Journal of Chromatography, 388 (1987) 295–305
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 164

EFFECTS OF ORGANIC SOLVENTS ON THE PARTITIONING OF ENZYMES IN AQUEOUS TWO-PHASE SYSTEMS

GÖTE JOHANSSON*,* and GERHARD KOPPERSCHLÄGER
Institute of Biochemistry, Section of Medicine, Karl Marx University, Leipzig (G.D.R.)
(Received October 22nd, 1986)

SUMMARY

Organic solvents (ethylene glycol, glycerol, dimethyl sulphoxide, dimethylformamide, dioxane, methanol and propanol-2, as well as sucrose and urea) have been included in aqueous two-phase (liquid–liquid) systems comprised of water, dextran and poly(ethylene glycol). The concentration of the organic solvent was in most cases 20% (w/w). The influence of these solvents on the phase-forming properties, the volume ratio, the freezing point and the partitioning of a polymer-bound ligand, Procion Red HE-3B poly(ethylene glycol), has been studied. The partition coefficients for alkaline phosphatase decrease with ethylene glycol, glycerol, sucrose and urea (factors of 0.25–0.5), but increase with the other substances (factors of 1.2–1.6). The temperature effects on the partitioning of alkaline phosphatase from calf intestine as well as of phosphofructokinase from yeast in systems containing ethylene glycol have been studied and compared with partitioning in standard systems, not containing solvents. The possible uses of the above systems for partitioning studies of enzymes are discussed.

INTRODUCTION

Aqueous two-phase systems, comprised of water, dextran and poly(ethylene glycol) (PEG), have been used for the purification of enzymes by liquid–liquid extraction[1,2]. During the last ten years the introduction of affinity ligands restricted to one of the liquid phases has greatly enhanced the selectivity of the partitioning of various enzymes between the phases[3,4]. Especially the reactive triazine dyes have been used as inexpensive enzyme ligands, which can be bound covalently to both PEG and dextran under mild alkaline conditions[5,6].

In the present work the effects of the partial exchange of water for solvents or other organic (water-soluble) substances were studied. Within such two-phase systems, enzymes could be partitioned at temperatures below zero. Moreover, the dielectric constants of the phases and other solvation-determining properties are

* Present address: Department of Biochemistry, Chemical Center, P.O. Box 124, S-22100 Lund, Sweden.

0021-9673/87/$03.50

changed which might influence the partitioning of enzymes and the enzyme–ligand interaction. The effects of the solvent and temperature on the partitioning of alkaline phosphatase from calf intestine (E.C. 3.1.3.1) and phosphofructokinase from baker's yeast (E.C. 2.7.1.11) have been determined using Cibacron Blue F3G-A and Procion Red HE-3B as PEG-bound dye affinity ligands.

MATERIALS AND METHODS

Chemicals

Poly(ethylene glycol) (PEG) with M_r = 6500–8000 and dextran with M_r = 500 000 were obtained from Serva (Heidelberg, F.R.G.). Procion Red HE-3B (I.C.I., Manchester, U.K.) and Cibacron Blue F3G-A (Ciba-Geigy, Basle, Switzerland) were bound to PEG and the products were purified as described earlier[7]. The solvents, sucrose and urea were of analytical grade. Biochemicals were provided by Boehringer (Mannheim, F.R.G.). Alkaline phosphatase was prepared from calf intestines as described by Kirchberger and Kopperschläger[8], and phosphofructokinase was prepared from baker's yeast according to Hofmann and Kopperschläger[9].

Enzyme assays

Alkaline phosphatase was determined in 1 *M* diethanolamine hydrochloride, pH 9.8, containing 1 m*M* magnesium chloride and 10 m*M* *p*-nitrophenyl phosphate, at 405 nm according to Haüsamen *et al.*[10]. Phosphofructokinase was assayed with a coupled optical test[9]. In both cases a Specol 21 spectrophotometer, at 366 nm, connected with a potentiometric recorder G1B1 (Zeiss, Jena, G.D.R.) was used.

Two-phase systems

The systems were prepared from stock solutions of dextran (20%, w/w) and PEG (40%, w/w), buffer (200 m*M* Tris–HCl, 40 m*M* magnesium chloride), water and organic additives. Solid urea and sucrose, respectively, were dissolved directly in the systems. Concentrations are given in % (w/w) except for salt which are given in mmol per kg system. Enzyme solution (5 μl) was added to the thermostatted system (4 g), equilibrated by careful shaking for 20 s, left for 15 min to settle at the same temperature and finally centrifuged for 2 min at 2500 *g* (same temperature) to obtain clear phases.

Partition coefficients

The partition coefficients, *K*, equal to the ratio of the concentrations of a solute in the upper and lower phase, respectively, were determined by removing samples from the two phases with micropipettes, usually 100 μl. The samples were diluted by factors of 2–21 depending on the solute concentration and analyzed either for enzyme activity or for Procion Red PEG (absorbance measurements at 540 nm). In the latter case, corrections were made for light scattering by using an equally diluted phase without ligand–PEG as a blank.

Volume ratio

The ratio between the volumes of the upper and lower phase was determined by equilibrating the systems (20 g) in calibrated graduated cylinders at given temperatures.

Transition point

The concentrations of the two polymers dextran and PEG at which the systems become homogeneous were determined by turbidometric titration at 0°C. Known weights of the systems (6.9% dextran, 5.1% PEG, 20% organic solute, 10 mmol/kg Tris–HCl and 2 mmol/kg magnesium chloride, pH 7.5) were mixed while shaking with a solution containing the above substances, except for dextran and PEG, both at 0°C, until the two phases just disappeared. The final concentrations of the polymers were calculated from the total weight.

Freezing point

The freezing points were determined by freezing the systems in acetone–solid carbon dioxide and by monitoring the temperature at which the gel-like solid was liquified.

RESULTS

A number of water-soluble organic solutes were included in an aqueous (liquid–liquid) two-phase system containing the two polymers dextran and PEG. In several cases a strong influence of the solute on the volume ratio was found (Table I). Especially methanol and propanol-2 gave rise to high volume ratios. In these systems the small lower phases was highly viscous and this can be interpreted as an effective exclusion of the dextran from the alcohol-rich upper phase. In the other systems the volume ratios indicated a more even distribution of the solute between the two phases. The transition point (from one to two liquid phases) also depended on the organic solute included (Table I). Urea and glycerol changed the transition point to higher values. On the other hand, the formation of two phases was enhanced by the aprotic solvents in the series dimethyl sulphoxide, dioxane, dimethylform-

TABLE I

EFFECTS OF VARIOUS ORGANIC SOLUTES (20%, w/w) ON THE VOLUME RATIO, TRANSITION POINT, FREEZING POINT AND PARTITION COEFFICIENT ($K_{L\text{-}PEG}$) OF PROCION RED HE-3B PEG (2% OF TOTAL PEG)

The two-phase systems contained 6.9% (w/w) dextran, 5.1% (w/w) PEG, 10 mmol/kg Tris–HCl, pH 7.5, 2 mmol/kg magnesium chloride, 20% (w/w) organic solute and water. Temperature: 0°C.

Organic solute	*Volume ratio*	*Transition point (% dextran/% PEG)*	*Freezing point (°C)*	$K_{L\text{-}PEG}$
–	1.51	4.68/3.46	− 0.3	18
Dimethyl sulphoxide	1.82	4.33/3.20	−13	26
Dimethylformamide	2.41	3.49/2.58	−12	18
Dioxane	2.12	3.96/2.93	−15	25
Ethylene glycol	1.57	4.66/3.45	−13	19
Glycerol	1.29	4.95/3.66	−11	16
Sucrose	1.21	4.70/3.47	− 5	19
Urea	0.78	6.15/4.54	−18	4
Methanol	3.6	1.56/1.15	− 6	23
Propanol-2	4.6	0.94/0.70	−10	23

TABLE II

EFFECTS OF VARIOUS ORGANIC SOLUTES (20%, w/w) ON THE PARTITION COEFFICIENT, K, OF ALKALINE PHOSPHATASE, AND THE AFFINITY PARTITIONING EFFECT, Δ log K CAUSED BY PROCION RED HE-3B PEG (2% OF TOTAL PEG)

System and temperature as in Table I.

Organic solute	*Log K*	*Δ log K*	*Δ log K/log $K_{L\text{-}PEG}$*
–	−0.28	0.98	0.78
Dimethyl sulphoxide	−0.57	0.90	0.71
Dimethylformamide	−0.68	1.06	0.75
Dioxane	−0.68	1.21	0.87
Ethylene glycol	−0.12	1.05	0.82
Glycerol	−0.09	0.59	0.49
Sucrose	−0.18	0.45	0.35
Urea	−0.09	0.13	0.22
Methanol	−0.74	1.55	1.1
Propanol-2	−0.89	1.32	1.0

amide and especially by methanol and propanol-2. The addition of 20% solute decreased the freezing point by 5–18°C (Table I).

The addition of solutes influenced the partitioning of alkaline phosphatase (Table II). The values of the partition coefficient K, of the enzyme generally decreased with increasing polymer concentration, *i.e.*, with increasing distance from the corresponding transition point. Δlog K, i.e., the difference between the log K values in the presence and in the absence of Procion Red HE-3B PEG, showed no direct correlation with either the distance from the transition point or the partition coefficient of dye–PEG (Table II).

A series of systems was prepared with compositions having the same relative distance from the limiting phase-forming concentrations (transition-point values plus 2.22% dextran and 1.64% PEG, respectively). As seen in Table III, the volume ratios show remarkable similarities with those in Table I, except for urea, methanol and propanol-2, which all gave higher values. The log K values for the enzyme increased relative to the values in Table II when the system composition was closer to the transition point, and *vice versa*. The Δ log K values were reduced in the case of aprotic solvents (dimethyl sulphoxide, dimethylformamide and dioxane) but were nearly the same as in Table II. The affinity partitioning effect, Δ log K, also depended on the temperature, increasing with decreasing temperature for systems containing ethylene glycol, dimethylformamide, dioxane and dimethyl sulphoxide. For the last two solvents a significant change occurred between zero and −10°C. The effect of glycerol, sucrose and urea on Δ log K with decreasing temperature was small.

The partitioning of Procion Red HE-3B PEG between the phases was only moderately changed when 33% of the water in the system was replaced by ethylene glycol. Also the concentration of ligand–PEG had a minor influence on its partitioning (data not shown).

The freezing point of a two-phase system can be reduced to −30°C by inclusion of ethylene glycol (Table IV). The freezing points determined are compared with values calculated from the freezing depression formula.

TABLE III

PARTITIONING OF ALKALINE PHOSPHATASE IN TWO-PHASE SYSTEMS WITH COMPARABLE COMPOSITIONS RELATIVE TO THE RESPECTIVE TRANSITION POINTS (TABLE I)

The systems contained dextran and total PEG as listed below, 10 mmol/kg Tris–HCl, 2 mmol/kg magnesium chloride, pH 7.5, and 20% organic solute. Affinity partitioning was carried out with a constant concentration of Procion Red HE-3B PEG (0.17% of total PEG). Enzyme concentration: 4 U per g system.

Organic solute	*System composition (% dextran/ % PEG)*	*Volume ratio at 0°C*	*log K*				*Δ log K*			
			−10	*0*	*20*	*40°C*	*−10*	*0*	*20*	*40°C*
–	6.90/5.10	1.5	–	−0.24	−0.06	0.00	–	0.98	0.86	0.80
Dimethyl sulphoxide	6.55/4.84	1.7	−0.58	−0.43	−0.16	−0.07	1.16	0.68	0.54	0.45
Dimethylformamide	5.71/4.22	2.5	−0.43	−0.20	0.00	0.06	0.80	0.66	0.56	0.52
Dioxane	6.18/4.57	2.0	−0.50	−0.36	−0.19	−0.16	1.41	1.02	0.96	1.01
Ethylene glycol	6.88/5.09	1.6	−0.28	−0.18	−0.01	0.07	1.12	1.05	0.76	0.66
Glycerol	7.17/5.30	1.3	−0.26	−0.18	−0.05	0.02	0.57	0.52	0.64	0.62
Sucrose	6.92/5.11	1.2	–	−0.19	−0.03	−0.03	–	0.45	0.60	0.63
Urea	8.37/6.18	1.1	−0.34	−0.28	−0.10	−0.05	0.08	0.18	0.17	0.22

TABLE IV

FREEZING POINTS OF TWO-PHASE SYSTEMS AS A FUNCTION OF THE CONTENT OF ETHYLENE GLYCOL

The systems contained 6.9% dextran, 5.1% PEG, water and ethylene glycol.

Concentration of ethylene glycol			*Freezing point (°C)*	
%(w/w)	*mol/kg system*	*mol/kg water*	*Determined*	*Calculated*
0	0	0	− 0.3	0
6.2	1.00	1.22	− 3.1	− 2.6
12.4	2.00	2.65	− 6.7	− 3.7
18.6	3.00	4.32	−11.3	− 8.3
24.8	4.00	6.32	−18.0	−12.0
31.0	5.00	8.77	−23.1	−16.4
37.2	6.00	11.81	−30.4	−22.2

The extraction curves for alkaline phosphatase, *i.e.*, the increase in Δ log K of the enzyme with increasing concentration of ligand–PEG (Fig. 1), were only marginally influenced by the temperature. Both with (33%) and without ethylene glycol, typical saturation curves were obtained as has been pointed out elsewhere[11,12]. The

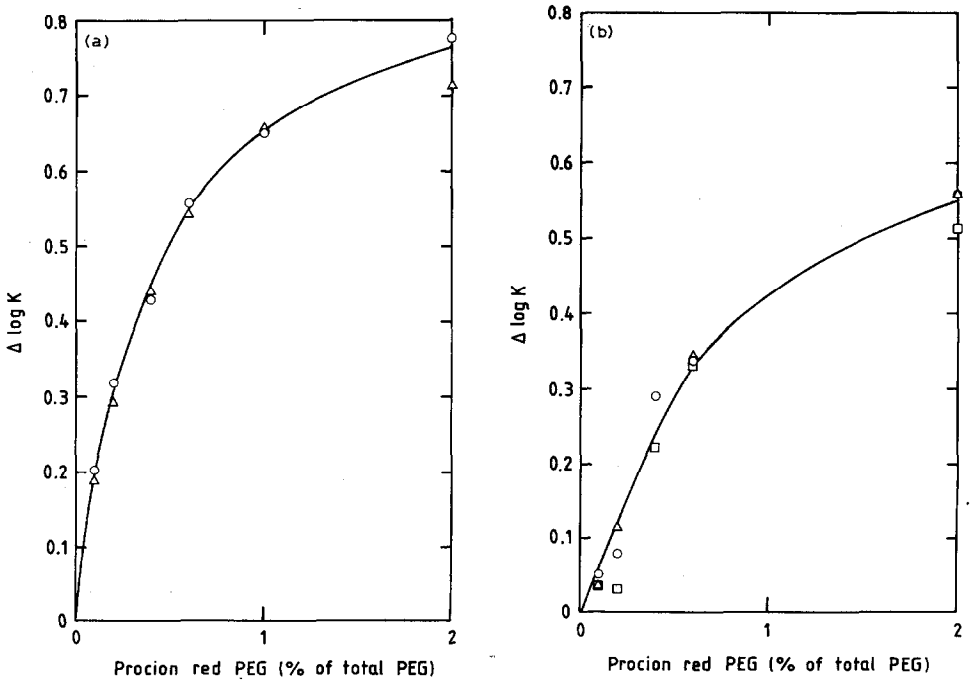

Fig. 1. Extraction curves for alkaline phosphatase at various temperatures. (a) Two-phase system with only water as solvent; (b) system containing 33% ethylene glycol. □, −23°C; ○, 0°C; △, 40°C. System composition: 6.9% (w/w) dextran, 5.1% (w/w) PEG, 10 mmol/kg Tris–HCl and 2 mmol/kg magnesium chloride, pH 7.5. L-PEG = Procion Red HE-3B PEG. Enzyme concentration: 4000 U/kg.

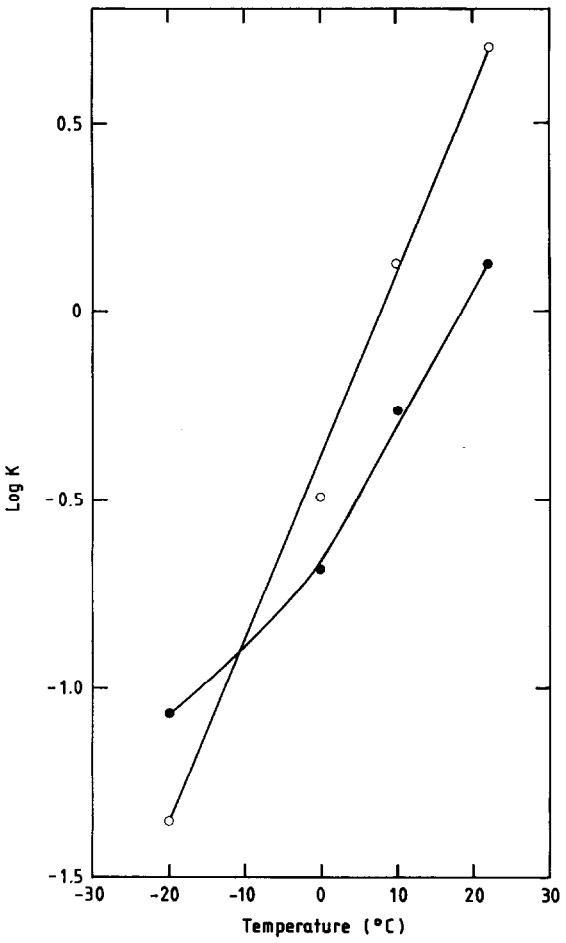

Fig. 2. Dependence of the partitioning of phosphofructokinase on the temperature. (●) Without dye-PEG; (○) with 0.1% Cibacron Blue F3G-A PEG. System: 5% PEG, 7.5% dextran, 50 mol/kg sodium phosphate buffer, pH 7.0 EDTA, 2-mercaptoethanol, and 33% ethylene glycol; 5–10 U of enzyme (dialyzed against buffer) per g system.

glycol reduced the maximum Δ log K value (saturation value) by one fourth and also shifted the concentration of ligand–PEG necessary to obtain half saturation from 0.4% of total PEG (for water) to 0.66–0.7% of total PEG (for 33% ethylene glycol). The values have been evaluated by an inverse plot technique as described earlier[11].

The partitioning of phosphofructokinase, from yeast, showed a strong dependence on the temperature in a phase system containing 33% ethylene glycol in the absence and in the presence of Cibacron Blue F3G-A PEG. The ligand–PEG caused an exclusion of the enzyme from the upper phase in both cases at −20°C (Fig. 2). The affinity partitioning effect decreased with increasing content of ethylene glycol (data not shown), but the extraction properties could to a great extent be restored by using an excess of Cibacron Blue F3G-A PEG (Figs. 3 and 4). The presence of

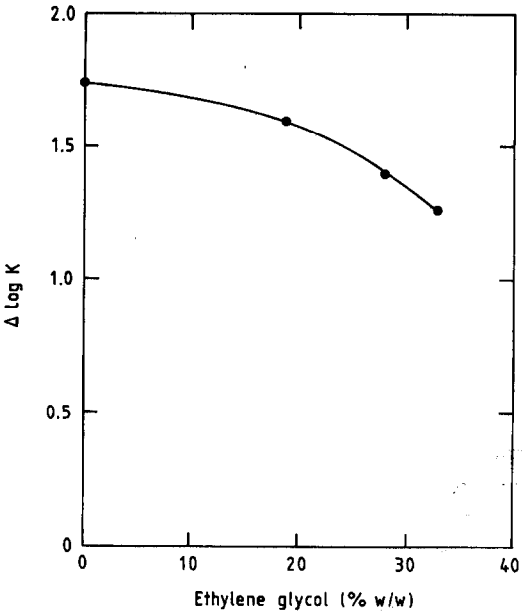

Fig. 3. Affinity partitioning effect, Δ log K, of phosphofructokinase in the presence of various concentrations of ethylene glycol. Systems as in Fig. 2 but with 5% of the total PEG as Cibacron Blue F3G-A PEG and with 0–33% ethylene glycol. Temperature: 0°C.

ethylene glycol gave rise to an extraction curve which was less steep than the one obtained in a water-based system.

DISCUSSION

Aqueous two-phase systems have long been used for the separation and study of cell components including proteins, nucleic acids, cell organelles and membranes[1,2]. They are generally employed at 0–4°C, and the partitioning of biological materials may be influenced by the addition of salts or by using polymer-bound groups. The results presented in this paper show that a considerable part of the water can be replaced by (water-soluble) organic substances without damaging effects on the two-phase system or its partitioning properties concerning proteins. The addition of organic solutes widens the possibilities of variation of important properties. These include partitioning below 0°C, changes in the dielectric constant of the two phases, influence on the interaction between (polymer-bound) ligand and protein and adjustment of association–dissociation equilibria between protein subunits. The addition co-solutes increases the possibilities of the liquid phases mimicing the solvent properties in various compartments of the living cell. By using the phase systems at very low temperatures, enzymes might also be kept in "frozen" conformational states which could even be separated from each other by partitioning.

The data obtained show that the organic substances have a strong influence on the phase-forming properties resulting from organic solute–water as well as organic solute–polymer interactions. For further insight, phase diagrams have to be

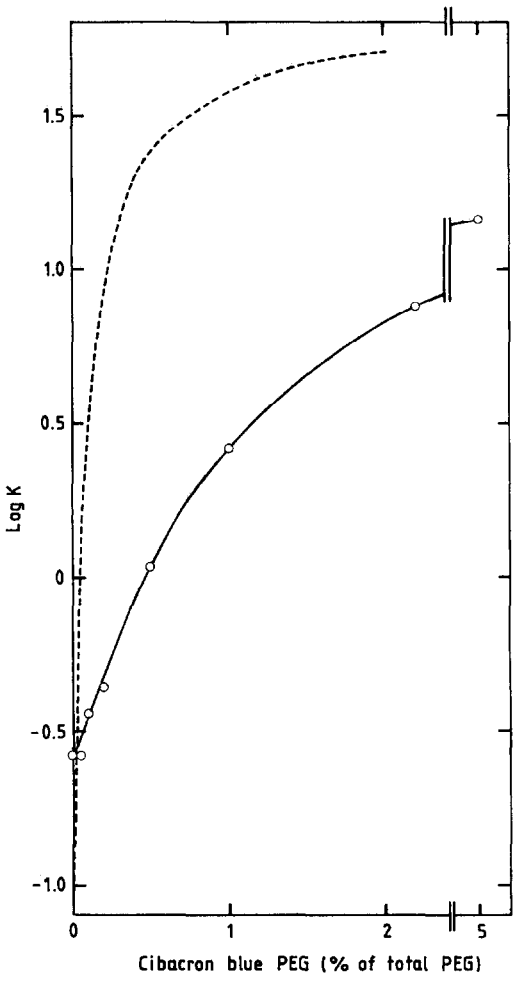

Fig. 4. Dependence of log K of phosphofructokinase on the concentration of Cibacron Blue F3G-A PEG in the presence of 33% ethylene glycol. Broken line indicates the corresponding extraction curve in the absence of ethylene glycol. System as in Fig. 2 but with 0.5 U enzyme per g system and 0–5% of total PEG in the form of dye–PEG. Temperature: 0°C.

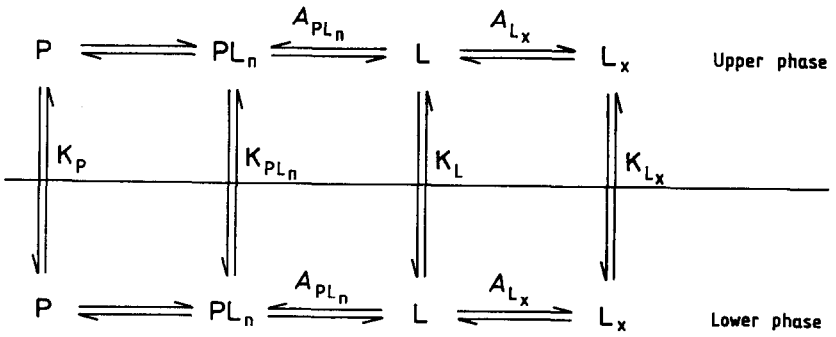

Fig. 5. Model of affinity partitioning including ligand–ligand interactions (see text).

determined showing the content of each component in the two phases. Knowledge of the distribution of the organic solute between the phases is of special interest. The data in Table III, with systems equally distant from the transition points, show however that the so-called tie-lines change their location in the vector space of the two polymers.

The influence on the affinity partitioning effect, $\Delta \log K$, has been measured for alkaline phosphatase and phosphofructokinase. The two solvents dimethyl sulphoxide and dimethylformamide, Table III, which both interact with water (strongly negative enthalpy of mixing), reduce $\Delta \log K$, as do the oligo-hydroxyl compounds glycerol and sucrose, and the diamino compound urea, in the case of alkaline phosphatase. The last three compounds may interact both with water and the polymers via multiple hydrogen bonds. This reduced influence of the ligand–PEG on the partitioning of an enzyme can depend on an interaction between the organic solute with either the binding site (on the enzyme molecule), the ligand or both. Thus, the binding constant for the ligand–enzyme interaction should be reduced which would lead to a less steep extraction curve, *i.e.*, higher ligand–PEG interaction to reach half saturation. The extraction of phosphofructokinase (Fig. 4) reveals this effect. If, however, not the binding strength but the maximum value of $\Delta \log K$ is decreased by the organic solute, without a corresponding change in the partition coefficient of the ligand–PEG itself, the effective ligand concentration may be drastically reduced by strong ligand–ligand or ligand–solvent (cage-structure) interaction.

An approach based on the model presented by Flanagan and Barondes[3] is given in Fig. 5. It is assumed that x ligands form an aggregate, that the association constants are the same in the two phases and that both ligand association and ligand–protein interactions are close to their saturation values.

For the concentration of protein–ligand–PEG (PL_n), the following relationship involving the concentrations of ligand–PEG (L) and protein (P) is fulfilled in both phases

$$[PL_n] = A_{PL_n}[L]^n[P] \tag{1}$$

where A_{PL_n} is the association constant. The ratio of eqn. 1 for the upper and lower phase, respectively, gives

$$K_{PL_n} = K_L^n K_P \tag{2}$$

where K is the partition coefficient of PL_n, L and P, respectively. Similarly, for the ligand–PEG complex:

$$[L_x] = A_{L_x}[L]^x \tag{3}$$

$$K_{L_x} = K_L^x \tag{4}$$

Combination of eqns. 2 and 4 gives

$$K_{PL_n} = K_{L_x}^{n/x} K_P \tag{5}$$

or

$$\log K_{PL_n} = n/x \log K_{L_x} + log\ K_P \quad (6)$$

By introducing $\Delta \log K_{max} = \log K_{PL_n} - \log K_P$, we obtain:

$$\Delta \log K_{max} = n/x \log K_{L_x} \quad (7)$$

$\Delta \log K_{max}$ is therefore reduced relatively to the apparent partition coefficient of the ligand–PEG by a factor $1/x$. To test the above (and similar) models, partitionings of enzymes with a known number of binding sites should be made with various concentrations of system components.

ACKNOWLEDGEMENTS

We thank Dr. Kirchberger and Miss Lorentz for valuable practical help and advice. G. J. acknowledges financial support from the Swedish Natural Science Research Council and the National Swedish Board for Technical Development.

REFERENCES

1 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Wiley, New York, 3rd ed., 1986.
2 H. Walter, D. E. Brooks and D. Fisher, *Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Applications to Biotechnology*, Academic Press, Orlando, FL, 1985.
3 S. D. Flanagan and S. H. Barondes, *J. Biol. Chem.*, 250 (1975) 1484.
4 M.-R. Kula, G. Johansson and A. F. Bückmann, *Biochem. Soc. Trans.*, 7 (1979) 1.
5 H. K. Kroner, A. Cordes, A. Schelper, M. Morr, A. F. Bückmann and M.-R. Kula, in T. C. J. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982, p. 491.
6 G. Kopperschläger and G. Johansson, *Anal. Biochem.*, 124 (1982) 117.
7 G. Johansson and M. Joelsson, *Biotechnol. Bioeng.*, 27 (1985) 621.
8 J. Kirchberger and G. Kopperschläger, *Prep. Biochem.*, 12 (1982) 29.
9 E. Hofmann and G. Kopperschläger, *Methods Enzymol.*, 90 (1982) 49.
10 T. U. Haüsamen, R. Helger, W. Rick and W. Gross, *Clin. Chim. Acta*, 15 (1967) 241.
11 G. Johansson, G. Kopperschläger and P.-Å. Albertsson, *Eur. J. Biochem.*, 131 (1983) 589.
12 G. Johansson, *Biochim. Biophys. Acta*, 451 (1976) 517.