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Aqueous two-phase systems with a liquid protein (bovine serum albumin) phase for partitioning of enzymes[1]

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Abstract

New aqueous liquid–liquid two-phase systems based on bovine serum albumin and sodium thiocyanate in combination with either poly(vinyl alcohol) or poly(ethylene glycol) were investigated. Phase diagrams are presented. Lactate dehydrogenase and some mitochondrial enzymes were partitioned in the systems. All the phase components used influenced, either positively or negatively, the activity of lactate dehydrogenase. The enzymes showed a strong preference for the albumin phase. Possible scientific and biotechnoligical uses are discussed.

Keywords: Aqueous two-phase systems; Partitioning; Proteins; Bovine serum albumin; Polyvinyl alcohol; Poly(ethylene glycol); Lactate dehydrogenase; Enzymes

1. Introduction

Aqueous two-phase systems have been extensively used for about 40 years for the fractionation of biological macromolecules and particles [1–3]. The systems can be divided into two categories: those containing a salt and a polymeric substance, and those which contain two polymers. The most common combination is poly(ethylene glycol) and dextran. However, many other polymer pairs have been used for partitioning of biopolymers [4]. Aqueous liquid–liquid systems can also be obtained by combining an aqueous solution of a protein with a synthetic or natural polymer [4–6]. Systems with one phase consisting of a concentrated protein solution are of interest for using enzymes in a protein-rich environment since it will mimic their natural milieu inside the cell [7]. The opposite phase can be used as a depot for the substrate and/or be a recipient for the product. Protein–polymer systems may therefore be useful as a base of enzyme reactors.

In this work, two-phase systems based on bovine serum albumin (BSA) in combination with either poly(vinyl alcohol) (PVA) or poly(ethylene glycol) (PEG) were studied. The phase compositions and the effect of the system components on the activity of lactate dehydrogenase were investigated. This, and also some mitochondrial enzymes, were included in the systems and their partitioning between the phases was determined.

[1] This paper is dedicated to Professor Gerhard Kopperschläger on the occasion of his 60th birthday.

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2. Experimental

2.1. Materials

Poly(vinyl alcohol) (88% hydrolysed, M_r 25 000) was purchased from Aldrich (Steinheim, Germany). Bovine serum albumin, fatty acid free and prepared from fraction V albumin, was obtained from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) (M_r 20 000) and lactate dehydrogenase (LDH) from porcine muscle were purchased from Serva (Heidelberg, Germany). Cytochrome *c*, fumarase, glutamate oxaloacetate transminase (GOT), glutamate dehydrogenase (GDH) and mitochondrial malate dehydrogenase (m-MDH) were obtained from Sigma. All other chemicals were of analytical-reagent grade.

2.2. Enzyme assays

LDH activity was measured photometrically at 340 nm according to Bergmeyer et al. [8] with an assay solution containing 2.0 m*M* sodium pyruvate, 0.20 m*M* NADH and 100 m*M* sodium phosphate buffer (pH 7.5). The solution was supplied with PVA, BSA and/or NaSCN. In the studies of the influence of pH, the molarity of the buffer (calculated on phosphorus) was kept constant at 100 m*M*. Fumarase [9], GOT [10], GDH [11] and m-MDH [12] were also measured photometrically.

2.3. Phase diagram

The phase diagram for the system PVA–BSA–100 m*M* NaSCN–water was constructed by weighing in 10-g systems from concentrated solutions of PVA (20%, w/w), BSA (250 g l^{-1}) and NaSCN (0.5 *M*). After equilibration at 23°C and settling, the phases were recovered and analysed. The content of BSA was determined by measuring the optical rotation of phases diluted 2.5-fold (4 g of phase diluted with water to 10 ml) using a AA-10 polarimeter (Optical Activity) equipped with a sodium lamp. The specific rotation for BSA was determined to be 56.8° ml g^{-1} dm^{-1}. The concentration of NaSCN was determined by measuring the electrical conductivity on solutions obtained by diluting 1 g of phase to 350 ml, with Metrohm Model 644 conductometer and correcting for the influence of the BSA. The concentration of PVA was determined via the refractive index (Zeiss immersion refractometer with an L1 prism) of the dilutions use for BSA determinations and correcting for the amount of BSA and NaSCN present.

The phase diagram for the system PVA–BSA–100 m*M* NaSCN–100 m*M* sodium phosphate buffer (pH 7.5)–water was constructed by weighing in 10-g systems from concentrated solutions as above but also using 0.5 *M* sodium phosphate buffer (pH 7.5 when diluted 1:10 with water). The phases diluted as above (1:2.5) and 2 ml were desalted by passing the solution through a mixed ion exchanger (IRA-400 in basic form and Dowex 50W-X4 in acidic form) with a 6-ml bed, and the column was rinsed with water. A total of 15 ml was collected. The concentrations of BSA and PVA were determined by measuring the absorbance at 280 nm and the refractive index. Both substances gave an increment in both cases. The absorption coefficient ε was 0.66 l g^{-1} cm^{-1} for BSA and 0.01884 l g^{-1} cm^{-1} for PVA. The increment in refractive index, Δn, was $1.714 \cdot 10^{-4}$ for 1 g l^{-1} BSA and $1.489 \cdot 10^{-4}$ for 1 g l^{-1} PVA. The salt contents of the phases were not measured. Phase diagrams were obtained by plotting the PVA versus BSA concentration.

The phase diagram for the system PEG (M_r 20 000)–BSA–100 m*M* NaSCN–5 m*M* sodium phosphate buffer (pH 7.5)–water was constructed via turbidometric titrations of two-phase systems with salt solution (100 m*M* NaSCN and 5 m*M* sodium phosphate) to determine the transition between one- and two-phase areas. The estimated transition points were checked by centrifugation of systems with slightly lower and higher polymer concentrations to verify the absence or presence of two liquid phases. The binodal curve generated by the transition points was used to estimate the positions of tie-lines for two-phase systems from the mass ratio between the upper and lower phases by applying the lever rule [13]. This ratio was obtained via the volume

ratio observed for 10-g systems in calibrated graduated centrifuge tubes and the determined densities of the phases.

2.4. Effect of phase components on LDH

The time dependence of enzyme activity in the presence of BSA, PVA or NaSCN and their mixtures was determined by making solutions of these substances and including LDH at low concentration (2 U ml^{-1}). The solutions were kept at 23°C and samples were withdrawn and measured for LDH activity after various times of incubation.

2.5. Partitioning of enzymes

LDH (2 U ml^{-1}) was included in two-phase systems (4 g) made from concentrated solutions of PVA, BSA and salts (as described above). The systems were carefully mixed and centrifuged for 5 min at 1000 *g* at 23°C. Samples of the phases were withdrawn and analysed for LDH content. The partition coefficient, *K*, of LDH is defined as the ratio of the enzyme activity between the upper and lower phases. The time dependence of *K* was determined by removing samples after various time intervals, each time preceded by mixing and centrifugation. The stable *K* values given were obtained after 1 h.

In the same way, mitochondrial enzymes and cytochrome *c* were partitioned in systems prepared from PEG (M_r 20 000), BSA and salts. *K* values for the enzymes were determined as above. In the case of cytochrome *c* the *K* values was obtained as the ratio between the concentration of protein determined by absorbance measurement at 550 nm.

3. Results and discussion

3.1. Phase diagram

The two polymer substances PVA, with M_r 25 000, and the protein BSA, with M_r 68 000, are incompatible in water when the concentrations exceed certain values. The incompatibility is enhanced by the presence of sodium thiocyanate (data not shown). Without salt, very high concentrations of PVA and BSA are needed to obtain two-phase systems. Addition of sodium thiocyanate reduces these concentrations strongly. By using 100 m*M* sodium phosphate buffer in addition to NaSCN, this effect is enhanced. The phase diagrams of systems containing BSA, PVA, NaSCN (100 m*M*) and water with and without sodium phosphate buffer (100 m*M*) at pH 7.5 and 23°C are shown in Fig. 1. The borderline (binodal curve) between one- and two-phase areas is the curved line on which all compositions of the upper phases (to the left) and of the lower phases (right) lie. The positive effect of phosphate on the phase formation is clearly seen in Fig. 1B. It is obvious, from Fig. 1A, that systems can be obtained with one phase containing a high concentration of BSA (>25%) while the other phase contains less than 1% of this protein. The BSA-rich phase is the denser one.

The phase diagram of systems containing BSA, PEG, NaSCN (100 m*M*), sodium phosphate buffer (100 m*M*) (pH 7.5) and water at 23°C is shown in Fig. 2. In this case, the lower phases contain more BSA compared with the PVA-containing system, when system compositions with a similar distance from the binodal curve are compared. Also, the slope of the tie-lines is considerably less for the PEG–BSA.

3.2. Effect of phase component on LDH activity

When included in the assay solution for LDH, the polymer PVA showed a negative effect on the enzyme activity (Table 1). BSA (2%), on the other hand, enhanced the activity by up to 18% and NaSCN (100 m*M*) increased the activity by as much as 76% (Table 1). Combining the phase components two by two showed that the effects were approximately additive. The same is true when PVA, BSA and NaSCN are used together provided that the mixtures were in one phase area of the phase diagram.

The positive effect of NaSCN on the LDH activity showed a maximum at 100 m*M* salt (Fig. 3). High concentrations (>300 m*M*) had a negative effect on the activity compared with thio-

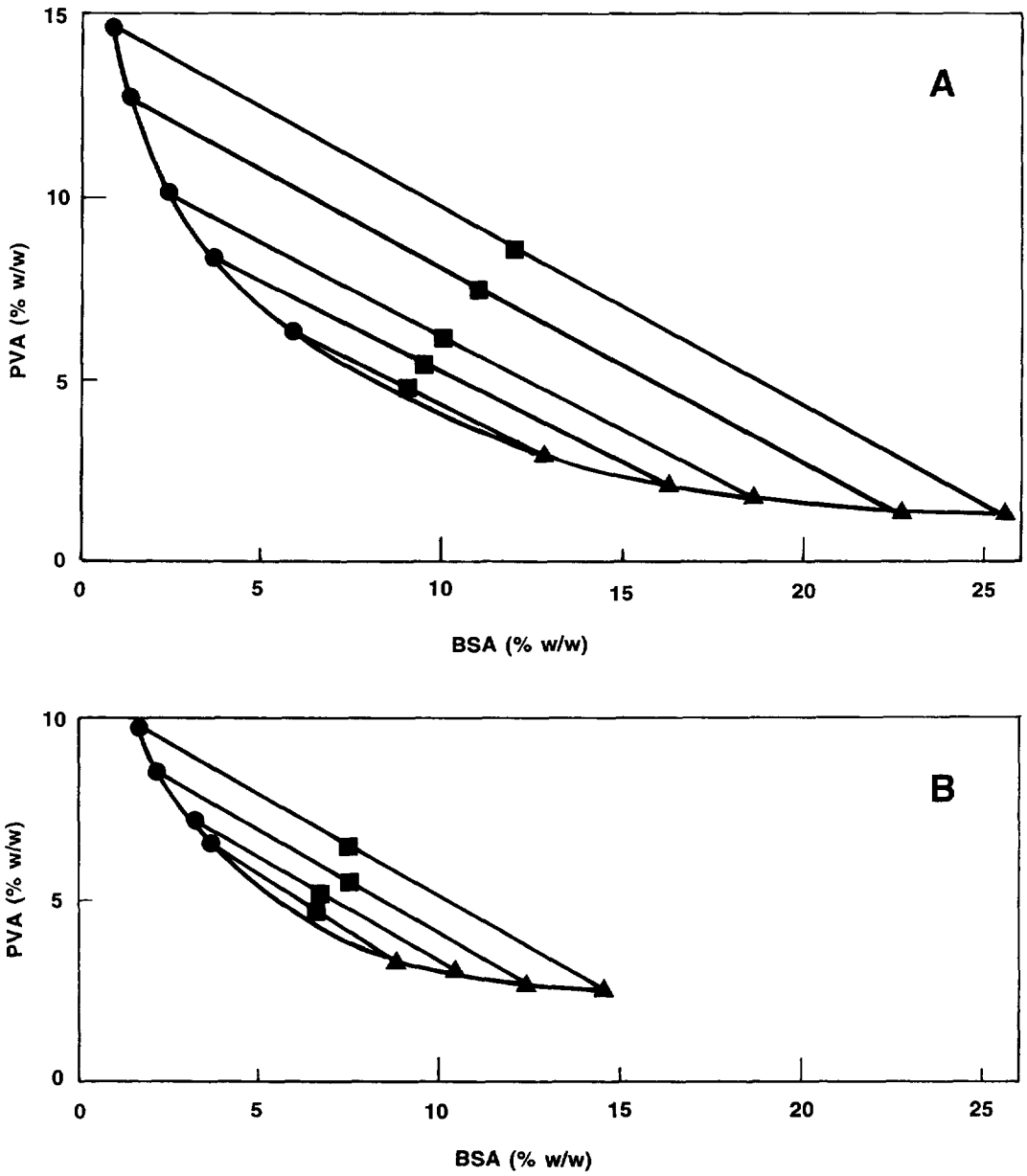

Fig. 1. (A) Phase diagram for the system PVA (M_r 25 000; 88% hydrolysed)–BSA–NaSCN (0.1 *M*) in water. (B) Phase diagram for systems containing all compounds as in (A) but also including 100 m*M* sodium phosphate buffer (pH 7.5). Temperature in both cases, 23°C. (■) Total composition of system; (●) composition of upper phase; (▲) composition of lower phase.

cyanate-free solution. No noticeable time dependence of this activation (with 100 m*M* NaSCN) in the time interval 1–80 min could be seen (Fig. 4).

3.3. Partitioning of LDH

The partition coefficient, K_{LDH}, of LDH is time dependent but it is stable after 1 h (Table

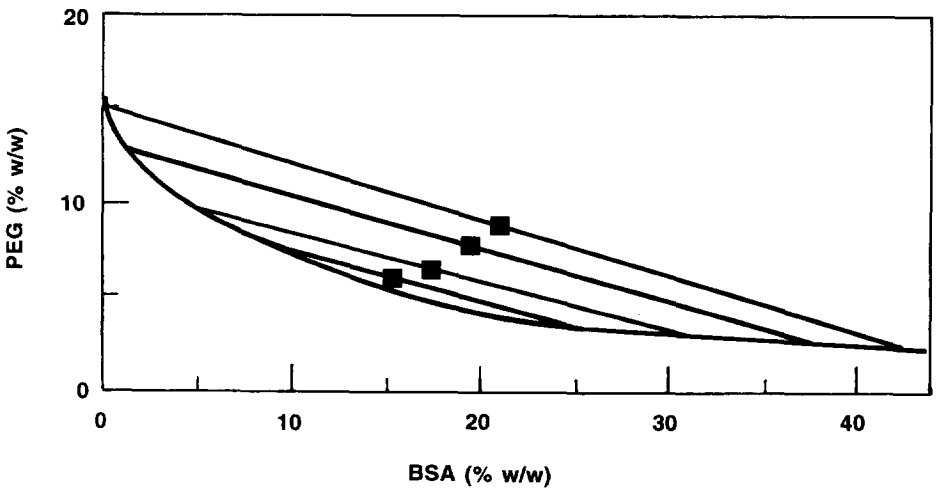

Fig. 2. Phase diagram for the system PEG (M_r 20 000)–BSA–NaSCN (0.1 M)–100 mM sodium phosphate buffer (pH 7.5) in water. (■) Total composition of systems. Temperature, 23°C.

2). K_{LDH} values in BSA–PVA–NaSCN (100 mM) systems are given in Table 3. To compare the values between systems with and without sodium phosphate buffer (100 mM, pH 7.5), the logarithm of the partition coefficient, log K_{LDH}, was plotted versus log K_{BSA} (Fig. 5). With both

Table 1
Effect of PVA, BSA and NaSCN, present in the assay solution, on the enzyme activity of lactate dehydrogenase at 23°C

Addition	Relative enzyme activity (%)
None	100 ± 2
9.7% (w/w) PVA	75 ± 6
100 mM NaSCN	176 ± 14
1.0% (w/w) BSA	109 ± 3
2.0% (w/w) BSA	118 ± 2
8.8% (w/w) PVA and 94 mM NaSCN	113 ± 4
1.0% (w/w) BSA and 100 mM NaSCN	193 ± 5
2.0% (w/w) BSA and 100 mM NaSCN	193 ± 4
9.7% (w/w) PVA, 1.0% (w/w) BSA and 100 mM NaSCN	107 ± 4
9.7% (w/w) PVA, 1.45% (w/w) BSA and 100 mM NaSCN	120 ± 7
9.7% (w/w) PVA, 1.94% (w/w) BSA and 100 mM NaSCN	71 ± 3

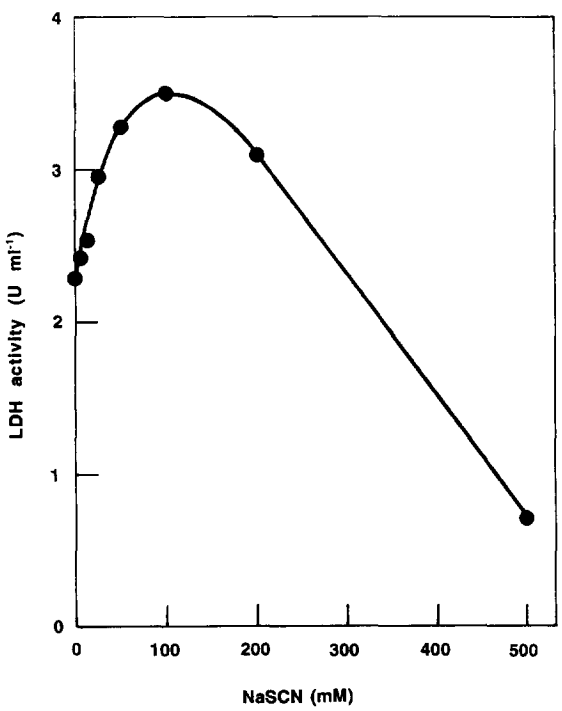

Fig. 3. Enzyme activity of lactate dehydrogenase (constant concentration) as a function of the concentration of NaSCN in the assay medium. Temperature, 23°C.

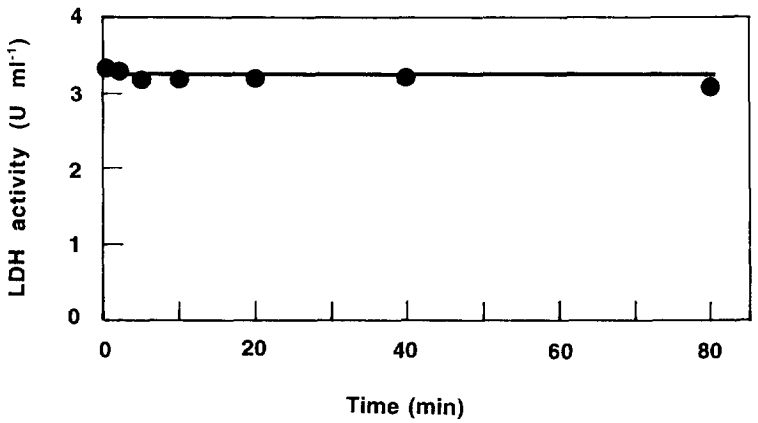

Fig. 4. Enzyme activity of lactate dehydrogenase (constant concentration) as a function of time when incubated in assay medium containing 100 m*M* NaSCN but no substrate (pyruvate). Temperature, 23°C.

Table 2
Time dependence of the partition coefficient, K_{LDH}, of LDH (6 u/ml) in a system containing 5.5% (w/w) BSA, 8.3% (w/w) PVA, 0.1 *M* NaSCN and 0.1 *M* sodium phosphate buffer (pH 7.5), at 23°C

Time (h)	K_{LDH}
0	0.093
0.5	0.050
1	0.046
2	0.046

Table 3
Partition coefficients, K_{LDH}, of lactate dehydrogenase (6 u/ml added) in systems containing various concentrations of BSA and PVA

System		Na phosphate buffer (m*M*)	K_{LDH}
BSA (%, w/w)	PVA (%, w/w)		
9.5	5.0	None	0.173
9.5	5.5	None	0.111
9.5	6.0	None	0.066
10.0	6.2	None	0.044
7.0	5.0	100	0.196
7.0	5.4	100	0.133
7.0	5.8	100	0.105
7.0	6.3	100	0.072
5.5	8.3	100	0.046

The two-phase systems contained PVA, BSA, 0.1 *M* NaSCN and either 0 or 100 m*M* sodium phosphate buffer (pH 7.5) at 23°C

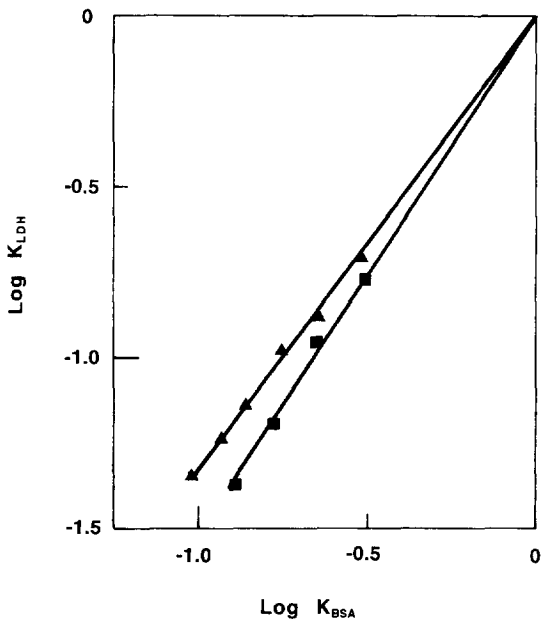

Fig. 5. Log K_{LDH} (data from Table 2) as a function of log K_{BSA} calculated from values in Fig. 1 and the densities of the phases. The two-phase systems contained (■) 0 or (▲) 100 m*M* sodium phosphate, together with PVA, BSA and 0.1 *M* NaSCN. Temperature, 23°C.

kinds of systems, with and without phosphate, a linear relationship was found between the two logarithmic *K* values. The systems without phosphate gave a more extreme affinity of the enzyme for the lower phase compared with the

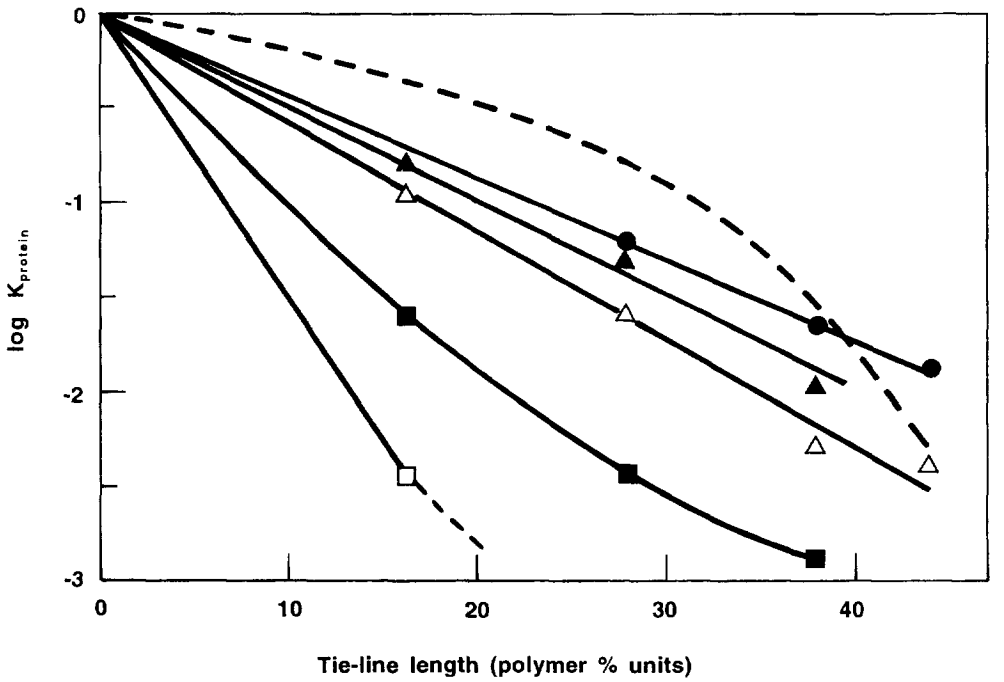

Fig. 6. Logarithm of partition coefficient, log $K_{protein}$, (●) cytochrome *c*, (□) fumarase; (■) GDH; (▲) GOT and (△) m-MDH, as function of the length of the tie-lines, taken from Fig. 2, of the systems and expressed as a percentage of polymer concentration. Broken line indicates log K_{BSA}. The two-phase systems contained PEG (M_r 20 000), BSA, 0.1 *M* NaSCN and 5 m*M* sodium phosphate buffer (pH 7.5). Temperature, 23°C.

system with phosphate. The ratio log K_{LDH}/log K_{BSA} was calculated to be 1.53 in the absence and 1.33 in the presence of phosphate. To obtain conditions such that 99.9% of the enzyme is in the BSA phase (at a volume ratio of 1) and if the above relationships hold outside the investigated range, a system with log $K_{BSA} = 0.01$ would be needed.

In the BSA–PEG–NaSCN (100 m*M*) system, a number of mitochondrial enzymes in addition to cytochrome *c* were partitioned (Fig. 6). Very low partition coefficients could be obtained. In the case of fumarase, some values were lower than 0.001. The log *K* values for these proteins were not linearly related to log K_{BSA}. Instead, the log *K* values gave fairly linear plots for several proteins versus the length of the tie-lines of the two-phase systems. With this kind of system it is possible to localize at least some enzymes to more than 99.9% in the lower phase (at equal phase volumes).

3.4. Enzyme reactor model

The two-phase systems with extreme partitioning of enzyme to the lower phase could be useful as the operating part of an enzyme reactor as depicted in Fig. 7. The upper phase is entered from the bottom of a column, filled with the same phase, but also containing a suspension of protein phase, containing the enzyme. By mixing in the lower part of the column the phase droplets are kept small and therefore the interface will be large and the mass transport favourable. The incoming top phase contains substrate for the enzyme which is transformed into a product when it enters the bottom phase droplets. Substrates, coenzymes and products of the enzymatic reaction partition relatively equally between the phases. The product diffuses back to the mobile phase and leaves the column with this phase.

This kind of enzyme reactor can be used as a

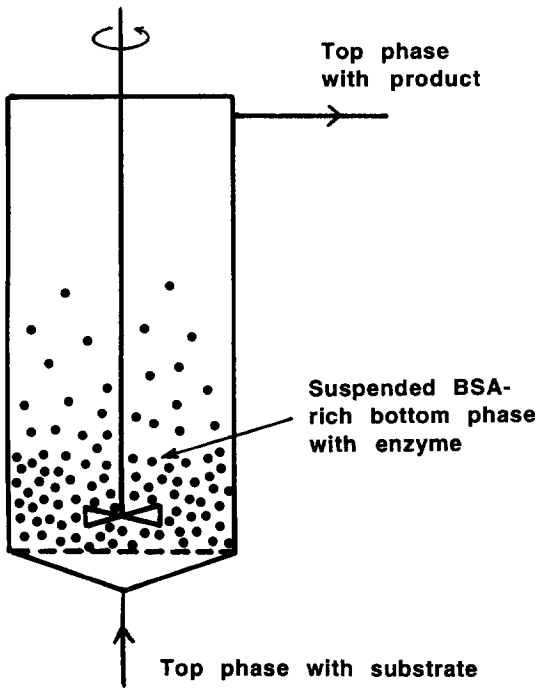

Fig. 7. Model of an enzyme reactor based on the BSA-PEG-NaSCN system.

model for the living cell. By using a protein phase, the natural environment of the enzymes can be mimicked and the function of enzymes and enzyme systems in concentrated form surrounded by another protein could be studied.

Acknowledgement

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