Artifacts in the Centrifugation of Ribosomal and Heterogeneous Ribonucleic Acid in '99%-Dimethyl Sulphoxide' Gradients

By IAN H. MAXWELL*

Department of Molecular Biology, University of Geneva, Geneva, Switzerland

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Increasing the total quantity of RNA loaded on 99%-dimethyl sulphoxide gradients profoundly altered the sedimentation pattern of heterogeneous RNA and (to a smaller extent) that of rRNA. Aggregation of small quantities of rRNA with fast-sedimenting heterogeneous RNA was also found to occur in these gradients.

The sedimentation analysis of RNA is complicated by variations in the secondary structure of different RNA species and by the tendency of RNA to form aggregates (Sedat & Sinsheimer, 1970). These problems would be avoided if all RNA species could be completely denatured. Strauss *et al.* (1968) reported that this can be achieved by conducting sedimentation in a high concentration of dimethyl sulphoxide (Me_2SO), and widespread use has since been made of this technique to study various problems (Tonegawa *et al.*, 1970; Holmes & Bonner, 1973), including that of the relationship of mRNA to heterogeneous nuclear RNA (Imaizumi *et al.*, 1973; Ruiz-Carrillo *et al.*, 1973; Knöchel & Tiedemann, 1975).

It is now shown here that the sedimentation pattern of heterogeneous RNA (hRNA) from animal cells is markedly affected by the total quantity of RNA applied to a 99%- Me_2SO gradient. The results also show that RNA aggregation is not eliminated in 99%- Me_2SO gradients. The interpretation of experiments in which a specific mRNA sequence has been detected in fast-sedimenting fractions from 99%- Me_2SO gradients must therefore be questioned.

Materials and Methods

Polyoma-virus-transformed hamster cells were grown and labelled in roller bottles at 37°C. After 22 h labelling with [^{14}C]uridine (55 mCi/mmol; New England Nuclear Corp., Boston, MA, U.S.A.), actinomycin D was added to 0.05 $\mu\text{g}/\text{ml}$, and labelling with [^3H]uridine (27 Ci/mmol; New England Nuclear Corp.) was carried out for 3 h. Total RNA extraction was performed by a modification of the method of Kirby & Cook (1967), including chloroform extractions. The RNA was purified and separated from DNA by salt precipitation and pelleting through

* Present address: Department of Anatomy, University of Colorado School of Medicine, 4200 E. Ninth Ave., Denver, CO 80220, U.S.A.

6M-NaBr (Kirby & Cook, 1967), before passage through Sephadex G-75.

^{14}C -labelled rRNA was isolated by method 1 of Kirby (1965), from cells labelled for 28 h with [^{14}C]uridine.

Linear gradients were prepared, consisting of 0–10% (w/v) chloral hydrate, 1% (v/v) water and 1 mM- Na_2EDTA in Me_2SO in a total volume of 36 ml in polyallomer tubes. RNA samples were dissolved in 0.25 ml of 1 mM- Na_2EDTA (aqueous) and mixed with 0.25 ml of Me_2SO and 0.50 ml of dimethyl-formamide (Strauss *et al.*, 1968), and then the total mixture was layered on a gradient. Centrifugation was performed at 95000g at 25°C in the SW27 rotor of a Beckman ultracentrifuge, for the times indicated. Radioactivity was measured in samples of gradient fractions, after precipitation with trichloroacetic acid on filter paper, by liquid-scintillation spectrometry with appropriate correction for channel overlap.

Results and Discussion

Total RNA was extracted from cultured hamster cells that had received a long-term label with [^{14}C]uridine, followed by a short label with [^3H]uridine in the presence of a low concentration of actinomycin D to suppress rRNA synthesis (Roberts & Newman, 1966). This enabled the sedimentation patterns of rRNA and non-ribosomal RNA (i.e. mRNA + heterogeneous nuclear RNA) to be determined simultaneously. Density gradients were centrifuged in the largest rotor that was available (Beckman SW27; total volume per gradient 37 ml). The density gradients consisted of 0–10% (w/v) chloral hydrate (Hastings *et al.*, 1968; Dubroff & Nemer, 1975), which was used in preference to sucrose because it dissolved more readily in Me_2SO , giving less viscous solutions. Essentially similar results were obtained in other experiments performed with sucrose. In preliminary experiments, up to 350 μg of rRNA was found to sediment in two distinct

Fig. 1. Effect of the quantity of RNA loaded on the sedimentation pattern of rRNA (^{14}C) and hRNA (^3H) in 99%- Me_2SO gradients

In (a) 20 μg and in (b) 200 μg of the same doubly labelled RNA preparation were centrifuged for 22 h. The direction of sedimentation is from left to right. $\bullet$ — $\bullet$, ^{14}C radioactivity; $\circ$ — $\circ$, ^3H radioactivity.

peaks, whereas an attempt to centrifuge 900 μg resulted in the 28S RNA (and some 18S RNA) becoming distributed throughout the lower part of the gradient. In subsequent experiments, in which hRNA was examined, the total quantity of RNA loaded per gradient was not more than 200 μg .

Fig. 1 shows the sedimentation analysis in Me_2SO of 20 μg and 200 μg of the same preparation of doubly labelled RNA. In both gradients, the rRNA (^{14}C radioactivity) sedimented in two peaks, but the larger load of RNA resulted in slower sedimentation of both peaks and in a pronounced broadening of the band of 28S RNA. A much more drastic effect of the increased RNA load on the sedimentation pattern of hRNA was observed. This is shown by the sharp fall in ^3H radioactivity between fractions 15 and 17

in Fig. 1(b). In contrast, in Fig. 1(a) the ^3H radioactivity showed a gradual smooth fall from fraction 15 to the bottom of the gradient (apart from a small 28S peak due to residual rRNA synthesis).

A similar alteration in the sedimentation pattern of hRNA with increase in the RNA load has been consistently observed with five different RNA preparations. The proportions of the total ^3H radioactivity loaded that were recovered in the fractions from the gradients shown in Figs. 1(a) and 1(b) were 71 and 55% respectively (compared with 90% for ^{14}C). In other experiments, the recovery in the gradient fractions ranged from 90 to 100% for ^{14}C and from 70 to 85% for ^3H , and the relative recoveries of ^3H and ^{14}C did not correlate with the quantity of RNA loaded per gradient.

Fig. 2. Isolation of fast-sedimenting hRNA from a 99%- Me_2SO gradient and re-sedimentation in a similar gradient

(a) A 200 µg sample of the same RNA preparation as that used for Fig. 1 was centrifuged for 18 h and the fractions indicated by the bar were pooled. (b) A sample (0.25 ml) of the pool was mixed with 0.25 ml of 1 mM- Na_2EDTA , containing 2 µg of ${}^{14}\text{C}$ -labelled rRNA, and with 0.5 ml of dimethylformamide. The mixture was layered on a fresh 99%- Me_2SO gradient and centrifugation was carried out for 17 h. The arrows indicate the positions of the 18S and 28S rRNA peaks in a simultaneously centrifuged gradient containing a sample (16 µg) of the original doubly labelled RNA preparation. The direction of sedimentation is from left to right. ●—●, ${}^{14}\text{C}$ radioactivity; ○—○, ${}^3\text{H}$ radioactivity.

A precise interpretation of the change in the sedimentation pattern of hRNA with increase in the RNA load is therefore made difficult by the variation in total ${}^3\text{H}$ recovery. However, it seems probable that the alteration in the sedimentation pattern was due partly to slower sedimentation of a fraction of the hRNA and partly to pelleting of some hRNA. Control experiments showed that the altered sedimentation pattern was not due to RNA degradation.

Although a substantial fraction of the hRNA showed anomalous sedimentation behaviour under the conditions of Fig. 1(b), it was possible that the

${}^3\text{H}$ -labelled RNA observed in the lower part of the gradient, which had sedimented beyond the rRNA region, genuinely represented large molecules sedimenting at rates determined by their molecular weights. If this were true, these molecules would be expected to re-sediment at the same rates in Me_2SO gradients in the presence of a small total load of RNA. This expectation was not confirmed (Fig. 2). A sample (200 µg) of the same RNA preparation as that used for Fig. 1 was centrifuged in an Me_2SO gradient for 18 h, and fractions containing fast-sedimenting ${}^3\text{H}$ -labelled RNA were pooled as indicated in Fig. 2(a). A sample of the pooled RNA

(without precipitation) was mixed with 2 μ g of 14 C-labelled rRNA, and the mixture was then sedimented in an Me₂SO gradient for 17 h. As shown in Fig. 2(b), the result was that most of the 3 H-labelled RNA was pelleted. Only 20% of the 3 H and 50% of the 14 C radioactivity loaded were recovered in the gradient fractions. The most probable explanation of this result is that the 3 H-labelled RNA had formed aggregates together with the added rRNA. Similar observations have been made by R. Perry (personal communication). It is also noteworthy that the peaks of 14 C-labelled RNA that were observed in Fig. 2(b) sedimented faster than the rRNA peaks in a sample of total RNA (16 μ g) centrifuged in a parallel gradient. This might be explained by partial release of rRNA from aggregates during sedimentation.

Evidence for aggregation or anomalously fast sedimentation in 99%-Me₂SO gradients has been reported by other workers with Sindbis-virus RNA (Simmons & Strauss, 1972), 28S rRNA (De Kloet *et al.*, 1974) and a fraction of bacteriophage ϕ X174 mRNA (Sedat & Sinsheimer, 1970). In contrast, the RNA of Sendai virus has been reported to sediment at an anomalously slow rate (Kolakofsky *et al.*, 1974), probably indicating more complete denaturation of Sendai-virus RNA than of the rRNA markers in 99% Me₂SO. The present results show that anomalous sedimentation in Me₂SO is readily observed with mammalian hRNA and is not a property restricted to certain viral RNA species.

The results presented in Fig. 1 show that 99%-Me₂SO gradients may be overloaded with RNA much more easily than has hitherto been realized. Numerous workers have employed gradients of small volume (e.g. 5.5 ml) to analyse substantial quantities of RNA. In such cases it is clearly not valid to use rRNA markers, centrifuged in parallel gradients (or even in the same gradient), to estimate the sedimentation coefficient of non-ribosomal RNA species. The possibilities of RNA overloading and/or aggregation are particularly relevant to the interpretation of experiments in which apparently giant RNA, isolated from 99%-Me₂SO gradients, has been reported to contain a specific mRNA sequence

(Imaizumi *et al.*, 1973; Ruiz-Carrillo *et al.*, 1973; Knöchel & Tiedemann, 1975).

In the experiments presented here, RNA was loaded on the gradients under the conditions originally described (Strauss *et al.*, 1968). It is possible that modification of these conditions (e.g. inclusion of a heat treatment) might overcome the problem of aggregation in some cases (Holmes & Bonner, 1973; Simmons & Strauss, 1972). However, the problem of overloading is probably inherent to the centrifugation of RNA in very high concentrations of organic solvents.

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References

- De Kloet, S. R., Mayo, V. S. & Andrean, B. A. G. (1974) *Arch. Biochem. Biophys.* **162**, 313–315
- Dubroff, L. M. & Nemer, M. (1975) *J. Mol. Biol.* **95**, 455–476
- Hastings, J. R. B., Parish, J. H., Kirby, K. S. & Cook, E. A. (1968) *Biochim. Biophys. Acta* **155**, 603–605
- Holmes, D. S. & Bonner, J. (1973) *Biochemistry* **12**, 2330–2338
- Imaizumi, T., Diggelmann, H. & Scherrer, K. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1122–1126
- Kirby, K. S. (1965) *Biochem. J.* **96**, 266–269
- Kirby, K. S. & Cook, E. A. (1967) *Biochem. J.* **104**, 254–257
- Knöchel, W. & Tiedemann, H. (1975) *Biochim. Biophys. Acta* **378**, 383–393
- Kolakofsky, D., Boy de la Tour, E. & Delius, H. (1974) *J. Virol.* **13**, 261–268
- Roberts, W. K. & Newman, J. F. E. (1966) *J. Mol. Biol.* **20**, 63–73
- Ruiz-Carrillo, A., Beato, M., Schutz, G., Feigelson, P. & Allfrey, V. G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3641–3645
- Sedat, J. W. & Sinsheimer, R. L. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 163–170
- Simmons, D. T. & Strauss, J. H. (1972) *J. Mol. Biol.* **71**, 599–613
- Strauss, J. H., Jr., Kelly, R. B. & Sinsheimer, R. L. (1968) *Biopolymers* **6**, 793–807
- Tonegawa, S., Walter, G., Bernardini, A. & Dulbecco, R. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 823–831