EUR 571.e

REPRINT

ASSOCIATION
EUROPEAN ATOMIC ENERGY COMMUNITY - EURATOM
UNIVERSITÉ LIBRE DE BRUXELLES - U.L.B.

RIBOSOMES FROM HeLa CELLS

by

D. WINCKELMANS, M. HILL and M. ERRERA

1964

Contract No. 007-61-10 BIAB

Reprinted from
BIOCHIMICA ET BIOPHYSICA ACTA
80 - 1964

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BIOCHIMICA ET BIOPHYSICA ACTA

BBA 8359

RIBOSOMES FROM HeLa CELLS

D. WINCKELMANS*, M. HILL** AND M. ERRERA

Laboratoire de Biophysique et Radiobiologie, Faculté des Sciences, Université libre de Bruxelles, Bruxelles (Belgique)

(Received July 23rd, 1963)

SUMMARY

An electron-microscope study of the HeLa-cell cytoplasmic and nuclear 74-S ribosomes has been performed. These behave similarly when cations are removed by versene: both break up into 30-S and 50-S subunits. In the heavy part of the centrifugation gradient used to isolate the ribosomes it has been shown that 74-S ribosomes are associated in polysomes and a rough calculation has shown that it is not unreasonable to believe that as many as 40 ribosomal units or more could exist in some polysomes.

INTRODUCTION

During studies of rapidly labelled RNA and its subsequent fate in HeLa cells, it became necessary to characterize the fractions obtained by differential gradient centrifugation. Although the digestion agents used to break up the cells and dissociate their constituents (deoxycholate and deoxyribonuclease) were chiefly chosen to obtain ribosomes, it was not certain that other cell fragments (chromosomal, mitochondrial, etc.) do not remain in the gradient and thus obscure the interpretation of the experiments. In the present study, ribosomes were found to be the only major visible elements.

This study deals with the comparison of nuclear and cytoplasmic ribosomes of HeLa cells and with the existence of polysomes in the cytoplasmic fractions. No attempt has yet been made by us to observe polysomes in the nuclear fractions by electron microscopy, although our observations do show ribosomal association and our biochemical studies point to the possibility of their existence[1].

MATERIALS AND METHODS

Preparation of cells

The cells were cultured as described in the preceding paper[1] in ϕ_{10} medium containing calf serum, yeast extract, amino acids and vitamins. In some instances, 1 μC of carrier-free ^{32}P was added per ml as inorganic phosphate for three generation times in order to increase the resolution of the particles in the gradients when the

* Present address: Laboratorio Internazionale di Genetica e Biofisica, Napoli, Italy.
** Present address: Institute of Biophysics, Czechoslovak Academy of Sciences, Brno, Czechoslovakia.

absorbancy was too low to observe well-defined peaks (*i.e.* when less than $4 \cdot 10^8$–$5 \cdot 10^8$ cells were used for preparing nuclear ribosomes or when the effect of ribonuclease on the distribution of ribosomes was studied).

Reagents

Tris–Mg^{2+} buffer: 0.05 M Tris plus 0.025 M KCl plus 0.005 M $MgCl_2$, pH 7.45.

Phosphate–Mg^{2+} buffer: 0.0011 M sodium phosphate plus $5 \cdot 10^{-4}$ M $MgCl_2$, pH 7.45.

Deoxyribonuclease (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5): Nutritional Biochemicals (2 times crystallized).

Ribonuclease (polyribonucleotide 2-oligonucleotidotransferase (cyclizing), EC 2.7.7.16): Sigma (5 times crystallized).

^{32}P carrier free: from Mol (Belgium).

Harvesting of cells

The dead cells were removed by light shaking before the medium was discarded or by washing them once in cold Eagle's solution. The cells were loosened from the Roux flasks with glass beads, washed again in Eagle's solution and then treated by one of the following procedures, depending on the final aim of the experiment. All preparations were done at 0–2°, unless otherwise stated.

Preparation of ribosomes from whole cells

Whole-cell preparations were made in the presence of bentonite (prepared according to FRAENKEL-CONRAT *et al.*[2]) to prevent the action of intracellular ribonuclease. The cells from 10 Roux flasks (about $4 \cdot 10^8$ cells) were dispersed in 1.5 ml Tris–Mg^{2+} buffer containing 0.5 mg bentonite plus 1 % sodium deoxycholate per ml and a few crystals of deoxyribonuclease. When the drop in viscosity of the suspension was complete (approx. 30 min) the homogenate (1.5 ml) was then layered on a 28 ml 15–30 % sucrose gradient made in Tris–Mg^{2+} buffer and centrifuged for 2 h in the SW 25 rotor of the Spinco model L preparative ultracentrifuge at 24 000 rev./min.

Preparation of cytoplasmic and nuclear ribosomes

Cells ($4 \cdot 10^8$) were homogenized by the method of HARRIS *et al.*[3] in a 0.1 % solution of Tween 80 at 4° for 25–45 min. The nuclei were separated by centrifugation for 5 min at $200 \times g$.

The cytoplasmic ribosomes were collected from the supernatant which was further centrifuged for 15 min at $3000 \times g$. This second supernatant was treated with 0.2 % sodium deoxycholate, and after 30 min at 0–2°, the ribosomes were concentrated by 2-h centrifugation in the rotor No. 40 of the preparative Spinco ultracentrifuge at 105 000 $\times g$. Up to 85 % of the cytoplasmic RNA was recovered in the ribosomal pellet. It was taken up either in 1 ml phosphate–Mg^{2+} buffer and centrifuged for 4.5 h on a 5–20 % sucrose gradient as in the preceding sections (70-S, 50-S, 30-S ribosomes), or in Tris–Mg^{2+} buffer and centrifuged for 2 h at 24 000 rev./min in a 15–30 % sucrose gradient prepared in the same buffer (cytoplasmic polysomes).

The nuclear ribosomes were prepared in 2 different ways, as follows.

(1) For electron-microscopy preparations, the nuclei were simply dispersed in 1.5 ml phosphate–Mg^{2+} buffer to which was added 0.5 mg of bentonite plus 1 % sodium deoxycholate per ml and a few crystals of deoxyribonuclease. After about 30 min, the preparation was layered on a 28-ml gradient prepared from 5–20 % sucrose made up in phosphate–Mg^{2+} buffer and spun in the SW 25 rotor of the Spinco centrifuge at 24 000 rev./min for 4.5 h.

(2) For further purification of the ribosomes, the nuclei were resuspended in 25 ml Tris–Mg^{2+} buffer and homogenized in an MSE blendor at 8000–9000 rev./min for 3 min. The homogenate was centrifuged at 15 000 $\times g$ for 15 min. To the supernatant, sodium deoxycholate was added to a final concentration of 0.5 %. After 30 min at 0–2°, the suspension was centrifuged in rotor No. 40 of the Spinco preparative centrifuge for 2 h at 105 000 $\times g$. To study the distribution of ribosomes in the gradient, the pellet was resuspended in 0.2 ml of phosphate–Mg^{2+} buffer and layered on a 5-ml 5–20 % sucrose gradient prepared in the same buffer. To study the dissociation of the ribosomes, a similar pellet was resuspended in 0.2 ml of 0.01 M versene pH 7.45 and layered on phosphate buffer containing no $MgCl_2$ (pH 7.45). In both cases the 0.2-ml suspensions were centrifuged for 90 min in the SW 39 rotor of the Spinco centrifuge at 39 000 rev./min.

Fixation for electron microscopy

After the absorbancy or ^{32}P profile in the gradient was established by analyzing the fractions in the usual manner, small aliquots of the selected fractions (0.2–0.4 ml) were dispersed in an equal volume of the following fixing solution: phosphate–Mg^{2+} buffer containing 12 % sucrose and 1 % osmium tetroxide. Fixation lasts for 10–20 h at 4° after which a droplet of the suspension was carefully transferred to a grid coated with collodion and carbon, and air-dried. In order to remove the dried sucrose the grids were immersed successively for 30 sec into sucrose solutions of decreasing concentration 12, 10, 5, 3 %) followed by a wash in buffer and lastly in distilled water. The grids were finally platinum shadowed by the standard procedure.

All the observations were made on a Hitachi model HS-G microscope at a magnification of $\times$16 000.

RESULTS

Total cell preparations

When total cell extracts were centrifuged in the 15–30 % sucrose gradient (the sedimentation profile is shown in Fig. 1) heavy fractions were observed under the electron microscope, and fraction No. 4 is presented in Plate 1. Many ribosomal aggregates of various sizes can be seen including chain-like formations in addition to a few isolated ribosomes.

When the whole cell extract was treated (1 h at 4°) before centrifugation by 0.5 μg of ribonuclease (Nutritional Biochemicals) per ml all the heavier components disappeared (Fig. 2). These ribosomal associations therefore behave similarly to those observed by WARNER *et al.*[4], NOLL *et al.*[5] and WETTSTEIN *et al.*[6].

Nuclear and cytoplasmic ribosomes

The sedimentation profiles (absorbancy) of these two fractions prepared in phosphate–Mg^{2+} buffer appeared as in Fig. 3 where the nuclear and cytoplasmic

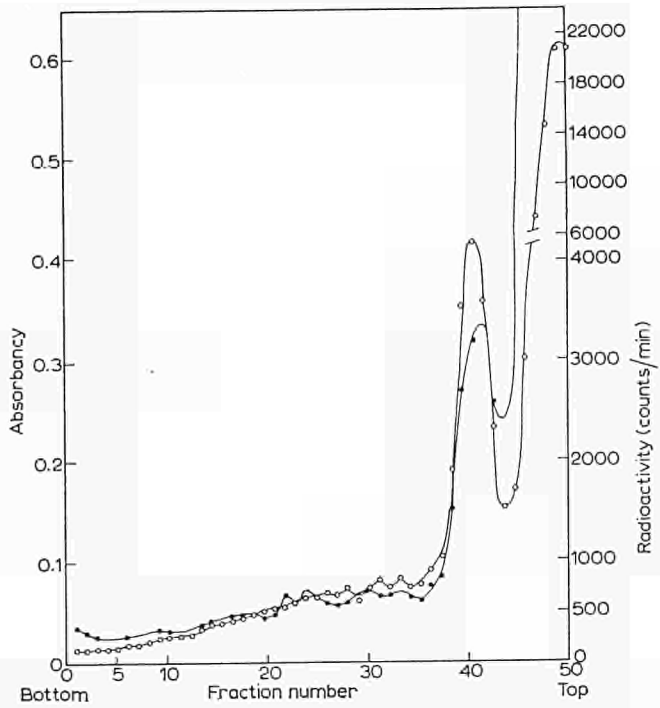

Fig. 1. Sedimentation profile of total-cell homogenate in a 15–30 % sucrose gradient. Abscissae: fraction number normalised as in HILL *et al.*[1]. Ordinates: left, absorbancy (●–●); right, counts/min (○–○).

Plate 1. Whole cells, Fraction 4 (Fig. 1), 46 000×.

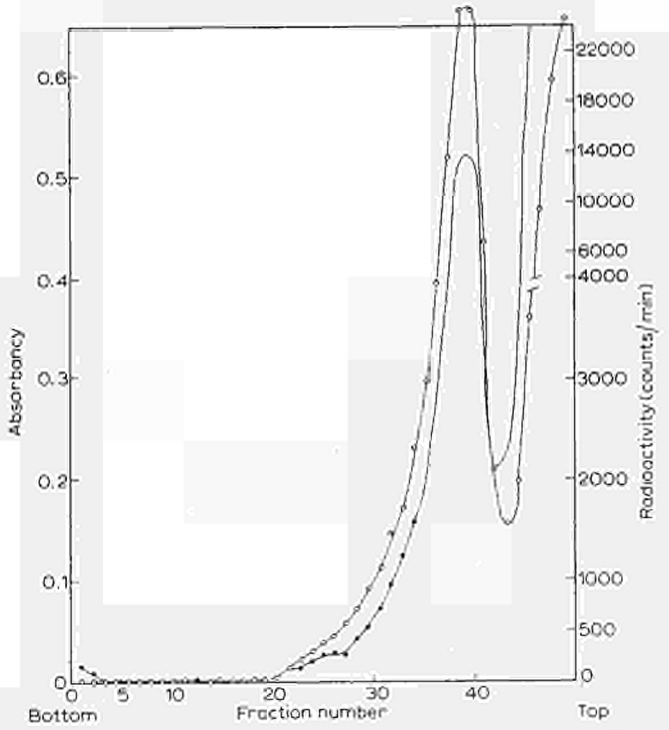

Fig. 2. Sedimentation profile of ribonuclease-treated total-cell homogenate in a 15–30 % sucrose gradient. Coordinates as in Fig. 1. ●–●, absorbancy; ○–○, radioactivity.

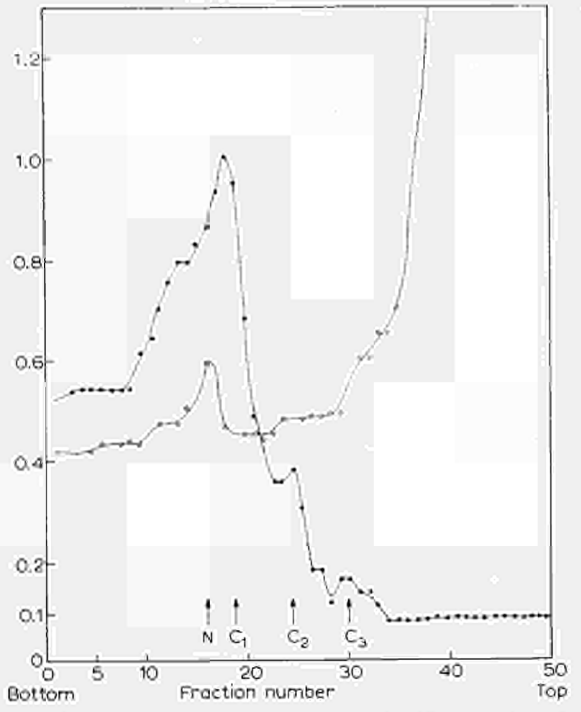

Fig. 3. Sedimentation profile (absorbancy) of cytoplasmic (C, ●–●) and nuclear (N, ○–○) ribosomes. Arrows N and C_1 C_2 C_3 indicate fractions which were selected for electron microscopy. The high absorbancy of the nuclear fractions at the top of the gradient is due to DNA digestion products liberated during the disruption of the isolated nuclei.

major peaks (order of 74 S) can be clearly seen; the minor cytoplasmic ribosomes (region of 50 S and 30 S) are also apparent. The upper part of the profile of the nuclear extract is obscured by the digestion products of the DNA which do not permit the localization of the lighter ribosomal fractions. The cytoplasmic and nuclear ribosomes sedimenting in the 74-S region are similar in size and shape (Plates 2 and 3)

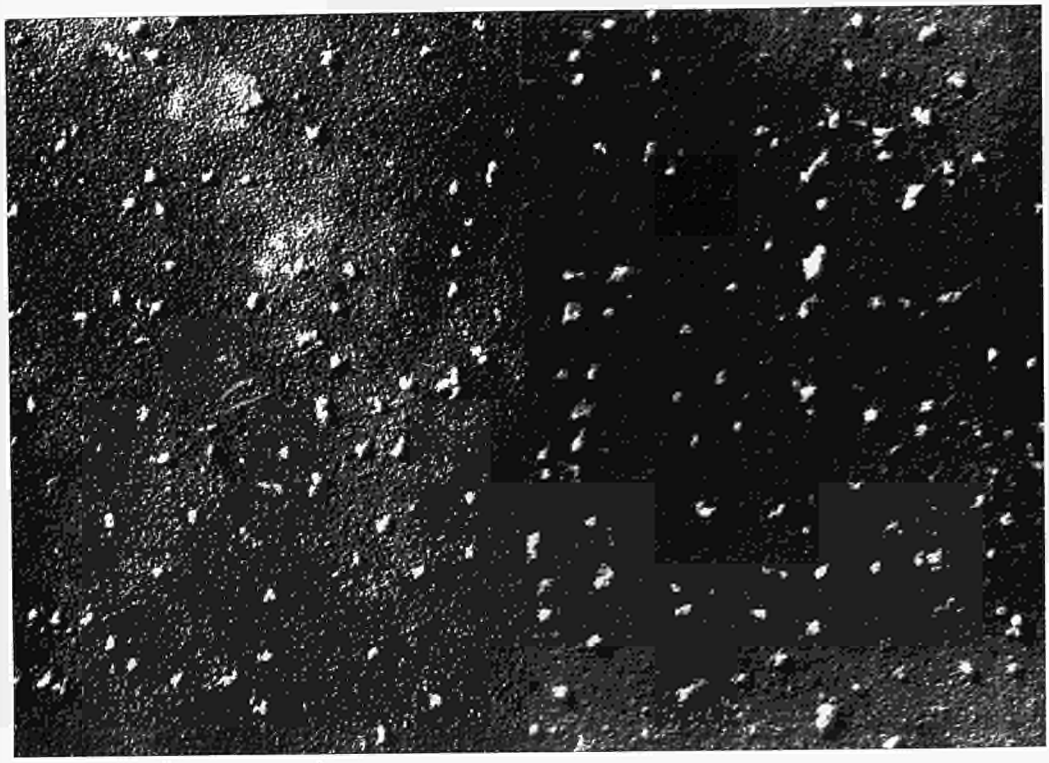

Plate 2. Cytoplasmic 74-S ribosomes (C_1 of Fig. 3), 46 000×.
Plate 3. Nuclear 74-S ribosomes (N_1 of Fig. 3), 46 000×.

and are difficult to distinguish one from the other. They also behave similarly when treated with versene: both types are seen to dissociate into two lighter components (Fig. 4). Plates 4 and 5 show cytoplasmic 50-S and 30-S ribosomes obtained as shown in Fig. 3.

Measurements made on these various ribosomes show the following dimensions:

Cytoplasmic 74-S ribosomes, large diameter	approx. 420 Ångstrom
Cytoplasmic 74-S ribosomes, small diameter	approx. 330 Ångstrom
Cytoplasmic 50-S ribosomes	approx. 340 Ångstrom
Cytoplasmic 30-S ribosomes	approx. 140 Ångstrom
Nuclear 74-S ribosomes, large diameter	approx. 470 Ångstrom
Nuclear 74-S ribosomes, small diameter	approx. 350 Ångstrom

The 74-S cytoplasmic ribosomes appear to have an ovoid shape, the smaller extremity of which has a diameter very close to the 50-S ribosomes. The long axis

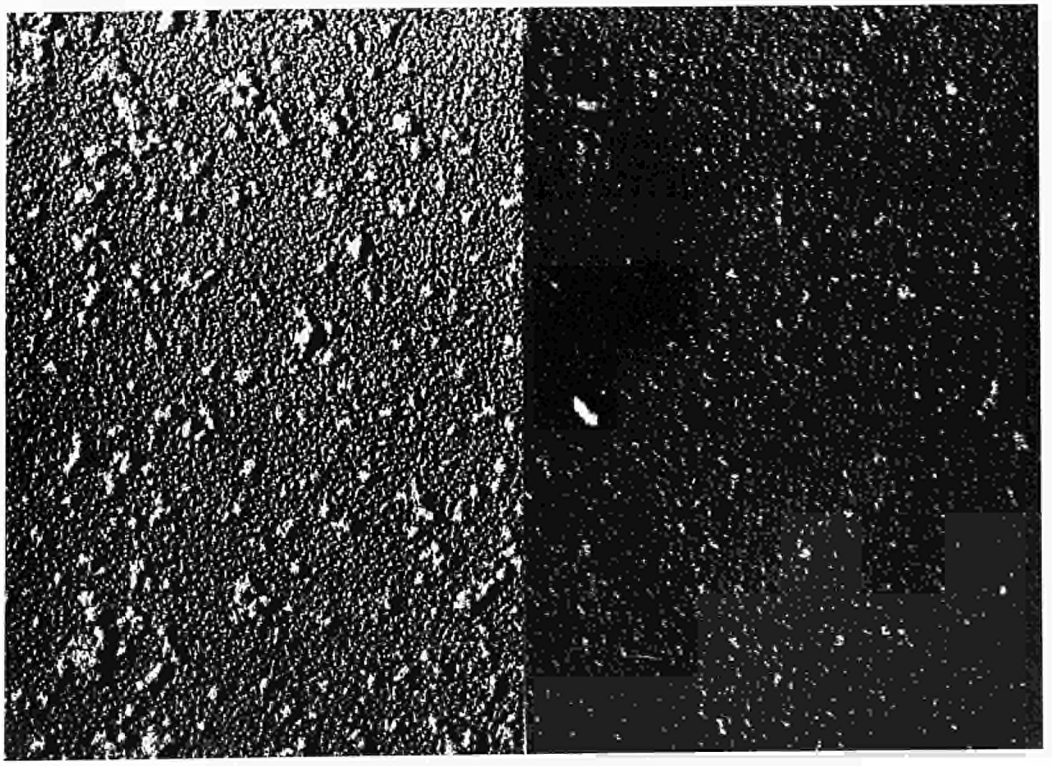

Plate 4. Cytoplasmic 50-S ribosomes (C_2 of Fig. 3), 46 000×.
Plate 5. Cytoplasmic 30-S ribosomes (C_3 of Fig. 3), 46 000×.

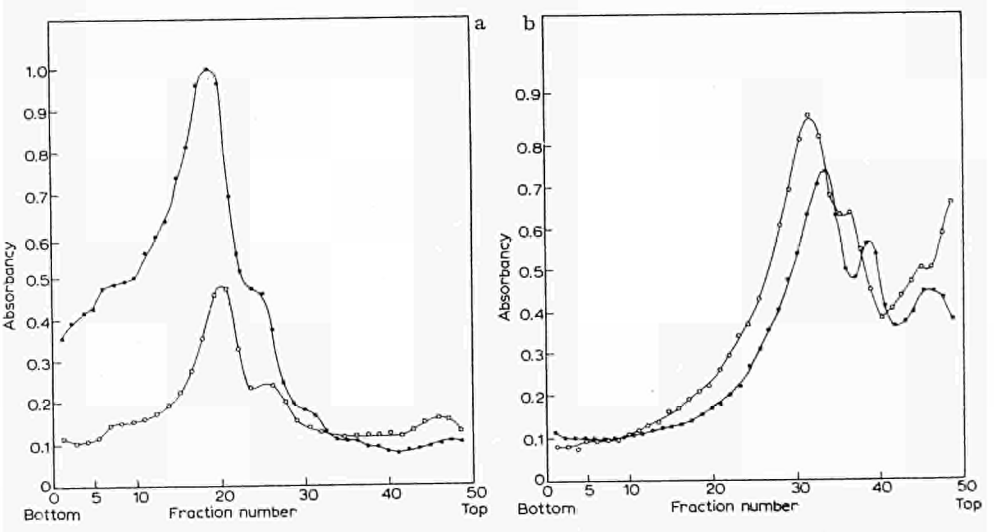

Fig. 4. Sedimentation profile (absorbancy) of cytoplasmic (●–●) and nuclear (○–○) ribosome pellets taken up in phosphate–Mg^{2+} buffer (a) and in versene (b).

of these 74-S particles is somewhat smaller than the sum of the diameters of the 30-S plus 50-S ribosomes. This suggests a deformation of the contact surfaces of these ribosomes which appear to have a mode of association very similar to that postulated for *Escherichia coli* and yeast[7]. Although the nuclear 74-S ribosomes are somewhat larger than the cytoplasmic ones, they are probably also constituted of a similar association.

The 74-S nuclear ribosomes have a similar morphology to thymus nuclear ribosomes[8–10]. The last authors found particles chiefly of approx. 260 Å in diameter, which sediment as 78-S ribosomes, but the preparations also contained smaller and larger particles (130–350 Å) which might be dissociation or aggregation products of the former. WANG[9], after treating the ribonucleoprotein particles with distilled water, found particles of 300 Å, 250 Å and 100 Å diameter, the larger ones probably being various combinations of the small ones.

When the cytoplasmic ribosomes were prepared in Tris–Mg^{2+} buffer and layered over a 15–30 % sucrose gradient, fractions could be collected in the lower half of the gradient and the electron microscopic observations showed a variety of clumps, chains and other types of ribosomal association (polysomes), and also some isolated ribosomes of the size of the 74-S category (Plate 6).

In these polysomes, the diameter of the 74-S units was of the order of 400 Å and these units were closely associated, and not more than 4–5 Å separate each member of a chain.

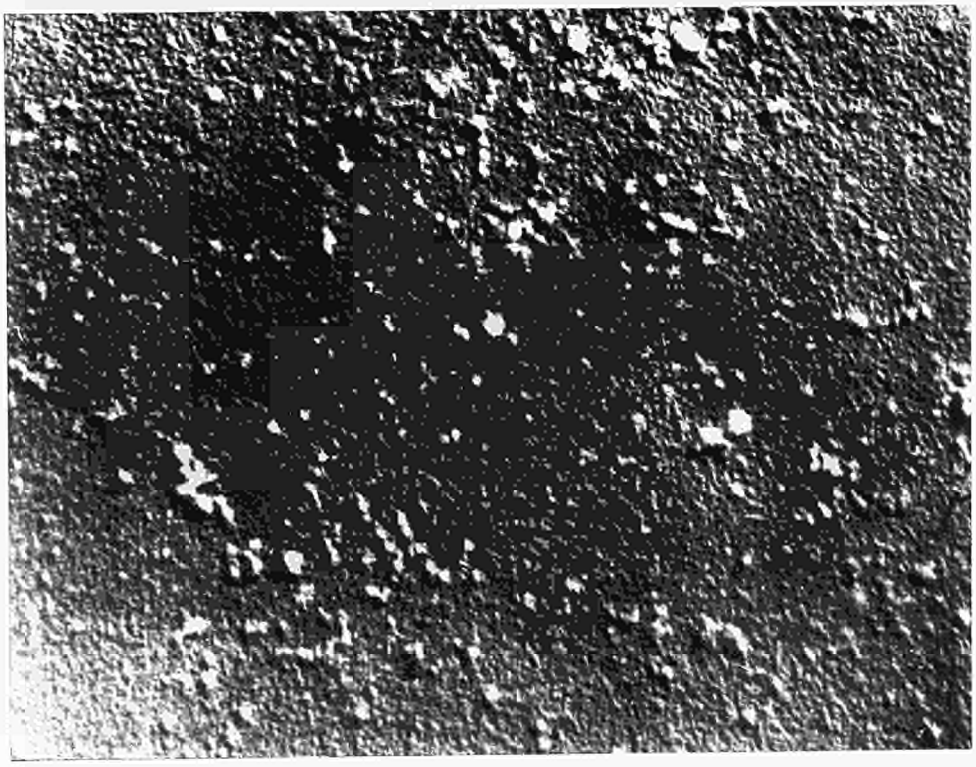

Plate 6. Cytoplasmic polysomes (Fraction 7) without ribonuclease treatment after density gradient centrifugation, 46 000 ×.

Rough estimations of the molecular weight of RNA formed rapidly and probably bound to several ribosomes, give a value of at least $2 \cdot 10^6$ M (ref. 1); this would give a chain length of the order of 20 000 Å which could carry some 40 or more ribosomal units. The larger aggregates of Plate 1 could very well consist of at least as many units, but the ones in Plate 6 have probably been partly de-aggregated.

When the ribosomal preparation was treated with 0.5 μg of ribonuclease per ml before gradient centrifugation, no more chains were observed, and, with the exception of a few minor aggregates of 2–3 units, all the ribosomes have the size of those observed in the 74-S peak (Plate 7).

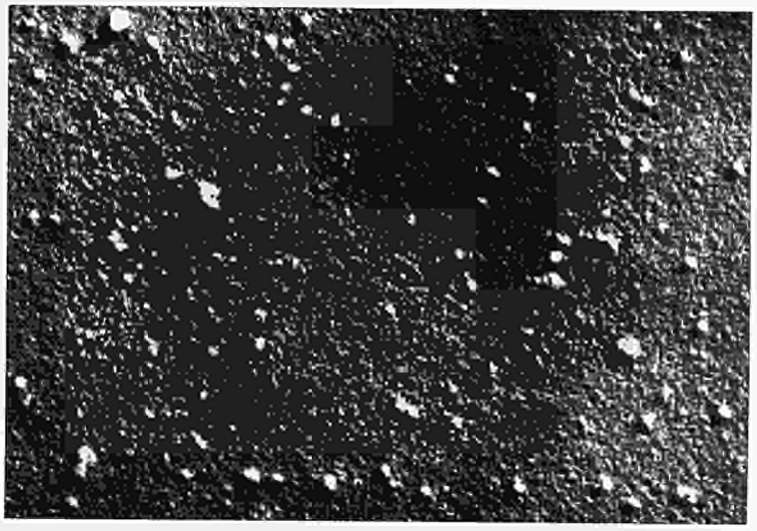

Plate 7. As Plate 6, but with ribonuclease treatment.

When the absorbancy profile of the sedimentation pattern was studied, it was seen (Fig. 5) that after ribonuclease reatment the absorbancy was lower in the peak (74-S region). This effect of ribonuclease has been observed several times and the relative proportion of ultraviolet absorbing constituents in 3 zones of the gradient has been tabulated (Table I). These experiments support the view that the various ribosomal units of the polysomes are held together by RNA and the preceding paper shows that this RNA becomes labelled very rapidly by radioactive precursors (m-RNA ?).

In another experiment, the last ribosomal pellet before gradient centrifugation was taken up in 0.01 M versene and centrifuged in a sucrose gradient made up in Tris buffer containing no $MgCl_2$ (Fig. 5).

The absorbancy of the heavy part of the gradient has dropped and, in addition, all the 74-S region has dissociated into lighter components (50 S and 30 S).

This experiment suggests that the link between the RNA strand which unites the 74-S ribosomes takes place at or near the contact surface between their 30-S and 50-S constituents and that cations (Mg^{2+} ?) participate in this union.

DISCUSSION

The similarity between nuclear and cytoplasmic ribosomes is striking; they both appear to have similar sedimentation constants (approx. 74 S) and both dissociate

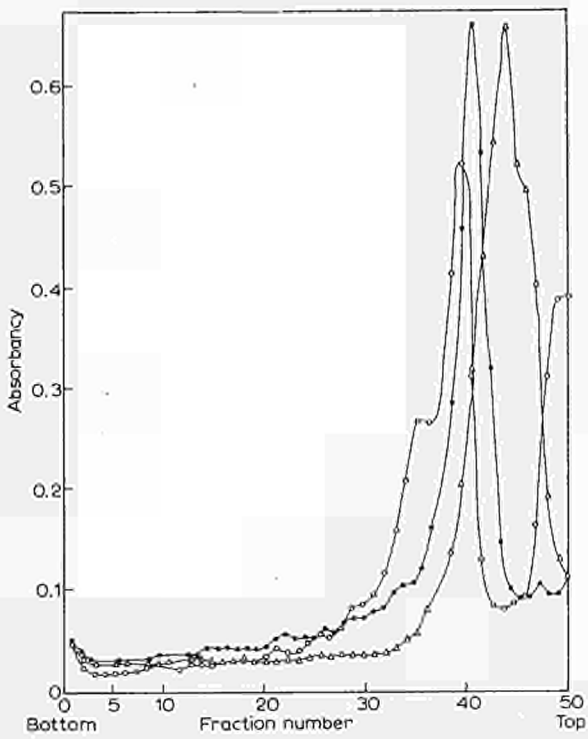

Fig. 5. Sedimentation profile of cytoplasmic ribosomes prepared (a) in Tris–Mg^{2+} buffer (untreated; ●–●); (b) in the same buffer to which ribonuclease was added before centrifugation (○–○); or (c) in Tris buffer containing versene instead of $MgCl_2$ (△–△).

TABLE I

PERCENT OF ULTRAVIOLET ABSORBING MATERIAL IN DIFFERENT REGIONS OF THE CENTRIFUGATION GRADIENTS

The 74-S region consisted of 5 fractions on each side of the 74-S peak; the other 2 regions being the heavier fractions (polysomes) and the lighter ones (surface). The ribonuclease was added before gradient centrifugation and the incubation was done for 1 h at 4°. In expts. 1 and 2, 0.5 μg of enzyme was used per ml, and in Expt. 3, 5 μg/ml.

	Polysomes		*74-S region*		*Surface*	
Control	27.9		61.3		10.8	
RNAase		17.3		49.6		33.1
Control	30.9		53.6		15.5	
RNAase		20.8		49.4		29.8
Control	29.6		58.2		12.2	
RNAase		17.7		41.3		41.0

in the same manner when the cations are removed by versene. It is not yet possible to give accurate sedimentation constants: a preliminary run done with the collaboration of Dr. HAMERS in Professor JENNER's laboratory has shown that the major peak of cytoplasmic ribosomes sediments with a constant of approx. 74 S (compared to 74 S of PENMAN *et al.*[11], to 78 S of ZIMMERMAN[12] for HeLa-cell ribosomes, and 78 S found for thymus nuclear ribosomes by POGO *et al.*[10] and that after dissociation these ribosomes sediment with constants of approx. 50 S and of approx. 30 S. A small amount of these constituents can already be seen in the presence of the magnesium

buffer, but perhaps with a slightly higher sedimentation constant. The slightly different sedimentation profiles seen for the nuclear and cytoplasmic ribosomes and for their dissociation products are not believed to be significant.

The dissociation pattern of the nuclear ribosomes is identical to the one observed for thymus nuclei by POGO[10].

The existence, in the heavier parts of the gradients, of ribosomal associations (clumps or chains), apparently made up of 74-S ribosomal units, points to the existence of polysomes. In the previous paper this part of the gradient was shown to be the most active in protein synthesis. This is a confirmation of what has been found for reticulocytes by WARNER *et al.*[4], by NOLL *et al.*[5] and WETTSTEIN *et al.*[6], and more recently by PENMAN *et al.*[11] for HeLa cells.

The fact that these associated ribosomes break up in ribonuclease also confirms the belief that the mode of union of these units involves RNA (m-RNA as postulated by the various authors just cited). Unfortunately the resolution of the electron microscope used has not enabled us to observe with certainty any RNA chain between the various units of the polysomes. The fact that these polysomes break up into 50-S and 30-S ribosomes when cations are removed suggests a close association of the linking RNA strand with the contact surfaces of the ribosomal subunits. A rough calculation presented in the text leads to the idea that association of as many as 40 ribosomal units per polysome might exist.

ACKNOWLEDGEMENTS

We thank Professor J. BRACHET most heartily for giving us permission to use his electron microscope and Professor R. JEENER and DR. HAMERS for the analytical centrifuge.

We are very grateful to Drs. F. VANDERHAEGHE AND P. VAN GANSEN for their advice in preparing the samples for electron microscopy.

This work was supported by the National Institutes of Health, U. S. Public Health Service, Grant R.G. 6214 and by Euratom, Contract 0.16.61.10 ABIB. One of us, M.H., is a Research Fellow of the International Atomic Energy Agency (Vienna).

REFERENCES

1 M. HILL, A. MILLER-FAURÈS AND M. ERRERA, *Biochim. Biophys. Acta*, 80 (1964) 39.
2 H. FRAENKEL-CONRAT, B. SINGER AND A. TSUGITA, *Virology*, 14 (1961) 54.
3 H. HARRIS, H. W. FISHER, H. RODGERS, T. SPENCER AND J. W. WATTS, *Proc. Roy. Soc. London, Ser. B*, 157 (1963) 177.
4 J. R. WARNER, S. KNOFF AND A. RICH, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 122.
5 H. NOLL, T. STAEHELIN AND F. O. WETTSTEIN, *Nature*, 198 (1963) 632.
6 F. O. WETTSTEIN, T. STAEHELIN AND H. NOLL, *Nature*, 197 (1963) 430.
7 J. K. KOEHLER, *Nature*, 194 (1962) 757.
8 J. H. FRENSTER, V. G. ALLFREY AND A. E. MIRSKY, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 432.
9 T. Y. WANG, *Arch. Biochem. Biophys.*, 97 (1962) 387.
10 A. O. POGO, B. G. T. POGO, V. C. LITTAU, V. G. ALLFREY AND A. E. MIRSKY, *Biochim. Biophys. Acta*, 55 (1962) 849.
11 S. PENMAN, K. SCHERRER, Y. BECKER AND J. E. DARNELL, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 654.
12 E. F. ZIMMERMAN, *Biochem. Biophys. Res. Commun.*, 11 (1963) 301.