PARTICLE FRACTIONATION IN LIQUID TWO-PHASE SYSTEMS

THE COMPOSITION OF SOME PHASE SYSTEMS AND THE BEHAVIOUR OF SOME MODEL PARTICLES IN THEM APPLICATION TO THE ISOLATION OF CELL WALLS FROM MICROORGANISMS

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The study of mass isolated particles like, for example, nuclei, mitochondria, chloroplasts and cell walls obtained from disintegrated cells is becoming more and more important in biochemistry and cell physiology. Such work often requires efficient fractionation methods. Generally centrifugation techniques are used, and differences in the size, density and form of the particles are utilized for their separation. It should, however, be of value if these methods could be complemented by methods where other properties of the particles—for example, the electrical charge and surface properties of the particles—determine the fractionation.

Some experiments described in a preliminary report[1] suggested the possible use of a liquid–liquid two-phase system for the separation of cell particles. If a number of particles are shaken in a two-phase system they will distribute themselves mainly according to their surface properties, thus collecting either in the one, or in the other phase, or at the interface. Principally the method resembles those partition methods used in protein fractionation. See the reviews by PORTER[2] and VON TAVEL AND SIGNER[3] for further references.

Flotation, which is used for particle concentration in ore dressing, is based upon the adhesion of particles at an air–water interface, and that method is also of interest in connection with this work. The literature on flotation is immense. Valuable general information may be obtained, for example, from the books by GAUDIN[4] and TAGGART[5].

The present paper will describe the theoretical background of the method, the composition of some selected phase systems, and the behaviour of some model particles in them. The results obtained might serve as a rough guide for the application to actual separation of cellular particles.

The method has been applied for the isolation of cell walls from microorganisms (*Chlorella* and *Aerobacter*). A description of these isolation procedures is included at the end of the paper.

THEORETICAL CONSIDERATIONS ON THE BEHAVIOUR OF PARTICLES IN A TWO-PHASE SYSTEM

If a low molecular substance is partitioned in a two-phase system a finite partition coefficient (C_1/C_2) is usually established. (C_1 = concentration in phase 1, C_2 = concentration in phase 2.) BRÖNSTED has pointed out[6] that the higher the molecular weight

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of the substance—other factors being kept constant—the more one-sided the distribution will be, and he deduced the following approximate formula

$$\frac{C_1}{C_2} \sim e^{\frac{M\lambda}{RT}}$$

where M is the molecular weight of the substance and λ a constant characteristic for the phase system and the substance. Experiments on the fractionation of macromolecules[7] have shown the usefulness of BRÖNSTED's theory. The theory is also applicable to large particles[6,8], *e.g.* of cellular dimensions. M in the formula above should then be replaced by the surface area of the particles[6], but the consequence will be that the tendency for a one-sided distribution will be still more pronounced. While for proteins phase systems can be chosen which will give a partition coefficient around unity[2,3], the coefficient for a cellular particle should in most cases be infinitive or zero.

Therefore, if a two-phase system is shaken with a number of identical particles we should, in most cases, expect to find practically all these either in one phase or in the other. Complete separation of two kinds of particles will therefore be achieved if the constant λ in the formula above has opposite signs for the two kinds of particles.

One often finds adsorption of particles at the interface. This phenomenon was theoretically explained by QUINCKE[9] and by DES COURDES[10], and has been experimentally studied by HOFMAN[11] and REINDERS[12] on powders of insoluble inorganic salts in water–organic solvent systems. Suppose that the surface free energy per unit area of a particle P (Fig. 1) in phase 1 is γp_1 and in phase 2 γp_2 and the interfacial free energy per unit area between the two phases is γ_{12}. Neglecting gravity, DES COURDES[10] showed that if

(1) $\gamma p_1 - \gamma p_2 \geqslant \gamma_{12}$ the particle will be stable in phase 2
(2) $\gamma p_2 - \gamma p_1 \geqslant \gamma_{12}$ the particle will be stable in phase 1
(3) $\gamma p_1 - \gamma p_2 < \gamma_{12}$ the particle will be stable at the interface.

When gravity is considered and the particle is denser than the phases the particle is pulled downwards by a force F (see Fig. 2), which depends upon the size and density of the particle and the densities of the two phases. This force is counteracted by a force upwards depending upon the vertical component of γ_{12} and the length of the line where the particle is in contact with the interface. Only when the latter

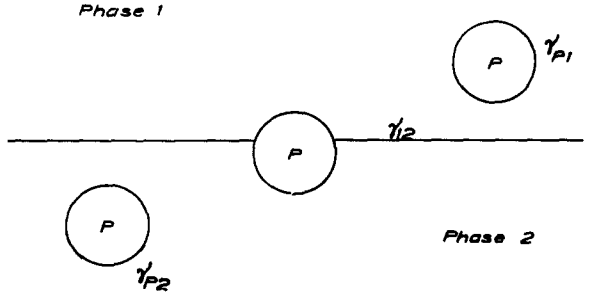

Fig. 1. A spherical particle in a two-phase system. γp_1, γp_2, γ_{12} are surface free energies per unit surface. To the left: $\gamma p_1 - \gamma p_2 \geqslant \gamma_{12}$. The particle is stable in phase 2. To the right: $\gamma p_2 - \gamma p_1 \geqslant \gamma_{12}$. The particle is stable in phase 1. In the middle: $|\gamma p_1 - \gamma p_2| < \gamma_{12}$. The particle is stable at the interface. Gravitational force is neglected.

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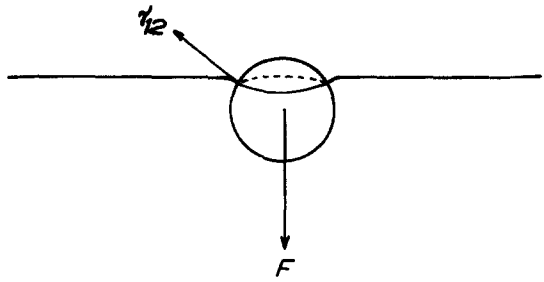

Fig. 2. A particle at an interface under the influence of gravity.

force equals the gravitational force will the particle remain at the interface. Equations that describe the relationship when this condition is fullfilled have been deduced for particles of different shapes in connection with flotation studies[4,5].

During the shaking process, however, conditions are rather different from those at a quiet interface. Drops of different sizes of the two phases are produced and move around randomly. The particles are therefore exposed to various forces, *e.g.* frictional and centrifugal, in a very complicated way. For a detailed discussion see ref. [4,5].

THE CHOICE OF PHASE SYSTEMS

The phase systems should be as inert as possible to the particles which are to be fractionated. This means usually that consideration must be paid to, among other properties, the water content, ionic strength, osmotic pressure, ability to dissolve out substances from the particles, denaturing effects upon proteins, and so forth. Since adsorption at the interface is utilized for separation the possible damaging effect of the interfacial tension must also be borne in mind. Generally, therefore, low interfacial tension should be desirable.

Different cell particles have highly different requirements upon the preservative properties of their environment. Likewise the aim of isolating a cell particle might put quite different requirements on the choice of media. Sometimes a very sensitive cell physiological property is studied, which requires a gentle treatment, but sometimes the particle is only isolated in order to extract a substance that is bound to it. It is, therefore, desirable to have a number of phase systems which present a large variation in chemical and physical properties.

The two-phase systems used in protein partition studies[2,3] are usually produced by mixing water, salt or a sugar, and a water-soluble organic solvent. The water content varies between 40–75 %. Although these systems should find application for cell particles too, it would be of value to have systems available with higher water content, *e.g.* 75–95 % (the water content of cells usually lies between these values), lower osmotic pressure, and lower salt concentration.

In order to achieve this, special attention has been paid to the use of polymers as components in the systems. Theoretical calculations on the thermodynamics of polymer solutions[13–15] and experiments[16,17] have shown that the mixing of different polymer solutions very easily produces two phases even at low concentration of polymers. This is partly explained as an entropy effect. As is well known, the free energy of mixing, ΔF_M, the heat of mixing, ΔH_M, and the entropy of mixing, ΔS_M, are related in the following way:

$$\Delta F_M = \Delta H_M - T\Delta S_M$$

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It has been shown theoretically that when two different polymer solutions are mixed, the value of ΔS_M is low compared to that obtained on mixing the same amount of their monomers[13–15]. Therefore, even a small positive value of ΔH_M is enough to make ΔF_M positive and prevent mixing. This usually means that a relatively small dissimilarity between two polymer compounds is enough to make them incompatible and that, for a certain pair of polymers, the higher the molecular weight the lower the concentration of them necessary for the production of two phases. Experiments by DOBRY AND BOYER-KAWENOKY[16] and KERN AND SLOCOMBE[17] showed that out of 58 pairs of polymers, soluble in non-aqueous solvents, all except 7 produced two phases. Experiments on water-soluble polymers by DOBRY[18] indicate the same phenomena that is confirmed by the present investigation.

All polymers studied here are non-ionic. Some of them have been chosen because they have been used in media for preserving isolated cell particles. Thus, dextran[19] and polyvinylpyrrolidone[20–22] have been used for liver mitochondria, and polyethylene glycol[23] for chloroplasts from algae. In these reports beneficial effects have been ascribed to the use of the polymers. It must, however, be pointed out that polymers may give undesired phenomena, such as complex formation and precipitation of proteins. This is especially to be expected in the case of polyelectrolytes. Although systems of polyelectrolytes like, *e.g.* complex coacervates, may also find application, they have not been studied at the present stage.

MATERIALS AND METHODS FOR THE ANALYSES OF THE PHASE SYSTEMS

Dextran was obtained from AB Pharmacia, Uppsala, in fractions with the following intrinsic viscosities and approximate mean molecular weights according to the manufacturer: 0.17 (M_w 20000), 0.27 (M_w 90000), 0.37 (M_w 179000, M_n 83000), 0.68 (M_w 1800000, M_n 280000). (M_w = weight av. mol. wt.; M_n = number av. mol. wt.).

The polyethylene glycols (*PEG*) used were the following "Carbowax" compounds obtained from Carbide and Carbon Chemicals Company, U.S.A. (av. mol. wts., according to the manufacturer, in parentheses): "Carbowax" polyethylene glycol 300 (285–315), 600 (570–630), 1540 (1300–1600), 4000 (3000–3700), 6000 (6000–7500), 20 *M* (15000–20000).

Methoxy polyethylene glycol (*MPEG*) 550, av. mol. wt. 525–575, and

Polypropylene glycol (*PPG*) 425, av. mol. wt. 400–450 were obtained from the same manufacturer.

Polyvinylpyrrilidon (*PVP*), Kollidon 20 and 30, with av. mol. wt. 11000 and 38000, respectively, were obtained from Badische Anilin und Soda Fabrik, Ludwigshafen, Germany.

Polyvinylalcohol (*PVA*) was obtained from Firma Wacher Chemie GMBH, Munich, Germany. The two following fractions were used: Polyviol 03/20, mol. wt. 13000 and Polyviol 13/20, mol. wt. 44–49 000.

Methylcellulose (*MC*), Methocel, 31.5% methoxyl, visc. 4149 cps, was obtained from Dow Chemical Company, U.S.A. Av. mol. wt. $\sim$300000.

Ethylhydroxyethylcellulose (*EHEC*), Modocoll E600 was obtained from Mo och Domsjö AB, Sweden. Av. mol. wt. $\sim$200000.

Phosphate buffer pH 7.1, 306.9 g K_2HPO_4 and 168.6 g KH_2PO_4 (Baker Analyzed) per l (36% w/w, 3 *M*).

The composition at the plait point was obtained by mixing weighed amounts of water and water solutions with known amount of components so that phase separation just occurs and so that the volumes of the two phases obtained were equal. Alternatively, the plait point was obtained from extrapolation of the middle point of tie lines near the plait point, in cases when a detailed phase diagram had been constructed.

ANALYSES FOR THE COMPOSITION OF THE PHASES

The systems PEG or PVP–phosphate–water: A concentrated solution (25% w/w) of the polymer was mixed with the phosphate buffer and water in suitable proportions in a separatory funnel. The funnel was repeatedly shaken in a thermostat. After the separation of the two phases was

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complete, their composition was determined in the following way. The water content was obtained by weighing before and after freeze-drying of a sample. The dry residue was dissolved in water and passed through a Dowex-50 column, and the salt content was determined by titrating the liberated phosphoric acid with 0.1 *M* NaOH using bromcresol green as indicator. The PEG or PVP content was obtained by subtraction.

The systems PEG, PVP, or PVA–dextran–water were analyzed similarly: The water content by freeze-drying, dextran by optical rotation in a Hilger polarimeter assuming $[\alpha]_D^{20} = +199°$, and PEG, PVP or PVA by subtraction. It should be noted that since the dextran fractions used are not monodisperse, a difference in the mean mol. wt. of the dextran in two conjugate phases is to be expected. The specific rotation must therefore be constant for different mol. wt. This is usually true for mol. wt. above 10000.

RESULTS FROM THE ANALYSES OF THE PHASE SYSTEMS

In Table I are listed some combinations of substances, which, together with water, can produce a liquid two-phase system. (Since water is always one of the components,

TABLE I

SOME COMBINATIONS OF TWO SUBSTANCES, WHICH, TOGETHER WITH WATER, CAN PRODUCE A LIQUID TWO-PHASE SYSTEM

PEG = polyethylene glycol; MPEG = methoxypolyethylene glycol; PPG = polypropylene glycol; PVP = polyvinylpyrrolidone; PVA = polyvinylalcohol; MC = methylcellulose; EHEC = ethylhydroxyethylcellulose; butylcellosolve = ethylene glycol monobutyl ether.

Potassium phosphate	+	PEG	PPG	+	glucose
Potassium phosphate	+	MPEG	PPG	+	glycerol
Potassium phosphate	+	PPG	PVP	+	dextran
Potassium phosphate	+	PVP	PVP	+	butylcellosolve
PEG	+	PPG	PVA	+	dextran
PEG	+	PVP	PVA	+	butylcellosolve
PEG	+	PVA	Dextran	+	butylcellosolve
PEG	+	dextran	Dextran	+	propylalkohol
PPG	+	PVP	Dextran	+	MC
PPG	+	dextran	Dextran	+	EHEC

it will later be omitted when a system is mentioned.) Table II gives the composition at the plait point of some selected systems. Phase diagrams have been constructed on a number of systems and Figs. 3–6 show some representative examples. Very near the critical mixing point the compositions of the two phases are almost identical. As the total composition departs from the critical mixing point, the composition of the two phases very quickly becomes different. The polymers then tend to concentrate in their respective phases, while the low molecular components, salt and water, are more evenly distributed. The larger the difference in molecular size between the two components the more unsymmetrical the phase curve will be (Fig. 4 and 6). By using fractions of a polymer with larger av. mol. wt. higher water content can be obtained in the system (Fig. 4 and 6). The polymer–polymer systems are thus composed of one phase rich in the one polymer and a second phase rich in the other polymer, both phases being rich in water.

These findings are in accordance with the theory on phase equilibria[13–15].

The systems can be complemented by adding other substances. Low molecular substances like salt or sugars can for example, be added to the polymer–polymer systems in order to obtain systems with desired ionic strength and osmotic properties.

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TABLE II

COMPOSITIONS OF PHASE SYSTEMS AT THE PLAIT POINT

Temp. *C*	*Component I* *(number indicates mol. wt.)*	*Component II* *(number indicates mol. wt.)*	*Composition (w/w) %*		
			I	*II*	*H_2O*
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 285–315	19.2	14.2	66.6
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 570–630	15.7	12.7	71.6
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 1300–1500	12.6	11.2	76.2
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 3000–3700	11.0	9.5	79.5
0.0°	$KH_2PO_4 + K_2HPO_4$	PEG 3000–3700	12.6	8.7	73.7
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 6000–7500	9.9	7.1	83.0
0.0°	$KH_2PO_4 + K_2HPO_4$	PEG 6000–7500	11.9	6.4	81.7
20.0°	$KH_2PO_4 + K_2HPO_4$	PEG 15000–20000	7.6	10.5	81.9
20.0°	$KH_2PO_4 + K_2HPO_4$	PVP 11,000	9.6	14.2	76.2
20.0°	$KH_2PO_4 + K_2HPO_4$	PVP 38,000	8.7	10.2	81.1
20.0°	Dextran 20000	PEG 3000–3700	10.0	7.6	82.4
20.0°	Dextran 20000	PEG 6000–7500	8.6	4.6	86.8
20.0°	Dextran 20000	PEG 15000–20000	4.1	5.2	90.7
20.0°	Dextran 90000	PEG 6000–7500	6.6	3.7	89.7
0.0°	Dextran 179000	PEG 6000–7500	5.5	3.8	90.7
20.0°	Dextran 179000	PEG 6000–7500	5.2	3.1	91.7
20.0°	Dextran 1800000	PEG 6000–7500	3.9	3.4	92.7
20.0°	Dextran 1800000	PEG 15000–20000	1.3	3.0	95.7
20.0°	Dextran 90000	PVP 38000	4.9	6.7	88.4
20.0°	Dextran 179000	PVA 42000	2	3	95
20.0°	Dextran 1800000	MC 300000	0.8	0.3	98.9
20.0°	Dextran 1800000	EHEC 200000	0.7	0.4	98.9

PEG = polyethylene glycol; PVP = polyvinylpyrrolidone; PVA = polyvinylalcohol; MC = methylcellulose; EHEC = ethylhydroxyethylcellulose.

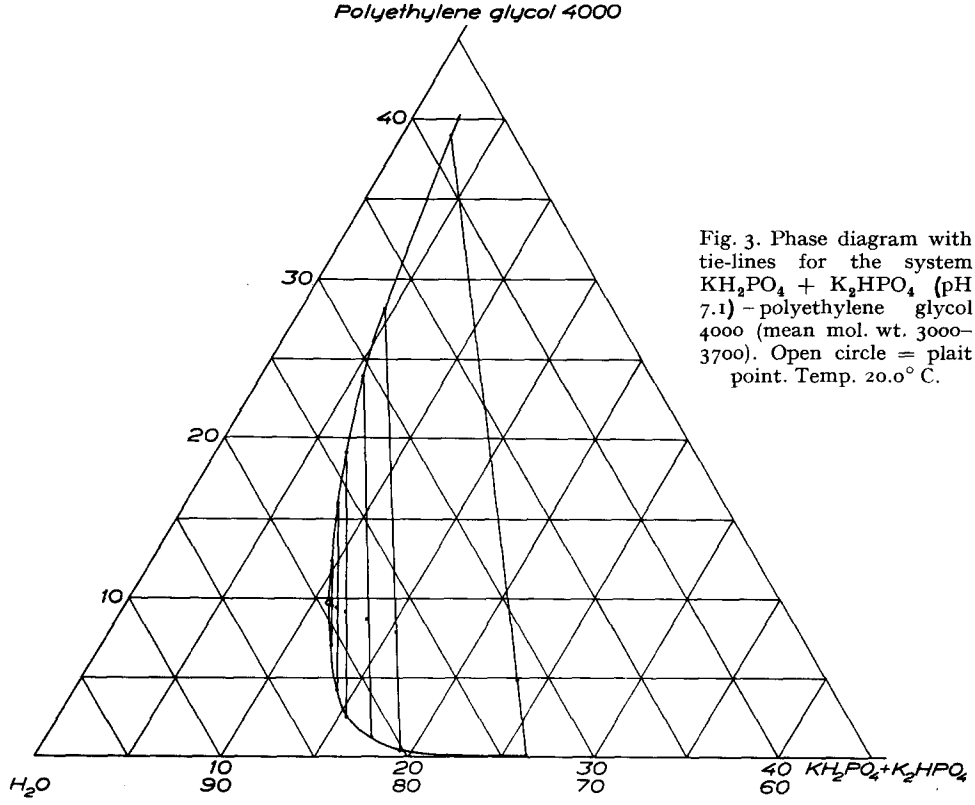

Fig. 3. Phase diagram with tie-lines for the system $KH_2PO_4 + K_2HPO_4$ (pH 7.1) – polyethylene glycol 4000 (mean mol. wt. 3000–3700). Open circle = plait point. Temp. 20.0° C.

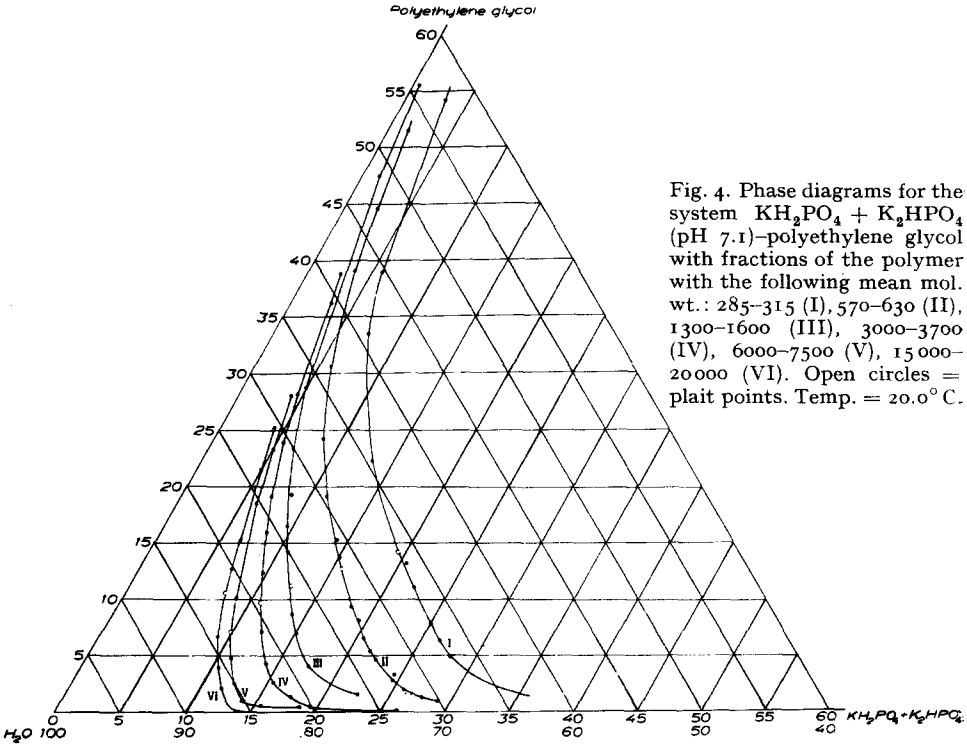

Fig. 4. Phase diagrams for the system $KH_2PO_4 + K_2HPO_4$ (pH 7.1)–polyethylene glycol with fractions of the polymer with the following mean mol. wt.: 285–315 (I), 570–630 (II), 1300–1600 (III), 3000–3700 (IV), 6000–7500 (V), 15000–20000 (VI). Open circles = plait points. Temp. = 20.0° C.

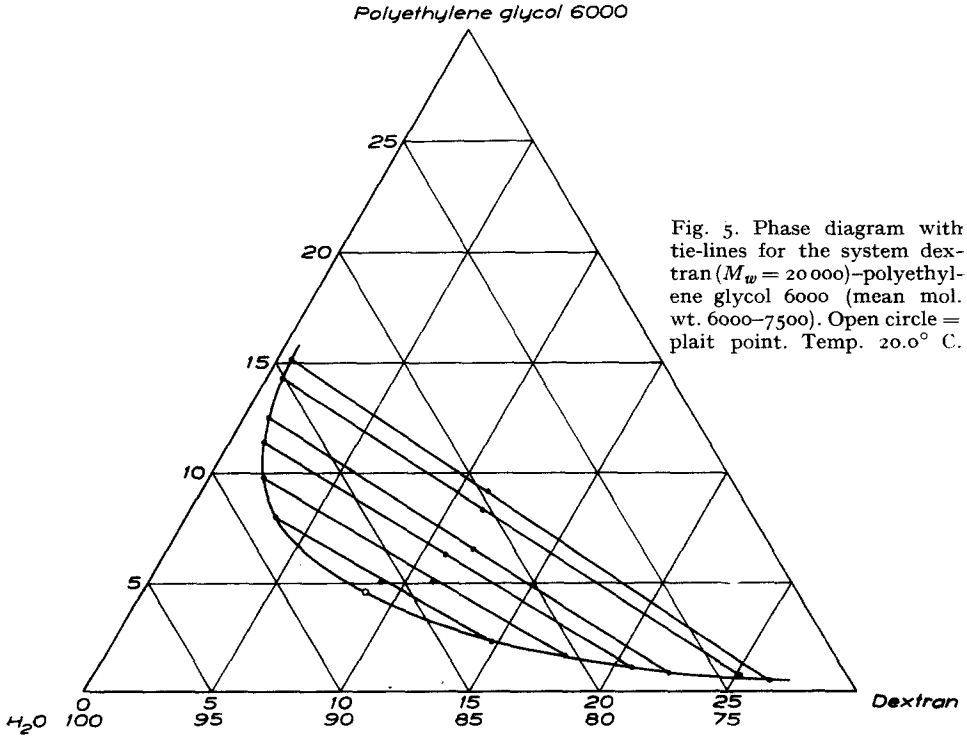

Fig. 5. Phase diagram with tie-lines for the system dextran ($M_w = 20000$)–polyethylene glycol 6000 (mean mol. wt. 6000–7500). Open circle = plait point. Temp. 20.0° C.

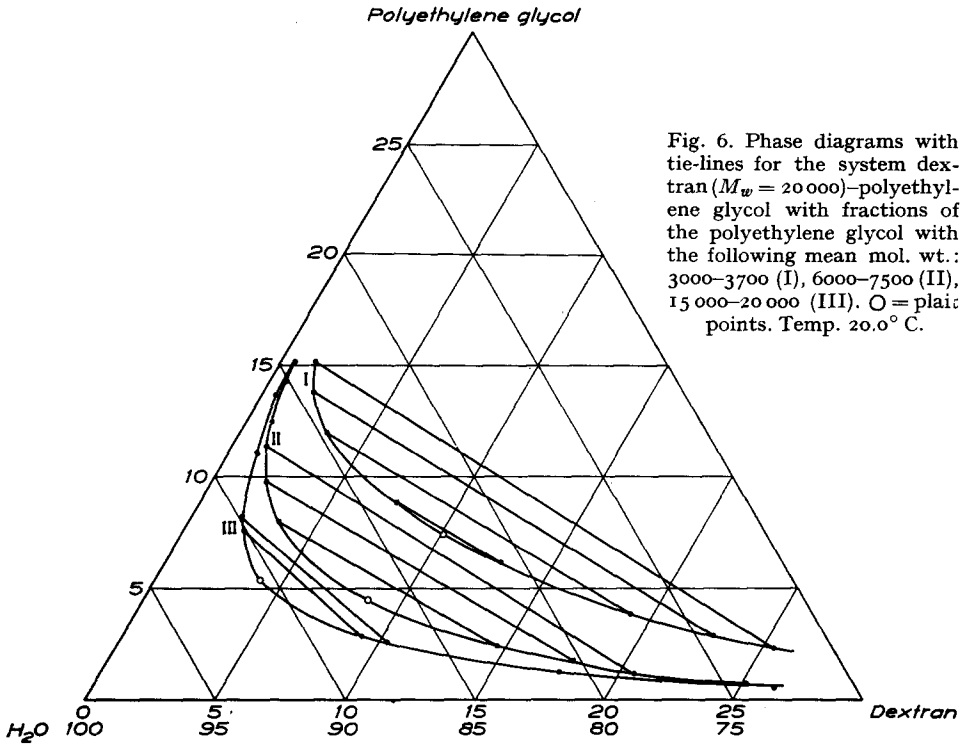

Fig. 6. Phase diagrams with tie-lines for the system dextran (M_w = 20 000)–polyethylene glycol with fractions of the polyethylene glycol with the following mean mol. wt.: 3000–3700 (I), 6000–7500 (II), 15 000–20 000 (III). O = plait points. Temp. 20.0° C.

The systems react differently to changes in temperature. The PEG or PVP–phosphate systems separate into phases at lower water content with lower temperature. The reverse holds for the systems PEG, PVP or PVA–dextran systems.

The time it takes for the phases to separate completely clear varies considerably and is mainly dependent upon the composition of the system in relation to the plait point and the viscosity. Very near the plait point the difference in density between the two phases is so small that it takes a long time for them to separate. On the other hand, far away from the plait point the viscosity is higher and the production of emulsion is sometimes considerable so that, after the main portions of the two phases have separated and a horizontal interface between them has been formed, small droplets of the one phase may remain in the other phase for a long time, from hours up to days. If the volumes of the two phases are fairly equal, emulsion usually occurs in both phases, but if the volumes are of different sizes emulsion usually occurs in the largest phase because it is only the small phase which forms droplets in the other.

At intermediate compositions, however, two clear phases are formed in the shortest time, compared with the two extreme cases considered above.

The viscosity of the polymer systems is higher than in low molecular systems but usually not inconvenient for practical work. Exceptions are the systems PEG–PVP, PEG–PVA and the other systems when their compositions are far away from the plait point so that the polymers are concentrated in their respective phases with concentrations up to 30–40%. Then more or less gel-like phases are produced. The rise in the viscosity caused by a higher molecular weight fraction of a polymer is

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partly compensated by the fact that a lower concentration of the polymer is necessary for the production of two phases.

Multiphase systems are produced when several components, all mutually incompatible, are mixed[16,17]. For example the system water–dextran–polyethyleneglycol–polypropyleneglycol or water–butylcellosolve–dextran–polyvinylalcohol gives three liquid phases over certain concentrations of the components.

THE BEHAVIOUR OF PARTICLES IN TWO-PHASE SYSTEMS

Model particles

Polystyrene particles were obtained from Dow Chemical Company, U.S.A. They were monodisperse and had 1 micron diameter.

Starch particles. Amend Potato Starch (Amend Drug & Chemical Co., New York, U.S.A.) was fractionally sedimented in the following way in order to obtain the smallest particles: The starch was suspended in water. The solution, which had a height of 12 cm, was allowed to stand for 4 h in the cold (+ 4° C). The upper 10 cm of the solution was carefully collected by a siphon leaving the larger particles in the bottom. The supernatant was collected and again allowed to stand for sedimenting. This procedure was repeated twice. The final supernatant was collected. This gives a suspension of mainly spherical starch particles with diameters between 3 and 9 μ. The mean diameter was 6 μ.

Cellulose particles were obtained in the same way as the starch particles from Munktell Cellulose powder (from Grycksbo Pappersbruk, Grycksbo, Sweden). The shape of the particles varies but most of them are rodlike, 1–3 μ thick and with a length of 10–20 μ.

Chlorella pyrenoidosa Pringsheim (F. E. Allison, USDA, Strain L-72a) was, unless otherwise stated, cultivated in the following solution (solution I): 0.25% KNO_3, 0.49% $MgSO_4 \cdot 7H_2O$, 0.25% KH_2PO_4 with the following microelements in p.p.m.: H_3BO_3 0.3, $ZnSO_4 \cdot 7H_2O$ 0.3, $MnSO_4 \cdot H_2O$ 1.3, $NaMoO_4 \cdot 2H_2O$ 0.05, $CuSO_4 \cdot 5H_2O$ 0.008, $CoCl_2 \cdot 6H_2O$ 0.04.

Scenedesmus obliquus No. 276/3a obtained from the Culture Collection of Algae and Protozoa of the Botany School, Cambridge, England.

Scenedesmus quadricauda obtained from the Institute of Physiological Botany, Uppsala, Sweden, were cultivated in the following solution (solution II): 0.4% NH_4NO_3, 0.2% K_2HPO_4 0.2% $MgSO_4$, + earth extract.

Aerobacter sp. strain 1912[24] were supplied by Dr. B. NORÉN, Institute of Physiological Botany, Uppsala, Sweden. They were grown in broth nutrient solution (beef extract 3 g, bactotryptone 5 g, glucose 1 g, water 1 l). The cells were used after an incubation of 24 h at 30° C.

Penicillum spores were obtained from agar plate cultures of *Penicillum notatum*.

All particles were thoroughly washed with water by sedimentation or centrifugation before they were used.

EXPERIMENTAL

The particles used here have, in almost all cases, a higher density than the two phases. The sedimentation of the particles is, except in systems very near the plait point, negligibly slow compared with the time it takes for the two phases to separate. If the particles are shaken in a two-phase system in a test tube one can usually, after the two phases have separated, determine in what phase the particles have gone by visual inspection of the colour or turbidity of the phases. However, since emulsification is sometimes considerable, one has to pay attention to this phenomenon if one wants to know in what phase the particles have gone. For example, particles present at the interface are attached to the small droplets forming the emulsion. These droplets loaded with particles may be suspended in one phase for a long time after the main bulk of the phases has separated. This phenomenon may then give the wrong impression that the particles are freely suspended in the phase. This difficulty may be overcome by varying the volumes of the two phases. As mentioned before, the formation of emulsion is dependent upon the mutual size of the volumes of the two phases so that it is usually the smallest phase which produces the droplets

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in the other phase. Therefore, if one shakes the particles in one test tube with a small bottom phase and a large top phase, and in another test tube with a large bottom phase and a small top phase, one can usually detect if the particles are freely suspended in one phase or not. It is advisable also to check by examination in a microscope.

Attention must also be paid to overloading phenomena. When the two phases are shaken, the interface formed has a large surface and a large capacity for adsorption. After the two phases have separated, the interfacial area has diminished to the final horizontal interface. If the number of adsorbed particles is large enough this horizontal interface can not hold up all the particles. Therefore, large aggregates of particles are seen to stream down from the interface.

Sometimes the adsorbed particles tend to aggregate and form "islets" at the horizontal interface and this may also cause aggregates of particles to stream down from the interface.

Measuring the collection of particles at the horizontal interface

When there is a distribution of particles between the bottom phase and the interface the respective numbers of particles are obtained in the following way. After the two phases have separated and a horizontal interface has been formed those particles which are free in the bottom phase will sediment to the bottom, while those that are attached to the interface will remain there. The particles at the interface are then collected by carefully sucking them up with a pipette placed just above the interface. Each portion of the particles thus separated is then washed twice in water by sedimenting in a tube before measuring the number. In the case when the particles are distributed between a top phase and the interface, the number in the top phase is obtained by taking out a known volume of newly-formed top phase and determining the concentration of particles. When the total top phase volume is known, the total number of particles in the top phase can be calculated The number of particles at the interface is then obtained by substraction from the total number of particles in the whole system.

The experiments reported in Fig. 7 were made in the following way. In tubes of 20 cm length and 3.5 cm diameter 5.00 g PEG 4000 was dissolved in 21.25 g H_2O. 10.00 g of water containing the particles was added, and the tubes were put into a thermostat at 20.0° ± 0.02° C. To the tubes were then added amounts of the phosphate buffer so that the desired compositions were obtained. (The smallest amount of the phosphate buffer which causes phase separation is 16.3 g, and this gives a composition near the plait point (Fig. 3). Further additions of the phosphate buffer then produce compositions more and more removed from the plait point.) The tubes were shaken in the thermostat a couple of times and were then allowed to stand until the particles in the bottom phases had sedimented to the bottom of the tubes. The particles at the interfaces were then taken away as described above.

The number of particles was obtained by counting suitably diluted suspensions in a Buerker counting chamber. 400–500 particles were usually counted.

RESULTS

In Table III are listed the various ways of behaviour of some particles in a number of systems. The composition of the systems was made in the neighbourhood of the plait point but still so far from it that the two phases separated so quickly that the sedimentation of the particles was negligible.

Table IV shows the behaviour of some particles in the phosphate–PEG and dextran–PEG systems with different fractions of the polymers. The three algae were cultivated in the same culture solution (solution II). The same results as in Table IV were obtained when the different algae were present together. The different behaviour is therefore reflecting different characters of the particles.

When *Chlorella* was shaken in a phosphate–PEG system with PEG 6000, 4000 or 1540 mixed with small amounts of PEG 600 or 300, the *Chlorella* went to the

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TABLE III

THE BEHAVIOR OF PARTICLES IN DIFFERENT PHASE SYSTEMS

(The substances in the particle columns indicate that the particle goes to the phase rich in that substance)

Phase system	*Polystyrene*	*Starch*	*Cellulose*	*Chlorella*	*Penicillum spores*
Phosphate + PEG	PEG	phosphate	phosphate	phosphate	I
Phosphate + MPEG	MPEG	phosphate	phosphate	MPEG	I
Phosphate + PPG	PPG	phosphate	phosphate	phosphate	I
Phosphate + PVP	PVP	phosphate	phosphate	phosphate	I
Phosphate — butylcellosolve	phosphate	phosphate	phosphate	phosphate	I
Phosphate — propylalcohol	propylalcohol	phosphate	phosphate	phosphate	I
PEG — PPG	PEG	PEG	PEG	PEG	I
PEG — PVP	PEG	PVP	PVP	PVP	I
PEG — PVA	PVA	PEG	PEG	PEG	I
PEG — dextran	PEG	dextran	dextran	PEG	I
PPG — PVP	PVP	PVP	PVP	PVP	I
PPG — dextran	PPG	dextran	dextran	dextran	I
PPG — glucose	glucose	glucose	glucose	glucose	I
PPG — glycerol	glycerol	glycerol	glycerol	glycerol	I
PVP — dextran	PVP	dextran	I (dextran)	PVP	PVP
PVP — butylcellosolve	PVP	PVP	PVP	PVP	I
PVA — dextran	PVA	dextran	I (dextran)	dextran	PVA
PVA — butylcellosolve	butylcellosolve	PVA (I)	I (PVA)	PVA	I
Dextran — butylcellosolve	dextran	dextran	dextran	dextran	I
Dextran — propylalcohol	propylalcohol	dextran	dextran	dextran	I
Glucose — butylcellosolve	glucose	glucose	glucose	glucose	I
Glucose — propylalcohol	glucose	glucose	glucose	glucose	I
Dextran — MC	MC	dextran	MC	dextran	MC
Dextran — EHEC	EHEC	dextran	EHEC	dextran	EHEC

PEG = polyethylene glycol (3000–3700); MPEG = methoxypolyethylene glycol; PPG = polypropylene glycol; PVP = polyvinylpyrrolidone (11 000); PVA = polyvinylalcohol (13 000); MC = methylcellulose; EHEC = ethylhydroxyethylcellulose. Mol. wt. of dextran, 20 000 for all systems except dextran — MC and dextran — EHEC, when it was 1800 000. I = interface.

TABLE IV

THE BEHAVIOR OF PARTICLES IN THE SYSTEMS PHOSPHATE–PEG AND DEXTRAN–PEG WITH DIFFERENT FRACTIONS OF THE POLYMERS

(The substances in the particle columns indicate that the particle goes to the phase rich in that substance)

Phase system (number indicates mol. wt.)		*Chlorella pyrenoidosa*	*Scenedesmus obliquus*	*Scenedesmus quadricauda*	*Aerobacter*
Phosphate — PEG	285–315	PEG	PEG	PEG	PEG
Phosphate — PEG	570–630	PEG	phosphate	I (PEG)	phosphate
Phosphate — PEG	1300–1600	phosphate	phosphate	phosphate	phosphate
Phosphate — PEG	3000–3700	phosphate	phosphate	phosphate	phosphate
Phosphate — PEG	6000–7500	phosphate	phosphate	phosphate	phosphate
Phosphate — PEG	15 000–20 000	phosphate	phosphate	phosphate	phosphate
Dextran 20 000 — PEG	3000–3700	PEG	PEG	PEG	PEG
Dextran 20 000 — PEG	6000–7500	PEG	PEG	PEG	PEG
Dextran 20 000 — PEG	15 000–20 000	dextran	dextran	dextran	dextran
Dextran 179 000 — PEG	15 000–20 000	dextran	dextran	dextran	dextran
Dextran 1 800 000 — PEG	15 000–20 000	PEG	PEG	PEG	PEG

In all these systems the starch and cellulose particles go to the phosphate-rich or dextran-rich phases, and the polystyrene particles go to the PEG-rich phases.

phosphate-rich phase. Conversely, in systems with PEG 600 or 300 mixed with small amounts of PEG 6000, 4000 or 1540, the *Chlorella* went to the PEG-rich phase. At intermediate mixtures of the high and low molecular fractions, systems could be produced in which the *Chlorella* had a strong tendency for adsorption at the interface. Very near the plait point of such a system *Chlorella* was found in both phases after they had separated. However, this principally interesting phenomenon must be studied in more detail before it can be decided if the presence of *Chlorella* in both phases is due to thermal motion of the particles, gravity, or heterogeneity of the particles.

The adsorption at the interface

If the composition of the systems is changed away from the plait point so that the difference between the two phases becomes larger, the particles are more and more adsorbed at the interface. This is a common phenomenon. However, different particles in the same system behave quite differently as do also one kind of particles in different systems. Fig. 7 shows the adsorption of *Chlorella*, starch, and cellulose particles, dependent upon the composition of the system phosphate–PEG. As ordinate is plotted the percentage of particles which remain at the horizontal interface after the two

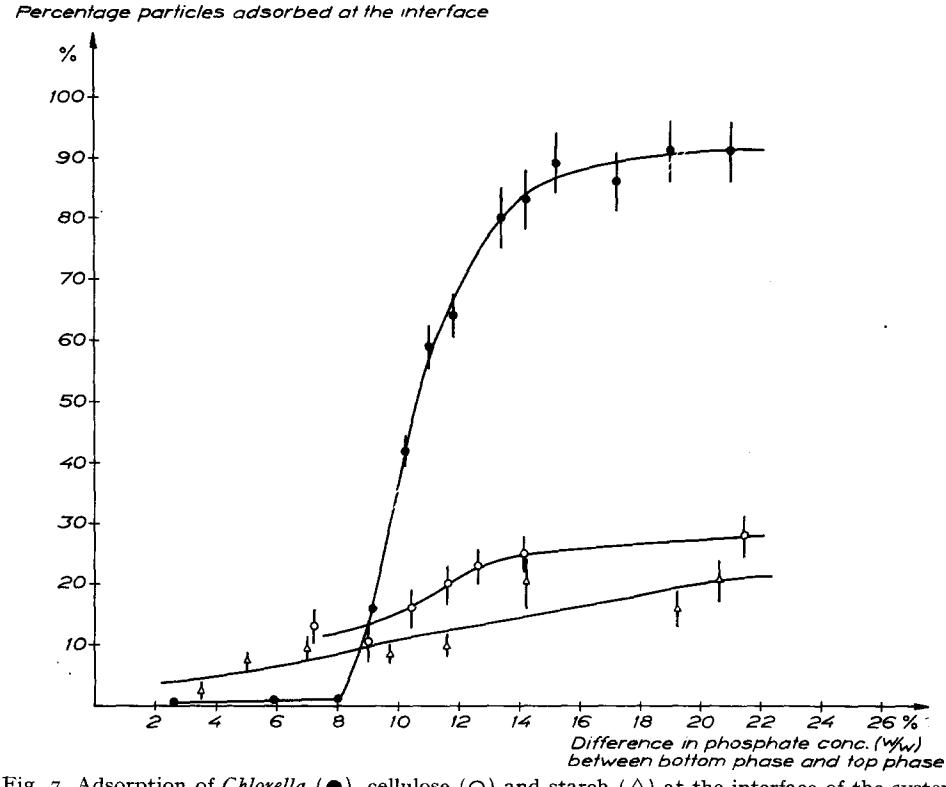

Fig. 7. Adsorption of *Chlorella* (●), cellulose (○) and starch (△) at the interface of the system phosphate–polyethylene glycol 4000 at different compositions. As abscissa is plotted the difference in phosphate concentration between the phosphate-rich phase (bottom phase) and the polyethylene glycol-rich phase (top phase), which is a measure of how far from the plait point the composition is removed. Compare with Fig. 3. Temp. 20.0° C.

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phases have separated. As abscissa is plotted the difference in phosphate concentration between the two phases, which is a measure of how far away from the plait point the system is. Since the total composition of the system was known, the difference in phosphate concentration between the two phases was obtained graphically from the phase diagram (Fig. 3). Similar curves are obtained if the difference in PEG or water content, or if the concentration of phosphate in the bottom phase, is plotted as abscissa. The experiment could not be continued at compositions further away from the plait point than described in Fig. 7 because then droplets of the PEG-rich phase adhered to the glass wall below the interface.

For *Chlorella*, starch, and cellulose particles, the following was found:

(1) For a fixed phase composition the number of particles, which are adsorbed at the interface, is proportional to the total number of particles, provided the number of particles is so low that no overloading occurs (Fig. 8).

(2) If the composition of the two phases is kept constant but the mutual size of the volumes of the two phases is varied—that is, at total compositions along a tie line—the number of particles which are adsorbed is not significally changed.

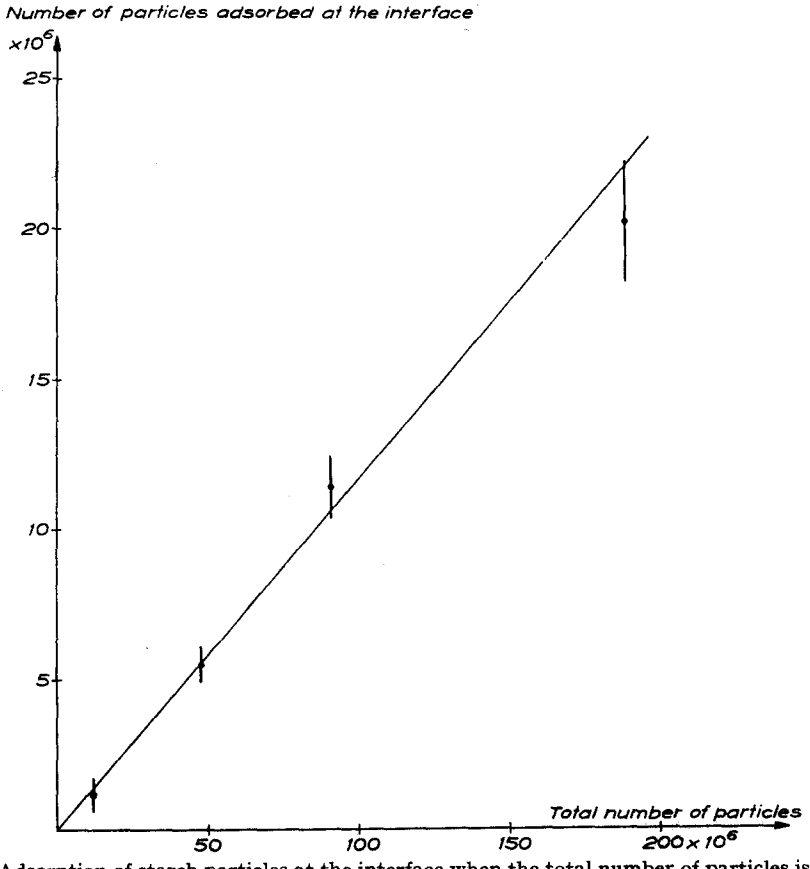

Fig. 8. Adsorption of starch particles at the interface when the total number of particles is varied, in the system phosphate–polyethylene glycol 4000. Total composition of the system (w/w): phosphate 13.2%, polyethylene glycol 8.8%, water 78.0%. Difference in phosphate concentration between bottom phase and top phase = 11%. Temp. 20.0° C.

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(3) If the top phase, together with the interface and the particles adsorbed there, is taken away and replaced by a new top phase, then new particles from the bottom phase are adsorbed at the interface. This can be repeated several times. Likewise, if the interface particles are shaken with a new bottom phase, part of them will go to the bottom phase. These phenomena, which resemble counter-current extraction, have, however, not been studied quantitatively in detail so far.

The adsorption at the interface can also be affected by adding a new component to the system, *e.g.*, the addition of phosphate or PPG to the PEG–dextran and the addition of phosphate to the dextran–PVA systems, causes the *Chlorella* to adsorb at the interface. As has already been described, the addition of a different fraction of the same polymer may also cause adsorption at the interface.

ISOLATION OF CELL WALLS FROM MICROORGANISMS

The use of two-phase systems has been applied to the isolation of cell walls from *Chlorella* and *Aerobacter*, and it is believed that with slight modifications it can be applied to other microorganisms as well. For previous work on the isolation of cell walls from bacteria see the review by SALTON[25].

It was found that the cell walls behave qualitatively similar to the whole cells. Thus, near the plait point of the system phosphate–PEG 4000, cell walls and starch granula from *Chlorella* stay in the salt-rich phase while the rest of the cell content collect at the interface. At compositions away from the plait point the cell walls, like the whole cells (see Fig. 7), go to the interface, while the starch grains remain mainly in the bottom phase. In the dextran–PEG 4000 system the cell walls go to the PEG-rich phase, the starch grains to the dextran-rich phase, and the rest of the cell content to the interface.

Cell walls from *Aerobacter* go to the salt-rich phase of the phosphate–PEG system. Near the plait point they are slightly contaminated by other particles. At a composition a little away from the plait point the cell walls remain uncontaminated in the salt-rich phase. The rest of the cell content goes to the interface.

PROCEDURE

Cell walls from Chlorella

(1) In the phosphate–PEG 4000 system:

10 ml of a suspension of *Chlorella pyrenoidosa* Pringsheim, previously washed once in H_2O and containing $6 \cdot 10^7$ cells, was shaken with Ballotini glass beads, 0.158 mm diameter, in a Mickle disintegrator for 5 min. The treated suspension was diluted to 70.4 ml H_2O, and added to 11.2 g PEG 4000 and 36.1 g phosphate buffer. The mixture was shaken by hand a couple of times in a 100-ml separatory funnel, in a thermostat at 20°. The area of the interface was 48 cm^2.

After the two phases had separated, the bottom phase was collected and shaken with another conjugate top phase with the same volume as before (which may be obtained from an identical phase system without the cell particles), plus 16.2 g phosphate buffer. The bottom phase was taken away, leaving the top phase and the interface with the cell walls in the funnel. These were shaken with a new conjugate bottom phase. The bottom phase was taken away and the cell walls at the remaining interface were collected, diluted with water, and centrifuged at 3500 r.p.m. for 15 min. This cell-wall preparation may occasionally be slightly contaminated with starch grains.

(2) In the dextran–PEG system:

The cells were disintegrated as above. 33.5 ml of a suspension of disintegrated cells were mixed with 22.5 g 30% w/w PEG 4000 solution and 44 g 25% w/w dextran 20 000 solution at 20°. After separation, the top phase was collected and shaken with a new conjugate bottom phase. The top phase was collected, and the cell walls centrifuged and washed with water. This cell-wall preparation is uncontaminated by starch grains.

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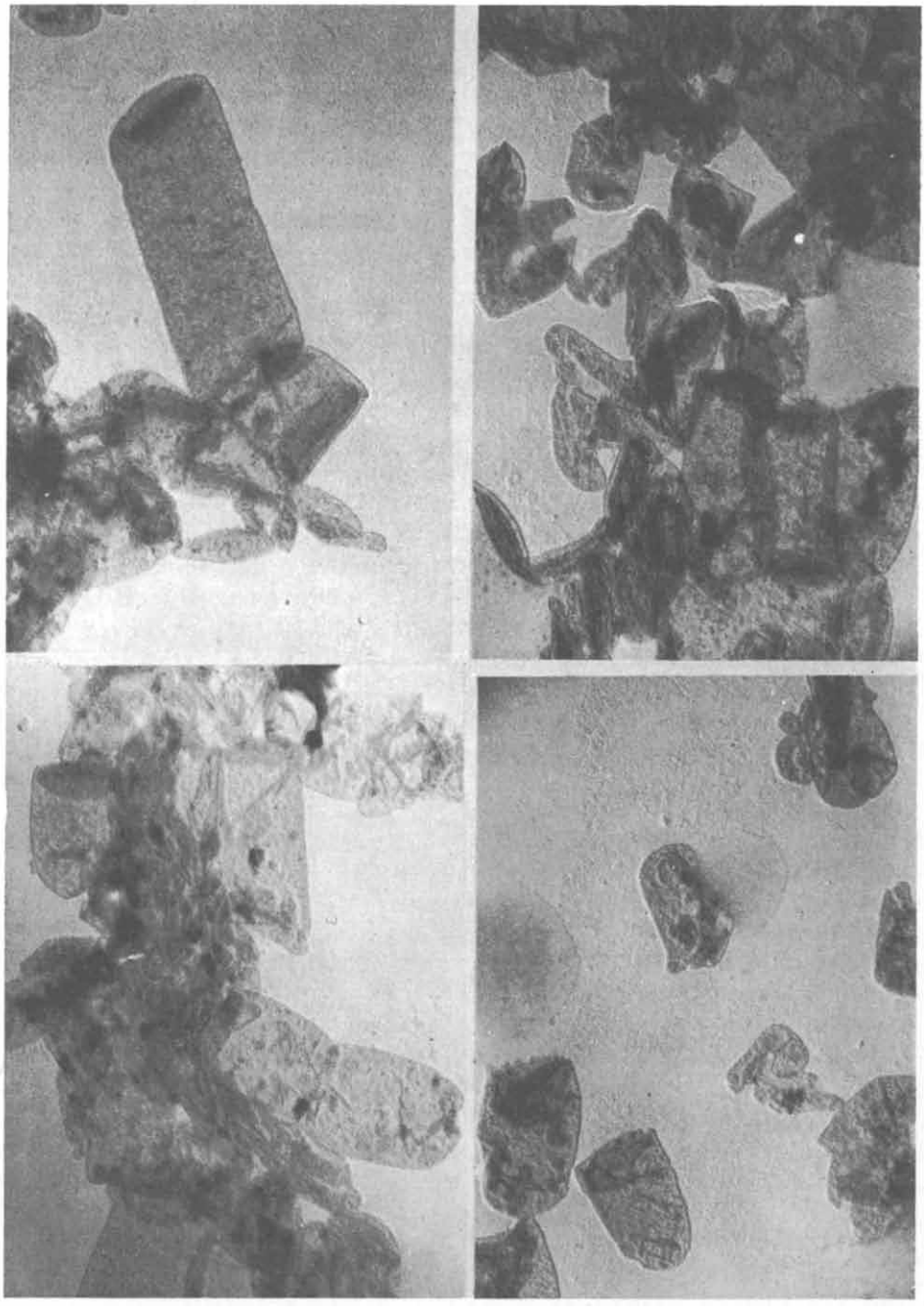

Fig. 9. Electron micrograph of cell walls of *Aerobacter*, isolated in the phosphate–polyethylene glycol 4000 system. Magnification 12000 ×. Shadowed with gold–manganin.

Cell walls from Aerobacter

The bacteria was disintegrated in the Mickle disintegrator according to [26]. 125 g of a suspension of disintegrated bacteria containing approx. 50 mg dry weight was shaken with 20 g PEG 4000

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and 71.5 g phosphate buffer in a separatory funnel. The surface of the interface was 80 cm^2. The bottom phase was collected and shaken with a new top phase of an equivalent phase composition. The bottom phase was collected and the cell walls in it were then washed by repeated centrifugations at 15000 *g* for 15 min. Fig. 9 shows electron micrographs of such a preparation. They were obtained by drying a suspension of the washed cell walls on a grid covered by a formvar film grid. It was shadowed with gold–manganin, and photographed in a modified Siemens electron-microscope which was run at 90 kV.

The cell walls have been used for studying the bacteriolytic activity of myxobacteria[27].

DISCUSSION

The following should be remembered in connection with the behaviour of particles in two phase systems. Since the surface properties are the main determining factors, the total composition of a particle does not give enough information if one wants to explain or predict how a particle will behave when it is shaken in a two phase system. Small amounts of substances adsorbed on the particle, either during their preparation or in the phase system, may therefore drastically change the behaviour of the particle. The prehistory of the particle is important. Of course, this is to be considered especially when one is dealing with particles of biological origin. Therefore, for example in the physiological state, the culture medium of cells or the way in which cell particles have been prepared must be standardized in order to get reproducible results.

On the other hand, the sensitivity to the surface properties of the particles may be utilized in order to get specific separation.

The above experiments suggest that separation of particles can be carried out in the following ways.

(1) By using different phase systems. Thus particles which go to the same phase in one system may go to opposite phases in another system (Table III). (Even the replacement of one polymer component by another molecular fraction of the same component may be used, see Table IV.) One then has to work near the plait point in order to avoid adsorption at the interface. This case is the simplest one, and fractionation can easily be performed on a large scale.

(2) By changing the composition of a system and utilizing different tendencies of the particles for adsorption at the interface (Fig. 7). In this case the area of the interface puts limitations as to the amount of substance which can be fractionated. However, because of the size of the particles, the adsorption capacity of the interface is comparatively large. If, for example, spherical particles with a density of 1 and with 1 μ radius are adsorbed at an interface to a closely packed layer, one particle thick, the capacity will be of the order of 0.1 mg/cm^2. For many purposes, therefore, a separatory funnel is enough for fractionation on a 10–100 mg scale. In order to fractionate larger quantities an arrangement like flotation would be possible and will be studied further. Likewise, the possibility of applying partition chromatography and counter current extraction will be investigated.

When a two-phase system is used for fractionation of cellular particles the main problem will, of course, be to find a phase system which can act as an adequate media for the particles and carry out separation in the same time. Only fractionation experiments on cellular particles, together with measurements of their characteristic activities before and after treatment in a phase system, will show how great the

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possibilities are to find such a system. However, from the experiments above the following may be pointed out.

(1) As is shown by the compositions of the phase systems described in this paper and those used for protein fractionation[2,3], there are phase systems available which present a great variation in chemical properties. By using polymers it is possible to obtain phase systems with high water content in both phases. If, then, low molecular substances are added to these systems it is believed that physiologically rather acceptable systems may be obtained. The systems, dextran–PEG, dextran– PVP, dextran–PVA, dextran–MC, and dextran–EHEC, seem from that point of view to be the most attractive ones. The concentrations of the polymers in these systems are of the same order as in solutions which have been used for preserving cell particles, or as plasma substitutes.

(2) Separation of particles may be effected in different systems. This is exemplified by the experiments reported in Table III and by the isolation of the cell walls from *Chlorella*, which could be isolated from either a salt–polymer or a polymer–polymer system.

Because of this and because of its experimental simplicity it is believed that the use of liquid two-phase systems for fractionation of cellular particles will serve as a useful complement to centrifugation methods.

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SUMMARY

1. The possibility of using liquid two-phase systems for the fractionation of particles of cellular dimension is discussed.

2. The compositions of some phase systems containing one or two water-soluble polymers as components are described.

3. The behaviour of some model particles in these phase systems has been investigated.

4. The phase systems have been used for the isolation of the cell walls from *Chlorella* and *Aerobacter*.

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INTRACELLULAR RIBONUCLEIC ACID COMPOSITION IN REGENERATING LIVER CELLS*

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Evidence exists in the literature that the nucleotide composition of ribonucleic acid (RNA) is characteristic of each liver cellular fraction[1,2,3,4]. On the other hand, the nucleotide composition of the RNA isolated from rat-liver tumor induced by 4-dimethylaminoazobenzene was shown to be more similar in all cell fractions[5]. The study of RNA composition was undertaken in fast growing liver in order to establish whether the changes observed in the intracellular RNA composition of liver tumor were characteristic of neoplastic growth or mainly of the high rate of mitosis of this tissue. Very few studies have been carried out on the RNA composition of regenerating rat liver. CROSBIE *et al.*[6] have reported that the nucleotide composition of the RNA isolated from cytoplasmic fractions of rat liver was not affected 26 hours post-hepatectomy. A very recent report by Cox[7] indicated that no changes could be detected 72 hours post-hepatectomy.

In this report results obtained on the nucleotide composition of RNA isolated from cell fractions of regenerating liver 18 and 72 hours post-hepatectomy are presented. 18 hours post-hepatectomy correspond to a period prior to the first wave of mitosis and 72 hours to the period of maximal cell concentration per unit of tissue[8]. Fasted and sham-operated animals were used as controls. The present study indicates a tendency to homogeneity in the nucleotide composition of particulate RNA in normal fast growing liver tissue.

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** Research Associate of the National Cancer Institute of Canada.

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