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Studies on aqueous polymer two-phase systems containing agarose

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Methods for high temperature preparation and separation of aqueous polymer two-phase systems containing agarose have been developed. A number of water-soluble polymers have been screened for polymer incompatibility and two-phase formation together with agarose. A method for analysis of the polymer phase composition based on chromatography at 70°C on Superose 12®, a highly cross-linked, 10 μm diameter agarose gel filtration medium, is presented. Phase diagrams for three agarose-poly(ethylene glycol) (PEG) and three agarose–dextran two-phase systems are described.

INTRODUCTION

The incompatibility of aqueous polymer solutions containing three or more components is a well known phenomenon. The polymers in these solutions can be of both natural and synthetic origin. Ternary two-phase systems containing one or two polymers and a low molecular weight solute, such as an inorganic salt, in addition to water, have been subject to comprehensive studies, with the dextran–poly(ethylene glycol) (PEG) system (Walter *et al.*, 1985; Albertsson, 1986) probably being the most thorougly studied example. Marginal differences in the chemical structures of the two polymers is sufficient to cause incompatibility, governed by repulsive forces between the monomeric polymer building blocks (Brooks *et al.*, 1985; Gustafsson *et al.*, 1986; Abbott *et al.*, 1990). Phase separation has been demonstrated for moderately high concentrations of polysaccharide pairs as closely related as amylose–dextran (Kalichevsky *et al.*, 1986) and amylose–amylopectin (Kalichevsky & Ring, 1987), respectively.

Agarose is a linear galactan with a low degree of substitution with sulphate, pyruvate, and methoxy groups obtained by fractionation of agar extracted from red seaweeds (Duckworth & Yaphe, 1971). Due to its ability to spontaneously form rigid macroporous gels upon cooling of aqueous solutions, agarose has found widespread use as a matrix material for electrophoretic analysis and preparative chromatography of biological macromolecules such as proteins and nucleic acids (Hjertén, 1961, 1962, 1964).

The present work was initiated with the aim of studying the effect of different water-soluble polymers and other solutes on the gel-forming properties of commercial agarose preparations. As a first step in this study, phase separations between agarose and other polymers in solution at high temperatures have been investigated. Preliminary experiments had shown that phase separation occurred after the addition of relatively low concentrations of certain polymers and was apparently irrespective of temperatures in the range 50–95°C. This encouraged a more detailed study of the compositions of selected agarose–polymer–water two-phase systems, a task which required the development of techniques for automated high-temperature gel filtration chromatographic analysis of the polymers in the separated phases. We now report on the design of this chromatographic analysis system and also on the phase diagrams obtained with agarose in mixtures with PEG and dextran, respectively.

Mixtures of gel-forming polymer solutions have previously been studied by other investigators: agarose and β-1,4-glycans (Dea & Rees, 1987), agarose/κ-carrageenan and gelatin (Watase & Nishinari, 1980),

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amylose–dextran (Kalichevsky *et al.*, 1986), and amylose–amylopectin (Kalichevsky & Ring, 1987). The latter two studies and that of Watase and Nishinari, reported phase separation.

MATERIALS AND METHODS

Materials

A single commercial preparation of agarose (Agarose Seakem CB, Lot No. 60784, FMC BioProducts Inc. (formerly Marine Colloids Inc.), Rockland, Maine, USA) was used during the entire investigation period. This preparation possessed the following characteristics (manufacturer's data): Moisture 8·5 (%w/w), sulphate 0·06 (%w/w), pyruvate 0·12 (%w/w), ash 0·6 (%w/w), gel strength 1060 (g/cm^2), and electroendosmosis 0·10. The weight average molecular weight was estimated as 120 000 from the intrinsic viscosity obtained using a Ubbelhode-viscometer at 80°C and adopting the Mark-Houwink equation for agarose reported by Rochas and Lahaye (1989).

PEG with molecular weights: 1000, 6000, 20 000, and 35 000 were obtained from KEBO LAB, Stockholm, Sweden. Dextran T-70, Dextran T-40, molecular weights 70 000 and 40 000, and a special dextran fraction with a weight average molecular weight of approximately 25 000 (here denoted Dextran X-25), were obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

The water used throughout this investigation was deionized and further purified by reversed osmosis (RO) (ELGA Ltd). In the following text this is referred to as RO-water.

Preparation of agarose-containing two-phase systems

Agarose, the second polymer and RO-water were weighed, to an accuracy of a tenth of a mg, in a DURAN culture tube (12 × 100 mm), fitted with a melamine screw cap and the PTFE-protected seal. The polymers were suspended in water by shaking and mixing on a vortex mixer until a homogenous suspension was obtained. The polymers were dissolved by autoclaving. The pressure was allowed to increase to 1·4 bars (121°C) in 10 min. The pressure was held at this value for another 10 min, whereafter the pressure was allowed to decrease to ambient within 10 min. The tube was inspected for leakage and the contents for complete dissolution to make sure that no refractive particles or lumps of aggregated agarose could be observed. The two-phase system was equilibrated at the desired temperature using an end-over-end mixer, mounted in a thermostated cabinet, for 2·5 or 5 h depending on the temperature. Separation of the mixture into two clear phases was achieved by centrifugation in an angle rotor, mounted into a thermostated cabinet, at 2500 *g* (5700 rpm, radius at the interfacial boundary), at equilibrium temperature ±2°C. Centrifugation was continued until two clear phases were separated, typically this required 6–24 h depending on the type of second polymer, temperature and polymer concentrations.

Determination of phase volumes

After centrifugation, the tube was held in a vertical position at equilibration temperature until the interfacial boundary became horizontal. The total volume and the interfacial boundary were marked on the tube with a diamond tip using special apparatus built for this purpose. The tube was placed in a vertical tube-holder with a vertical slit through which the diamond tip horizontally could reach the glass surface of the tube. The diamond tip could be adjusted in the vertical position and fixed at the interfacial boundary or at the total volume meniscus. By gently turning the tube in the holder, a thin mark was created that circles the tube. The accuracy of this engraving is better than ±0·25 mm, which corresponds to 2% of the phase volume.

The phase system was allowed to cool to room temperature. Since agarose is included in each system, both phases gelled upon cooling. The gelled phases were carefully removed separately from the tube and saved for analysis of polymer content. This procedure was facilitated by the fact that the lower phase often contains a considerably higher agarose concentration. The empty tube was carefully washed with RO-water and dried at 80°C. The phase volumes were determined, after equilibrium to room temperature (20–23°C), by filling the tubes with ambient temperature RO-water, firstly to the phase boundary mark and secondly to the mark for the total volume meniscus. After each addition of water the tubes were weighed. The phase volumes were obtained by using the value of 1·00 g/ml (21°C) for the density of water.

For the accuracy of these volume determinations, including all steps in this process, an error of less than 5% can be expected.

Phase composition analysis

The chromatographic system

The compositions of the separated phases were analysed by gel filtration chromatography at 70°C on a Superose® 12 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The chromatographic system was a modified FPLC system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (Fig. 1), consisting of: an eluant container, a degasser (Erma optical, ERC 3310), a

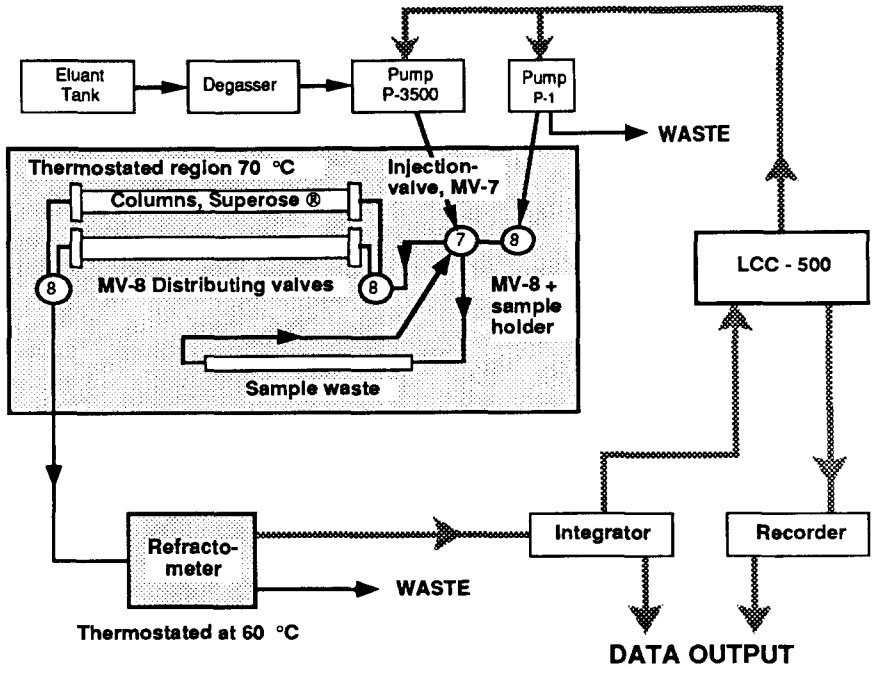

Fig. 1. System for size exclusion chromatographic analysis of polymers at elevated temperatures.

piston pump (Pharmacia P-3500), a sample holder (Pharmacia MV-8), an empty column tube for sample waste (Pharmacia HR-16/30), an injection valve (Pharmacia MV-7), two distributing valves (Pharmacia MV-8), a chromatographic column (Pharmacia HR-16/50), a refractometric detector (LDC Refractomonitor III), a recorder (Pharmacia Rec 482), a liquid chromatography controller (Pharmacia LCC-500) and an integrator (Shimadzu C-R3A). Valves, samples and chromatographic columns were thermostated at 70°C in a incubator water bath (Memmert). The refractometric detector was thermostated separately at 60°C using a circular bath (Haake D-8L).

The use of standards

In this study the internal standardization technique has been utilized, in which a known quantity of a substance, similar to the analytes in the sample but distinguishable on analysis, is added to the sample. The advantage of this technique is that no control of sample volume is needed, and that only the proportion between the solute of interest and the internal standard need to be determined. These proportions are then standardized by the use of external standards. These standards are prepared in a concentration series that cover the range of concentrations for the analyte, or at concentrations similar to the concentration of the analytes in the samples. These standards are treated in the same way as the sample including the addition of the internal standard, thus giving the standardized proportion.

The correlation between polymer concentration and area response from the refractometric readings deviated from linearity since the performance of the column changed somewhat over a series of a 300–400 chromatographic runs. The column life span in this application, agarose analysed on an agarose column matrix at 70°C, is limited to about 400 runs. Therefore several columns had to be used during the investigation and each measured point was standardized separately. The analysis of the polymer content in the external standards was performed immediately after the analysis of the polymer contents in each phase.

Sample preparation

The gelled phases were removed from the test tubes one by one and analysed separately. An entire phase was dissolved in RO-water in a screw capped bottle by heating on a boiling water bath until no refractive particles could be observed. The sample was kept on the boiling water bath for an additional 10 min to ensure complete dissolution. To each sample, a small volume of an internal standard containing low molecular weight poly(ethylene glycol) P(LMW-PEG), PEG 1000, was accurately added with a dispenser (Gilson D-5000). The solution was kept in a thermostated cabinet at 80°C for up to 15 min prior to chromatography.

Preparation of external standard

The external standard was prepared to give the same concentrations of polymers as in the sample. The contents were dissolved in RO-water in a screw capped bottle by heating in a boiling water bath until no refractive particles could be observed, usually after 10 min of agitation. The standards were kept on the boiling water bath for an additional 10 min to ensure complete dissolution. The standards were prepared

parallel to the samples and treated the same way, including addition of LMW-PEG internal standard.

Chromatography

The polymer concentrations of each phase were determined after separation by analytical gel filtration on Superose® 12 packed in Pharmacia HR 16/50 columns to a bed height of 500 mm. The concentration of each polymer was determined by integration of the separated peaks. A typical chromatography was performed at 100 ml/h (50 cm/h) corresponding to a running time of 1 h and is shown in Fig. 2. The back pressures were normally in the range 1–1·5 MPa. The column life length was typically 300–400 chromatographic cycles.

Calculations

The amount of polymer in each peak was determined according to the internal standard method for every sample and the corresponding standard. The samples and standards were chromatographed five times each and the results were averaged. The results for the amount of analytes, agarose and PEG or dextran in the analysed phase samples, were corrected by use of the standards. The concentrations of agarose and PEG or dextran in the upper and lower phase were calculated from the amount of polymers in each phase and the phase volumes, respectively. These data were used to prepare the phase diagram.

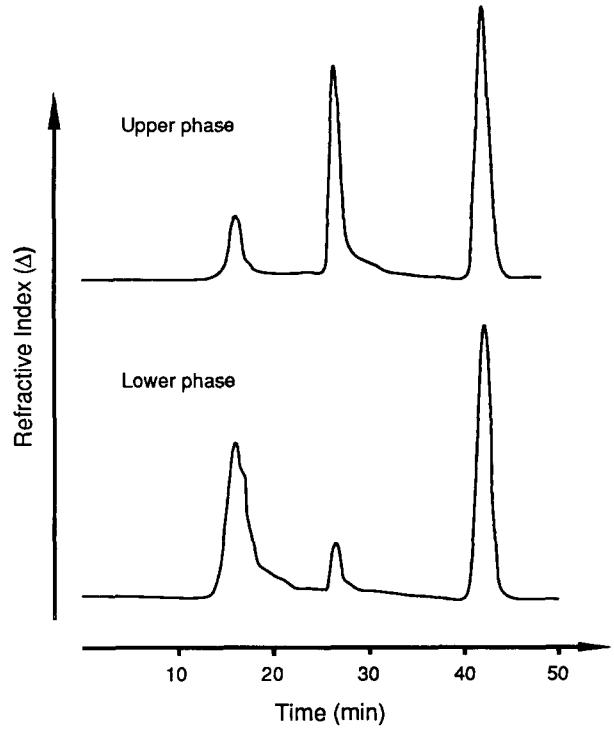

Fig. 2. Chromatogram for the top (a) and bottom (b) phases of an agarose–PEG 20 000 (3·2; 2·6 %w/w) system. Peaks in order left to right: Agarose, PEG 20 000, and PEG 1000.

RESULTS AND DISCUSSION

The main polymer properties affecting the formation of an aqueous two-phase polymer system with transparent, non-turbid phases separated by a distinct interfacial boundary, are low chemical heterogeneity, high purity, good solubility and a relatively narrow molecular weight distribution. Table 1 lists those commercially available polymers that were investigated for incompatibility with agarose in aqueous solution. Of these, one synthetic polymer, PEG, and one polysaccharide polymer, dextran, were chosen for a more detailed investigation of their phase diagrams because of their low polydispersity. They also gave rise to phase separation with agarose at low total polymer concentrations.

A common way to establish the phase-diagram for an aqueous two-phase system is to add small quantities of solution I containing one of the polymers to solution II containing the other polymer. When the mixed solution becomes 'cloudy', turbid, or opalescent, a 'cloud-point' is noted and an amount of the aqueous solvent is added so that the solution becomes transparent again. The

Table 1. Polymers investigated for incompatibility with agarose in aqueous solutions at 80°C[a]

poly(ethylene glycol) (PEG)		*poly(sucrose)*	
PEG 6000	+	Ficoll 70	+
PEG 20 000	+	Ficoll 400	+
PEG 35 000	+	*poly(1-6 glucan)*	
poly(vinyl pyrrolidon) (PVP)		Dextran T10	−/+
PVP K25	+	Dextran X25	+
PVP K30	+	Dextran T40	+
PVP K60	+	Dextran T70	+
PVP K90	+	Dextran T110	+
poly(vinyl alcohol) (PVA)		Dextran sulphate 500	+
PVA 14 000	+	Linear poly(acrylamide)	−
PVA 49 000	+		
PVA 100 000	+		

process is repeated so that a series of cloud-points, with increasing concentrations of polymer I and decreasing concentration of polymer II is obtained, representing the binodal line which will asymptotically reach the lowest concentration of polymer II. The entire process is then repeated starting with polymer solution I and the addition of polymer solution II. This will give a second series of cloud-points representing the part of the binodal line which will asymptotically reach the lowest concentration of polymer I. By combining the results from the two series and plotting the data in a phase diagram, a binodal line can be fitted. For obvious reasons it is not possible to perform this procedure in a reproducible way at high temperatures, 80– ~100°C, due to loss of vapour and the difficulty of making small precise additions of the viscous agarose solution. Instead, solutions of polymer blends were prepared that gave rise to aqueous two-phase systems. The polymers were dissolved at 120°C, equilibrated for 2·5 h at 80°C or 5 h at 60°C in an end-over-end mixer, and finally phase separated by centrifugation at 80°C or 60°C, respectively.

The method described for the preparation of two-phase systems resulted in gelled phases that were fully separated and had the characteristic opalescence of pure agarose gels. Systems that are not fully separated, due to too short centrifugation time show cloudiness near and around the interfacial border. The data of the two-phase systems that were studied in detail are listed in Table 2 and the corresponding phase diagrams are shown in Figs 3–8. The standard error for the five sample runs, and for the five standard runs that were used to calculate each value, was typically in the range 0·01–0·10% (w/v). Polymer recoveries were in the range 95–105%. The polymer concentrations were plotted in the phase diagrams in such a way that the polymer that partitions to the most dense phase, i.e. the bottom phase, was plotted as abscissa and the concentration of the polymer that partitions to the less dense phase, i.e. the top phase, was plotted as ordinate. In two of the phase diagrams (Figs 3 and 4) a single upper-phase value is plotted. The bottom phases of these systems were analysed, but no reliable results were obtained due to viscosity effects resulting from the high agarose concentration. The results for the top phases were in order and match the other data and the binodal line. The binodal lines were fitted manually via Bezier-functions in a computer program (Canvas™ 3·0, Deneba Software, Miami, Florida).

The equilibration temperature was 80°C for all systems investigated, except for a limited study of the temperature dependence in the phase diagrams for two agarose–PEG systems (Figs 9 and 10). In accordance with the results obtained by Albertsson (1986) for the dextran–methyl cellulose system, the binodal shift between 80°C and 60°C is negligible.

Table 2. Systems studied

	Temperature (°C)	Fig.
Phase diagrams		
Agarose–poly(ethylene glycol) 6000	80	3
Agarose–poly(ethylene glycol) 20000	80	4
Agarose–poly(ethylene glycol) 35000	80	5
Agarose–Dextran X25	80	6
Agarose–Dextran T40	80	7
Agarose–Dextran T70	80	8
Equilibrium temperature dependence		
Agarose–poly(ethylene glycol) 6000	60/80	11
Agarose–poly(ethylene glycol) 20000	60/80	12
Study of equilibration time		
Agarose–poly(ethylene glycol) 20000	60/80	13

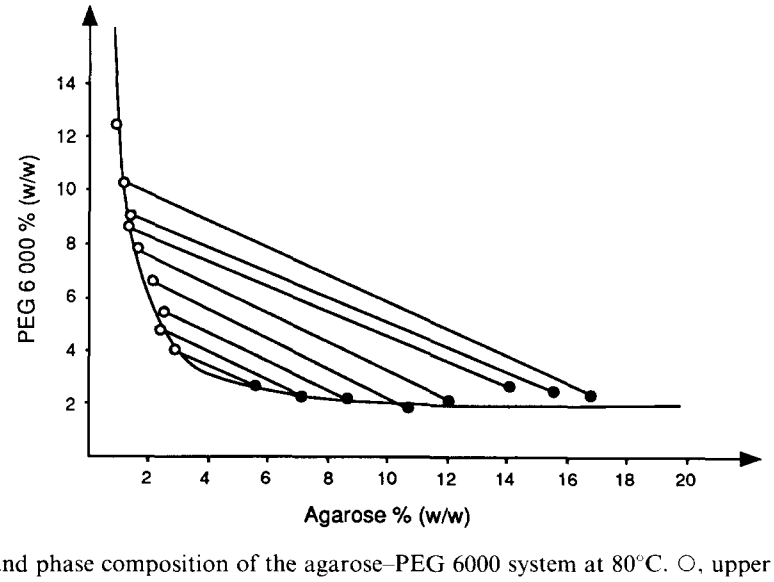

Fig. 3. Phase diagram and phase composition of the agarose–PEG 6000 system at 80°C. ○, upper phase; ●, lower phase.

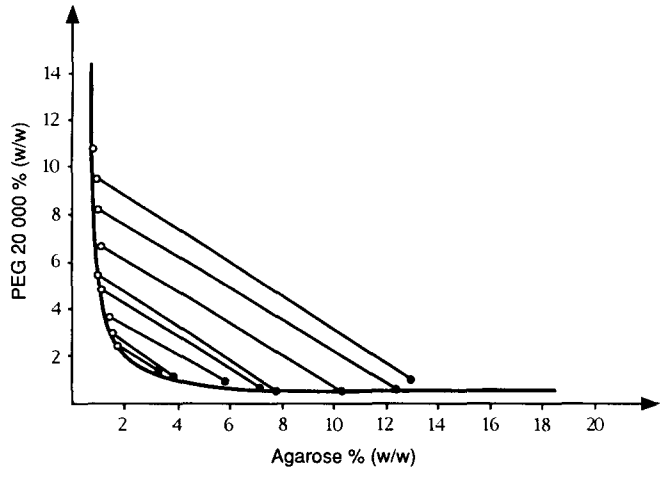

Fig. 4. Phase diagram and phase composition of the agarose–PEG 20 000 system at 80°C. ○, upper phase; ● lower phase.

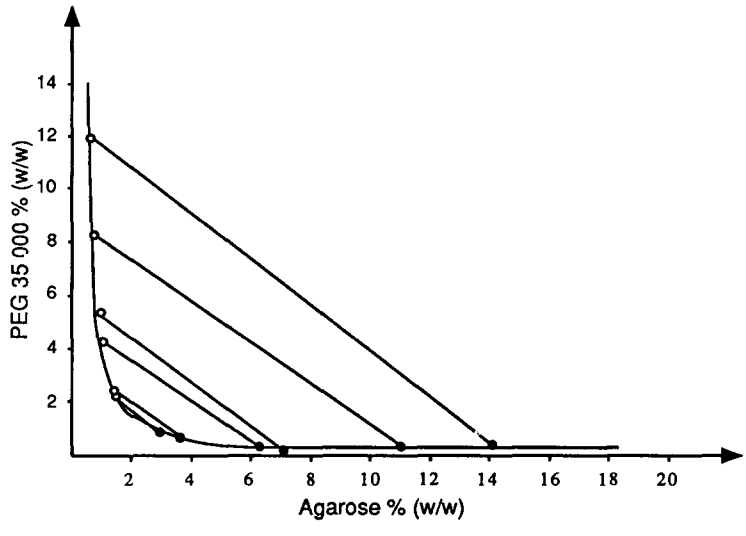

Fig. 5. Phase diagram and phase composition of the agarose–PEG 35 000 system at 80°C. ○, upper phase; ● lower phase.

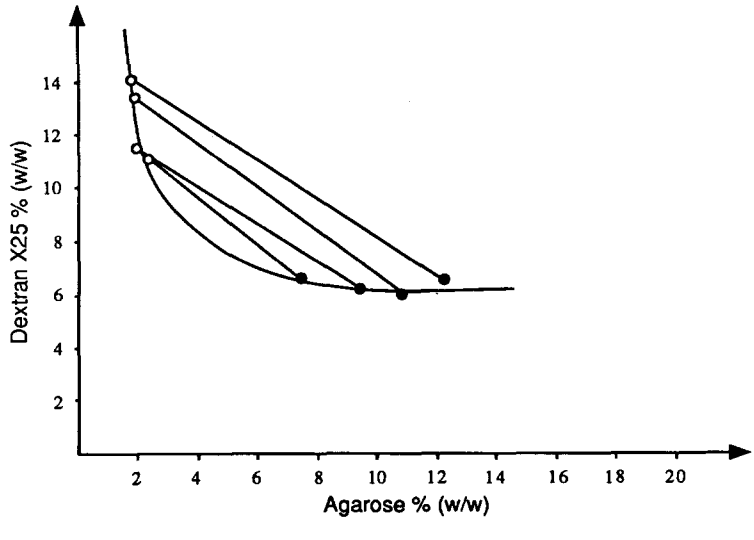

Fig. 6. Phase diagram and phase composition of the agarose–Dextran X25 system at 80°C. ○, upper phase; ● lower phase

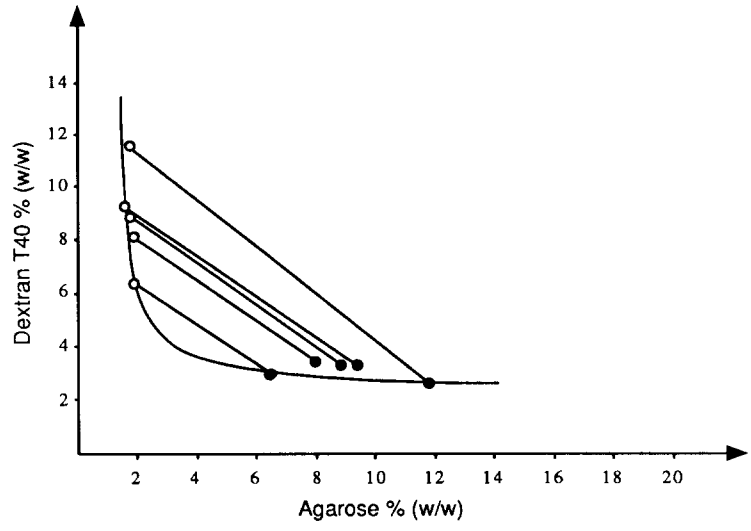

Fig. 7. Phase diagram and phase composition of the agarose–Dextran T40 system at 80°C. ○, upper phase; ● lower phase.

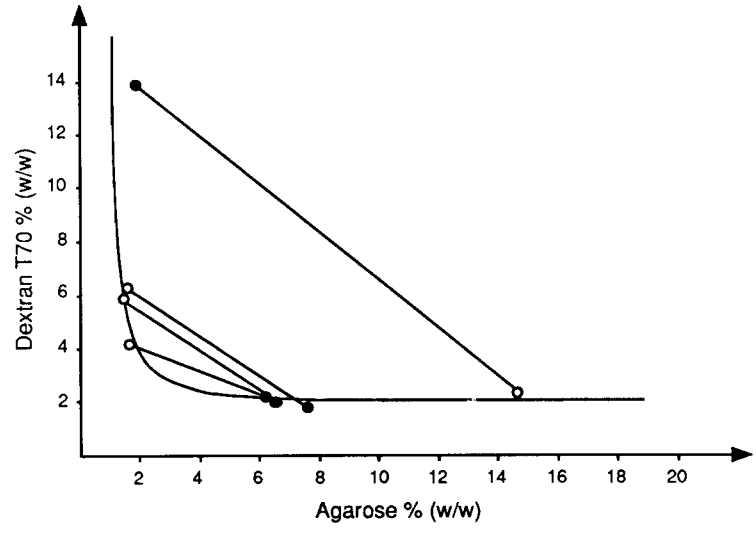

Fig. 8. Phase diagram and phase composition of the agarose–Dextran T70 system at 80°C. ○, upper phase; ● lower phase.

The equilibration time requirement was studied for the agarose–PEG 20 000 system at 60 and 80°C, respectively. Two polymer solutions within the one-phase regime were mixed and equilibrated by end-over-end mixing at each temperature for 2·5 h and 5 h, respectively. The phases were separated by centrifugation. Subsequent phase composition analysis showed an almost perfect fit with the original binodal line, indicating 2·5 h be a sufficient equilibration time (Fig. 11).

It is well known that in any aqueous polymer two-phase system, the position of the binodal in a phase diagram depends on the molecular weights of the polymers. The higher the polymer molecular weight, the closer to the axis the binodal will be positioned. This means that phase separation occurs at decreasing polymer concentrations with increasing molecular weight of either, or both, of the two polymers. In this study, the molecular weight of the agarose component was kept constant, by using the same agarose preparation with a weight average molecular weight of 120 kD in all experiments, whilst the molecular weights for PEG and dextran were varied.

The phase diagram of the agarose–PEG 6000 system (80°C) (Fig. 3) resembles that of the dextran (Mw 460 000)–PEG 6000 system (20°C) described by Albertsson (1958). Both systems are ternary, consisting of water, a polysaccharide, and a synthetic polymer, PEG. The polysaccharide-rich phase is in both cases the bottom phase, the slope of the tielines are in the same order and the curvature of the binodal line is similar. The main difference is that the dextran concentration in the upper phase of the dextran–PEG systems asymptotically reaches zero concentration when the total polymer concentration, and especially the PEG

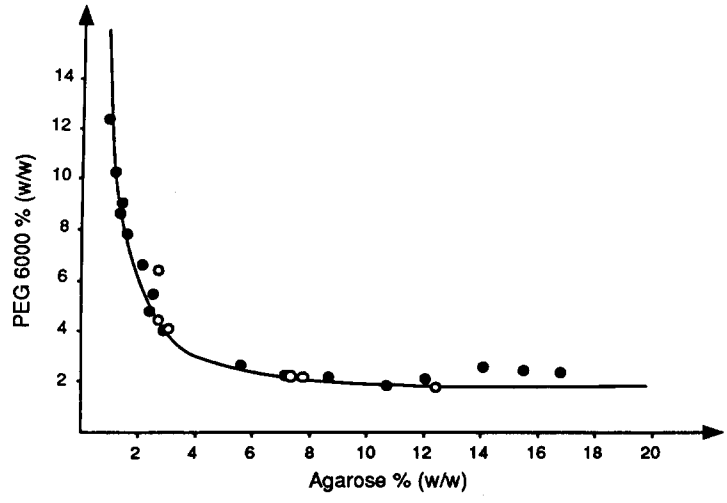

Fig. 9. Equilibration temperature dependence on the position of the binodial line for the agarose–PEG 6000 system. ○, 60°C; (●), 80°C.

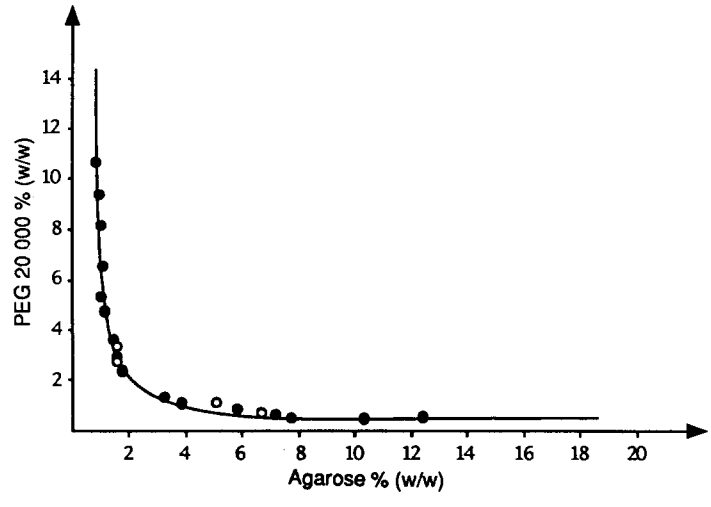

Fig. 10. Equilibration temperature dependence on the position of the binodial line for the agarose–PEG 20 000 system. ○, 60°C; ●, 80°C.

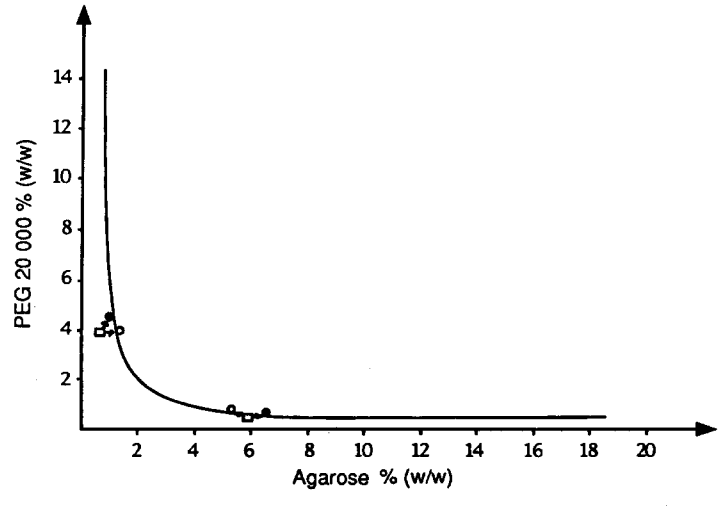

Fig. 11. Investigation of the effect of equilibration time for the agarose–PEG 20 000 system at 60°C, 5 h, and 80°C, 2·5 h. Analysed phase compositions resulting from mixing of two one-phase systems superimposed onto the phase diagram. ○, equilibration temperature 60°C; ●, equilibration temperature 80°C; □, compositions of the original one-phase systems.

concentration, is increased. This is not the case for the agarose–PEG system, where the agarose concentration asymptotically reaches a value of 0·7% (w/v) when the total polymer concentration is increased. The agarose concentration in the top phase is almost unaltered when PEG with higher molecular weights are used (Figs 4 and 5).

The phase diagram of the agarose–dextran (Mw 25 000) system (80°C) (Fig. 6) resembles that of the dextran (Mw 460 000)–Ficoll system (Mw ~ 400 000) (20°C) described by Albertsson (1958), both systems being ternary and consisting of water and two polysaccharide polymers. Ficoll is a polymer obtained by random polymerisation of sucrose. The agarose-containing system is, however, phase separated at lower polymer concentrations in spite of the much lower molecular weights of the polymers. This can be interpreted as being due to a larger apparent structural difference between the polymers in the dextran–agarose system than in the dextran–Ficoll system. An interesting finding in the agarose–dextran (Mw 70 000) system is that, at increased polymer concentrations, the phases change place with one another so that the agarose-rich bottom phase becomes the top phase and vice versa (Fig. 8). This indicates the small density difference between the phases in this system.

At the critical point of the phase diagram, the upper and lower phases are identical, having the same density. Thus, systems positioned close to the critical point require prolonged centrifugation time, in some cases up to 48 h, in order to give complete phase separation. Prolonged centrifugation times are also required for phase systems with high concentrations of polymer, especially for agarose, because of increased viscosity.

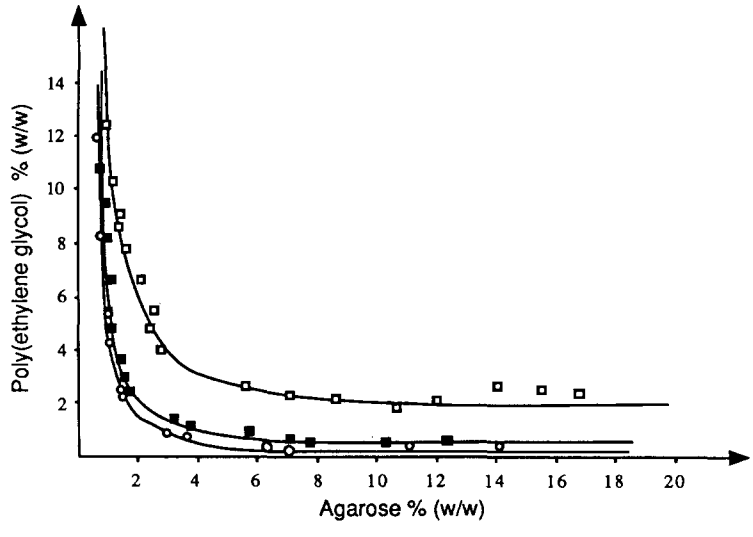

Fig. 12. Molecular weight dependence of the agarose–poly(ethylene glycol) systems on the binodial line position. □, agarose–PEG 6000; ■, agarose–PEG 20 000; (○), agarose–PEG 35 000.

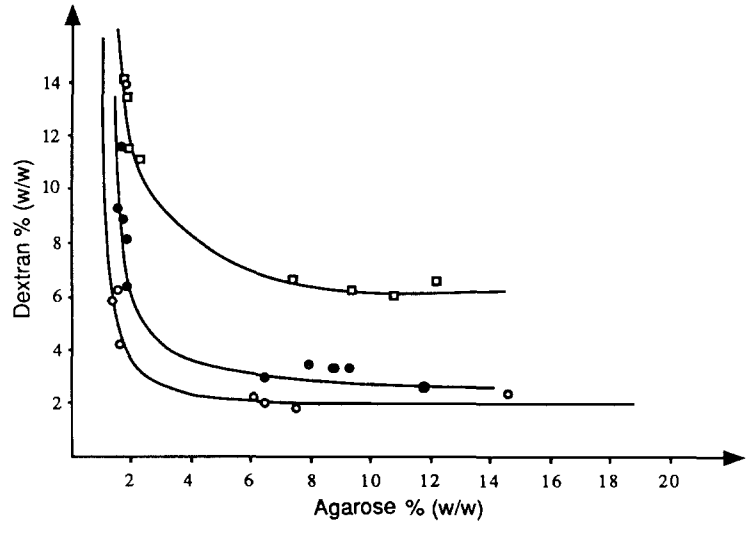

Fig. 13. Molecular weight dependence of the agarose–dextran systems on the binodial line position. □, agarose–Dextran X25; ●, agarose–Dextran T40; (○), agarose–Dextran T70.

Extended centrifugation times means, on the other hand, that the polymers in solution are held at elevated temperatures for longer periods of time with an increased risk of thermal degradation. This is observed for the agarose–PEG 6000 system (Fig. 3) as a slight increase in the PEG concentration at high agarose concentrations, indicating that the system might have changed due to thermal degradation. The risk is apparent for acid labile polysaccharides such as agarose and dextran. For synthetic polymers, such as PEG, no indication of thermal degradation could be observed. To avoid a lowering of pH due to the presence of dissolved CO_2, the RO-filtered water used for preparing the polymer solutions was routinely aspirated with N_2-gas.

The experiments confirm the expected results that the binodal line position is shifted towards lower polymer concentrations with increasing molecular weight of PEG (Fig. 12) and dextran (Fig. 13). The line asymptote close to the abscissa, at low concentrations of PEG or dextran, is changed accordingly. But for low concentrations of agarose, the binodal asymptote close to the ordinate remains almost unaltered since the average molecular weight and the molecular weight distribution of the agarose preparation used in this study was kept constant.

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