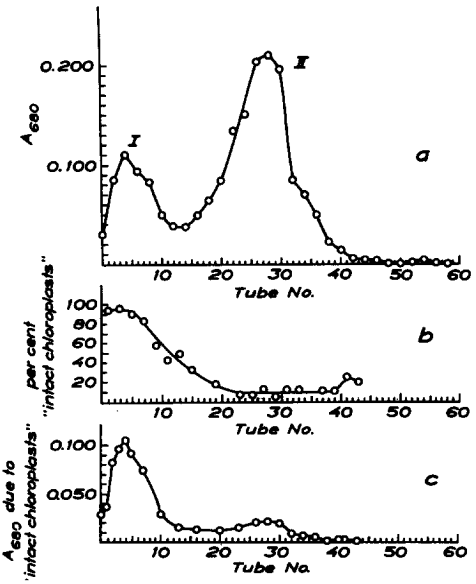

Fig. 1. Counter-current distribution of spinach chloroplasts in a phase system of dextran-polyethylene glycol-sucrose-buffer. 58 transfer.
a) Absorbancy of each fraction diluted 4.5 times.
b) Percentage "intact" chloroplast in fractions determined by phase contrast microscopy.
c) Absorbancy due to "intact" chloroplasts. This curve is calculated from curves a and b assuming that "intact" and "broken" chloroplasts have the same absorbance.

We make the reasonable assumption that the chloroplasts which are classified here as "intact" and which are found in peak I are bounded by the chloroplast membrane while the "broken" chloroplasts have more or less lost their membranes. The two kinds of particles would have different surface properties and would therefore exhibit different distribution behaviour. This could also explain why two distinct peaks are obtained. Even a puncture or slight damage to a small part of the chloroplast membrane might alter the surface properties anough to produce a sharp change in the partition behaviour.

The best evidence that the chloroplasts classified as "intact" resemble the chloroplasts in the cell more closely that do the "broken ones is that the "intact" chloroplasts are converted to "broken" chloroplasts upon standing. We studied the kinetics of this conversion from "intact" to "broken" chloroplasts in the initial chloroplast preparation and measured the time after which the number of "intact" chloroplasts had decreased to its half value. At room temperature this time was less than 10 min. In the cold (1° C) the "half-life" of "intact" chloroplasts suspended in 0.4 M sucrose, and 0.01 M Tris-HCl, pH 8, was about 3 hr. If they were suspended in the phase system, however, it was more than 6 hr. Thus, the polymers have a protective effect.

We also studied the decay of the "intact" chloroplasts of peak I in Fig. 1a. These were even more stable, exhibiting a "half-life" of more than 100 hr at 1° C. Thus the purified "intact" chloroplasts are more stable than the "intact" chloroplasts of the initial mixture. This might indicate either that decay-promoting substances have been removed or that the chloroplasts of peak I are a selection of chloroplasts which are more stable than the average "intact" chloroplast of the starting preparation.

Other experiments with the same phase system under the same conditions show that there is a considerable variation in the relative amounts of chloroplast material in the two peaks from preparation to

preparation. The ratio between the areas under peak I and II thus varied from 1/10 to 2/3.

The results of this study indicate clearly the potential usefulness of CCD in polymer phase systems for the analysis of mixtures of cell particles like chloroplasts. It seems probable that further work along this line will provide more information about the complexity of chloroplast preparations. The broadness of peak II indicates heterogeneity of the material in this peak and more transfers might indicate that this population of chloroplasts contains several distinct classes of different types. It appears promising to analyze biochemically the different fractions and to correlate function with structure. Photophosphorylation is not adversely affected by the presence of the polymers.

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