Partition of Macromolecules and Cell Particles in Aqueous Two-Phase Systems Based on Hydroxypropyl Starch and Poly(ethylene Glycol)

SISSI STURESSON,[1] FOLKE TJERNELD,*[,2] AND GÖTE JOHANSSON[2]

[1]Hyclone AB, S-223 70 Lund, Sweden; and [2]Department of Biochemistry, University of Lund, PO Box 124, S-221 00 Lund, Sweden

Received September 11 1989; Accepted January 4, 1990

ABSTRACT

The partition behavior of proteins, nucleic acids, cell membranes, cell organelles and whole cells has been studied in liquid–liquid two-phase systems composed of water, poly-(ethylene glycol), and an hydroxypropyl starch. The properties of the systems are in many respects comparable with the traditional poly(ethylene glycol)-dextran systems, but the cost is reduced to around one-fifth.

Index Entries: Hydroxypropyl starch; partition of cell particles; partition of macromolecules; two-phase systems; liquid–liquid extraction.

INTRODUCTION

Aqueous two-phase systems based on hydroxypropyl starch (Aquaphase PPT) and poly(ethylene glycol) (PEG) have been introduced (*1,2*) to

*Author to whom all correspondence and reprint requests should be addressed.

reduce the cost for large-scale partition procedures, especially extraction of enzymes by use of polymer-bound affinity ligands (*3*). These systems are designed to replace the traditional dextran–PEG two-phase systems. It was found that the new kind of system worked well for affinity partition of a number of enzymes and that both PEG and Aquaphase PPT could be used as ligand carrier (*1*). The two kinds of systems have been compared in their separating properties when used for countercurrent distribution of yeast enzymes (*1*) and serum proteins (*4*). In the present work, the possible use of Aquaphase–PEG two-phase systems as a tool for partition and separation of a number of macromolecules and particles of biomedical and biotechnical interest, has been studied.

MATERIALS AND METHODS

Chemicals

Poly(ethylene glycol) with a mol wt of 7000–9000, PEG 8000, and 3000–3700, PEG 3400, were purchased from BP Chemicals (Hythe, UK), and with mol wt of 17,000–20,000, PEG 20000, was purchased from Serva (Heidelberg, FRG). Aquaphase PPT, mol wt 35,000, was obtained from Perstorp Biolytica (Lund, Sweden). Dextran T-500, mol wt 500,000, was purchased from Pharmacia (Uppsala, Sweden), and Cibacron blue F3G-A from Serva (Heidelberg, FRG). Procion yellow HE-3G was a kind gift from Swedish ICI Co (Göteborg, Sweden). All other chemicals were of analytical grade.

Proteins

Bovine serum albumin, fraction V, and rabbit IgG were obtained from Sigma Chemical Co (St. Louis, MO).

Enzymes

Protein extract from *E. coli* was obtained by sonification of a cell suspension followed by centrifugation for 30 min at 50,000 × g. Soluble extract of bakers' yeast was obtained as described earlier (*5*). Glucose-6-phosphate dehydrogenase, from yeast, and lactate dehydrogenase, from rabbit muscle, were purchased from Boehringer (Mannheim, FRG).

Nucleic Acids

Deoxyribonucleic acid (DNA), from calf thymus, and ribonucleic acid (RNA) from bovine liver, were purchased from Sigma (St. Louis, MO).

Membranes

Synaptic membranes from calf brain cortex were prepared according to Olde and Johansson (*6*).

Chloroplasts

Chloroplasts, class II (without envelope), were isolated from spinach leaves according to Andersson et al. (*7*),

Cells

Yeast cells were obtained from fresh commercial press yeast (bakers' yeast) that was suspended in water, and the cells were allowed to sediment. *E. coli* K12, was a gift from Dr. Hahn-Hägerdal, Department of Applied Microbiology, University of Lund. *E. coli* JM105 pUR 291 was a gift from Dr. L. Bülow of the Department of Pure and Applied Biochemistry, University of Lund.

PEG Derivatives

The textile dyes, Cibacron blue F3G-A and Procion yellow HE-3G, were coupled to PEG and the dye–PEG was purified as described by Johansson and Joelsson (*8*).

Countercurrent Distribution

Countercurrent distribution (CCD) was performed in a centrifugal CCD machine containing 60 chambers (*9*). Each chamber contained 2 mL two-phase system. In each cycle, 1 min mixing and 2 min centrifugation were performed followed by transfers of the upper phases. The experimental details have been described elsewhere (*10*).

Analysis

Protein was determined according to Bradford (*11*), with bovine serum albumin as standard. Lactate dehydrogenase was assayed according to Bergmeyer (*12*), and glucose-6-phosphate dehydrogenase according to Noltman et al. (*13*), both photometrically at 340 nm. β-Galactosidase was analyzed according to Craven et al. (*14*), and nucleic acids were measured photometrically at 260 nm. The concentrations of membranes and cells were determined by measurement of the light scattering as the apparent absorbance at 400 nm (synaptic membranes), 620 nm (yeast cells), or 680 nm (chloroplasts). The partition coefficient, K, is defined as the

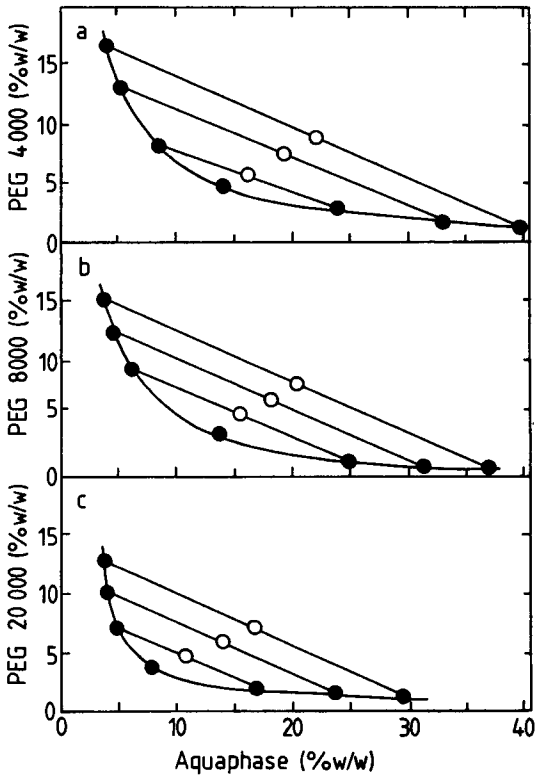

Fig. 1. Phase diagrams for mixtures of Aquaphase PPT and PEG of various mol wts: a, 3400; b, 8000; and c, 20,000, in water. Temperature, 23°C.

ratio between the concentration of the partitioned material in the upper and lower phases, respectively. The concentrations were determined by diluting samples from the phases and determining the material as described above.

Phase Diagram

The phases of a number of two-phase systems were analyzed for content of Aquaphase PPT by polarimetry, and of PEG by refractometry as described elsewhere (*3*).

RESULTS

A number of cell constituents were partitioned in the PEG–Aquaphase PPT system and compared with the partition in similar two-phase systems based on dextran 500 and PEG. Phase diagrams for systems (at 23°C) composed of Aquaphase PPT and PEG of three mol wt are presented in Fig. 1.

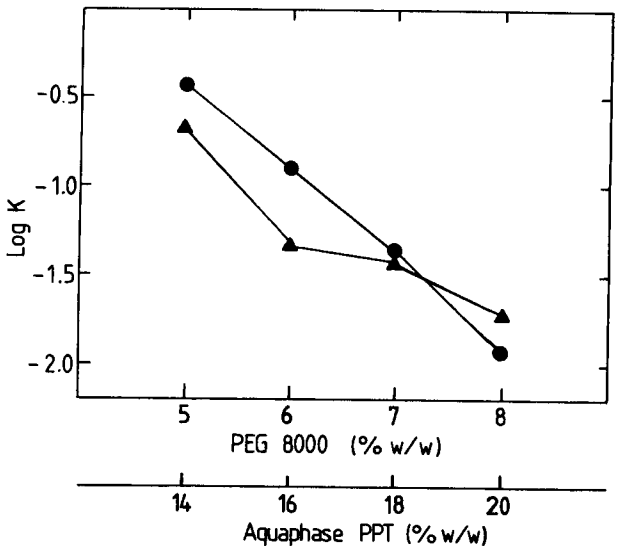

Fig. 2. Partition of lactate dehydrogenase from rabbit muscle (▲) and glucose-6-phosphate dehydrogenase from yeast (●) as function of the concentration of Aquaphase PPT and PEG 8000. The systems contained, beside the two polymers, water, 50 m*M* sodium phosphate buffer, pH 7.0, and 2 U/mL of the enzymes. Temperature, 23°C.

Partition of Proteins

In PEG–dextran systems, the polymer concentration can be used to influence the partition of proteins (*15*). Figure 2 shows the effect of polymer concentration on the partition of glucose-6-phosphate dehydrogenase and lactate dehydrogenase in phase systems based on Aquaphase PPT and PEG 8000. As in PEG–dextran systems, the enzymes are partitioned more to the bottom phase with increasing polymer concentration.

Bovine serum albumin showed a higher partition coefficient in the PEG–Aquaphase system compared with the partition in a corresponding PEG–dextran system (Table 1). The salt and pH values tested otherwise showed a similar relative (factorial) effect on the partition. Immunoglobulin G (IgG) (Table 1) had lower partition coefficient than the albumin in PEG–Aquaphase. Part of the IgG (28%) precipitated in the PEG–Aquaphase system, and this precipitate was collected at the interface. When IgG was introduced to the PEG–dextran system, as much as 80% was precipitated.

Two enzymes present in crude extract of *E. coli*, β-galactosidase and glucose-6-phosphate dehydrogenase, (Table 2) were strongly affected in their partition by varying the salt content, the mol wt of PEG, or by using PEG-bound affinity ligands.

Table 1
Partition of Bovine Serum Albumin (BSA) and Immunoglobulin G (IgG)

		Partition coefficient		
		Aquaphase -PEG		Dextran -PEG
pH	Buffer	BSA	IgG	BSA
5.0	sodium acetate	0.34	0.005	0.085
6.0	sodium phosphate	0.56	0.02	0.18
7.0	sodium phosphate	1.50	0.11	0.41
8.0	sodium phosphate	2.14	0.12	0.73
7.0	sodium phosphate + 100 m*M* NaCl	0.21	0.24	0.06
7.2	Tris-HCl	1.10	<0.005	0.08
8.0	Tris-HCl	1.47	<0.005	0.10

The two-phase systems contained either 14% (w/w) Aquaphase PPT, 5% (w/w) PEG 8000, or 8% (w/w) dextran 500, 6% (w/w) PEG 8000 and, in both cases, 10 m*M* buffer. The protein content was 5g L^{-1}. Temperature, 23°C.

Table 2
Partition of β-Galactosidase (β-Gal) and Glucose-6-Phosphate Dehydrogenase (G6PDH) Present in an Extract of *E. coli*

Enzyme	Strain of *E. coli*	Type of PEG	Additions	Partition coefficient
β-Gal	JM105 pUR291	3600	0.01 *M* $(NH_4)_2SO_4$	45
		3600	0.01 *M* $NaNO_3$	4.1
		20000	0.01 *M* $(NH_4)_2SO_4$	0.26
		20000	0.01 *M* $NaNO_3$	<0.01
G6PDH	K12	8000	None	0.27
		8000	0.1 *M* KI	0.008
		8000	1% (of total PEG) Cibacron blue F3G-A PEG	1.6
		8000	1% (of total PEG) Procion yellow HE-3G PEG	50

System composition: 19% (w/w) Aquaphase PPT, 7% (w/w) PEG, 16% soluble extract of *E. coli*, 1.7 m*M* sodium phosphate buffer, pH 7.0, and salt or PEG derivatives. Temperature, 23°C.

Partition of Nucleic Acids

The partition of bovine RNA and DNA in the PEG–Aquaphase system is shown in Table 3. The high-mol wt DNA had a much more extreme partition coefficient than the RNA, and the former macromolecule is also more influenced by the kind of salt present in the system.

Table 3
Partition of DNA (from calf thymus) and RNA (from bovine liver)

Ionic composition	Partition coefficient	
	DNA	RNA
Buffer A	250	2.8
Buffer A + 0.1 *M* NaCl	17	0.82
Buffer B	100	N.D.
Buffer B + 0.1 *M* NaCl	73	N.D.
Buffer C	101	2.5
Buffer C + 0.1 *M* NaCl	97	0.73

System composition: 13% (w/w) Aquaphase PPT, 5% (w/w) PEG 8000, buffer, and eventually, salt as well as nucleic acid (0.5g L^{-1}). Buffers: A, 10 m*M* sodium phosphate buffer, pH 7.0; B, 10 m*M* lithium phosphate buffer, pH 7.0; and C, 10 m*M* sodium acetate buffer, pH 5.0. Temperature, 23°C. N.D. = not determined.

The heterogeneity of the two nucleic acids has been tested by CCD in a system where the DNA partitions to the upper phase (Figs. 3a,b). When a mixture of DNA and RNA was analysed in the same way (Fig. 3c), part of the two nucleic acids (60%) appeared in the expected positions while the rest was found inbetween these two positions in the CCD diagram.

Separation of Proteins and Nucleic Acids

When a crude extract of bakers' yeast was analysed by CCD in a PEG–Aquaphase two-phase system, a considerable separation of protein (measured according to Bradford) from nucleic acids (main absorbance at 260 nm) was achieved (Fig. 4).

Partition of Membranes

Synaptic membranes, in a PEG–Aquaphase system with only sodium phosphate buffer, partitioned to the upper phase. By use of increasing concentration of sodium chloride, the membranes were transfered to the interface between the phases, and to the lower phase (Fig. 5).

Partition of Cell Organelles

The effect of salt composition on the partition of chloroplasts in PEG–Aquaphase systems is shown in Fig. 6. In the presence of 10 m*M* sodium phosphate buffer, the chloroplasts partitioned to the top phase. By addition of 25 m*M* sodium chloride to this system, the chloroplasts were located

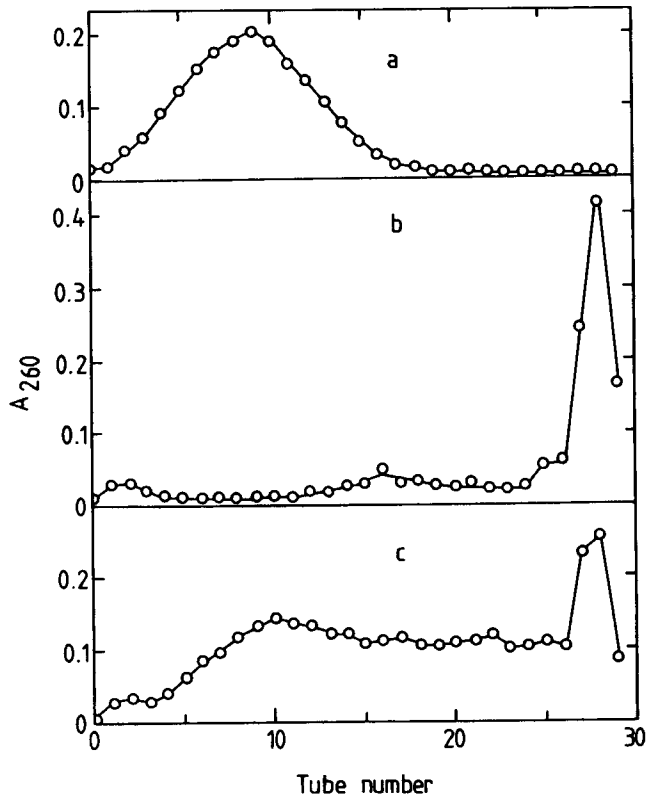

Fig. 3. Centrifugal countercurrent distribution of A: RNA from bovine liver; B: DNA (high molecular weight) from calf thymus; and C: a mixture of the DNA and RNA. The two-phase system contained 13% (w/w) Aquaphase PPT, 5% (w/w) PEG 8000, 100 m*M* NaCl and 10 m*M* sodium acetate buffer, pH 4.8. The sample, 1.5 mg of each nucleic acid was initially included in systems No 0, and 29 transfers were carried out. Temperature, 4°C. The measurements were made after 20 dilution of the phase systems.

almost totally at the interface. Increasing concentrations of NaCl gave a distribution between the interface and the lower phase.

Partition of Cells

At 10 m*M* sodium phosphate buffer, pH 7.0, the yeast cells were found mainly in the upper phase of the PEG–Aquaphase system whereas they were excluded from this phase in a PEG–dextran system (Table 4). By increasing the concentration of sodium chloride, the cells could be partitioned first to the interface, and then to the lower phase in the PEG–Aquaphase system. In the dextran–PEG system, the cells distributed between the interface and the lower phase at all tested concentrations of sodium chloride (Table 4).

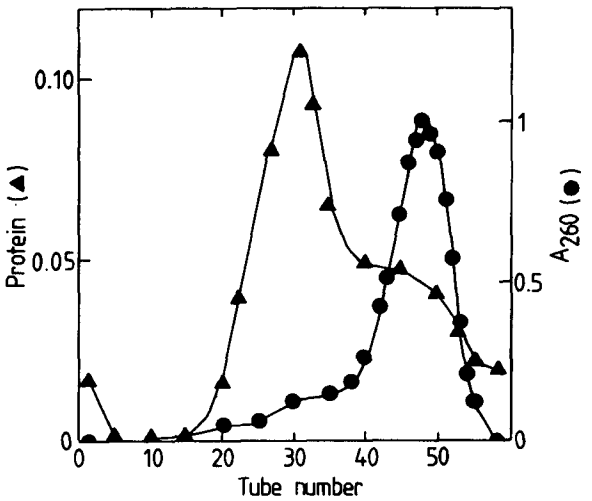

Fig. 4. Centrifugal countercurrent distribution of soluble extract from baker'yeast. The two-phase system contained 13% (w/w) Aquaphase PPT, 5% (w/w) PEG 8000 and 25 m*M* sodium phosphate buffer, pH 7.0. The sample was initially included in systems No 0-2, and 55 transfers were carried out. Temperatures, 4°C. The distribution of protein (according to Bradford) and UV absorbing material (at 260 nm) were determined after 20 dilution. The protein concentration is given in g L^{-1}

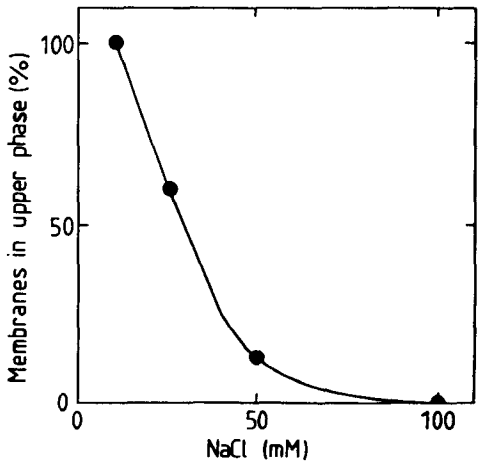

Fig. 5. Partition of synaptic membranes from calf brain cortex between the upper phase and the interface (+lower phase) as a function of the concentration of sodium chloride. Composition of two-phase system: 13% (w/w) Aquaphase PPT, 5% (w/w) PEG 8000, 10 m*M* sodium phosphate buffer, pH 7.5, 0-100 m*M* NaCl and membranes (corresponding to 50mg protein/L). Temperature, 23°C.

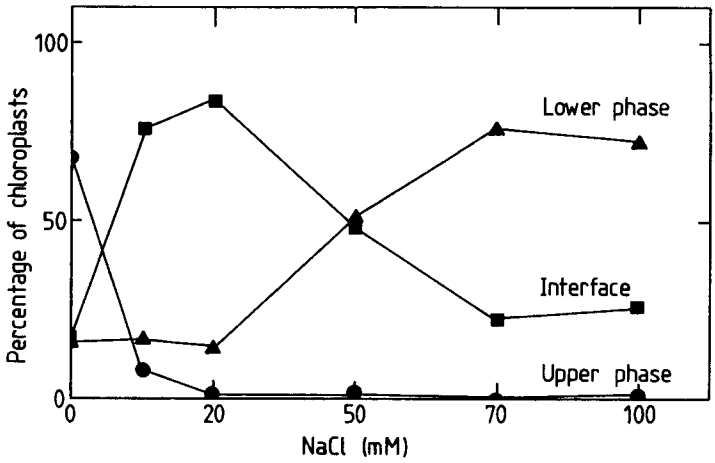

Fig. 6. Partition of broken chloroplasts (class II) from spinach. System composition: 13% (w/w) Aquaphase PPT, 4.5% (w/w) PEG 8000, 10 m*M* sodium phosphate buffer, pH 7.4, and 3 mg chloroplasts/mL. Temperature, 23°C.

Table 4
Partition of Yeast Cells (*Saccharomyces cerevisiae*) in Two Kinds of Aqueous Two-Phase Systems

	System A			System B		
Concentration of NaCl (m*M*)	% in T	% at I	% in B	% in T	% at I	% in B
0	97	3	0	0	16	84
12.5	89	11	0	0	21	79
25	20	80	0	0	18	82
50	1	98	1	0	25	75
75	0	62	38	0	16	84
100	0	33	67	0	36	64

System A: 13% (w/w) Aquaphae PPT, 5% (w/w) PEG 8000, 10 m*M* sodium phosphate buffer; System B: 5% (w/w) dextran 500, 4% (w/w) PEG 8000 and buffer as in system A. Both systems contained 0–0.1 *M* NaCl and 1% (w/w) yeast cells. Temperature, 23°C. T = top phase, I = interface, and B = bottom phase.

DISCUSSION

Until today, a vast number of data has been accumulated on the separation of proteins and other macromolecules in the aqueous two-phase systems composed of water, dextran, and PEG. In the present work, we have demonstrated the close similarity between the PEG–dextran system and a new, cheaper two-phase system based on PEG and an hydroxy-

propyl starch (Aquaphase PPT). The new system can, as shown here, replace the traditional PEG–dextran system.

Partition of Proteins

Enzymes partitioned more strongly towards the lower phase with increasing concentrations of PEG and Aquaphase PPT. The same effect is also generally found in PEG–dextran systems (*15*). This effect can, therefore, be used in both kinds of systems to adjust the partition of proteins between the two phases.

Several other ways of influencing the partition of enzymes and other proteins work equally well in both systems (see Tables 1 and 2, Fig. 2, and reference (*15*)). The partition of the two enzymes studied (β-galactosidase and glucose-6-phosphate dehydrogenase) in crude extract of *E. coli*, was strongly influenced by the mol wt of PEG, by the kind of salt included in the system, and by the use of PEG-bound affinity ligand present in the upper (PEG-rich) phase in the PEG–Aquaphase PPT system. All these methods of steering the partition can, therefore, be applied for improving the separatory capacity of both kinds of system.

Partition of Nucleic Acids

The partition of nucleic acids, as in the PEG–dextran system (*16*), is strongly influenced by varying the salt composition of the system. In both kinds of systems, the partition coefficients change more strongly on variation of the salt composition than what is observed for proteins. This reflects the high charge density on the nucleic acid molecules that is responsible for the sensitivity toward the electrostatic interactions in the system (*17*). Systems where the high-mol wt DNA partitioned strongly toward the upper phase when RNA was evenly distributed between the phases, were easily found. The salt compositions earlier used in PEG–dextran systems to affect the partition of nucleic acids (*16*) gave similar results when used in PEG–Aquaphase systems. The large difference in partition of DNA and RNA, as demonstrated by CCD technique (Fig. 3), did not lead to the almost complete separation which was expected. Instead, the CCD experiment with a mixture of DNA and RNA demonstrated a partial complexation of the two nucleic acids. This might be caused by a hybridization, since DNA and RNA were both of bovine origin.

The nearly total fractionation of proteins from nucleic acids demonstrated by CCD (Fig. 4) is an example of group separations that may be of interest in a number of downstream processes.

Partition of Membranes, Cell Organelles, and Cells

The partition of biological membranes, as demonstrated with synaptic membranes, is sensitive to the concentration of salt in the PEG–Aqua-

phase PPT system. A similar salt dependence has been observed in PEG-dextran systems (*6,18*).

The PEG-Aquaphase PPT systems have some unique properties when compared with the PEG-dextran systems. It has earlier been shown (*1*) that the glyceraldehydephosphate dehydrogenase (present in extract of bakers' yeast) by CCD, with the former system, was split in two isoenzymic forms when this was not the case in PEG-dextran systems. Another interesting advantage is that serum proteins show higher solubility in PEG-Aquaphase compared with PEG-dextran systems. This has been demonstrated via the studies of the partition of serum albumin and IgG (Table 1). When IgG was added to the PEG-dextran system, a greater part was precipitated, but only minor precipitation was observed in the PEG-Aquaphase system.

Other unique properties of the new system were found in the partition of large biological particles, such as cells and cell organelles. Yeast cells (Table 4) partitioned between the interface and the lower phase in the PEG-dextran system almost independently of the salt concentration, whereas in PEG-Aquaphase, they could effectively be moved between all three compartments by this method of steering the partition. Chloroplasts showed a similar partition behavior. This large flexibility in adjustment of the partition of cell particles makes the PEG-Aquaphase system useful for separation of cells and organelles. The reason for the acceptance of the particles in the upper phase of the new system might be the relatively high concentration of Aquaphase PPT also in this phase (Fig. 1). Microorganisms, e.g., yeast, have a cell wall built of carbohydrates, and the outer surface of cell organelles is partly covered with sugar residues. These particles may therefore be better solvated in a PEG solution if this also contains some of the starch derivative.

Large-Scale Use

The ease in the scaling up of these two-phase systems has been pointed out by several research groups (*3,19,20*). The use of aqueous two-phase extraction in a large-scale has almost exclusively been carried out with PEG-salt systems. This is partially a consequence of the high price of fractionated dextran. The main disadvantage of PEG-salt systems is that, the high salt concentration prevents the binding of enzymes to affinity ligands. The selective separations which can be carried out by using affinity partition are therefore, in most cases, limited to two-polymer systems. The partition experiments in this and earlier works (*1*) indicate that PEG-Aquaphase systems can replace the PEG-dextran system, both for partition of biopolymers, and cell particles. Furthermore, the huge knowledge of the steering of partition, established for the PEG-dextran systems, can, with minor modifications in most cases, be applied for the new system.

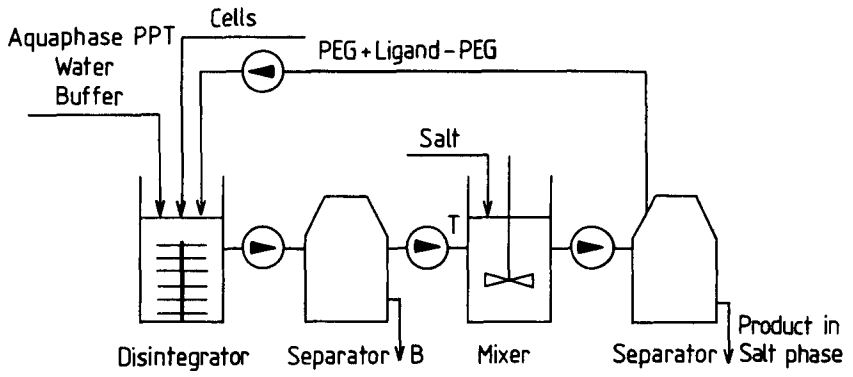

Fig. 7. Scheme for affinity extraction with separation and recovery of product, and ligand-PEG (together with unsubstituted PEG).

Since the cost of Aquaphase PPT is considerable lower than for fractionated dextran (a factor of around 0.1), the PEG–Aquaphase PPT system reduces the cost of the two-polymer systems, making them more economically favorable in large-scale processes. A possible arrangement for combined disintegration, affinity extraction, and final separation of product, e.g., an enzyme, from the polymer-bound affinity ligand (by formation of a PEG–salt system) is shown in Fig. 7. This scheme covers the most important parts of the downstream process, and it is based on a highly specific affinity extraction into the upper (PEG-rich) phase while debris is separated with the first bottom (Aquaphase-containing) phase.

Alternative Systems

Beside hydroxypropyl starch, other inexpensive polymers have been tried for aqueous two-phase extractions. Crude dextran has been used together with PEG for large-scale enzyme purifications (*21*). The viscosity of the bottom phases of these systems was impractically high, but it was reduced by a partial hydrolysis of the crude dextran. A soluble cellulose derivative, ethylhydroxyethyl cellulose (EHEC), has been used to generate aqueous two-phase systems, both with dextran and hydroxypropyl starch (22). With EHEC, it is possible to obtain phase systems with low polymer concentration. However, the high viscosity of EHEC in water limits the usefulness of this polymer in biotechnical processes.

An important advantage in the two-polymer aqueous phase systems based on dextran, hydroxypropyl starch, or ethylhydroxyethyl cellulose, is the biodegradability of these polymers. The waste problem caused by the salt in the PEG/salt systems can be avoided by the use of inexpensive two-polymer phase systems.

During the preparation of this work, a successful extraction of β-galactosidase from a crude protein fraction of *E. coli* by using PEG–Aquaphase PPT as well as PEG–potassium phosphate systems has been presented by Schwenzer and Kopperschläger (*23*).

ACKNOWLEDGMENTS

We thank Ingrid Elmroth, Håkan Enwall, and Åsa Westrin for technical assistance, and Ann-Christin Wikander for the artistic work. This study has been supported by a grant from the National Swedish Board for Technical Development, STU (FT).

REFERENCES

1. Tjerneld, F., Berner, S., Cajarville, A., and Johansson, G. (1986), *Enzyme Microb. Technol.* **8,** 417.
2. Gustafsson, Å., Wennerström, H., and Tjerneld, F. (1986), *Fluid Phase Equilibr.* **29,** 365.
3. Tjerneld, F., Johansson, G., and Joelsson, M. (1987), *Biotechnol. Bioeng.* **30,** 809.
4. Birkenmeier, G., Kopperschläger, G., Albertsson, P.-Å., Johansson, G., Tjerneld, F., Åkerlund, H.-E., Berner, S., and Wickström, H. (1987), *J. Biotechnol.* **5,** 115.
5. Kopperschläger, G. and Johansson, G. (1982), *Anal. Biochem.* **124,** 117.
6. Olde, B. and Johansson, G. (1985), *Neurosci.* **15,** 1247.
7. Andersson, B., Åkerlund, H.-E., and Albertsson, P.-Å (1976), *Biochim. Biophys. Acta* **423,** 122.
8. Johansson, G. and Joelsson, M. (1985), *Biotechnol. Bioeng.* **27,** 621.
9. Åkerlund, H.-E. (1984), *J. Biochem. Biophys. Meth.* **9,** 133.
10. Johansson, G., Andersson, M., and Åkerlund, H.-E. (1984), *J. Chromatogr.* **298,** 483.
11. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248.
12. Bergmeyer, H. U. (1970), *Methoden der Enzymatischen Analyse* vol. 1, 2nd ed., Verlag Chemie, Weinheim/Bergstr., p. 441.
13. Noltman, E. A., Gubler, C. J., and Kuby, S. A. (1961), *J. Biol. Chem.* **236,** 1225.
14. Craven, G. R., Steers, Jr., E., and Anfinsen, C. B. (1965), *J. Biol. Chem.* **240,** 2468.
15. Albertsson, P.-Å (1986), *Partition of Cell Particles and Macromolecules,* 3rd ed., Wiley, New York.
16. Albertsson, P.-Å. (1965), *Biochim. Biophys. Acta* **103,** 1.
17. Müller, W. (1985), in *Partitioning in Aqueous Two-Phase Systems,* Walter, H., Brooks, D. E., and Fisher, D., eds., Academic, Orlando, p. 227.
18. Gierow, P., Sommarin, M., Jergil, B., and Larsson, C. (1986), *Biochem. J.* **235,** 685.

19. Hustedt, H., Kroner, K. H., and Kula, M.-R. (1985), in *Partitioning in Aqueous Two-Phase Systems*, Walter, H., Brooks, D. E., and Fisher, D., eds., Academic, Orlando, 529.
20. Veide, A., Smeds, A.-L., and Enfors, S.-O. (1983), *Biotechnol. Bioeng.* **25,** 1789.
21. Kroner, K. H., Hustedt, H., and Kula, M.-R. (1982), *Biotechnol. Bioeng.* **24,** 1015.
22. Tjerneld, F. (1989), *Separations Using Aqueous Phase Systems*, Fisher, D. and Sutherland, I. A., eds., Plenum, New York, p. 429.
23. Schwenzer, B. and Kopperschläger, G. (1989), *Biomed. Biochim. Acta* **48,** 33.