Partition of Cell Particles in Three-phase Systems

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Three-phase systems consisting of water and three polymers, dextran, Ficoll and poly(ethylene glycol), have been used for partition of some cell particles. Particles of biological origin are stable in these systems due to the high water content, 60–75 %. The partition behaviour of a particle is strongly affected by introducing charged polymers in the three-phase system. By varying the amount of charged polymer the affinity of particles for the different phases can successively be changed. In this way *Chlorella pyrenoidosa*, *Saccharomyces cerevisiae* and subchloroplast particles have each been shown to consist of more than one fraction. These fractions can be separated from each other by a single partition step. By introducing hydrophobic groups to one of the polymers, the partition of cell particles can in some cases be affected. Subchloroplast particles and mitochondria have been resolved into several fractions, differing in hydrophobicity, with such three-phase systems.

One of the methods used for studying the composition of biological materials is liquid-liquid extraction with biphasic systems obtained by mixing aqueous solutions of two suitable polymers.¹ Such dextran-poly(ethylene glycol)-water two-phase systems have been used for separation of biological macromolecules and cell particles.^{1,2} The homogeneity of cell particles such as chloroplasts,^{3,4} red blood cells,⁵ mitochondria,⁶ and *Chlorella pyrenoidosa*⁷ has been studied mostly by the technique of counter-current distribution.¹ These investigations have shown that such particles consist of several subpopulations.

The partition of a substance between the two phases depends on pH as well as type and concentration of salt included in the system.^{8,9} The partition can also be adjusted by using a polymer, which bears a few covalently bound groups, e.g. trimethylamino-poly(ethylene gly-

col), poly(ethylene glycol)-sulfonate or palmitoyl-poly(ethylene glycol).^{10,11} The two former polymers are ionic while the latter one is hydrophobic.

By mixing aqueous solutions of three different polymers a system with three phases can be obtained.^{1,12,13} The study of several proteins in these systems has shown that due to the high water content they can partition between all the phases without losing their biological activity. The three isoenzymes of enolase have by this method been enriched each in one of the phases in a single partition step.¹³

The three-phase systems ought to have advantages compared to two-phase systems also for the analysis of heterogeneous populations of particles. In this work the partition of some particles of biological origin such as cells, chloroplast fragments, and mitochondria has been investigated in three-phase systems consisting of water, dextran, Ficoll, and poly(ethylene glycol).

EXPERIMENTAL

Materials

Dextran T 500, batch No. 5996 ($M_w = 5 \times 10^5$) and Ficoll, batch No. 4720 ($M_w = 4 \times 10^5$) were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. M_w = weight average molecular weight.

Poly(ethylene glycol) (PEG) with $M_n = 4000$ and $M_n = 6000$ were obtained from Union Carbide, New York, as Carbowax. M_n = number average molecular weight.

Trimethylamino-PEG (TMA-PEG) and PEG-sulfonate (S-PEG) prepared from Carbowax 4000¹⁴ were gifts from Dr. G. Johansson. Deoxycholate-PEG (DC-PEG), the ester between PEG and deoxycholic acid, was prepared from Carbowax 6000 by Dr. G. Johansson (internal communication).

Microorganisms. *Sarcina lutea* was cultivated in a medium containing Nutrient Broth, pH 7.3 and 0.5 % sodium chloride. *Chorella pyrenoidosa* was cultivated as described by Albertsson and Baird.¹⁵ Bakers' yeast, *Saccharomyces cerevisiae*, standard yeast and special yeast, was obtained from Jästbolaget, Sollentuna, Sweden.

Before partition all these particles were washed twice by centrifugation and resuspension in 0.010 M potassium phosphate buffer, pH 6.8.

Subchloroplast particles. Class II chloroplasts were fragmented just before use with the help of a Yeda press by a method described by Andersson and Åkerlund.¹⁶

Mitochondria from rat liver were prepared according to Ericson⁶ and the mitochondria were kept in a 0.32 M sucrose solution.

All further chemicals were of analytical grade and the water was double distilled in a quartz apparatus.

Methods

The three-phase system. The three-phase system used contained 7 % dextran, 12 % Ficoll, and 12 % PEG with $M_n = 4000$ (including substituted PEG) and 0.5 mM potassium phosphate buffer pH 6.8.

4.00 g systems were prepared by mixing sample, water solutions of dextran 20 %, Ficoll 40 % and/or a solution containing both charged and uncharged PEG, the total amount of PEG being 40 %. The last solution was prepared from water solutions of PEG and substituted PEG, both 40 %. The solutions were then mixed in proportions required to yield the desired amount of charged PEG in the system. To minimize the variations in composition from time to time the highest concentration of charged PEG in the system was chosen as the concentration of charged PEG in the stock solution. For example, if the maximal concentration of TMA-PEG to be used in the system was 20 %, the solutions of TMA-PEG and PEG, both 40 %, were mixed in the proportion of 1:4. Thus a series of systems with the same polymer concentration but differing in percentage of substituted PEG were obtained.

All concentrations are given in per cent weight per weight. The systems were well

mixed by turning them upside down 40 times and allowed to settle at room temperature for 30 min. After that, they were mixed again and now allowed to settle for 90 min.

Each of the three phases is enriched in one of the polymers, the bottom phase in dextran, the middle phase in Ficoll and the top phase in PEG. The ratio between the volumes of the top phase, the middle phase and the bottom phase in these systems is 1.60:1.50:0.70. The composition of each phase (Table 1) was determined by a method described in a previous paper.¹³

Determination of the concentration of cells in each phase. After separation of the three phases 0.200 ml from the top phase and 0.200 ml from the middle phase were withdrawn and each aliquot was diluted with 1.50 ml of distilled water. The material at the interface between the top phase and the middle phase (interface_{TM}) was collected in a test tube and 1.50 ml of distilled water was added. The high viscosity of the bottom phase makes withdrawal from this phase difficult. After removing the excess of the two upper phases the bottom phase was diluted with 5.25 ml of distilled water and mixed well to ensure a homogeneous suspension. The material at the interface between the middle phase and the bottom phase (interface_{MB}) was included in the bottom phase suspension, also this depending on the high viscosity (when cells from bakers' yeast were partitioned the bottom phase had to be further diluted). The concentration of particles in these suspensions was determined by measuring the absorbance at a suitable wavelength (Table 2) using a Zeiss spectrophotometer, PMQ II.

The distribution of particles is given as the amount of particles in each phase expressed as the percentage of the total amount of particles in the system. The amount of particles in a phase was calculated from the concentration of particles and the volume of the phase. The amount of material at the interface_{TM} is obtained as the difference between the total amount and the sum of amounts in the three phases, including the interface_{MB}. These values agree quite well with the measured concentrations at the interface_{TM}.

The percentage in each phase was plotted versus the amount of substituted PEG with a separate curve for each phase.

Table 1. Composition of the phases in a three-phase system in which 10 % of the PEG was replaced by TMA-PEG. All concentrations are given in per cent weight by weight.

Phase	PEG + TMA-PEG/%	Ficoll/%	Dextran/%	Water/%
Top	22	3	0	75
Middle	7	25	1	67
Bottom	0	3	37	60

Table 2. Wavelength used for absorption studies

Type of cell particle	Wavelength/nm
<i>Sarcina lutea</i>	400
Yeast cells	400
<i>Chlorella pyrenoidosa</i>	465
Subchloroplast particles	680
Mitochondria	529

RESULTS

Partition of a relatively homogeneous particle

Sarcina lutea.. The distribution of the coccus *Sarcina lutea* in a three-phase system containing varying amounts of TMA-PEG is shown in Fig. 1. The total amount of cells in a system corresponded to an absorbance of A_{400} (1 cm)=8.0. In a system containing no TMA-PEG 87 % of the material is found in the bottom phase and 13 % is found in the middle phase. When PEG is successively replaced by TMA-PEG the affinity of the coccus for the three phases changes so that the cells successively move from the bottom phase over the middle phase to the interface_{TM} and the top phase. The bottom phase curve decreases linearly while the middle phase curve goes through a maximum when the concentration of TMA-

Fig. 1. The effect of TMA-PEG on the partition of *Sarcina lutea*. Cell content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○.

PEG is 0.20 % of the total amount of PEG. When 1.00 % of TMA-PEG is used in the system, 34 % of the material is found in the top phase and 59 % is found at the interface_{TM}. At higher contents of positively charged PEG in the system, *Sarcina lutea* partition only between the top phase and the interface_{TM}. It is noticeable that a minute variation in the concentration of TMA-PEG has a great effect on the partition of this organism.

It has earlier been shown that a suspension of *Sarcina lutea* consists of relatively homogeneous cells.³ The extraction profile of the coccus (Fig. 1) is therefore characteristic for

Fig. 2. The partition of two types of bakers' yeast in three-phase systems containing TMA-PEG. Cell content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○. (A) Standard yeast; (B) Special yeast.

a suspension of homogeneous particles. Furthermore, this extraction profile is analogous to that of a single protein.¹³

Partition of heterogeneous particles

Yeast cells. Two types of bakers' yeast, *Saccharomyces cerevisiae*, were investigated: so called standard yeast and special yeast. These two kinds of yeast are known to consist of more than one strain.² In these partition experiments the concentration of cells in the system was 0.05 g/kg.

The partition pattern of a cell-suspension from standard yeast is shown in Fig. 2 A. It is seen that the cells are extracted from the bottom phase in two distinct steps by the upper phases when the amount of TMA-PEG in the system is increased. The middle phase curve has two different maxima: one at 6 % TMA-PEG and another at 11 % TMA-PEG. This indicates the presence of at least two types of cells in the standard yeast.

Cells from special yeast (Fig. 2 B) partition quite differently from cells of standard yeast. In systems containing 15 % TMA-PEG 75 % of the cells of standard yeast are found in the top phase while most of the material from special yeast, 80 % of the cells, still is in the bottom phase. On the other hand the cells of standard yeast move from the bottom phase via the middle phase to the top phase within a smaller interval. This indicates that the cells of special yeast are more heterogeneous and the diagram also shows a more complex partition pattern. The material in the bottom phase is extracted into the other phases in three steps and both the middle phase curve and the interface_{TM} curve show three distinct peaks. This means that in a system containing 17 % TMA-PEG one fraction of cells ought to be concentrated in the bottom phase, another at the interface_{TM} and a third one in the top phase.

To verify this assumption the following separation experiment was made. Three 4.00 g systems containing 17 % TMA-PEG were weighed out, two with cell-suspension from special yeast and one with only buffer (blank system). After separation the top phase from one of the yeast-containing systems and the

Fig. 3. Separation of the three fractions of cells in special yeast in a three-phase system with 17 % of the PEG replaced by TMA-PEG.

top phase from the blank system were interchanged. The three systems were gently mixed again and after separation each phase was analyzed (Fig. 3). The fraction from the original top phase remains up to 88 % in the top phase and more than 90 % of the material in the middle phase and the bottom phase is still in these phases. The material at the interface_{TM} is the same. The experiment shows that three rather pure fractions can be obtained in just one single partition step. The above results with yeast cells agree quite well with experiments made in two-phase systems.²

Chlorella pyrenoidosa. It has earlier been shown by partition that normally grown *Chlorella* is composed of two major sub-populations differing in stage of maturation or age. Cells that have just divided differ in their partition from older cells.⁷

When *Chlorella* was distributed in the three-phase systems a complete extraction from bottom to top phase was not achieved by using only positively charged PEG. To complete the extraction a negatively charged polymer, S-PEG, had to be included in the system. The total cell content in a system corresponded to an absorbance of A_{465} (1 cm) = 2.0.

Fig. 4A shows the partition of *Chlorella* taken from a growing culture. Three major fractions are indicated. An older population of *Chlorella*,

Fig. 4. The effect of TMA-PEG and S-PEG on the partition of *Chlorella pyrenoidosa*. Cell content in the bottom phase $\square$, in the middle phase $\bullet$, in the top phase $\blacktriangle$ and at the interface between the top and middle phase $\circ$. (A) *Chlorella* from a growing culture; (B) *Chlorella* which has been stored.

which had been standing in 10 mM potassium phosphate buffer pH 6.8 for three months, was also investigated (Fig. 4B). This three months old population had quite different partition properties. No material at all was found at the interface_{TM} and more of the positively charged PEG was needed for extracting the cells from the bottom phase. Also this aged population of *Chlorella* consists of three major fractions. The proportion of these fractions is, however, different from that in the young population. This indicates that the *Chlorella* changes its surface properties with time.

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A one month old population of *Chlorella* was subjected to an extraction experiment, carried out as the one described with yeast cells. Also in the case of *Chlorella* the three main fractions could easily be separated; one in the bottom phase, another at the interface_{TM} and a third one in the top phase.

Subchloroplast particles. Mechanical fragmentation of chloroplasts give rise to a very heterogeneous mixture of particles.¹⁶ When these fragments are partitioned in three-phase systems the extraction curves indicate the presence of three major fractions (Fig. 5). In this respect

Fig. 5. The effect of TMA-PEG on the partition of fragments from Class II chloroplasts. Fragment content in the bottom phase $\square$, in the middle phase $\bullet$, in the top phase $\blacktriangle$ and at the interface between the top and middle phase $\circ$.

the results agree with results from counter-current distribution of the fragments.¹⁶ The three-phase system contained polymers and 2.5 mM sodium phosphate buffer pH 7.4, 2.5 mM sucrose and 0.5 mM sodium chloride. The amount of fragments in a system corresponded to 50 μ g chlorophyll.

Fig. 6 shows a separation of the three major fractions of subchloroplast particles in a system in which 15 % of the PEG is replaced by TMA-

Fig. 6. Separation of the three major fractions of subchloroplast Class II particles in a three-phase system with 15 % of the PEG replaced by TMA-PEG.

PEG. In this experiment each phase was shaken with new pure blank phases. Some biological activities of the fragments in each phase and at the interface_{TM} were controlled: Nicotinamideadenine dinucleotide phosphate reduction, with dichlorophenolindophenol – isoascorbate as electron donor, and dichlorophenolindophenol reduction, with water as electron donor.¹⁶ These investigations show that the subchloroplast particles retain their biological activity in the system.

Partition with DC-PEG. In all experiments described above the effect of a charged polymer on the partition of different cell particles has been investigated. However, polymers with other covalently bound groups can be used. One group which has been substituted to the PEG is deoxycholate and this group will give the polymer a hydrophobic character. Since the PEG-chain is the dominant part of the molecule, it partitions as PEG itself. In these systems a substance is partitioned under conditions of zero interfacial potential in the system.⁸ This means that the electrical charge of a partitioned substance does not influence its partition behaviour.

It has earlier been observed that a hydrophobic PEG can be used to influence the partition of some proteins while the polymer has no effect on others.¹¹ In analogy with proteins, the partition of some cell particles can be affected by DC-PEG. By a successive replacement of PEG by DC-PEG in the three-phase system the

Fig. 7. Partition of chloroplast Class II fragments in three-phase systems containing various amounts of DC-PEG. Fragment content in the bottom phase $\square$, in the middle phase $\bullet$, in the top phase $\blacktriangle$ and at the interface between the top and middle phase $\circ$.

distributions of chloroplast fragments and mitochondria from rat liver can be affected while the distributions of cells from bakers' yeast and *Sarcina lutea* are unaffected. In these experiments the materials were partitioned in three-phase systems containing 8 % dextran, 8 % Ficoll and 8 % (PEG + DC-PEG), the latter two with $M_n = 6000$.

The effect of DC-PEG on the partition of chloroplast fragments is shown in Fig. 7. The extraction curves obviously indicate the presence of two major fractions. The three-phase system contained except polymers 50 mM potassium sulfate, 2.5 mM sodium phos-

phate buffer pH 7.4, 2.5 mM sucrose, and 0.5 mM sodium chloride. The amount of fragments in the system corresponded to 50 μg chlorophyll. The biological activity of the fragments in these systems was controlled as mentioned above. The fragments retained their biological activity, but the activity of the material from the top phase decreased somewhat.

The partition diagram of mitochondria from rat liver (Fig. 8) indicates the presence of three fractions. With higher concentration than 10 % DC-PEG the partition is stabilized, 23 % of the material is found at the interface_{MB}, 43 % of the material is found at the interface_{TM} and

Fig. 8. The effect of DC-PEG on the partition of mitochondria from rat liver. Mitochondria in the bottom phase $\square$, in the middle phase $\bullet$, in the top phase $\blacktriangle$ and at the interface between the top and middle phase $\circ$.

the rest of the material, 34 %, is found in the top phase. Different forms of mitochondria from rat liver have been observed earlier.⁶ The three-phase system contained except polymers 0.2 M sucrose and the total amount of organelles corresponded to an absorbance of A_{520} (1 cm) = 2.5. The mitochondrial material in each phase and at the interface_{TM} was also controlled for some biological activities: succinate dehydrogenase, an inner membrane-bound enzyme, and fumarase, a matrix enzyme.⁶ It was found that also the mitochondria retained their biological activity in these systems.

Each type of cell particle, which was been partitioned in the three-phase systems, was analyzed under light microscope but no broken cells have been observed.

DISCUSSION

The distribution of a substance in a two-phase system can be expressed by its partition coefficient K (=concentration in the upper phase divided with the concentration in the lower phase). The partition coefficient is related to the net charge, Z , of the substance *via* eqn. (1)¹

$$\ln K = \ln K^0 + Z\psi\gamma \quad \text{eqn. (1)}$$

where ψ is the electrical interfacial potential, K^0 is the partition coefficient under zero interfacial potential or when Z is zero and γ is a constant including thermodynamic constants and temperature. A system with three liquid phases can be treated as a combination of three different two-phase systems. On this basis the quantitative models for two-phase systems^{1,9} have been applied to three-phase systems.¹⁷

In the three-phase system used here, 22 % (w/w) of the PEG + TMA-PEG is found in the top phase, 7 % (w/w) is located in the middle phase and the bottom phase contains less than 0.5 % (w/w). This unequal distribution of the charged PEG gives rise to differences in the electrical potential both between the top- and middle phase and between the middle- and bottom phase.¹⁷ The top phase is therefore positive compared to the middle phase which in turn is positive compared to the bottom phase.

In a system containing uncharged PEG and potassium phosphate buffer the ions of the

latter will direct the partition. The two anions of this buffer, H_2PO_4^- and HPO_4^{2-} , differ in their partition properties⁹ but they have both higher affinity for the bottom phase than for the other two phases. The interfacial potentials, ψ_{MB} and ψ_{TM} , are in this case working in the same directions as with TMA-PEG, but they have lower absolute values. Replacing the uncharged PEG with charged TMA-PEG in the phosphate containing system will increase the electrical potentials across the interfaces further. The resulting interfacial potential will be a mean of the interfacial potentials coming from TMA-PEG and potassium phosphate, and weighed with respect to the concentration of these species. Due to the unequal distribution of the TMA-PEG between the phases, a small amount of this charged PEG increases ψ_{TM} drastically to a certain stable value. ψ_{MB} , on the other hand, increases slowly with increasing amount of TMA-PEG, but will reach almost the same stable value as ψ_{TM} when the percentage of TMA-PEG in the system is high enough. Thus, increasing the amount of charged PEG in systems with constant concentration of buffer will change the interfacial potential across the interfaces, but to different values.

Since cells have a large number of charges on their surface their partition properties are strongly affected by charged polymers. It has been shown by particle-electrophoresis that most cells are negatively charged.¹⁸ That is why all investigated cells except *Chlorella pyrenoidosa* are extracted from the bottom phase over the middle phase to the top phase with increasing amount of TMA-PEG in the three-phase system. In the case of fresh *Chlorella* the cells are extracted from the top phase to the lower two phases with increasing amount of S-PEG. The reason for this effect of S-PEG on the partition is that the interfacial potentials, ψ_{TM} and ψ_{MB} , arisen from the unequal distribution of this negatively charged polymer, are working in the opposite direction of those interfacial potentials arisen from the unequal distribution of the TMA-PEG.

However, the partition properties of *Chlorella* are changed during storage. In a system containing no charged PEG the aged cells have lower affinity for the top phase and this is probably due to a decrease in the value of K^0 . This means that there might be a change in

the hydrophobicity of the cell with time so that *Chlorella* in its growing state has a more hydrophobic surface. When the aged *Chlorella* was partitioned the extraction steps were much sharper. This higher sensitivity towards changes in the interfacial potentials of the system can be explained by a change in the number of charges. If so, the cells increase their surface charge by storage. Greater number of charges on the surface of stored cells would decrease the tendency of aggregation and would explain why no material was found at the interfaces when these aged cells were distributed. The above agrees with results from cell electrophoresis of *Chlorella* where it is found that the cells change their surface properties during their life-cycle.¹⁸

When the three-phase technique is applied for partition of proteins their partition dependence on pH has been investigated.¹³ This technique can also be used to investigate the distribution of cell particles. However, the isoelectric points, pI, of particles are often low. Low values of pH may either destroy the particles or increase the possibility of particle aggregation. The method described in this paper, where the interfacial potentials are varied, is thus preferable since it is possible to work at a constant and moderate pH.

By attaching groups other than charged ones to one of the polymers in the three-phase system other specific properties of a particle can be used for separation purposes. The substitution of deoxycholate on PEG makes this polymer hydrophobic. The more hydrophobic a membrane is the greater its affinity for the top phase will be. From a mixture of cell particles fractions can successively be extracted into the top phase by enhancing its hydrophobicity by adding increasing amounts of DC-PEG to the system.

When DC-PEG was used for partition of fragments from Class II chloroplasts two different fractions were found while three different fractions were found when TMA-PEG was used. In the former case the particles were partitioned at constant interfacial potentials and therefore the two fractions differ only in their hydrophobicity. In the latter case, however, the particles distribute both according to their hydrophobicity and to their number of charges. Under these circumstances one of the

fractions, which was also obtained using DC-PEG, is probably split up into two fractions. These apparently differ in their charge density but not in their hydrophobic character.

It has been found that cell particles are not damaged and their biological activities are retained in these three-phase systems even at very high polymer concentrations. In spite of the higher viscosity of the three-phase system it has clear advantages compared to the two-phase system. In the former systems particles partition between the three phases as well as the two interfaces. That gives five locations, which under ideal conditions, can yield five fractions from a heterogeneous population of cells. If the partition properties of the different fractions are unequal enough, they can be separated from each other by a single, yet simple and not so time-consuming operation.

The affinity of a cell for a phase can be changed by introducing substituted groups onto any of the three polymers. Thus a certain desired property of a cell can be utilized for separation purposes and the cell can be directed to any of the three phases or two interfaces. So can, for example, one fraction in a cell mixture be directed to the middle phase and "impurities" can at the same time be extracted from the middle phase by the two other phases and the two interfaces. Two subpopulations, on the other hand, can easily be separated from each other by directing them to one phase each, the top phase and the bottom phase. The middle phase will then serve as a spacer phase and can serve as acceptor of "impurities" from the other two phases.

The three-phase technique can also be applied for analytical purposes. By studying the middle phase distribution curve for a partitioned particle suspension the presence of heterogeneity can be discovered. A homogeneous particle distributed in three-phase systems gives rise to only one peak in the middle phase curve. If there is several constituents in a mixture the curve has several maxima. For example a particle suspension consisting of two subpopulations with different partition properties yields a curve with two peaks. Thus, the number of well defined subpopulation fractions is equal to the number of peaks in the middle phase curve.

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