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Aqueous two-phase systems with increased density for partition of heavy particles

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ABSTRACT

The density of aqueous two-phase systems obtained from water, dextran and poly(ethylene glycol) (PEG) was increased by addition of metrizamide, which had a greater affinity for the upper (PEG-rich) phase than for the lower (dextran-rich) phase. The density of both phases could be increased to 1.2 g ml^{-1}. Above certain concentrations of metrizamide the dextran phase became the lighter one. Isopycnic phases were obtained by careful adjustment of the composition of the system, and the effect of metrizamide on phase composition was characterized. The systems were tested by partition of starch grains. Fractionation of mature pollen grains and starch grains was achieved by a counter-current distribution process using these systems.

INTRODUCTION

Fractionation of particles of biological origin can in many instances be achieved by partitioning between the two phases of aqueous two-phase systems [1–3]. Multi-step partition, *e.g.*, counter-current distribution (CCD) according to Craig [4], has been used for high resolution. When large and dense particles are to be studied, sedimentation might interfere with the partition. The possibility of increasing the density of both phases by addition of a "density maker", metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose], normally used for medical radiology and density gradients [5], was therefore investigated as a means of reducing undesirable sedimentation in dextran–poly(ethylene glycol)–water two-phase systems.

EXPERIMENTAL

Chemicals and materials

Dextran T-500 (MW = 500 000) and Sephacryl-300 were obtained from Pharmacia (Uppsala, Sweden), poly(ethylene glycol) (MW = 3400) from BP Chemicals (Hythe, U.K.) and metrizamide (centrifugation grade) from Nyegaard (Oslo, Norway). ^{32}P-labelled sodium phosphate was obtained from Amersham (Amersham, U.K.).

Mature pollen grains were harvested from *Hordeum vulgare* grown in a green-

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house. Starch grains were prepared from potato tubers by homogenization in 25 m*M* sodium phosphate buffer (pH 7.5) and washing by sedimentation mainly as described by Ohad *et al.* [6].

Density

The densities of the phases were determined by using a 5-ml pycnometer, weighed on an analytical balance.

Sedimentation rate

The relative rate of sedimentation of starch grains in various liquid media was determined by following the change in light scattering with time when the particles were sedimenting in a 3-ml cuvette. The apparent absorbance was followed by using a potentiometric recorder attached to the photometer (Hitachi 100-60). The time for sedimentation was taken from when the content of the cuvette had just been mixed to the inflection point detected by the recorder.

Two-phase systems

Systems with a total weight of 3 g were prepared from concentrated polymer solutions [20% dextran and 40% poly(ethylene glycol) (PEG)]. All systems contained 25% metrizamide. After temperature equilibration the phases were analysed for the content of metrizamide (by absorbance measurements at 240 nm). The concentrations of PEG and dextran in the two phases were determined by gel filtration on a column of Sephacryl-300 (25 × 1.5 cm I.D.) and the eluate was monitored with an Optilab (Vällingby, Sweden) Multiref 902B refractometer. The partition of phosphate was determined by including 0.33 μCi of $^{32}PO_4^{3-}$ per gram of the system. Samples (20 μl) were mixed with 8 ml of scintillation cocktail (Ready Safe; Beckman, Fullerton, CA, U.S.A.) and counted in a Beckman LS1801 scintillator. The concentration of pollen or starch grains in the CCD fractions was determined by measuring the light scattering as the apparent absorbance at 600 nm. Each fraction was diluted with 0.5 ml of water to break the phases and 300-μl (pollen) or 200-μl (starch grains) portions were analysed for the apparent absorbance with an ELISA spectrophotometer.

Two-phase systems with and without metrizamide were used to partition starch grains prepared as described above (5 g kg^{-1} system, dried for 1 min on filter-paper) at various pH values. The systems without metrizamide were composed of 8% dextran, 7% PEG and 0.3 mol kg^{-1} potassium phosphate buffer. The metrizamide-containing systems were composed of 5% dextran, 3% PEG, 25% metrizamide and 0.3 mol kg^{-1} potassium phosphate buffer. Material in the top and bottom phases was determined after 30 min of settling as described above by taking 0.5-ml aliquots from each phase and diluting them with 0.4 ml of water prior to analysis.

Counter-current distribution

The composition of the two-phase systems was either 2.5% dextran, 2.5% PEG, 25% metrizamide and 0.3 mol kg^{-1} K_2HPO_4 (for pollen) or 5% dextran, 3% PEG, 25% metrizamide and 0.3 mol kg^{-1} potassium phosphate buffer (pH 6.8) (for starch grains). The ratio between the volumes of the upper and lower phases was 0.72 at 21 ± 0.5°C (for the pollen experiment) and 0.90 at 23 ± 0.5°C (for the starch grains experiment). Each chamber of the thin-layer CCD apparatus [7] contained 0.90 ml of

bottom phase and 0.65 ml of top phase (pollen) or 0.75 ml of bottom phase and 0.68 ml of top phase (starch grains). A volume of 0.79 ml of the system was stationary and material collecting at the interface including 0.11 ml of bottom phase was therefore transferred together with the upper phase in the case of pollen or stationary in the case of starch grains. Pollen grains (50 mg) or starch grains (100 mg, dried for 1 min on filter-paper) were loaded in the first two chambers (Nos. 0 and 1) and 24 transfers (pollen) or 22 transfers (starch grains) were carried out by mixing for 20 s (pollen) or 1 min (starch) and settling for 8 min (pollen) or 5 min (starch). Theoretical curves and partition ratios, $G = \hat{\imath}/(55 - \hat{\imath})$ where $\hat{\imath}$ is the peak position, were calculated according to Craig [4].

RESULTS AND DISCUSSION

The mean density of the two-phase systems in Table I was increased from 1.03 to 1.20 g ml^{-1} by including metrizamide at up to 25% of the total weight of the system. Higher concentrations gave a strongly viscous dextran phase, making the systems less suitable for extraction. The difference in the densities between the two phases was similar, however, *i.e.*, <0.05 g ml^{-1}, in systems with and without metrizamide.

The phase diagram for a dextran–PEG–metrizamide–K_2HPO_4 system is shown in Fig. 1. The tie-line of one two-phase system is shown where the polymer content of the phases was determined. The inclusion of metrizamide made the PEG-rich phase denser than the dextran-rich phase. This was caused by the higher concentration of metrizamide in the former phase, probably owing to direct PEG–metrizamide interaction. The percentages of metrizamide, dextran and PEG are given in Table I.

Effect of K_2HPO_4 on the phase system

K_2HPO_4 strongly affected the composition of the phases and their volume ratio. Also, the concentration of polymers necessary to produce two phases at a given temperature varied drastically. This can be clearly seen in Table II, which shows

TABLE I

VOLUME RATIOS, DENSITIES OF PHASES AND THE PERCENTAGES OF METRIZAMIDE (MA), DEXTRAN (DX) AND PEG IN THE PHASES OF SYSTEMS WITH DIFFERENT CONCENTRATIONS OF THE TWO POLYMERS

The total concentation of metrizamide was 25% (w/w) in all systems. Temperature, 20°C.

Total composition		Volume ratio (top/bottom)	Density (g ml^{-1})		Phase composition					
					Top phase			Bottom phase		
DX (%)	PEG (%)		Top	Bottom	DX (%)	PEG (%)	MA (%)	DX (%)	PEG (%)	MA (%)
2.0	3.0	0.25	1.16	1.19	N.D.[a]	N.D.	19	N.D.	N.D.	29
3.0	2.5	0.72	1.18	1.23	6.9	0.3	20	<0.1	3.9	32
3.5	3.0	0.58	1.18	1.21	10.8	0.2	17	<0.1	5.0	32
4.5	2.0	1.20	1.20	1.22	9.2	0.2	20	<0.1	4.0	34

[a] N.D.: not determined.

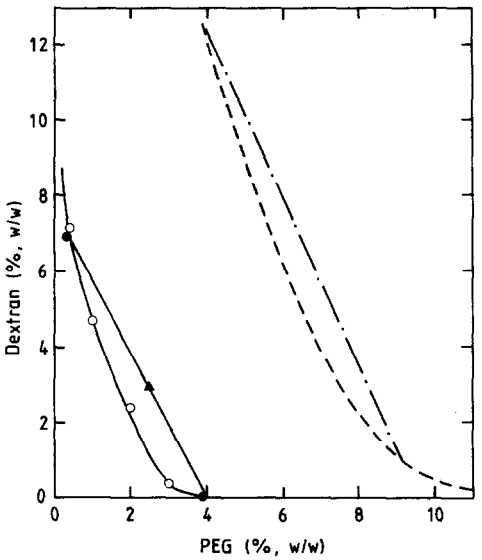

Fig. 1. Phase diagram for a dextran–PEG–metrizamide–K_2HPO_4 system at 20°C. The concentration of metrizamide was 25% (w/w) and that of the phosphate 0.3 mol kg^{-1} of system. ○, Points on the binodial curve obtained by turbidimetric titration. The total composition (▲) and the compositions of the phases (●) of one of the systems in Table I are shown. For comparison, the binodial curve (– – –) for a dextran–PEG system (from ref. 1) at the same temperature is given together with a tie-line (–·–).

the critical temperatures for the phase transition (from two to one phase) of a metrizamide-containing system, depending on the content of phosphate. The critical temperature was increased by as much as 30°C when the phosphate concentration was changed from 0.27 to 0.30 *M*. Also, the volume ratio was strongly dependent on the phosphate concentration and temperature (Table III). Inclusion of 4% (w/w) of sucrose in the system had no effect on either the phase transition or the volume ratio.

TABLE II

EFFECT OF PHOSPHATE ON THE CRITICAL TEMPERATURE OF PHASE TRANSITION (DIM POINT) FOR A SYSTEM CONTAINING 5% DEXTRAN, 2% PEG, 25% METRIZAMIDE AND VARIOUS AMOUNTS OF K_2HPO_4

Content of K_2HPO_4 (mol kg^{-1})	Critical temperature (°C)
0.27	2
0.28	14
0.29	29
0.30	33

TABLE III

EFFECTS OF PHOSPHATE AND TEMPERATURE ON THE VOLUME RATIO

Systems as in Table II.

Content of K_2HPO_4 (mol kg^{-1})	Temperature (°C)	Volume ratio (top/bottom)
0.28	0	1.4
	5	1.8
	10	2.7
	20	One phase
0.29	0	1.2
	5	1.4
	10	1.7
	20	3.0
0.30	0	1.0
	5	1.2
	10	1.4
	20	2.4

Isopycnic phases

A system composed of 7% dextran, 5% PEG, 25% metrizamide and 0.3 mmol kg^{-1} K_2HPO_4 at 20°C was found to have phases with equal densities. On decreasing the temperature to 0°C the dextran-rich phase became the denser one. An increased concentration of the phosphate, on the other hand, caused the PEG-containing phase to be the denser one at room temperature.

Sedimentation of starch grains

The sedimentation rate of dense particles was reduced within the systems containing metrizamide because of the increased density and also owing to a higher viscosity. The sedimentation rate of starch grains from potato tubers (60 μm diameter, 1.4 g ml^{-1}) was reduced by a factor of six when water was replaced with polymers (5% dextran and 2% PEG, which gives one phase at 20°C). When 25% of metrizamide was also included the sedimentation rate was only 2% of that in water. This strong decrease in the rate indicates that there is also an increase in the viscosity.

Partition of starch grains

The pH dependence of the partition of starch grains was studied in two-phase systems with and without metrizamide. Wihout metrizamide the apparent partition was not altered in the pH range 2.3–7.9. All material was found in the lower phase. However, this does not reflect a true solvating-directed partition as the sedimentation rate for the particles through the upper phase is too high. By including metrizamide (25%) and reducing the concentrations of polymer to around half, the starch grains partitioned between the two phases (and the interface) and their distribution could easily be adjusted by changing the pH of the system (Fig. 2). The starch grains partitioned mainly between the interface and one of the phases, which is the usual

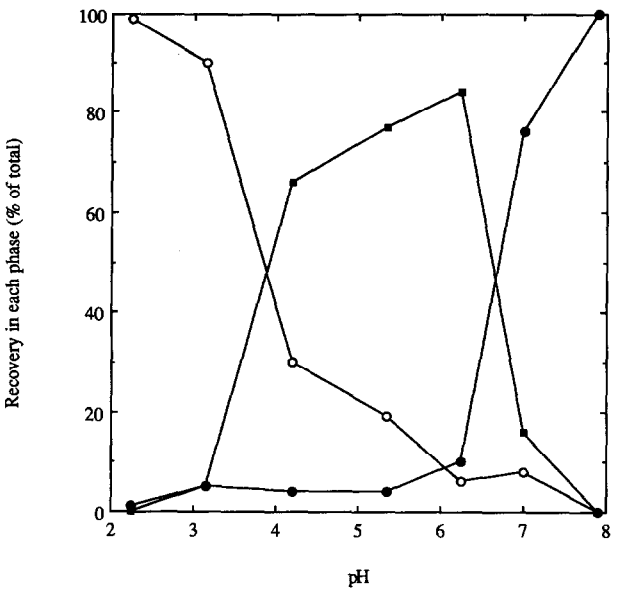

Fig. 2. Starch grains (5 g kg^{-1} of system) partitioned in metrizamide-containing two-phase systems at different pH values at 23°C. The systems were composed of 5% dextran, 3% PEG, 25% metrizamide and 0.3 mol potassium phosphate buffer per kg system. The amount of starch grains in the phases was determined via the apparent light scattering in diluted samples (see Experimental). Percentage of starch grains (●) in top phase, (■) at the interface and (○) in the bottom phase.

partitioning behaviour for particles [1,2]. In the present metrizamide-containing systems the dextran-rich phase was the upper one (checked by using small amounts of dye-labelled dextran and PEG). On increasing the pH the phosphate ion is increasingly charged, and this is known to influence the partition of negatively charged particles and proteins in favour for the PEG-rich upper phase in ordinary dextran-PEG systems [1,8]. However, in the metrizamide-containing systems (Fig. 2), the (negatively charged) starch grains show the opposite behaviour and they are more extracted into the PEG-rich phase with increasing pH.

On increasing the pH the distribution of potassium phosphate showed a small but distinct change in favour of the dextran-rich phase (Table IV). This is similar to what has been observed for the ordinary PEG-dextran systems [8]. The partition

TABLE IV

PARTITION COEFFICIENT, K, OF POTASSIUM PHOSPHATE BUFFER, LABELLED WITH ^{32}P, AT VARIOUS pH VALUES IN THE SAME SYSTEM AS IN FIG. 2 TOGETHER WITH THE VOLUME RATIO BETWEEN THE PHASES (TOP/BOTTOM)

pH	Volume ratio	$K_{phosphate}$
2.25	1.32	1.18
4.20	1.12	1.44
6.25	0.95	1.44
7.90	0.81	1.80

coefficient of the potassium phosphate is the mean of the hypothetical partition coefficients the ions should have had if they could partition independently of the electrostatic forces [9]. The inversion of the "phosphate effect" on the partition of particles indicates that potassium ions have a greater affinity for the dextran phase than the phosphate ions have, thereby reversing (compared with the metrizamide-free systems) the polarity of the electrical double layer at the interface. The change in pH, by varying the relative amounts of primary and secondary phosphate, affected the volume ratio (top/bottom) of the systems containing metrizamide, the volume ratio decreasing with increasing pH (Table IV). This variation shows that a redistribution of the components takes place, making the PEG-rich phase larger but also less attractive for phosphate and especially potassium ions relative to the dextran-rich phase. No variation in the volume ratio (= 1.40) was observed in normal systems.

Counter-current distributions

The metrizamide-containing systems were tested by performing CCDs with pollen grains and starch particles.

Fresh mature pollen grains (diameter 50 μm; density 1.2–1.3 g ml^{-1}) from *Hordeum vulgare* (barley) were showed to be heterogeneous when analysed in a thin-layer CCD apparatus [1,2]. As can be seen in Fig. 3, at least one major and three minor peaks can be fitted to the experimental data. Redistribution of material from fractions 2 and 10–25 (pooled) gave G values of 0.05 and 4, respectively. This

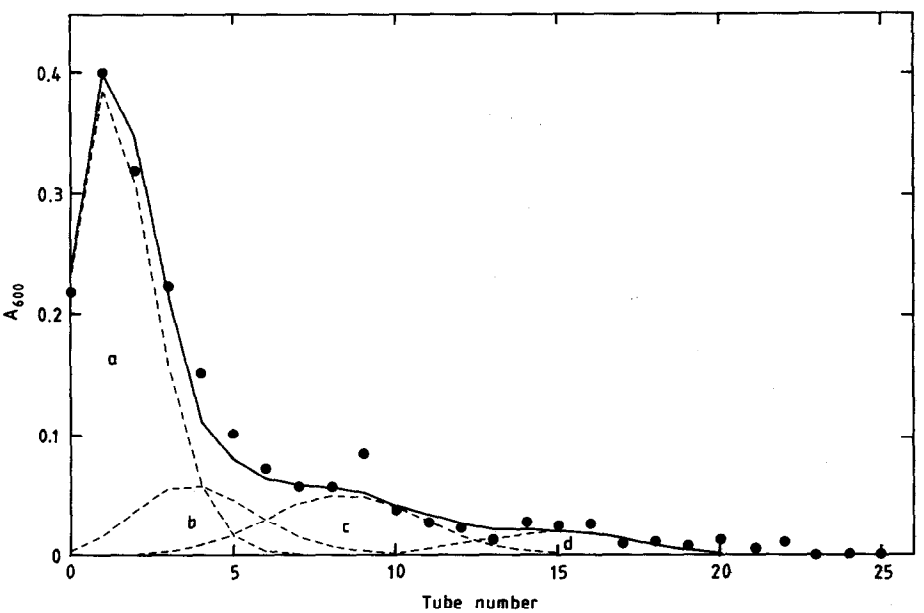

Fig. 3. Thin-layer CCD of mature pollen from *Hordeum vulgare*. The systems were composed of 2.5% dextran, 2.5% PEG, 25% metrizamide and 0.3 mol K_2HPO_4 per kg system. Experimental data are given under Experimental. The interface material was mobile. Theoretical curves for homogeneous substances (– – –) and their sum (———) are fitted to the experimental data (●). The G values for the theoretical curves are (a) 0.07, (b) 0.20, (c) 0.55 and (d) 1.63.

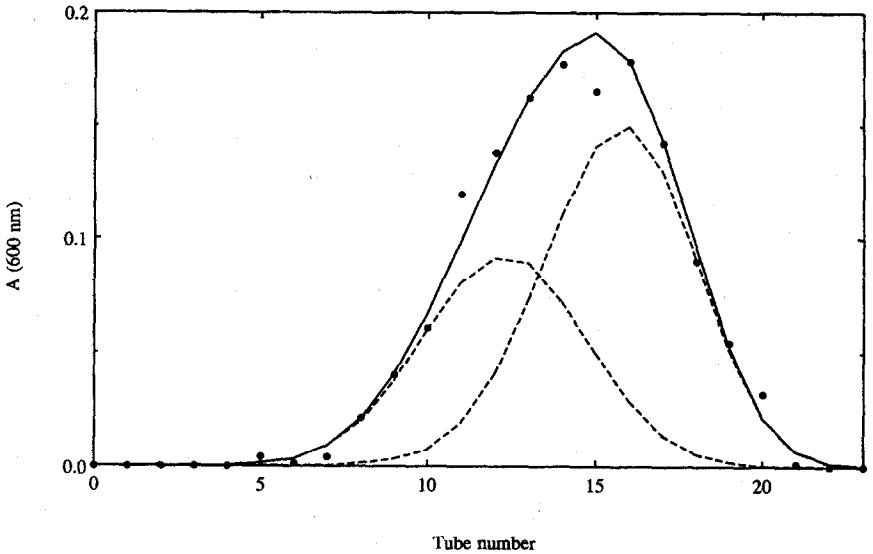

Fig. 4. Thin-layer CCD of starch grains from potatoes at pH 6.8. The composition of the two-phase system was the same as in Fig. 2. The amount of starch grains in the fractions was determined via light scattering measured as the apparent absorbance at 600 nm as described under Experimental. The material at the interface was stationary in this instance. (●) Experimental values; (– – –) theoretical curves for homogeneous substances with G values of 1.15 and 2.20, respectively, and (———) their sum.

demonstrates that the pollen grain population consists of several subfractions differing in the surface properties of the grains. No morphological differences between the fractions could be detected in the light microscope after staining with orcein [10].

Another CCD was performed by using the system described above for the partition of starch grains at pH 6.8 (Fig. 4). Starch grains show a slightly higher density (1.4 g ml^{-1}) than the pollen grains but have approximately the same diameter (60 μm). The starch grains gave rise to a much broader peak than expected for homogeneous particles, which might be due to various charge densities of the negative groups on the surface of the grains. No difference in the size of the starch grains could be observed when the different fractions were studied under the light microscope.

The aqueous two-phase systems with increased density of the two phases will be useful for the partition of various dense hydrophilic particles, both for fractionation and for studies of their surface properties. Examples of particles of interest, in addition to pollen grains and starch, are fish eggs, plant seeds, air-pollutant particles and minerals.

CONCLUSIONS

By addition of metrizamide "density maker", the density (and viscosity) of aqueous dextran–PEG two-phase system can be adjusted so as to reduce strongly the sedimentation of biological particles when they are partitioned between the phases. Metrizamide, however, affects the phase formation, the relative positions of the upper and lower phases and the partition-modifying properties of salts.

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