The Behaviour of Heterogeneous Nuclear Ribonucleic Acid in Partially and Completely Denaturing Conditions

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It is shown that the heterogeneous nuclear RNA (HnRNA) synthesized in the presence of actinomycin and at low and high temperatures sediments in low-ionic-strength sucrose gradients between the rRNA components, similar to the unmethylated RNA synthesized under 'step-down' conditions. If the ionic strength is increased then the HnRNA sediments more rapidly than 28S rRNA, with a large proportion about the 45S precursor rRNA position. Initially this was thought to be due to aggregation of the HnRNA; however, centrifugation and electrophoresis in completely denaturing conditions suggest that the molecular weight of this species of RNA is very large. The experiments reveal that HnRNA is conformationally unstable relative to the nucleolar RNA and that the slower sedimentation rate of HnRNA in 5mM-EDTA-Tris base-sucrose gradients reflects the greater expansion of the HnRNA relative to the nucleolar RNA. The implications of this finding are discussed.

When animal cells are given a short incubation with a radioactive precursor of RNA there are several species of RNA that become labelled rapidly. The bulk of the RNA synthesized in growing cells is rRNA and this fact is reflected in the newly synthesized RNA, which is in large part precursor to rRNA (Scherrer *et al.*, 1963). There are several conditions which appear to decrease or eliminate precursor rRNA synthesis, for example incubation of cells at lower (Stevens & Amos, 1971) or higher (McCormick & Penman, 1969; Warocquier & Scherrer, 1969) temperatures than the optimum, or treatment of cells with certain anti-metabolites (Perry, 1963). While precursor rRNA synthesis, which is thought to be confined to the nucleolus, is eliminated extra-nucleolar RNA synthesis continues. Incubation of cells at high densities ('step-down') also decreases, but does not eliminate, nucleolar RNA synthesis (Bramwell, 1970). Analysis of nRNA by a variety of methods has suggested that extra-nucleolar RNA is DNA-like in base composition, is heterogeneous with respect to sedimentation in sucrose gradients of medium ionic strength and is apparently of very high molecular weight (Houssais & Attardi, 1966; Fujisawa & Muramatsu, 1968; Penman *et al.*, 1968; Shearer & McCarthy, 1970).

Previously published work suggested that so-called giant heterogeneous nRNA (HnRNA) was an artifact brought about by the physical characteristics of the newly synthesized RNA (Bramwell & Harris, 1967; Mayo & De Kloet, 1971; Bramwell, 1972).

Examination of total nRNA isolated from cells subjected to 'step-down' has shown that the bulk of the rapidly labelled RNA sediments in sucrose

gradients of low ionic strength between the 28S and 18S rRNA species (Bramwell, 1972). This conflict with the results of other workers has now been resolved by a re-examination of the properties of the RNA in denaturing conditions and it has been possible to demonstrate that different conformational states of HnRNA exist in situations where nucleolar RNA synthesis is decreased.

Experimental

Cultivation of cells

HeLa cells were grown in suspension to a density of 1.5×10^6 – 2.0×10^6 cells/ml. 'Step-down' conditions were provided by resuspending the cells at a concentration of 10×10^6 – 15×10^6 cells/ml in Eagle's medium plus 6% (v/v) rabbit serum (Bramwell, 1970).

Labelling of RNA

Heterogeneous nRNA was labelled by exposure of HeLa cells to [3 H]uridine precursor for a period of 20 min after 60 min in 'step-down' conditions, except where otherwise stated.

Extraction of nRNA

RNA was extracted from HeLa-cell nuclei as described by Bramwell (1972). After lysis of the nuclei at 4°C with 2% (w/v) sodium dodecyl sulphate at pH 6, the viscous solution was extracted repeatedly with phenol saturated with water at 4°C until the supernatant was perfectly clear and no denatured protein remained at the interface (five or six times).

This exhaustive treatment is essential if reproducible results are to be obtained. Only then were the combined nucleic acid fractions precipitated with alcohol and subjected to treatment with deoxyribonuclease. After this procedure, the solution was no longer viscous and was made 10mM with respect to EDTA buffered at pH 5.4 with Tris-HCl. Sodium dodecyl sulphate was again added to 2% (w/v) and the solution extracted with phenol saturated with water at 4°C until no denatured protein was visible (two or three times). After ethanol precipitation the DNA fragments and glycogen were extracted with 2M-NaCl at 0°C. The precipitate was then dissolved in water and reprecipitated twice to remove traces of NaCl.

Extraction of total RNA

Total RNA was extracted from HeLa cells by lysing the washed cells with 2% (w/v) sodium dodecyl sulphate and proceeding exactly as for the rRNA fraction described above. (Total RNA refers to high-molecular-weight RNA and excludes tRNA.)

Density gradients

Linear sucrose gradients (25–5%, w/v) were prepared in 5mM-EDTA (free acid, adjusted to pH 5.4 with Tris base). Linear gradients were formed from 95% fully deuterated dimethyl sulphoxide containing 10% (w/v) sucrose and 5mM-EDTA-Tris-base buffer, pH 5.4, and 95% normal undeuterated dimethyl sulphoxide containing only 5mM-EDTA-Tris base, pH 5.4. Centrifugation was carried out on a Spinco L2-65 in a SW65 rotor for the stated times. Fractions (0.2ml) were collected by using the Isco density-gradient fractionator model 180 and a LKB Ultrarac fraction collector, and the radioactivity of each was measured in a Packard Tri-Carb model 3375 liquid-scintillation counter, after the addition of 10ml of a toluene-based scintillant.

Reagents

EDTA, phenol (A.R.), formamide and agarose (electrophoresis grade) were obtained from BDH, Chemicals Ltd., Poole, Dorset, U.K. Sodium dodecyl sulphate was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. This brand was proved to be a more efficient deproteinizer than others that are more highly purified. Tris (A grade, heavy-metal free) and urea (A grade) were obtained from Calbiochem, San Diego, Calif., U.S.A. Ribonuclease-free sucrose was obtained from Cambrian Chemicals Ltd., Croydon, London, U.K. Deoxyribonuclease I was an electrophoretically purified enzyme from Worthington Biochemical Co., Freehold, N.J., U.S.A. This was further purified with bentonite

as described by Bramwell (1972). This was found to be necessary, since this brand of deoxyribonuclease was proved to degrade rRNA. This fact has since been confirmed by other workers (Lee *et al.*, 1973; Holmes & Bonner, 1973). Actinomycin D was obtained from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Deuterated dimethyl sulphoxide was obtained from Fluorochem, Glossop, Derbyshire, U.K. [^3H]Uridine (sp. radioactivity 29 Ci/mmol), [^{14}C]uridine (sp. radioactivity 508 mCi/mmol) and L-[^3H]methionine (sp. radioactivity 9 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed as described by Bramwell (1972). Formamide-agarose gels were prepared by dissolving 0.1g of agarose (BDH Chemicals Ltd., 'for electrophoresis') in 20ml of 80% (v/v) formamide-water containing 5mM-EDTA-Tris-base buffer at pH 5.4. The solution was heated to dissolve the agarose, cooled and pipetted into gel tubes (12cm $\times$ 6mm diam.) and left to set for 18 h at 4°C. Before running the gels they were inverted or the tops sliced off so that the gel surface was absolutely flat before the sample was applied. Optimum resolution was obtained if no more than 10 μl of sample was applied to each gel.

Results

Patterns of RNA synthesized in the presence of actinomycin D

HeLa cells (200ml) growing in suspension culture were incubated at 37°C in the presence of actinomycin D (0.05 $\mu\text{g/ml}$) for 30 min. [^3H]Uridine was added (5 $\mu\text{Ci/ml}$), the suspensions were incubated for a further 60 min and total RNA was extracted.

Fig. 1 shows the pattern of radioactivity incorporated into RNA under the above conditions, obtained from a sucrose gradient containing only 5mM-EDTA-Tris and run at 25°C. The main peak of radioactivity in this buffer sediments at approximately 26S relative to the rRNA markers.

If the same sample of RNA is applied to a gradient containing 50mM-NaCl then the pattern of incorporation is considerably altered, and a proportion of the radioactivity is found in the pellet at the bottom of the tube (Fig. 1). Thus the pattern of radioactivity incorporated into RNA in the presence of low concentrations of actinomycin D is not very different from that shown in Fig. 2, where the cells had been exposed to 'step-down' conditions, apart from a decrease in the material sedimenting faster than 28S RNA. This confirms the earlier conclusions (Bramwell, 1972) that the material sedimenting

Fig. 1. Sucrose-density-gradient-sedimentation pattern of the incorporation of $[^3\text{H}]$ uridine ($5\ \mu\text{Ci/ml}$) into total cell RNA in the presence of actinomycin D ($0.05\ \mu\text{g/ml}$) for 60 min

The gradient contained 5 mM-EDTA-Tris base, pH 5.4. ●, Pattern of radioactivity obtained by centrifugation of a sample of the RNA on a gradient containing 50 mM-NaCl, superimposed on the pattern from the low-ionic-strength gradient (○). Centrifugation at 25°C was for 120 min at 60000 rev./min in 5 mM-EDTA-Tris base and 90 min at 60000 rev./min in 50 mM-NaCl + 5 mM-EDTA-Tris base. — E_{254} ; ○ and ●, radioactivity.

between the two peaks of rRNA represents RNA synthesized in the nucleoplasm, since actinomycin D preferentially inhibits nucleolar RNA synthesis (Perry, 1963).

Effect of temperature variation on the synthesis of RNA

Nucleolar synthesis of rRNA precursors is sensitive to changes in the physical environment of the cells, particularly to changes in temperature. At temperatures below 20°C , and at 42°C and above, nucleolar function is considerably impaired (McCormick &

Fig. 2. Sucrose-density-gradient-sedimentation pattern of the incorporation of $[^3\text{H}]$ uridine into nRNA extracted from cells exposed to 'step-down' conditions for 60 min and then labelled with $[^3\text{H}]$ uridine ($5\ \mu\text{Ci/ml}$) for a further 30 min

The gradient contained 5 mM-EDTA-Tris base, pH 5.4. Centrifugation at 25°C was for 120 min at 60000 rev./min. — E_{254} ; ○, radioactivity.

Fig. 3. Sucrose-density-gradient-sedimentation pattern of the radioactivity incorporated into total RNA during an 18 h incubation at 17.5°C with $[^3\text{H}]$ uridine ($5\ \mu\text{Ci/ml}$)

—, E_{254} ; ○, ^3H radioactivity incorporated, ○. Centrifugation at 25°C was for 120 min at 60000 rev./min. The gradients contained 5 mM-EDTA-Tris base, pH 5.4.

Fig. 4. Sucrose-density-gradient-sedimentation pattern of the radioactivity incorporated into total RNA during an 18 h incubation at 37°C with [^{14}C]uridine (0.1 $\mu\text{Ci/ml}$) followed by a 3 h incubation with [^3H]uridine (5 $\mu\text{Ci/ml}$) at 42°C

Radioactivity: $\circ$, ^3H ; $\bullet$, ^{14}C ; Centrifugation was for 120 min at 60000 rev./min at 25°C. The gradients contained 5 mM-EDTA-Tris base, pH 5.4.

Penman, 1969; Warocquier & Scherrer, 1969; Stevens & Amos, 1971).

HeLa cells (200 ml) growing in suspension culture at a density of 1×10^6 cells/ml were spun down at low speed (600 g) and resuspended in fresh medium at 17.5°C. [^3H]Uridine was added at a concentration of 5 $\mu\text{Ci/ml}$ and the cells were incubated for 18 h at 17.5°C. Total RNA was extracted as described above and analysed on sucrose gradients containing 5 mM-EDTA-Tris base, at 25°C. Fig. 3 shows the pattern of radioactivity obtained from such a gradient. In cells growing at 37°C for 18 h almost all the radioactivity would be in rRNA, but at 17.5°C the radioactivity incorporated into RNA sediments in a pattern similar to that obtained after actinomycin D treatment. Fig. 4 shows the pattern of radioactivity incorporated into RNA when the cells were incubated at 42°C for 3 h. The pattern is almost identical with those of the previous two experiments. Therefore in all three situations (low concentrations of actinomycin D, incubation at 17.5°C and at 42°C) the type of RNA being synthesized is the same and appears to represent nucleoplasmic RNA. The synthesis of the rRNA precursor sedimenting at 45S is considerably

Fig. 5. Sucrose-density-gradient-sedimentation pattern of the radioactivity incorporated into total RNA during a 30 min incubation with [$\text{Me-}^3\text{H}$]methionine (10 $\mu\text{Ci/ml}$) and [^{14}C]uridine (0.1 $\mu\text{Ci/ml}$)

The gradients contained (a) 5 mM-EDTA-Tris base, pH 5.4, or (b) 50 mM-NaCl + 5 mM-EDTA-Tris base, pH 5.4. Centrifugation at 25°C was for 90 min for (a) and 60 min for (b) at 60000 rev./min. —, E_{254} ; radioactivity incorporated: $\circ$, ^3H ; $\bullet$, ^{14}C .

decreased, although not completely eliminated, and minor shoulders are apparent at 32S and 18S.

Methylation of newly synthesized RNA

Nucleoplasmic RNA has been found to be unmethylated by several workers (Greenberg & Penman, 1966; Saponara & Enger, 1966; Zimmerman & Holler, 1967; Muramatsu & Fujisawa, 1968).

Fig. 6. Sucrose-density-gradient-sedimentation patterns of the radioactivity incorporated into total RNA during an incubation with [$\text{Me-}^3\text{H}$]methionine ($10\mu\text{Ci/ml}$)

The incubation was for 3h at 42°C (●), and in another experiment in the presence of actinomycin D ($0.05\mu\text{g/ml}$) for 4h at 37°C (○). —, E_{254} . Centrifugation was for 3h at 45000 rev./min at 25°C . The gradients contained 5mM-EDTA-Tris base, pH 5.4.

The pattern of methylation of newly synthesized RNA analysed in a sucrose gradient under conditions of low ionic strength as described above was therefore examined.

HeLa cells (200ml) were spun down and resuspended in Eagle's medium (200ml) for 16h. [$\text{Me-}^3\text{H}$]Methionine and [^{14}C]uridine were added at concentrations of $10\mu\text{Ci/ml}$ and $0.1\mu\text{Ci/ml}$ respectively. After 30min the cells were harvested and total RNA was extracted as described above. The pattern of radioactivity incorporated into RNA obtained from a sucrose gradient containing only 5mM-EDTA-Tris base is shown in Fig. 5(a). It is apparent that there is a striking difference between the methionine- and uridine-labelled RNA. The bulk of the ^3H radioactivity is in a peak sedimenting faster than 28S rRNA, with a smaller peak corresponding to 18S rRNA, whereas the [^{14}C]uridine distribution is similar to that seen in Fig. 2. There is thus no evidence, apart from a slight shoulder, that the nucleoplasmic RNA (defined, as in the previous section, as that sedimenting between 28S and 18S rRNA in conditions of low ionic strength) is methylated. This agrees with the work of others which shows that nucleoplasmic or 'polydisperse' RNA is unmethylated. Fig. 5(b)

shows a similar sample of RNA subjected to centrifugation on a sucrose gradient containing 50mM-NaCl. At this higher ionic strength the [^{14}C]uridine-pulse-labelled RNA shows a marked tendency to sediment more rapidly, leaving only a small proportion where the main peak of radioactivity had been in the gradient containing only 5mM-EDTA-Tris base.

The methyl-labelled RNA species are likely to be those formed in the nucleolus as precursors to rRNA. Attempts to isolate high-molecular weight [^3H]methylated RNA species from cells incubated at either 17.5° or 42°C or in the presence of actinomycin D ($0.05\mu\text{g/ml}$) were therefore unsuccessful. Fig. 6 shows that low-molecular-weight RNA (i.e. tRNA) is methylated under such conditions, but very little material of high molecular weight is methylated. Most of the tRNA was extracted with 2M-NaCl, and the residual pattern indicates that the small amount that remains has a very high specific radioactivity compared with that of the high-molecular-weight species of RNA.

These results once again show that the heterogeneous RNA from HeLa-cell nuclei can be made to sediment in gradients of low ionic strength at a rate that suggests that its molecular weight is intermediate in value between that of the two rRNA components. However, these conditions are only partially denaturing and so the behaviour of heterogeneous RNA was examined on polyacrylamide gels and density gradients in conditions that totally denature RNA. Thus centrifugation in dimethyl sulphoxide (Strauss *et al.*, 1968) and electrophoresis in acrylamide-formamide gels (Staynov *et al.*, 1972) or urea-acrylamide gels (Reijnders *et al.*, 1973) provide the necessary conditions. Fig. 7(a) shows the pattern of radioactivity obtained by centrifuging a sample of ^3H -pulse-labelled RNA on a dimethyl sulphoxide gradient. The bulk of the radioactivity is associated with material sedimenting faster than 28S. This result is at variance with an earlier result (Bramwell, 1972) where the peak of radioactivity centred about the 28S rRNA marker. This is now explicable in terms of the slight degradation of RNA if dissolved at low concentrations and left for prolonged periods of time (i.e. 48–72h) in dimethyl sulphoxide. Fig. 7(b) shows the pattern obtained from a sample of rapidly labelled RNA which had been stored for 1 week at -20°C in dimethyl sulphoxide at a concentration of $0.06\mu\text{g/ml}$. There is now a large peak of radioactivity sedimenting only slightly ahead of the 28S rRNA marker.

Gel electrophoresis should allow a higher degree of resolution than density-gradient centrifugation, and electrophoresis in formamide with a supporting buffer provides a completely denaturing system. Fig. 8(a) shows the pattern of radioactivity obtained from a sample of ^3H -labelled RNA (30min) after electrophoresis on an agarose-formamide gel. It is

Fig. 7. Dimethyl sulphoxide-density-gradient-sedimentation pattern of the radioactivity incorporated into total RNA during an incubation at 37°C with $[^3\text{H}]\text{uridine}$ ($10\mu\text{Ci/ml}$) for 30 min

^{14}C -labelled rRNA was added as a marker to give the same concentration of RNA/gradient. (a) An RNA sample ($100\mu\text{l}$) dissolved in 95% (v/v) dimethyl sulphoxide ($45\mu\text{g/ml}$) immediately before centrifugation. (b) An RNA sample ($200\mu\text{l}$) which had been stored for 1 week at -20°C in 90% (v/v) dimethyl sulphoxide ($45\mu\text{g/ml}$). Radioactivity: $\circ$, ^3H ; $\bullet$, ^{14}C . Centrifugation was for 16 h at 50000 rev./min at 25°C .

very similar to that in the dimethyl sulphoxide gradients, with a predominant peak of 45S RNA, some material of apparently higher molecular weight and some of lower. There is little material between

Fig. 8. Formamide-agarose-gel electrophoresis of RNA

(a) Total RNA from cells labelled for 30 min with $[^3\text{H}]\text{uridine}$ ($10\mu\text{Ci/ml}$) at 37°C and for 18 h with $[^{14}\text{C}]\text{uridine}$ ($0.1\mu\text{Ci/ml}$). (b) Total RNA from cells labelled for 120 min with $[^3\text{H}]\text{uridine}$ ($5\mu\text{Ci/ml}$) in the presence of actinomycin D ($0.05\mu\text{g/ml}$, added 30 min before $[^3\text{H}]\text{uridine}$) at 37°C . (c) Total RNA from cells labelled for 18 h with $[^{14}\text{C}]\text{uridine}$ ($0.1\mu\text{Ci/ml}$) at 37°C and for 3 h with $[^3\text{H}]\text{uridine}$ $10\mu\text{Ci/ml}$ at 42°C . Electrophoresis was for 90 min for (a) and (b) and 120 min for (c) at 100V at 22°C . —, E_{265} ; radioactivity: $\bullet$, ^3H ; $\circ$, ^{14}C

the 28S and 18S RNA, and it is thus similar to the pattern obtained on sucrose gradients of moderate ionic strength (Fig. 5b).

As shown above the RNA synthesized in the presence of actinomycin D and at 42°C behaves in the

same way as pulse-labelled RNA on sucrose gradients of low ionic strength.

Figs. 8(b) and 8(c) show samples of RNA synthesized in the presence of actinomycin D and at 42°C, analysed on agarose-formamide gels. Again the distribution is very similar to that from pulse-labelled RNA, with several clearly defined characteristic peaks of radioactivity.

Discussion

Both sets of denaturing conditions (i.e. dimethyl sulphoxide gradients and formamide-agarose gels) indicate that the HnRNA has a higher molecular weight than is apparent from the low-ionic-strength sucrose gradients. This is contrary to the results of Bramwell (1972) but agrees with the view generally held. The earlier finding appears to be due to chain scission occurring when the RNA was left in a totally denatured and expanded form for a prolonged period of time.

The sucrose gradients in 5mM-EDTA-Tris-base buffer apparently provide only partially denaturing conditions; there is no loss of hypochromicity (M. E. Bramwell, unpublished work), but there must be considerable loss of tertiary structure, since the sedimentation velocity is much decreased as compared with gradients in 50mM-NaCl. The HnRNA must therefore be much more sensitive than the 45S rRNA precursor to the lowering of the ionic strength, and this is shown in Fig. 5, where the methylated RNA species are unchanged relative to rRNA markers.

The electrophoresis patterns obtained on gels containing 6M-urea and 50mM-sodium acetate-sodium phosphate buffer are also in partially denaturing conditions (Bramwell, 1972); again there is no loss of hypochromicity, but there must be expansion of the molecules, since their mobilities are decreased considerably compared with electrophoresis in the absence of urea (M. E. Bramwell, unpublished work). Thus the HnRNA moves much more slowly than 45S RNA on such gels, but in completely denaturing conditions its mobility is relatively increased and it gives several peaks from 45 to 28S RNA.

The patterns compare very favourably with those obtained for nRNA after treatment with dimethyl sulphoxide before electrophoresis (Scott & Kuhns, 1972) and, surprisingly, for the polyribosomal RNA from KB cells (Kumar & Lindberg, 1972). The relative homogeneity of the HnRNA when examined under different conditions may well reflect the success in preventing both degradation and aggregation during the extraction and analysis of the highly labile nascent RNA. All species of mature RNA (i.e. rRNA, tRNA and stable mRNA) probably have a certain defined secondary and tertiary structure which is an integral part of their biological function.

This structure must depend on base sequence and probably requires the RNA chain to be folded in a specific way. Newly synthesized RNA might not have assumed the correct configuration before it was extracted, and might thereafter only achieve a limited secondary structure. Support for this hypothesis comes from the 'melting' profile of the RNA that binds tightly to methylated-albumin-kieselguhr columns (Bramwell, 1970). This profile gives a lower degree of hypochromicity during the first heating than on reheating, indicating that some annealing must have taken place during heating. Also, Birnboim (1972) has shown that 28S rRNA aggregates if it is heated with dimethyl sulphoxide. This procedure completely denatures the RNA, and subsequent precipitation with ethanol and solution in aqueous media permits only a limited renaturation of its original structure, suggesting that the final structure of an RNA molecule depends on an external imposition of shape as well as its intrinsic primary composition. The conformational instability of HnRNA may simply be due to the fact that it is newly synthesized or it may be related to its (A+U)-rich DNA-like base composition (the A·U base pairs being less stable than the G·C base pairs). A similar effect has been observed in the electrophoresis of mitochondrial RNA at different temperatures (Groot *et al.*, 1970).

It is known that the secondary and tertiary structure of the RNA from the bacteriophages F2 and R17 is important in specifying the correct proteins which are encoded in its base sequence (Fukami & Imahori, 1971; Lodish, 1970) and it is thus possible that the apparent flexibility in the shape of HnRNA and its probable product, mRNA, are important factors in regulation at the level of translation or alternatively at a post-transcriptional stage where the RNA is either degraded intranuclearly or converted into a more stable form.

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References

- Birnboim, H. C. (1972) *Biochemistry* **11**, 4588-4591
- Bramwell, M. E. (1970) *J. Cell Sci.* **6**, 53-72
- Bramwell, M. E. (1972) *Biochim. Biophys. Acta* **281**, 329-337
- Bramwell, M. E. & Harris, H. (1967) *Biochem. J.* **103**, 816-830
- Fujisawa, T. & Muramatsu, M. (1968) *Biochim. Biophys. Acta* **169**, 175-187
- Fukami, H. & Imahori, K. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 570-573
- Greenberg, H. & Penman, S. (1966) *J. Mol. Biol.* **21**, 527-535

- Groot, P. H. E., Aaij, C. & Borst, P. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1321–1327
- Holmes, D. S. & Bonner, J. (1973) *Biochemistry* **12**, 2330–2337
- Houssais, J. F. & Attardi, G. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 616–623
- Kumar, A. & Lindberg, U. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 681–685
- Lee, M. J., Penman, M. & Malt, R. A. (1973) *Biochim. Biophys. Acta* **294**, 461–463
- Lodish, H. F. (1970) *J. Mol. Biol.* **50**, 689–702
- Mayo, V. S. & De Kloet, S. R. (1971) *Biochim. Biophys. Acta* **247**, 74–79
- McCormick, W. & Penman, S. (1969) *J. Mol. Biol.* **39**, 315–333
- Muramatsu, M. & Fujisawa, T. (1968) *Biochim. Biophys. Acta* **157**, 476–492
- Penman, S., Vesco, C. & Penman, M. (1968) *J. Mol. Biol.* **34**, 49–69
- Perry, R. P. (1963) *Exp. Cell Res.* **29**, 400–406
- Reijnders, L., Sloof, P., Sival, J. & Borst, P. (1973) *Biochim. Biophys. Acta* **324**, 320–333
- Saponara, A. G. & Enger, M. D. (1966) *Biochim. Biophys. Acta* **119**, 492–500
- Scherrer, K., Latham, H. & Darnell, J. E. (1963) *Proc. Nat. Acad. Sci. U.S.* **49**, 240–248
- Scott, J. F. & Kuhns, V. L. (1972) *Anal. Biochem.* **47**, 471–480
- Shearer, R. W. & McCarthy, B. J. (1970) *J. Cell. Physiol.* **75**, 97–106
- Staynov, D. Z., Pinder, J. C. & Gratzer, W. B. (1972) *Nature (London) New Biol.* **235**, 108–110
- Stevens, R. H. & Amos, H. (1971) *J. Cell Biol.* **50**, 818–829
- Strauss, J. H., Kelley, R. B. & Sinsheimer, R. L. (1968) *Biopolymers* **6**, 793–807
- Warocquier, R. & Scherrer, K. (1969) *Eur. J. Biochem.* **10**, 362–370
- Zimmerman, E. F. & Holler, B. W. (1967) *J. Mol. Biol.* **23**, 149–161