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Simultaneous accumulation of low-molecular-mass RNA at the interface along with accumulation of high-molecular-mass RNA on aqueous two-phase system partitioning

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Abstract

On partitioning in the potassium phosphate–PEG aqueous two-phase system, the simultaneous accumulation of the low-molecular-mass RNA at the interface along with accumulation of the coexisting high-molecular-mass RNA was quantitatively shown. The low-molecular-mass RNA was inherently partitioned between the top and bottom phases if partitioned alone. However, the low-molecular-mass RNA was caught in the interface to a significant extent if partitioned with the coexisting high-molecular-mass RNA. The degree of the accumulation of low-molecular-mass RNA increased with an increase in the content of the coexisting high-molecular-mass RNA, while the high-molecular-mass RNA was mostly accumulated at the interface.

Keywords: Partitioning; Aqueous two-phase systems; RNA

1. Introduction

During the partition of biomaterials in aqueous two-phase systems, the phenomenon that a significant amount of biomaterials is accumulated at the interface is often observed [1–6]. This phenomenon is reproducible and applicable as an easy and effective method for condensation or separation [1–3] because it sometimes collects almost all the target at once in a short time. On the contrary, the accumulation at the interface sometimes causes interference with the recovery of the target in the phases by an undesirable entrapment [7]. The accumulation at the interface can arise from the selective partition to the interface and also it can be attributable to the formation of a third phase by the precipitation from the phase as a result of its supersaturation [8]. The accumulation phenomenon of the former is expressed as ‘adsorption at the interface’ by Albertsson [4–6,8], on the partitioning of particles, such as bacterial cells and cellular organelles. In the systems on the identical tie-line, a soluble substance usually partitions based on the concentration-ratio, which represents a steady partition coefficient regardless of the concentration initially added and in spite of the phase volume ratio. On the other hand, when the adsorption accompanies the partition, the amount ‘adsorbed’ at the interface is proportional to the amount partitioned to the one phase regardless of the concentration initially added and in spite of the phase volume ratio [8]. In this case, the partition coefficient changes along with phase volume ratio but the ‘amount-ratio’ between the ‘adsorbed’ amount and the amount partitioned to one of the phases does not change in spite of the phase volume ratio. The nature of this interesting phenomenon, however, has not been fully

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investigated in detail. Most of the observations about the accumulation are only for the particles partitioning.

Previously, we quantitatively demonstrated the accumulation of RNA along with partition as a unique example of the accumulation of a soluble substance. In some two-phase systems [9], the high-molecular-mass RNA is accumulated at the interface. The high-molecular-mass RNA is strictly 'adsorbed' at the interface in the potassium phosphate–PEG systems with PEG of a restricted molecular mass (1000–3000) [10]. The 'adsorbed' amount is proportional to the added amount, regardless of the concentration and the phase volume ratio [11]. It is interesting that the accumulation behavior of a soluble substance along with partition is similar to the unique phenomenon of the particles, which is described above [8].

On the other hand, the low-molecular-mass RNA fundamentally partitioned between the phases as a typical solute. On the partition in the presence of the high-molecular-mass RNA, the low-molecular-mass RNA mainly partitioned between the phases but was also caught in the interface to a significant degree at the same time. The accumulation of a low-molecular soluble substance along with partition is unique. In this paper, the interesting phenomenon of the simultaneous accumulation of the low-molecular-mass RNA along with accumulation of the high-molecular-mass RNA, depending on the coexistence of the high-molecular-mass RNA, has been investigated in detail.

2. Experimental

2.1. Materials

Polyethylene glycol 1540 (M_r=1500, expressed as PEG1500 in this study) and polyethylene glycol 4000 (M_r=3000, expressed as PEG3000) were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade. Potassium phosphate was a mixture of K_2HPO_4 and KH_2PO_4 with a molar ratio of 10 to 7 [12].

2.2. RNA preparation

Seven preparations of RNA were used in this study. RNA preparation I was a commercially available reagent (RNA 109223; Boehringer Mannheim GmbH, Mannheim, Germany) extracted from yeast. It showed a broad band on agarose electrophoresis that corresponded to a low-molecular-mass RNA of less than 70 bases. RNA preparations II, III, IV, V, VI and VII were prepared from baker's yeast by phenol–chloroform extraction and ethanol precipitation with diethylpyrocarbonate [13]. These RNA preparations II–VII were extracted from the same origin, baker's yeast. The methods for the extraction and the purification were fundamentally the same although the number of phenol extractions, ethanol precipitations and fractionations and other minor treatments for separation were not consistent among the preparations. RNA preparations II, III, IV and V were natural cellular 'total RNA', which contained all types of RNAs; ribosomal RNAs, tRNA and mRNA. RNA preparations VI and VII were also natural cellular RNA mixtures but increased in the low-molecular-mass RNA content by the fractional extractions, as follows. A pellet, by ethanol precipitation of the total RNA preparation, was suspended in 3 *M* sodium acetate solution. The fraction with a high content of low-molecular-mass RNA was obtained from the supernatant of the suspension after centrifugation at $3000\times g$ for 10 min. The high-molecular-mass RNA contents of the preparations were as follows: RNA preparation I, 0%; preparation II, 72%; preparation III, 73%; preparation IV, 75%; preparation V, 75%; preparation VI, 40%; preparation VII, 1.3%.

The RNA preparations were suspended in sterile distilled water and stored at −80°C. The RNA preparations contained less than 2% DNA as measured by Burton's method [14] and had A_{260}/A_{280} values greater than 2.0.

2.3. Analysis

The RNA concentration was determined using the GPC-mode HPLC system as described in a previous paper [11]. In order to describe the apparent difference of RNA partitioning depending on its molecular

mass, the RNA was demonstrated to be isolated as two groups: 'high-molecular-mass RNA' comprised of 26S and 17S rRNAs and 'low-molecular-mass RNA' comprised of 5.8S and 5S rRNAs, tRNAs and lower molecular-mass RNAs.

2.4. Two-phase systems

The two-phase systems used were potassium phosphate–PEG1500 and potassium phosphate–PEG3000 systems with 5 m*M* Na_2HPO_4 and 5 m*M* NaH_2PO_4, as shown in Fig. 1. The two-phase system in a graduated test tube with a total weight of 2.5–10 g was mixed with an RNA preparation by shaking upside down at 4°C. The two phases separated by centrifugation at 200×*g* for 2 min or at 600×*g* for 10 min were separately withdrawn at 4°C. RNA concentrations and phase volumes were determined for the initial mixture and top and bottom phases. The RNA amount accumulated at the interface was estimated from the difference in the amount between that initially added and partitioned in the phases. A series of systems with various phase volume ratios on the identical tie-line were prepared by mixing the solutions of the top phase and bottom phase which were partitioned beforehand without RNA and withdrawn separately, with adequate ratios. Every instrument, which came into contact with the RNA, and all the solutions except the RNA preparation were autoclaved before use.

3. Results and discussion

3.1. Partitioning of low-molecular-mass RNA

Fig. 2A shows the partition behavior of the low-molecular-mass RNA (RNA preparation I) in the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 aqueous two-phase system. It partitioned between the top and bottom phases even for the initial concentration up to 1.75 mg/ml. Its partition coefficient and percental distribution were constant in spite of the added concentration. The clear solutions of the top and bottom phases with the clear interface were observed even upon its partitioning at a high initial concentration. The examined two-phase system gave about 24% (w/w) PEG1500-containing top phase and about 21% (w/w) potassium phosphate-containing bottom phase. The low-molecular-mass RNA of preparation I was partitioned in both phases being soluble in each phase solution. At the maximum 2.2 mg/ml low-molecular-mass RNA was soluble still in the 21% (w/w) potassium phosphate-

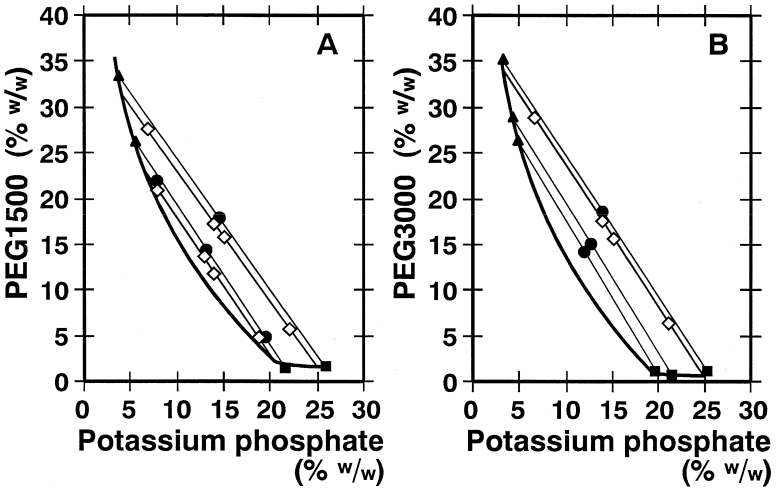

Fig. 1. Phase diagram of the potassium phosphate–PEG1500 system (A) and the potassium phosphate–PEG3000 system (B) with 5 m*M* Na_2HPO_4 and 5 m*M* NaH_2PO_4 at 4°C. Total compositions of the systems (●); composition of the top phase (▲); composition of the bottom phase (■). Total composition of systems with various phase volume ratios on the tie-line used for the partitioning study is shown (◇).

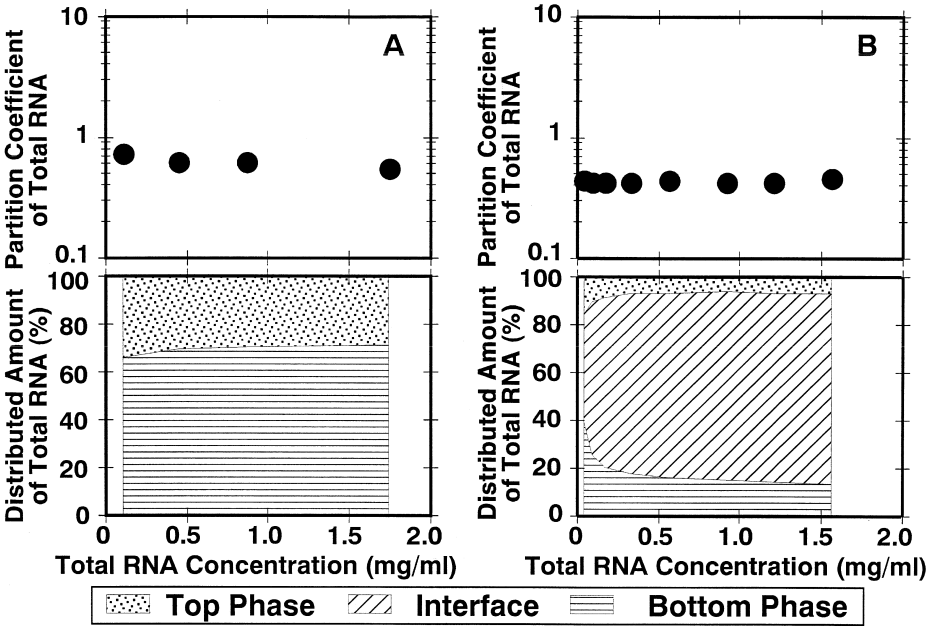

Fig. 2. Effect of the RNA molecular-mass on the accumulation accompanying the partition in the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system. (A) Low-molecular-mass RNA (RNA preparation I); (B) natural cellular RNA (RNA preparation II). RNA partition behavior is expressed in two indications. The upper figures show the partition coefficient, the ratio of the RNA concentration in the top phase to the RNA concentration in the bottom phase. The lower figures show the RNA amounts partitioned to the top phase, the interface and the bottom phase as a percentage of the total RNA amount initially added, being represented by the areas in spot, slanting shade and horizontal shade, respectively.

containing bottom phase. The low-molecular-mass RNA was inherently soluble up to a high concentration and partitioned between phases.

On the other hand, the partitioning became different in the case of the natural cellular 'total RNA' that contained low- and high-molecular-mass RNAs together, as shown in Fig. 2B. The white material, just like suspended white clouds, was found at the interface of the phases. Almost all the material collected at the interface was the high-molecular-mass RNA, which corresponded to more than 90% of the added amount of the high-molecular-mass RNA. The significant accumulation of the high-molecular-mass RNA is recognized as 'adsorption' along with partition [11]. The concept of the word 'adsorption' represents the selective partitioning to the interface [8]. The high-molecular-mass RNA partitioned only to the interface and bottom phase, consequently, its inherent partition coefficient could not be evaluated. Therefore, the RNA partition behavior will be represented by the 'distributed amount', hereafter in this paper, to show it actually. The partition coefficient of the natural cellular total RNA was also constant in spite of the added concentration but showed lower values than the case of the partition of low-molecular-mass RNA alone.

3.2. Simultaneous accumulation of high- and low-molecular-mass RNA

Natural cellular total RNA typically consists of 70–80% high- and 20–30% low-molecular-mass molecules if prepared without any fractionation and any decomposition. In order to investigate the effect of the molecular-mass content of the RNA on its accumulation along with partition, a series of natural cellular RNA was prepared with various contents of high-molecular-mass RNA from 5% to 78% by mixing the several natural cellular RNA preparations. Fig. 3 shows the partition in the potassium phosphate–PEG1500 systems with various phase volume ratios. The accumulation of the low- and

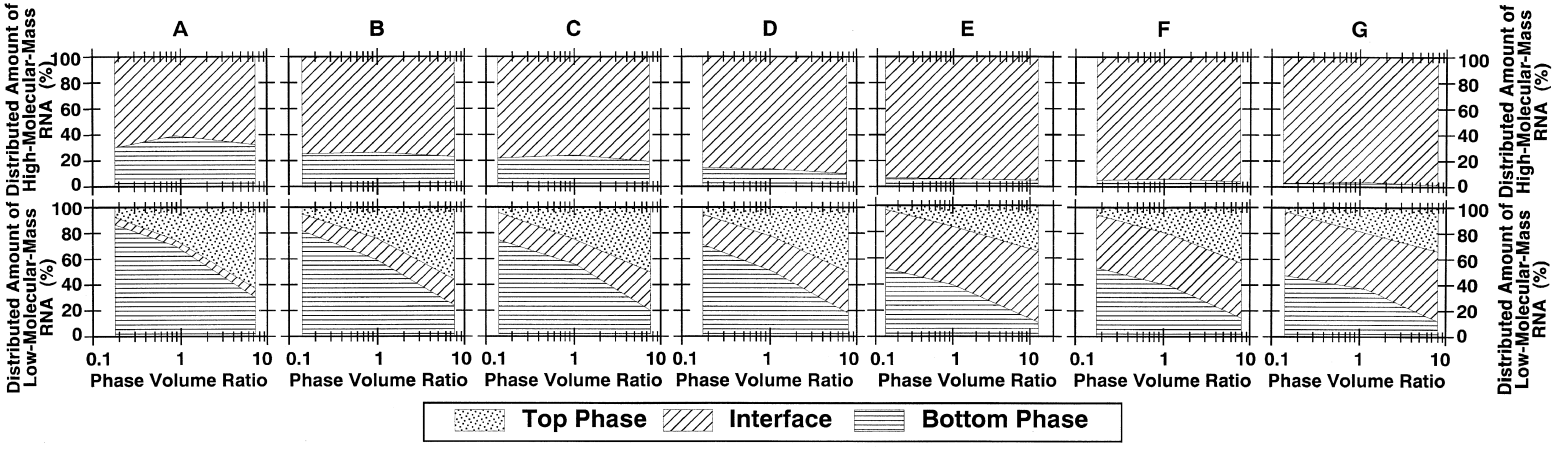

Fig. 3. Effect of the high-molecular-mass RNA content on the RNA accumulation in the potassium phosphate–PEG1500 system. The systems with different phase volume ratios on the identical tie-line, which the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system lay on, were examined. A series of the high-molecular-mass RNA contents was achieved by mixing the natural cellular RNAs (RNA preparations III, IV, VI and VII). RNA was added at the initial concentration of 0.2 mg total RNA/ml. The high-molecular-mass RNA content was as follows: 5% (A), 23% (B), 39% (C), 52% (D), 61% (E), 69% (F) and 78% (G). The RNA amounts partitioned to the top phase, the interface and the bottom phase as a percentage of the total RNA amount initially added, are shown separately for the high- and low-molecular-mass RNA.

high-molecular-mass RNA increased with an increase in the high-molecular-mass RNA content coexisting in the preparation. The natural total RNA preparation that was completely extracted from the yeast cells, such as in Fig. 3F and G, contained 70–80% high-molecular-mass. In the case of these preparations, more than 95% of the high-molecular-mass RNA and 40–50% of the low-molecular-mass RNA were simultaneously accumulated at the interface. On the other hand, the preparation that contained only 5% high-molecular-mass RNA, shown in Fig. 3A, had about 65% and 6% accumulation of the high- and low-molecular-mass RNA, respectively.

The percental distribution of the high-molecular-mass RNA and the accumulation of the low-molecular-mass RNA were almost constant in spite of the phase volume ratio in each preparation. On the partition of the lower content of the high-molecular-mass RNA, such as Fig. 3A–C, the accumulation of the high-molecular-mass RNA was relatively poor. The remainder of the high-molecular-mass RNA partitioned only to the bottom phase, whereas the low-molecular-mass RNA partitioned between phases. The partition coefficient of the low-molecular-mass RNA was relatively constant (0.3–0.7) in spite of the high-molecular-mass RNA content in the preparation and slightly decreased with an increase in the phase volume ratio for each preparation (data not shown). Thus the percental distribution of the low-molecular-mass RNA is found to be dependent on the phase volume ratio. On the contrary, the percental distribution of the high-molecular-mass RNA and the accumulation of the low-molecular-mass RNA depended on the high-molecular-mass RNA content. The fact that the partition of the high-molecular-mass RNA along with accumulation is dependent on the amount-ratio was shown in a previous paper [11]. It is interesting that the low-molecular-mass RNA inherently partitions on the concentration-ratio basis between the phases but seems to be accumulated at the interface based on the amount-ratio, simultaneously.

3.3. Accumulation of various RNA mixtures

The correlation between the RNA molecular-mass content and the RNA accumulation along with partition is summarized in Fig. 4A from the data of Fig. 3. The accumulation of the natural cellular RNA with various high-molecular-mass RNA contents is separately shown for the high- and low-molecular-mass RNA. The simultaneous accumulation of the high- and low-molecular-mass RNA was obviously increased with an increase in the high-molecular-mass RNA content in the preparation.

Fig. 4B shows the case of the mixture of the low-molecular-mass RNA preparation (RNA preparation I) and the natural cellular RNA (RNA preparation II), which were obtained from the different origins to one another. The accumulation of the low-molecular-mass RNA increased with an increase in the high-molecular-mass RNA content, also in this case. The accumulation of the low-molecular-mass RNA was enhanced by the increase in the high-molecular-mass RNA content, even if the accumulated amount of the low-molecular-mass RNA introduced from the RNA preparation II was eliminated, as shown in Fig. 4B. The accumulation of the low-molecular-mass RNAs from different origins increased with an increase in the high-molecular-mass RNA content.

The conformation of RNA molecules in the two-phase solutions is unknown. More than 80% of the cellular total RNA is ribosomal RNA. Within a ribosome of a eukaryotic cell, it is known that the 5.8S rRNA and 28S rRNA (26S rRNA in the case of yeast) form complementary base pairs at the specific site in its bigger subunit. Further, an existence of any other sites, which have complementarity between the native molecules, can be estimated among the natural RNAs. From the results of Fig. 4A, there can be the following possibility. Some of the complementary base pairs between the high- and low-molecular-mass RNA can be kept or rebuilt during the partition. And the base-paired RNA would go to the interface, acting as a high-molecular-mass RNA. However, from the data of Fig. 4B, the complementary base pairs between the RNA molecules do not mainly contribute to the simultaneous accumulation for the following reason. Preparation I alone had a different origin from the other preparations. Moreover, preparation I contained only molecules broken down into ones much smaller than natural RNA. There is very little possibility that preparation I originally has and still keeps specific binding sites with the high-mole-

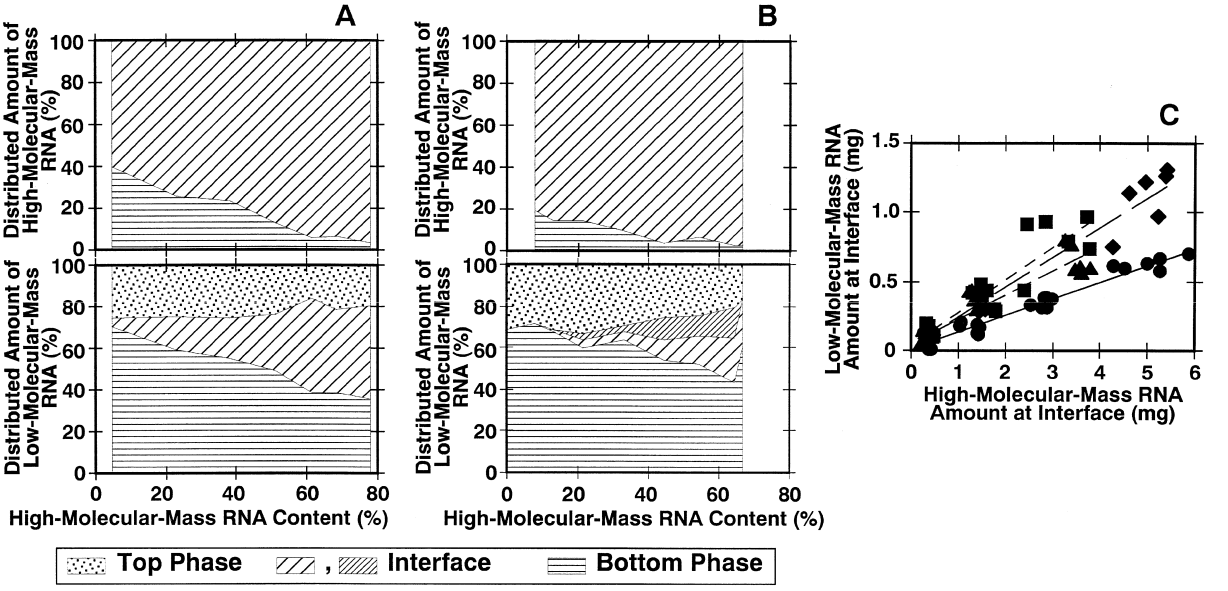

Fig. 4. Accumulation of various RNA preparations and their mixtures in the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system. A series of the high-molecular-mass RNA contents was achieved by mixing the RNA preparations. (A) Mixture of the natural cellular RNA preparations from the same origin (RNA preparations III, IV, V, and VI) at the initial concentration of 0.2 mg total RNA/ml. (B) Mixture of the natural cellular RNA (RNA preparation II) and the low-molecular-mass RNA (RNA preparation I), obtained from different origins to one another, at the initial concentration of 0.1–1.3 mg total RNA/ml. The RNA amounts partitioned to the top phase, the interface and the bottom phase as a percentage of the total RNA amount initially added, are shown separately for the high- and low-molecular-mass RNA. The amount of the low-molecular-mass RNA introduced from preparation I, shown in denser slanting shade, is estimated based on the assumption that the constant fraction of the low-molecular-mass RNA from preparation II is accumulated at the interface. (C) Relationship of the accumulated amounts between the high- and low-molecular-mass RNA for the natural cellular RNA preparation II (●), III (■), IV (♦) and V (▲). Data from the systems with various phase volume ratios (0.11–42) on the identical tie-line, which the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system lay on, are plotted together. RNA was added at the initial concentration of 0.05–0.7 mg total RNA/ml.

cule of natural RNA with such considerable frequency. From Fig. 4B, preparation I, broken-down RNA, also showed simultaneous accumulation along with 'adsorption' of the high-molecular-mass RNA. Hence the main reason for the simultaneous accumulation of the RNAs is not the complementary binding at the specific site(s) between the RNA molecules. The simultaneous accumulation of RNAs will occur by the affinity of the molecular surfaces, which is general for RNA in spite of their molecular-mass and independent of their sequences. The affinity between RNA molecular surfaces, which will be exposed under the partitioning condition where the high-molecule is accumulated at the interface but common among RNA molecules, will totally act as a main driving force for the simultaneous accumulation.

The relationship between the high- and low-molecular-mass RNA accumulation among the natural cellular RNA preparations is shown in Fig. 4C. The relationship between the high- and low-molecular-mass RNA accumulations was linear in each preparation. This relationship was maintained in spite of the initial RNA concentration and regardless of the phase volume ratio in each preparation. The slopes of the accumulated amounts of the low- to high-molecular-mass RNA were not identical among preparations. The difference in the slopes would reflect that the affinity between RNA molecules is affected by a difference in molecular-mass content and by a minor difference in the preparation process. It must be noted that the simultaneous accumulation of the low-molecular-mass RNA has a proportional relation to the accumulation of high-molecular-mass RNA.

3.4. Influence of system composition

The RNA partition is strictly affected by the molecular-mass of PEG which constructs the potassium phosphate–PEG two-phase systems near the binodials. Partitioning in the systems with PEG1000–3000 near the binodials shows a high-molecular-mass RNA accumulation at the interface for the examined systems with PEG300–20 000 [10]. The PEG1000–1500 systems especially exhibit a significant accumulation of the high-molecular-mass RNA. When the systems are removed from the binodials, all of the PEG1000–3000 systems show remarkable accumulation at the interface, not only for the high-molecular-mass RNA but also for the low-molecular-mass RNA [11]. In the system where the partition is accompanied by adsorption the high-molecular-mass RNA shows an 'amount-ratio' dependent partitioning in spite of the PEG molecular-mass, i.e. the partition ratio of the high-molecular-mass RNA between the bottom phase and the interface is constant regardless of the phase volume ratio [11].

Fig. 5 demonstrates the relationship between the high- and low-molecular-mass RNA accumulation in the systems where the compositions are far from the binodial. The accumulated amount of the low-molecular-mass RNA linearly increased with that of the high-molecular-mass RNA. The relations between them were almost identical in spite of the PEG molecular-mass and proportional regardless of the phase volume ratio. The two-phase systems with the same initial concentrations of polymers and salts but different molecular-mass polymers will develop two phases of similar compositions other than the PEG molecular-mass. Then the partitioned phases fundamentally have similar physical properties. The RNA accumulation depending on the PEG molecular-mass seems not to be strictly based on the specific interaction between certain molecular-mass PEGs and RNA but a result of the given situation from the dynamic partitioning in the two-phase system. Any factor, which is not specific for the PEG molecular-mass among systems with PEG1000–3000, will provide the conditions for the RNA accumulation.

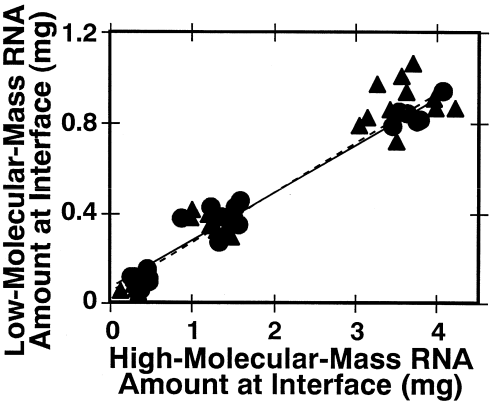

Fig. 5. Effect of PEG molecular-mass on the relation between the high- and low-molecular-mass RNA accumulation in the potassium phosphate–PEG system. The natural cellular RNA (RNA preparation II) was added at 0.05–0.5 mg total RNA/ml in the system with PEG1500 (●) or PEG3000 (▲). Data from the systems with various phase volume ratios (0.15–8) on the tie-line, which the 15% (w/w) potassium phosphate–16% (w/w) PEG1500 system lay on, are plotted together.

3.5. Solubility and accumulation

In Fig. 6, the solubility of RNA in an equilibrated

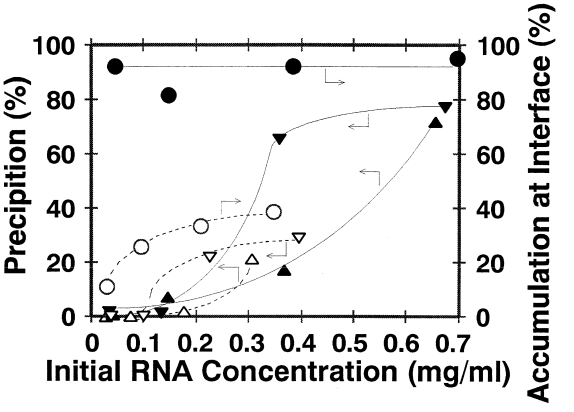

Fig. 6. RNA solubility and accumulation in the phase solutions of the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system. Precipitation of the high- (▲) and low- (△) molecular-mass RNA in the top phase solution and precipitation of the high- (▼) and low- (▽) molecular-mass RNA in the bottom phase solution are estimated from the initial concentration and the supernatant concentration after 10 h standing at 4°C. Partitioning in the 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system is examined to show the accumulation of the high- (●) and low- (○) molecular-mass RNA. Natural cellular RNA (RNA preparation II) is examined.

two-phase solution is shown. The 14% (w/w) potassium phosphate–12% (w/w) PEG1500 system was partitioned without RNA to be equilibrated at 4°C. The top and bottom phase solutions were separately withdrawn. The RNA preparation was added to each solution and allowed to stand for 10 h at 4°C. In the equilibrated top phase solution, which contained about 21% (w/w) PEG1500, almost all of the high- and low-molecular-mass RNA did not precipitate up to 0.3 mg/ml. For the equilibrated bottom phase solution, which contained about 24% (w/w) potassium phosphate, the RNA up to 0.2 mg/ml remained in solution. Above 0.3 mg/ml, the RNA precipitated into the bottom of the solution showing the salting-out effect. With respect to the low-molecular-mass RNA, the above data were in conflict with the data in Fig. 2A, where the low-molecule partitioned to both phases and was soluble up to a much higher concentration than in Fig. 6. This would reflect the effect of the coexisting high-molecular-mass RNA and the time-dependent precipitation of RNA. As also shown in Fig. 6, when both phase solutions were mixed with the RNA at the same concentration together to form the two-phase system, the high- and low-molecular-mass RNA were easily accumulated at the interface. It must be noted that the RNA is significantly accumulated on the two-phase system partitioning at a low concentration where the RNA is soluble enough in the individual solutions. From these results, the RNA accumulation at the interface of the two-phase system is not the result of precipitation in the top phase nor the result from salting-out in the bottom phase. The accumulation only arose when the RNA was allowed into the partitioning process in the two-phase system. The partitioning accompanied by accumulation is achieved during the situation of the dynamic partitioning process, not by the interaction observed in the phases partitioned in equilibrium. During the dynamic partitioning process, simultaneous ‘co-accumulation’ of the high- and low-molecular-mass RNA would occur. On the RNA partition in high concentration, the accumulation might be enhanced by a time-dependent salting-out effect after the original partitioning to the phases and the interface. Anyway, without the processing of the two-phase partitioning, no accumulation nor adsorption occurs.

4. Conclusion

In the potassium phosphate–PEG aqueous two-phase system, the low-molecular-mass RNA inherently partitioned between the top and bottom phases if partitioned alone, but it was caught in the interface if partitioned together with the high-molecular-mass RNA. In the system where most of the high-molecular-mass RNA was accumulated at the interface, the degree of simultaneous accumulation of the low-molecular-mass RNA was dependent on the high-molecular-mass RNA content in spite of the initial concentration and irrespective of the phase volume ratio. The simultaneous accumulation of the high- and low-molecular-mass RNA occurs during the partitioning process, and salting-out from the phase solution is not the major driving force for the RNA condensation at the interface. This indicates a possibility that a weak affinity of the low molecular polymers can be enhanced by that of the coexisting high molecular analogous polymers. The simultaneous accumulation of analogous polymers is suggestive of applying this phenomenon to an actual downstream processing.

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