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The dynamics of phase partition

A study of parameters affecting rat liver organelle partitioning in aqueous two-polymer phase systems

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Separation of subcellular organelles by two-phase partition is thought to reflect differential partition of the organelles between the two phases or between one of the phases and the interface. Studies by Fisher and colleagues [Fisher & Walter (1984) Biochim. Biophys. Acta **801**, 106–110] suggest that cell separation by phase partition is a dynamic process in which the partition changes with time. This is mainly due to association of the cells with sedimenting droplets of one phase in the bulk of the other. Rat liver organelle partition was studied to determine whether the same dynamic behaviour is observed. Partition was clearly time-dependent during 24 h at unit gravity, and was also affected by altering the volume ratio of the two phases and the duration of phase mixing. These results indicate that, as with cells, the partition of organelles between phases is a dynamic process, and is consistent with the demonstration that organelles adhere to the phase droplet surfaces. Optimization of the volume ratio between phases may lead to significant processing economies. Organelle sedimentation in the upper phase was significantly faster than in the iso-osmotic sucrose. Theoretical modelling of apparent organelle sizes indicates that aggregation occurs in the poly(ethylene glycol)-rich upper phase. This phenomenon is likely to limit the use of this technique in organelle separations unless means can be found to decrease aggregation.

INTRODUCTION

Partition between two aqueous polymer phases can be used for the separation of biological particles such as cells and organelles (Albertsson, 1971; Albertsson *et al.*, 1982; Fisher, 1981). The particles generally partition between one of the two phases and the interface between them. Particles that differ in their partitioning behaviour can be separated either in a single step, or more usually with multiple extraction techniques. The basis for separation is believed to be related to differences in charge and/or hydrophobicity of the membrane surfaces. It thus provides an additional means of separation to the more commonly used techniques such as centrifugation.

The way in which cells and organelles partition is generally considered to be dependent on the polymer concentrations, pH and the concentration and type of salts present in the phase system (Albertsson *et al.*, 1982; Morris & Peters, 1982). Partition has also been considered an equilibrium process, but studies showing the time-dependency of erythrocyte partition suggest that it may be a dynamic one (Raymond & Fisher, 1980; Fisher & Walter, 1984). Raymond & Fisher (1980) attribute this effect to the association of erythrocytes to small droplets of lower phase present in the upper phase, which slowly sediment long after the horizontal interface has formed. The cells are carried by these settling microdroplets to the interface, and their apparent partition into the upper phase thus decreases with time.

In the present study we have investigated whether the partition of organelles is dynamic and if a similar droplet-associated sedimentation mechanism operates.

An understanding of the basis for partition is necessary before the design and operation of successful multi-stage extraction techniques can be achieved. These tests have been performed with single-tube partitions in unit gravity; the effects of enhanced gravity require further consideration.

EXPERIMENTAL

Materials

Dextran T 500 (batch 16027) was obtained from Pharmacia (Uppsala, Sweden). Breox PEG 6000 was obtained from Hythe Chemicals (Southampton, U.K.). All other reagents were standard A.R. grade.

Preparation of phase mixtures

Phase systems (150 g) were prepared containing 3.3% (w/w) dextran T 500, 5.4% (w/w) PEG 6000, 10 mM-sodium phosphate buffer, pH 7.4, 0.26 M-sucrose, 0.05 mM-Na_2EDTA and 1 mM-ethanol. When a phase mixture had equilibrated to 4 °C, it was shaken and allowed to separate overnight at 4 °C into two phases. This phase-system composition was established during previous studies on rat liver homogenate (Heywood-Waddington *et al.*, 1984).

Stock solutions of the polymers, 20% (w/w) dextran T500 and 40% (w/w) PEG 6000, were used to prepare the phase systems, and were stored at −20 °C before use. An accurate determination of the dextran concentration was obtained by measuring optical rotation (model 141 polarimeter; Perkin–Elmer, Beaconsfield, Bucks., U.K.).

Abbreviation used: PEG 6000, poly(ethylene glycol) of M_r approx. 6000.

Sample preparation

Fed male Sprague–Dawley rats (150–200 g) were stunned and killed by cervical dislocation. The liver was immediately removed and 0.5 g of perilobular tissue was minced with a scalpel blade. The tissue was then disrupted, with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.), in 10 ml of ice-cold PEG-rich upper phase. Water (0.4 g) had previously been removed by evaporation with a stream of N_2, to allow for the water content of the liver tissue. The tissue received ten strokes of a loose-fitting (type A) pestle, followed by ten strokes of a tight-fitting (type B) pestle. Fibrous material was removed with a 50 μm-mesh gauze filter.

Single-partition studies

In order to determine the effect of time on the partition of liver organelles, a series of partitioning tubes (1 cm internal diam.) were prepared containing 2 ml of lower phase and 1.8 ml of upper phase. Freshly prepared homogenate (0.2 ml) was added to each tube, the contents were mixed by 15 inversions of the tube, and the organelles were allowed to partition for various periods. During partitioning the tubes were placed in a cold cabinet at 4 °C. When each tube had partitioned for the required time (20 min to 24 h), a sample (1 ml from the upper sector of the upper phase) was removed and stored at 4 °C. After all the samples had been collected, they were stored for future analysis at −20 °C.

A similar procedure was followed to determine the effect of phase volume ratio on partition. The volume of the lower phase in a series of partitioning tubes was varied between 2 ml and 0.04 ml, the upper-phase volume remaining constant at 1.8 ml plus 0.2 ml of homogenate. The time allowed for partitioning was 40 min.

The effect of mixing intensity was examined by following a shorter time course than the one described above, 20 min to 4 h, for three mixing conditions: three, 15 or 30 tube inversions.

Sedimentation of organelles

Gravity sedimentation of organelles was studied in two different media: PEG-rich upper phase and iso-osmotic sucrose. Freshly prepared homogenate (0.2 ml) was added to 1.8 ml of medium, the sample having been homogenized in the same medium. The upper phase was taken from a phase system that had been separated at 1 °C. Although this will give slightly different upper-phase composition, it should ensure that at 4 °C there are no lower-phase droplets present. The tube contents were mixed by 15 inversions of the tube, and a sample (1 ml) from the upper sector of the liquor was removed after various time periods and stored before analysis as for single-partition studies. The experiments were performed in a cold-room at 4 °C.

Analytical methods

The distribution of the organelles was determined by assaying the samples for the following marker enzymes: γ-glutamyltransferase, EC 2.3.2.2 (plasma membrane) (Smith *et al.*, 1979); β-*N*-acetyl-D-glucosaminidase, EC 3.2.1.30 (lysosomes) (Peters & Shio, 1976); neutral α-D-glucosidase, EC 3.2.1.20 (endoplasmic reticulum) (Peters & Shio, 1976); lactate dehydrogenase, EC 1.1.1.27 (cytosol) (Peters & Shio, 1976). All assays, except that for γ-glutamyltransferase, were performed on an Automatic Chemistry Unit (Aura Systems; Pye–Unicam, Cambridge, U.K.) (Shah *et al.*, 1984). These marker enzymes were chosen to represent organelles with a range of partition ratios (Morris & Peters, 1982) and also the cytosolic fraction. The percentage of enzyme activity in the samples can be used as an indication of the degree of sedimentation in a single-phase system, or of the partition ratio (the ratio between the amount of substance in the top phase and that at the interface or bottom phase) in a two-phase system.

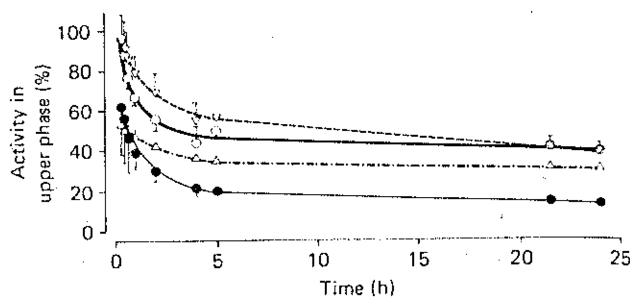

Fig. 1. Variation of partition in the upper phase of liver organelles with time

Results are means ± ranges for four experiments (two experiments only at 5, 21.5 and 24 h). Marker enzymes, with organelles shown in parentheses, are: ○, γ-glutamyltransferase (plasma membrane); ▽, β-*N*-acetyl-D-glucosaminidase (lysosomes); ●, neutral α-D-glucosidase (endoplasmic reticulum); △, lactate dehydrogenase (cytosol).

RESULTS

The change in the partitioning behaviour of the four marker enzymes with time is shown in Fig. 1. The behaviour was examined after the formation of the initial interface, by which time the organelles were differentially distributed between the two phases. After this, the partition of liver organelles appears to be time-dependent: after 24 h the activity of the membrane markers in the upper phase had decreased by 60–80% of the value at 20 min. The activity of the cytosolic marker in the upper phase also decreases with time, but to a lesser extent. The rate of decrease of partition in the upper phase is non-linear for all the marker enzymes.

Organelle sedimentation in PEG-rich upper phase was studied to establish if the decrease in apparent partition with time was due to a partitioning phenomenon or to gravity sedimentation. The percentage of suspended activity was found to decrease with time: 40–70% had settled after 24 h (Fig. 2). However, the initial rate of decrease is faster in the complete phase mixture than in the upper phase only, leading to most of the material reaching the interface within only 4 h. When iso-osmotic sucrose is used as the suspending medium (Fig. 3), the percentage of settled material is much lower than in PEG-rich upper phase and does not exceed 20% even after 24 h. In both iso-osmotic sucrose and upper phase, the rates of sedimentation are linear except for γ-glutamyltransferase, where at least two rates are observed.

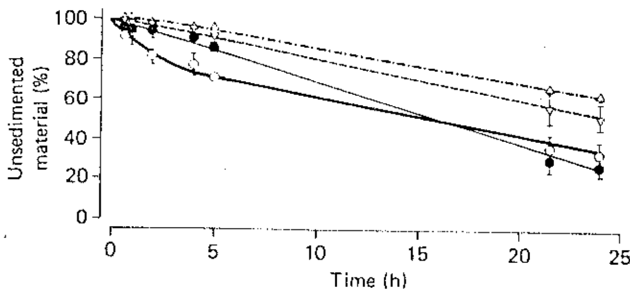

Fig. 2. Sedimentation of liver organelles in upper phase (no lower-phase droplets present)

Results are means ± ranges for three experiments. Further details are as in Fig. 1 legend.

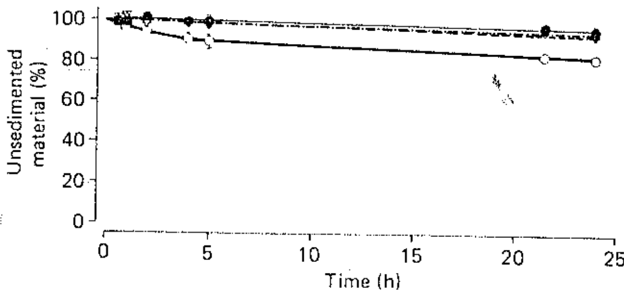

Fig. 3. Sedimentation of liver organelles in iso-osmotic sucrose

Results are means ± ranges for four experiments. Further details are as in Fig. 1 legend.

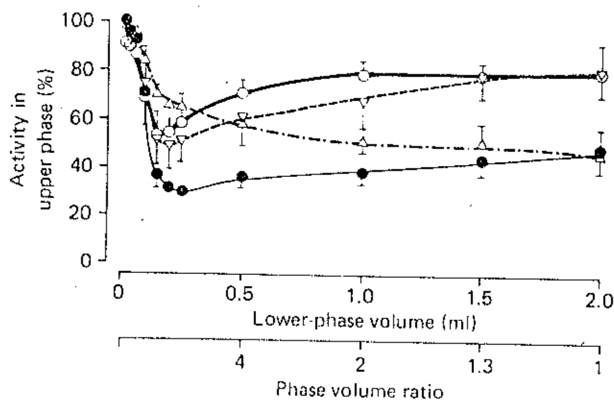

Fig. 4. Partition of liver organelles in the upper phase as a function of lower-phase volume

Results are means ± S.D. for five experiments. Further details are as in Fig. 1 legend.

The effect on partitioning behaviour of varying the lower-phase volume is shown in Fig. 4. Looking at the graph from right to left shows that a decrease in the volume initially causes a decrease in apparent partition of the organelle marker enzymes into the upper phase; a minimum value is reached at a volume ratio of 10:1, which is followed by an increase in activity in the upper phase. In contrast, the cytosolic marker enzyme, lactate dehydrogenase, shows a progressive increase in activity in the upper phase with decreasing lower-phase volume.

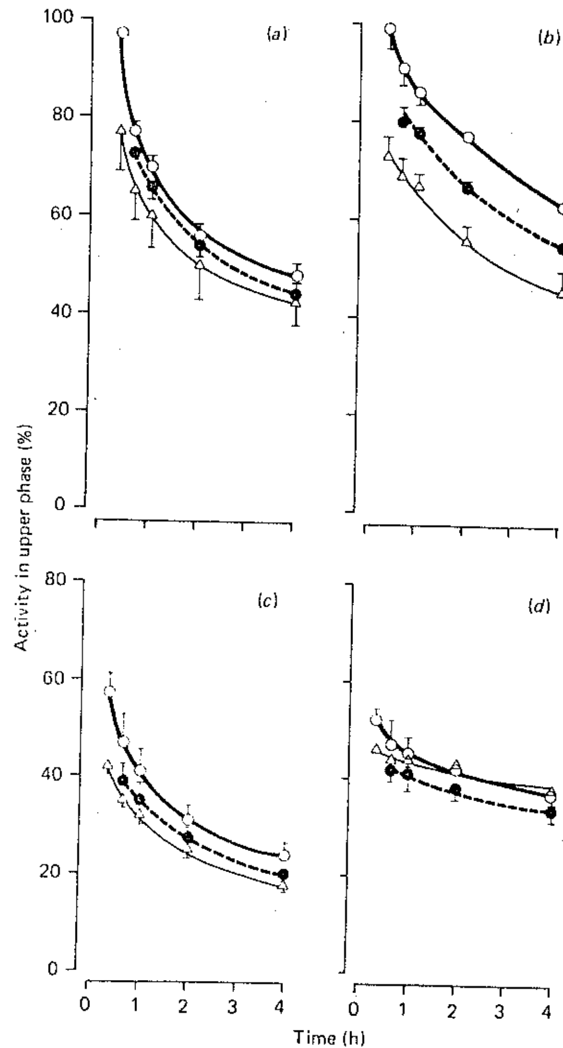

Fig. 5. Influence of mixing on the partition of γ-glutamyltransferase (*a*), β-*N*-acetyl-D-glucosaminidase (*b*), neutral α-D-glucosidase (*c*) and lactate dehydrogenase (*d*)

Results are means ± ranges for three experiments. Number of inversions of partitioning tube: ○, 30; ●, 15; △, 3.

The experimental variation is large, but a consistent trend was always observed.

Fig. 5 shows the effect of varying the degree of mixing on the apparent partition of the organelle and cytosolic marker enzymes. The partition of the lysosomal marker enzyme into the upper phase decreases as the amount of mixing is decreased. The other organelles show a similar trend, but to a lesser extent. The cytosolic marker does not exhibit this trend, but has similar upper-phase activities for each mixing intensity at each time point.

DISCUSSION

Analysis of the organelle settling curves will allow determination of the size of the sedimenting species. The species size should reflect the degree of organelle aggregation or association with lower-phase microdroplets. In order to compare the size of the species sedimenting in upper phase, iso-osmotic sucrose and complete phase system, it is necessary to take into

Table 1. Apparent organelle diameters calculated from their sedimentation rates in various media

The values were calculated by using the method shown in the Appendix. T_i is the time taken for particles to settle. The density of the subcellular organelles was taken as 1.1 g·ml⁻¹ (Ridge, 1978; Evans, 1979; Beaufay *et al.* 1974); densities of iso-osmotic sucrose, upper phase and lower phase were 1.034, 1.046 and 1.084 g·ml⁻¹ respectively. The viscosity of iso-osmotic sucrose is 1.97 mPa·s (Ridge, 1978), and that of upper phase 52.7 mPa·s. The temperature was 4 °C.

		Stokes settling diameter (μm)		
Medium	T_i (h)	Plasma membrane	Lysosomes	Endoplasmic reticulum
Complete phase mixture	1	92	77	83
	4	41	43	43
	> 24	7	12	11
Upper phase only	2–3	44	—	—
	> 24	11	10	12
Iso-osmotic sucrose	2–3	8	—	—
	> 24	0.9	0.6	0.6

account the different densities and viscosities of the media. This can be done by using Stokes Law and Curtain Theory (see the Appendix) to calculate the diameters of the various species from their sedimentation rates (Table 1).

The calculated diameters of the organelles in iso-osmotic sucrose are similar to those obtained by measurement from electron micrographs (not shown). Comparison of the apparent lysosome diameters in iso-osmotic sucrose (0.6 μm) and in upper phase only (10 μm) indicates that aggregation, as well as possibly swelling, is occurring in upper phase. Similar aggregation of endoplasmic reticulum and plasma membrane is indicated (Table 1). The variation of apparent size with settling time reflects the heterogeneity of the aggregates and organelles themselves.

The rate of organelle sedimentation in upper phase only is lower than the initial rate observed in the complete partition mixture, which indicates that sedimentation of the organelle aggregates does not totally account for the decrease in apparent partition with time. The shape of the settling curves obtained in the partition mixture suggests that there are several sizes of sedimenting species. The diameters of the species involved can be estimated by fitting three sedimentation rates to the settling curves (see the Appendix). The diameters of the species that settle during the first 4 h (41–92 μm) are greater than the diameter of the aggregated membrane settling in upper phase only (Table 1). The most likely explanation is that the dextran-rich microdroplets present in the upper phase of the complete phase system are associated with the organelles and facilitate their transport to the interface (Raymond & Fisher, 1980). The diameter of the species still settling after 24 h are 7–12 μm. These values are obtained from calculations based on the species being either droplet-associated or aggregated membrane; it is difficult to distinguish between these, because of their similarity in density.

Thus it appears that organelles (aggregated/unaggregated) settle in association with dextran-rich microdroplets present in the upper phase, with the size of the droplets remaining in suspension decreasing with time. During the period 4–24 h only small droplets (< 12 μm) will remain, and the sedimenting species is likely to be either unaggregated membrane on these droplets or aggregated membranes alone. Fig. 6 is a phase-contrast micrograph of upper phase from a complete phase mixture containing rat liver homogenate. It shows aggregated organelles attached to the interface of dextran-rich microdroplets present in the PEG-rich upper phase after 45 min partitioning. The average size of lower-phase microdroplets shown is less than might be expected: this is probably due to movement of the larger droplets out of the field of view during placement of the cover-slip over the sample.

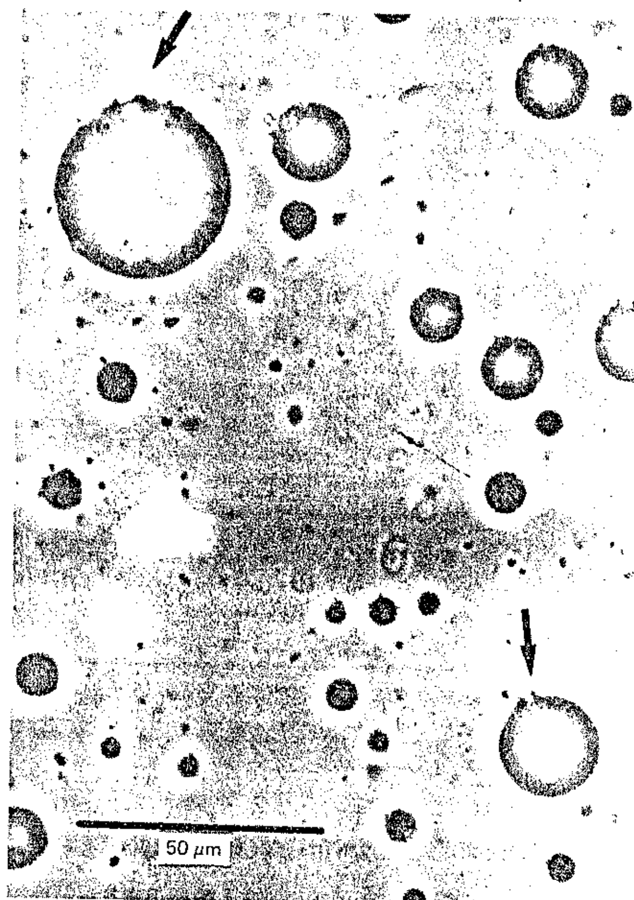

Fig. 6. Light micrograph (phase contrast) of organelles (↑) attached to dextran-rich droplets present in the upper phase from a complete phase mixture

The decrease in partition of soluble lactate dehydrogenase is likely to reflect sedimentation of enzyme adsorbed to membranes, but some sedimentation of aggregated protein could have occurred. The volume of the microdroplets is so small that it is not thought that settling of these has a significant effect on partition of soluble components.

Raising the volume ratio in the phase mixture from unity by decreasing the lower-phase volume is likely to cause less violent streaming of the lower phase through the upper phase during and after the mixing step. This would allow membranes to remain attached to the larger

descending droplets and reach the interface at a greater rate. Initially a decrease in partition into the upper phase is observed with decreasing lower-phase volume. However, a point is reached when further decreases in volume cause an increase in the amount of membrane in the upper phase. This is probably due to insufficient microdroplet surface area to transport the membrane to the interface. At very low volumes of lower phase there are so few droplets that nearly all the activity remains in the upper phase. From a practical point of view, it is interesting to note that the lower-phase volume can be decreased by 75% and similar differences in organelle partition still be obtained. Soluble material partitions such that the ratio of concentrations in the lower and upper phases remains constant; thus a decrease in lower-phase volume should cause an increase in the amount of material in the upper phase. This is observed for the cytosolic marker enzyme, lactate dehydrogenase.

The trend observed on variation of the degree of mixing can also be explained in terms of droplet-mediated transport to the interface. When a high degree of mixing is used, the droplets of lower phase formed in the upper phase are likely to be smaller and so take longer to settle to the interface. At a given time the partition into the upper phase would therefore be greater; the lysosomal marker enzyme shows this effect to the greatest extent. The soluble marker was unaffected by mixing, since its partition is not droplet-dependent. There is a small decrease in lactate dehydrogenase partition with time, which may be due to adsorption of activity on membranes.

The observations made in this study indicate that a similar droplet-associated mechanism of partition operates for organelles as for whole cells (Raymond & Fisher, 1980). It was also noticed that contact with upper phase appears to cause aggregation of the rat liver membranes. This could be contributing to the limited resolution of organelles obtained by toroidal-coil two-phase chromatography (Sutherland *et al.*, 1984). The components in the phase mixture causing the aggregation are at present unknown, but it is clear that these considerations should be taken into account in organelle subcellular fractionation by phase partition. The observation that a decreased lower-phase volume can be used to obtain similar relative partitioning behaviour of organelles could save expensive dextran-rich lower phase, especially when working on large-scale industrial preparations.

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APPENDIX

Curtain Theory (I. A. Sutherland, S. D. Flanagan, D. Heywood-Waddington & D. Fisher, unpublished work)

The proportion (P) of particles (quantity A_i, diameter d_i, density ρ_i) in the sampled liquor (viscosity μ_0, density ρ_0, height L_0), at time t, is given by:

$$P \doteq \sum_{i=1}^{n}\left[A_i\left(1-\frac{t}{T_i}\right)_{0<t<T_i}\right] \qquad (1)$$

where T_i is the time taken for all the particles to settle out of the sampling zone (L_0). This sedimentation time, T_i, can be approximated, by using Stokes Law, to:

$$T_i = \frac{18\mu_0 \cdot L_0}{(\rho_i-\rho_0)\cdot g \cdot d_i^2} \qquad (2)$$

where g is acceleration due to gravity. For example, if there are predominantly two groups of particles A_1 and A_2 of diameter d_1 and d_2, the settling curve would be as shown in Appendix Fig. 1.

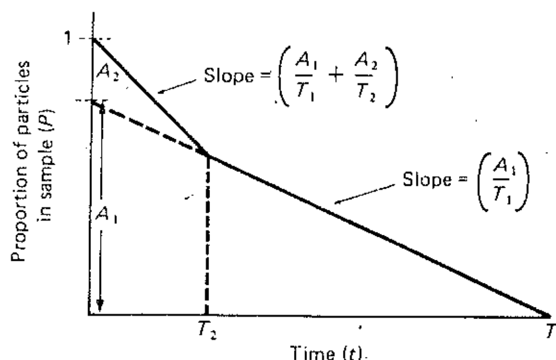

Fig. 1. Variation, with time, of proportion of particles in sampling zone

The curve shown is for the sedimentation of a mixture of two species.

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