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by

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STUDIES ON RAPIDLY LABELLED RIBONUCLEIC ACID IN HeLa CELLS

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SUMMARY

A study of the sedimentation pattern in sucrose gradients of rapidly-labelled RNA (5–30 min labelling with [^{14}C]adenine) was done on whole extracts of HeLa cells. Two regions of radioactivity have been found: a lighter region sedimenting in the range of 4 to 20-S particles and a heavier heterogeneous region sedimenting from about 30 S to the bottom of the gradients even when 30 % sucrose was used. The nature of the various types of RNA's likely to sediment in these regions is discussed. The heavier heterogeneous region contained very small amounts of DNA, but no definite evidence for the existence of DNA–RNA hybrids was obtained. On the other hand, the heavy heterogeneous region is active in amino acid incorporation and consisted of a variety of polysomes; their characterization by electron microscopy is described in a further paper. These polysomes, after a short labelling period, consist of unlabelled 74-S ribosomes to which is attached rapidly-labelled RNA; it was shown that it takes roughly 5 min for [^{14}C]adenine to become integrated into RNA sedimenting with the polysomes. Comparison of autoradiographic and sedimentation analysis shows that a large proportion of the polysomes might be formed in the cell nucleus.

INTRODUCTION

The problem of the biosynthesis of RNA's is far from being solved in spite of the progress made in recent years. After the demonstration by BRACHET[1] of the role of the cell nucleus in cytoplasmic RNA synthesis, many authors have approached the problem from a variety of angles which have led to the concept of three major classes of RNA's, which it is not necessary to define here[2]: messenger RNA's, ribosomal RNA's and aminoacid transfer RNA's.

The requirement of a DNA primer for m-RNA synthesis seems obvious, as does its dependence on nuclear function. These relationships do not seem quite as clear for r-RNA and s-RNA. In the case of r-RNA synthesis it was shown by PERRY *et al.*[3–5] that the nucleolus plays an important role in cytoplasmic RNA synthesis which is mostly ribosomal, but this does not exclude the participation of nucleolar associated DNA, as pointed out by the authors and as shown on plant cells by RHO AND BONNER[6].

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The fact that r-RNA is capable of hybridizing with DNA reinforces the idea that its synthesis is DNA dependent[7]. In the case of s-RNA the situation is still less clear, but as the incorporation of pseudo-UTP into an s-RNA-like molecule is DNA dependent, and since s-RNA also hybridizes with small sequences of DNA, there are reasons to believe that at least part of s-RNA synthesis is also DNA dependent[8,9].

We therefore thought it worth while to approach the problem of RNA synthesis by studying the distribution of cellular RNA in various fractions after brief exposure to a tracer, and to investigate its ultimate fate. The present study concerns only the rapidly-formed RNA components (5–30 min) analysed by sedimenting a whole-cell homogenate in a sucrose gradient; therefore in these experiments all the rapidly labelled RNA can be accounted for.

MATERIALS AND METHODS

Cell cultures

HeLa cells were used in all the present studies. We are grateful to the Institut Pasteur du Brabant for giving us the cell line and for helping us in making the culture media. The cells were cultured in the media described previously[4,5,10] in Roux flasks. The complete medium (ϕ_{10}) used for breeding the cells was changed to Eagle's medium 2 h before the start of an experiment. Cells were usually taken when they were nearing the end of the logarithmic phase of growth, but were still in monocellular layers.

RNA labelling

The cells were labelled in Roux flasks for the appropriate time with [^{14}C]adenine, dissolved in Eagle's medium (1 μC/ml). They were incubated with the precursor in a water bath at 37° and were chilled to 0–2° immediately after, and washed twice with cold Eagle's solution containing 200 μg of non-labelled adenine per ml.

Reagents used routinely

Tris–Mg^{2+} buffer: 0.05 M Tris, 0.025 M KCl, 5 mM $MgCl_2$, pH 7.45.

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

[8-^{14}C]Adenine sulphate: Amersham, 28.3 mC/mmole.

L-[^{14}C]Lysine monohydrochloride: Amersham, 307 mC/mmole.

Homogenization

The labelled monolayers of cells were washed in Eagle's medium, harvested with glass beads, collected by a 5-min centrifugation at 1500 rev./min, then washed again with cold Eagle's solution containing non-labelled adenine. Usually, for good absorbancy measurements of the centrifugation fractions, about $5 \cdot 10^7$ cells were needed. They were dispersed in 1.2–1.5 ml Tris–Mg^{2+} buffer or phosphate–Mg^{2+} buffer to which was added sodium deoxycholate (5 % in H_2O) and bentonite (5 mg/ml), prepared according to FRAENKEL-CONRAT[11] in a final concentration of 1 % and 0.5

mg/ml respectively. The buffers were adjusted to pH 7.45. The cells were dispersed by pipetting, and a few crystals of deoxyribonuclease were added in order to decrease the viscosity; after 30–60 min, the homogenate was ready for gradient centrifugation.

Sucrose gradient centrifugation

Linear sucrose gradients were prepared, according to BRITTEN AND ROBERTS[12], from 14.2 ml of 5 % (or 15 %) sucrose and 13.4 ml of 20 % (or 30 %) sucrose made up in the same buffer used for homogenization. The cell homogenates were layered on top of the sucrose gradients and were centrifuged in 34 ml lucite tubes in the swinging bucket rotor 25 of a Spinco model L preparative ultracentrifuge at 24 000 rev./min. When 5–20 % sucrose gradients were used, the duration of centrifugation was of 4.5 h; with the 15–30 % sucrose gradients, the centrifugation lasted for only 2 h. After centrifugation, 45–50 fractions were collected in 10-ml test tubes and assayed for absorbancy (0.15 ml plus 0.75 ml H_2O) and the remainder (about 0.35 ml) was prepared for radioactivity counting. To enable the graphs to be compared, the abscissae representing the gradient were always taken of a constant length independently of the number of fractions. Afterwards, for simplicity's sake, the abscissae were divided into 50 arbitrary fractions.

Radioactivity counting

To an aliquot of each fraction (about 0.35 ml), 0.3 ml of a bovine serum albumin solution (0.4 mg/ml in Tris or phosphate buffer) and 2.0 ml of 5 % trichloroacetic acid were added. The suspensions were filtered on 25 mm Millipore filters (HA 0.45 μ), washed twice with 2 ml of 5 % trichloroacetic acid, then dried at 37° in the oven for 2 h. The Millipore filters carrying the radioactive precipitates were then put into standard counting vials containing 5 ml of scintillation solution (0.5 % P.O.P. and 0.05 % P.O.P.O.P.). Counting was done in a model 725 Nuclear Chicago scintillation counter.

RESULTS

Pulse labelling

Typical profiles of the centrifugation gradients are presented in Fig. 1. These result from 5- and 10-min [^{14}C]adenine incorporation and the total cell homogenate was fractionated on a 5–20 % sucrose gradient. The peak of absorbancy is in the 74-S region (ribosomes)[13], the absorbancy in the light part of the gradient being obscured by the digestion products of DNA. A preliminary run with the analytical ultracentrifuge kindly performed by Dr. HAMERS indicated that the major peak of cytoplasmic ribosomes had a sedimentation constant of 74 S, and it is assumed that in these runs the major peak consisted essentially of this type of ribosomes. The smaller peaks in the lighter region had sedimentation constants of about 50 S and about 30 S (ribosomal constituents). Another peak at 16 S was also detected (free RNA?). Two distinct regions of radioactivity were observed. A light fraction remained near the surface of the gradient in the 4 to 20-S region which almost certainly contained some s-RNA either in its final form or as a precursor. The lighter part of the gradient was not studied further, and it was not possible for us to identify the heterogeneous 6 to 20-S fractions with a base distribution reminiscent of HeLa

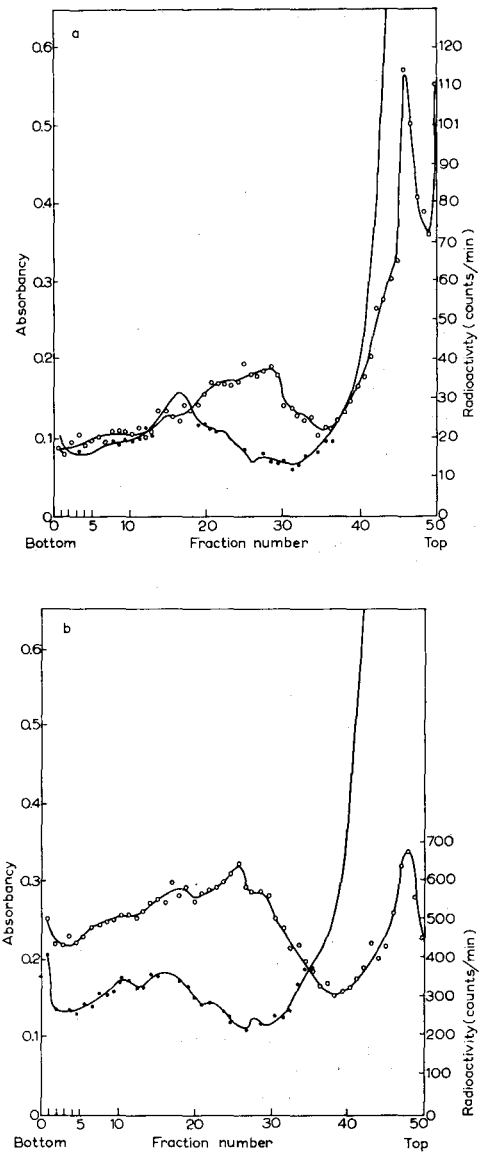


Fig. 1. [8-^{14}C]Adenine-labelled HeLa cells homogenized and centrifuged for 4.5 h in 5–20 % sucrose gradient at 24 000 rev./min. a: 5-min pulse; b: 10-min pulse. Abscissae: normalized fraction number (see text). Ordinates: left, absorbancy (●–●); right, counts/min (○–○).

DNA demonstrated by PENMAN *et al.*[13] because these RNA's would probably be confluent with the lighter peaks.

A heavy fraction contained radioactivity which sedimented as far as the bottom of the tube and consisted of heteregeneous components with a wide and irregular peak in the region sedimenting between the 50-S and the 30-S particles (Figs. 1a, 1b, and 3a, 3c). This RNA was very sensitive to ribonuclease (Fig. 3c) and disappeared rapidly when the cells were homogenized in the absence of an inhibitor of this

enzyme. This was observed repeatedly in attempts to characterize nuclear and cytoplasmic rapidly-labelled RNA by density gradient centrifugation. In the nuclear fraction the main peak is in the 4-S region with irregular tailing in the 25 to 30-S. In the cytoplasmic fraction only a small 4-S peak was observed in 10-min incubated cells.

This newly formed RNA can reasonably be assumed to be the heavy RNA of PERRY[14] and the 45-S and 35-S RNA's of SCHERRER *et al.*[15]. There could still be some free RNA sedimenting as far as the 74-S zone because both these authors found that "new" RNA still sedimented to the bottom of their gradients. The heavier part of this heterogeneous fraction (> 74 S) has been analysed further in a 15–30 % sucrose gradient to obtain more details. Fig. 2 shows that a considerable part of the rapidly-labelled RNA (about 40–50 %) still migrates faster than the 74-S components. This is unlikely to be free RNA, because the single-stranded regions would probably be mechanically broken during the preparation of the samples. This RNA could be bound to cellular structures or other macro-molecules.

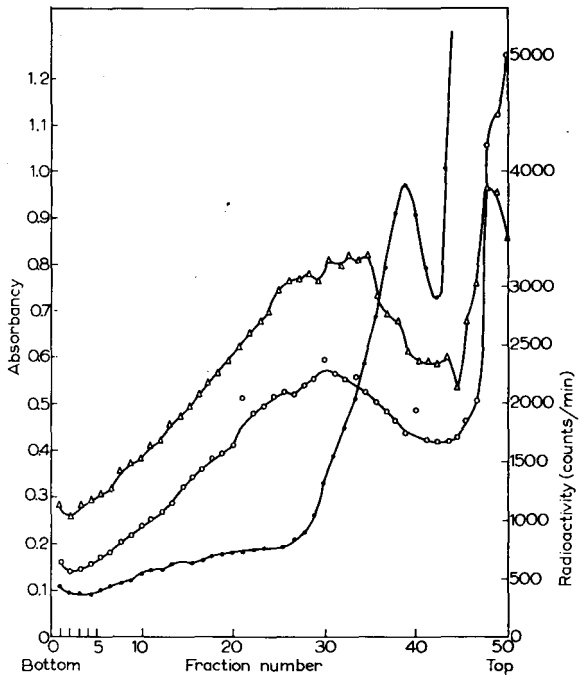


Fig. 2. [8-^{14}C]Adenine labelled HeLa cells (30-min pulse) with and without actinomycin D, homogenized and centrifuged in a 15–30 % sucrose gradient for 2 h at 24 000 rev./min. Coordinates: as in Fig. 1. ●–●, absorbancy; ○–○, radioactivity of actinomycin-treated; △–△, radioactivity of untreated HeLa cells.

PERRY[14,16] showed that in the presence of very small amounts of actinomycin D ($5 \cdot 10^{-8}$ M) there was a decrease in all the radioactivity of 30-min labelled RNA's sedimenting faster than 10 S, as well as an inhibition of nucleolar labelling; upon further incubation of these cells in non-radioactive medium no activity appeared in ribosomes. In an attempt to localize this ribosomal precursor RNA in fractions from

whole cells, a run was made in which actinomycin D was used under the conditions described by PERRY[14]: the cells were preincubated in Eagle's solution for 90 min, actinomycin D was added to a final concentration of $5 \cdot 10^{-8}$ M for 30 min, and finally the cells were incubated for another 30 min in the presence of actinomycin D and of [8-^{14}C]adenine as in the previous runs (Fig. 2). Actinomycin D decreased the activity along the whole of the gradient sedimenting faster than 4–10 S without any particular localization. As this treatment is presumed, as in PERRY's experiments, completely to inhibit the formation of 28-S and 16-S ribosomal RNA, one may conclude that adenine after a brief pulse goes partly to a heterogeneous or heterogeneously-bound precursor of r-RNA.

Analysis of the heavy components

Further experiments were done to obtain information on these heavy fractions and to find out what other types of cell constituents they might contain which might bind some of the new RNA.

a. Experiments with [^{3}H]thymidine-labelled cells permitted us to establish the existence of small amounts of DNA. However no conclusive evidence for the presence of DNA–RNA hybrids was obtained.

b. A functional analysis of the cell components sedimenting faster than the 74-S ribosomes demonstrated their activity in amino acid uptake. This evidence for the existence of polysomes was completed by a study of the kinetics of formation of these ribosomal complexes.

c. Parallel to these studies some of the fractions were observed in the electron microscope[17].

a. Presence of DNA:

(1) In the first experiment of this series the cells were prelabelled for 24 h with [^{3}H]thymidine before being transferred to Eagle's medium for 2 h. They were then further labelled in the usual manner for 10 min with [^{14}C]adenine. Analysis of the gradient (Fig. 3a) showed a significant thymidine radioactivity in the heavy region accompanying the labelled RNA, the greatest quantity being digested by the deoxyribonuclease before centrifugation and remaining near the surface of the gradient or becoming acid soluble. The amount of DNA remaining undigested amounted to less than 0.1 % of the total thymidine-labelled deoxyribonucleotides still precipitable by trichloroacetic acid.

(2) The second experiment, done only with [^{3}H]thymidine-labelled cells without further RNA labelling, demonstrated that some undigested DNA remained in the heavy part of the gradient (Fig. 3b). When deoxyribonuclease was added to the centrifuged fractions before precipitating the acid insoluble components on the Millipore filter, only about 20 % of the activity of the heavy compounds labelled with the thymidine was digested. When ribonuclease was added simultaneously with the deoxyribonuclease, no further digestion of the thymidine-labelled compound occurred and no evidence for DNA–RNA hybrids could be obtained. The residual radioactivity could be due to unspecific labelling by [^{3}H]thymidine degradation products.

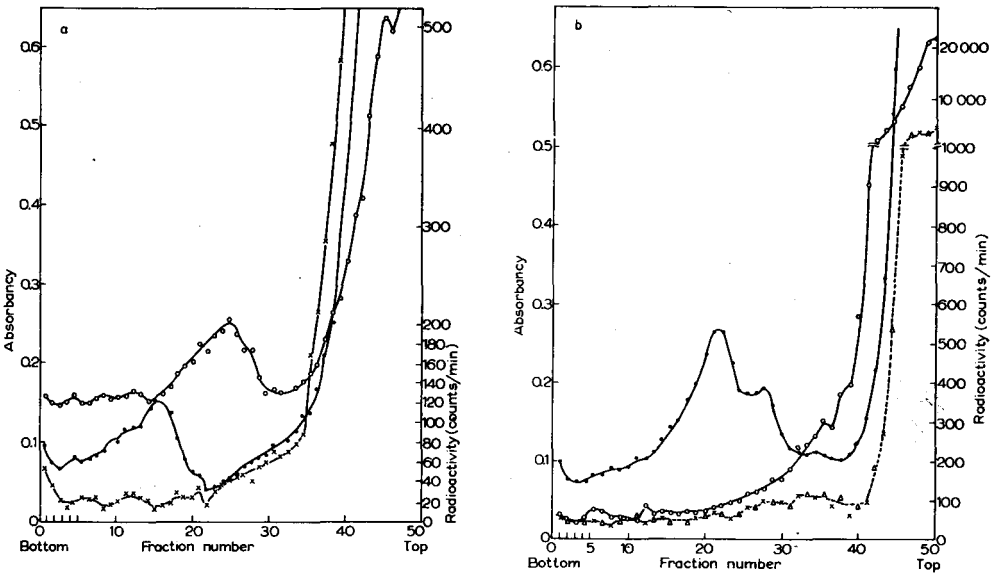
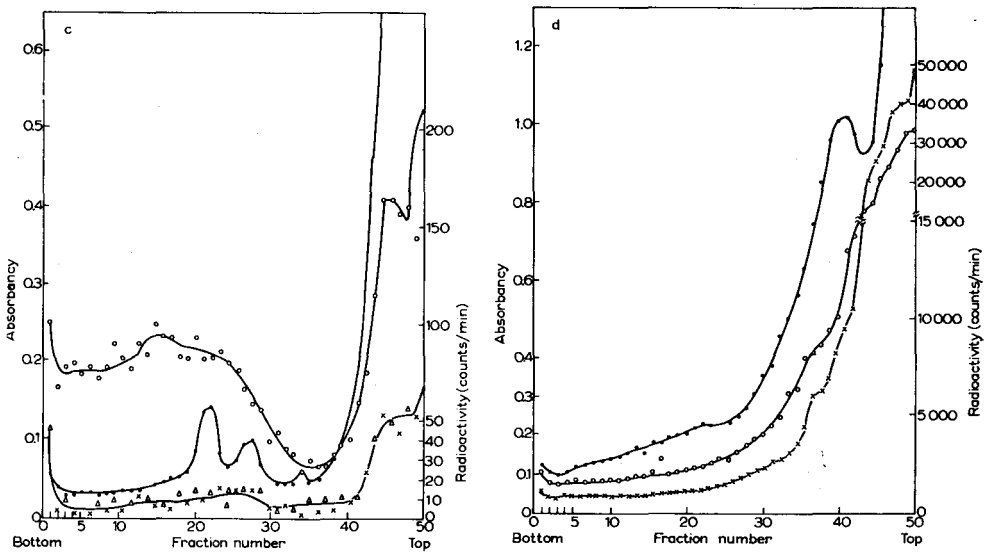


Fig. 3. a: Double-labelled HeLa cells, [^{3}H]thymidine: 24 h; [8-^{14}C]adenine: 10 min. Significant but small thymidine radioactivity was still seen in the heavy part of the gradient. ●–●, absorbancy; ○–○, radioactivity in [^{14}C]RNA; ×–×, radioactivity in [^{3}H]DNA.
b: [^{3}H]Thymidine-labelled HeLa cells (24 h). ●–●, absorbancy; ○–○, no deoxyribonuclease, radioactivity; ×- - -×, deoxyribonuclease after centrifugation, radioactivity; Δ- - -Δ, deoxyribonuclease plus ribonuclease after centrifugation, radioactivity.



c: [^{14}C]Adenine-labelled cells (30 min). ●–●, absorbancy; ○–○, untreated; ×–×, ribonuclease after centrifugation; Δ–Δ, deoxyribonuclease plus ribonuclease after centrifugation. In cases a–c the cell homogenate was sedimented in a 5–20 % sucrose gradient and centrifuged for 4.5 h at 24 000 rev./min. Coordinates as in Fig. 1.
d: [^{3}H]Thymidine-labelled HeLa cells (24 h). ●–●, absorbancy; ○–○, deoxyribonuclease before centrifugation, radioactivity; ×–×, deoxyribonuclease plus ribonuclease before centrifugation, radioactivity. The cell homogenate was sedimented in a 15–30 % sucrose gradient and centrifuged for 2 h at 24 000 rev./min. Coordinates as in Fig. 1.

(3) In the third experiment, cells labelled in the usual manner by [8-^{14}C]adenine for 10 min were homogenized and analysed on the sucrose gradient. After centrifugation, 5 μg of ribonuclease or 5 μg ribonuclease plus 10 μg of deoxyribonuclease were added per ml to alternate fractions and these were incubated for 30 min at 37° before being assayed on the Millipore filters after trichloroacetic acid precipitation. They were compared with controls run in parallel from the same batch of cells. The ribonuclease treatment digested about 90 % of the labelled macromolecules sedimenting in the heavy part of the gradient, and the addition of deoxyribonuclease to the ribonuclease did not increase the amount of RNA digested in this fraction (Fig. 3c): again it was not possible to obtain evidence for any significant amount of RNA–DNA hybrid.

(4) A last experiment was performed on a 24-h [^{3}H]thymidine-labelled culture: 0.5 μg of ribonuclease was added per ml immediately after the homogenization together with the deoxyribonuclease added routinely (Fig. 4d). There was a significant shift of the radioactivity to the lighter part of the gradient when ribonuclease was used, indicating that the existence of small amounts of DNA–RNA hybrids must not be excluded.

The failure of the preceding experiments could be due to traces of ribonuclease in the sucrose used to prepare the gradients. Both enzymes are active when added directly to the homogenate before centrifugation, whereas in the deoxyribonuclease controls only the one enzyme is present.

b. Presence of polysomes: In addition to the electron-microscope studies of various centrifuged fractions, the existence of polysomes was established by observing their disappearance in the presence of ribonuclease[17] and by demonstrating their activity in amino acid uptake. A preliminary run with the analytical ultracentrifuge showed heavy components to sediment as well-defined peaks in the 90-S and 118-S region for cytoplasmic preparations, and in the 111-S region for nuclear preparations.

Evidence for amino acid uptake in the heavy parts of the gradient

It has been shown in reticulocytes that haemoglobin synthesis takes place in polysomes[18]: these are associations of ribosomes, presumably along a m-RNA strand[13,19–21]. It was therefore important to study the pattern of protein synthesis or at least of amino acid uptake in the various parts of the gradient. To this effect, the cells after incubation in Eagle's medium for 2 h, were further incubated for 2.5, 5, 10 and 30 min in Eagle's medium (prepared without lysine) to which was added 10 μC of [^{3}H]lysine per ml. After 2.5 min, the uptake was hardly above background, but for longer incubation times there was a measurable uptake of lysine in the region where ribosomal complexes were found in the electron-microscope studies. The results are shown in Fig. 4, and lysine uptake is calculated for unit absorbancy in the same fraction ("relative specific activity"). It can be seen that maximum relative activity was found in the heavier fraction and reached almost 60 % of the 30-min value after 5 min incubation. These results are similar to those mentioned above and confirm the fact that polysomes are the site of protein synthesis. The minimum of specific activity in the 74-S ribosome region is striking.

In the lighter parts of the gradient (difficult to analyse on account of deoxy-

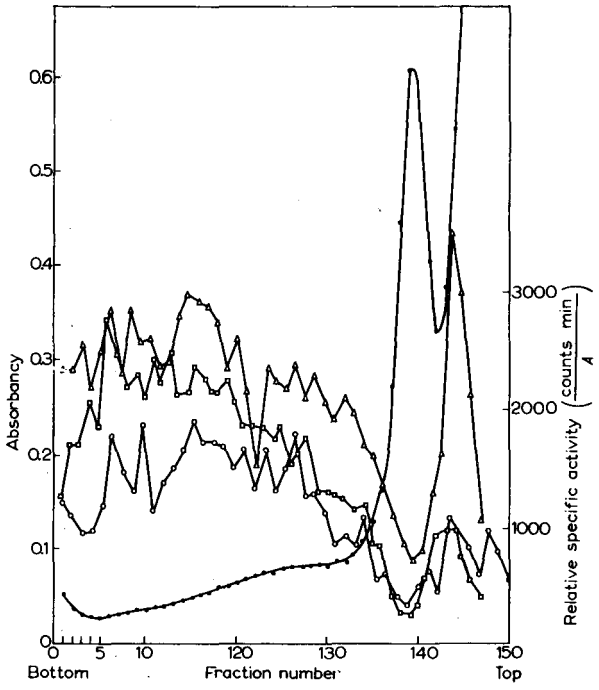


Fig. 4. Kinetics of lysine uptake by HeLa cells. The cells were homogenized and centrifuged for 2 h on a 15–30 % sucrose gradient. The results are plotted as relative specific activity (counts/min per unit of absorbancy for each run). Other coordinates as in Fig. 1. ●–●, absorbancy; ○–○, 5 min; □–□, 10 min; △–△, 30 min.

ribonuclease-digestion products) the relative specific activity increased with time, the highest increase taking place in the part of the gradient where the absorbancy was minimum (about 30–50 S). It is not yet possible to discuss the nature of this radioactivity peak, but this new protein remaining bound to physical structures obviously needs further investigation. Lysine being one of the prominent ribosomal amino acids (10–12 % of the total amino acid composition of the ribosomes, according to WANG[22]) it would not be astonishing if part of this radioactivity consists of new ribosomal proteins. Near the surface of the gradient appreciable amounts of labelled acid-insoluble material was found, probably representing newly released proteins.

Kinetics of polysomes labelling

To study the kinetics of adenine uptake into polysomes, cells were given [^{14}C]-adenine for various periods of time and thereafter analysed by density gradient centrifugation in a 15–30 % sucrose gradient. The results are shown in Figs. 5 and 6. It can be seen that after 10 min incubation, the radioactivity was still relatively low, but a rough graphic calculation shows that 40 % sedimented in the region of the gradient heavier than the 74-S ribosomes. After 20 min, approx. 47 % was in this region, and after 30 min, approx. 65–70 %. The kinetics of uptake in fractions 15, 25, 35, 38 and 47 is plotted in Fig. 6. These fractions were chosen arbitrarily, except for fraction 35, which is in the region where ribosome dimers are expected to sediment, and for fraction 38 (74-S monomers). If one extrapolates these various

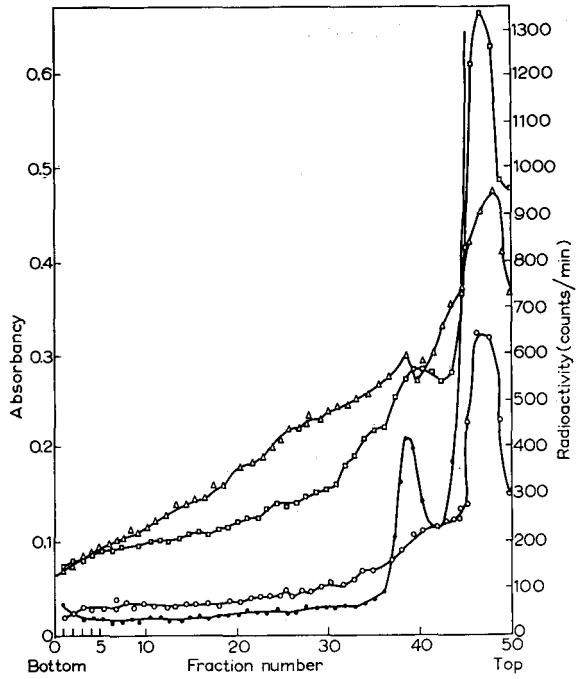


Fig. 5. Kinetics of polysome labelling in HeLa cells. Cells were incubated for 10, 20, 30 min with [8-^{14}C]adenine, homogenized, put on a 15–30 % sucrose gradient and centrifuged for 2 h at 24 000 rev./min. ●–●, absorbancy; ○–○, radioactivity after 10 min; □–□, after 20 min; △–△, after 30 min incubation. Coordinates as in Fig. 1.

curves for zero uptake into the heavy fractions, they all extrapolate down to 4–6 min, after which the activity begins to rise. This indicates that it takes approx. 5 min for the labelling of polysomes from existing ribosomes and new RNA. The present experiments do not prove that inside the cell, the polysomes are formed in such little time. The newly labelled RNA could very well become attached in polysomal form to ribosomes which become accessible only after the homogeneisation. Experiments to determine if the labelling of the polysomes occurs before or after homogenisation are under way. The rise in the relative specific radioactivity of fraction 38 (74 S) is slower than in the polysomal fractions on account of the bulk of pre-existing 74-S ribosomes. In fraction 42 (20–50 S?) the rise in radioactivity apparently starts from time zero — it might well be free RNA before it becomes bound to the polysomes; however we believe that more refined kinetic studies than the present ones are needed in order to discover the relationship which may exist between lighter and heavier fractions.

Could polysomes be formed in the cell nucleus?

Earlier radioautographic experiments[10] have demonstrated that after 10 min incubation, more than 95 % of the radioactivity of labelled adenine or other nucleosides resides in the nucleus. Table I gives a comparison of the cytological and gradient distribution of rapidly-labelled RNA.

If the labelling of polysomes, as studied in the experiments described in Figs.

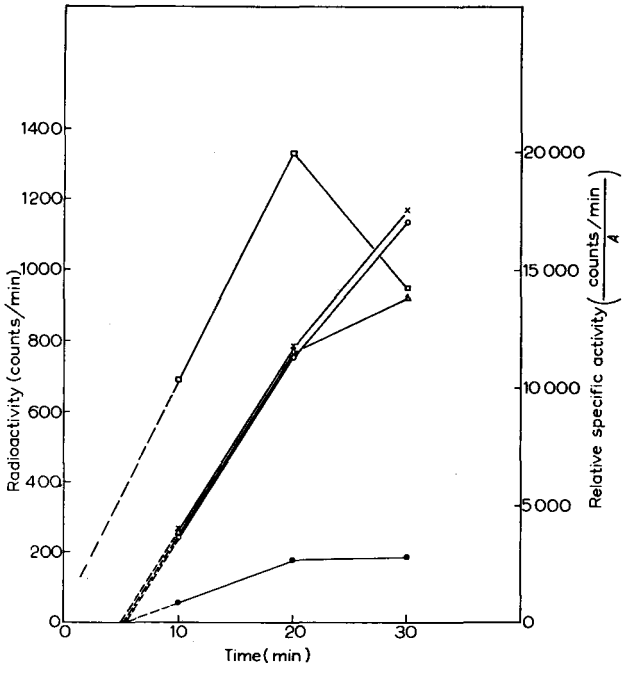


Fig. 6. Kinetics of polysome labelling in HeLa cells. Fractions 15 (×–×), 25 (○–○), 35 (△–△), 38 (●–●) and 47 (□–□) plotted as relative specific radioactivity, (activity counts/min per unit of absorbancy). The uptake of adenine into polysomes seems to follow identical kinetics for dimers or polymers and indicates that these have presumably all been formed during the experiment. The lower relative specific activity of the monomers is taken to indicate that many monomers present at the beginning of the labelling period have not become attached to newly formed RNA. Fraction 42 (sedimenting in the 10 to 16-S region) cannot be expressed in relative specific activity on account of the high absorption due to DNA digestion products. Abscissae: time (min). Ordinates: left, counts/min (Fraction 42); right, counts/min per unit of absorbancy (other fractions).

TABLE I

PER CENT RADIOACTIVITY IN CELL NUCLEUS COMPARED TO ACTIVITY OF POLYSOMES

Figures in Column 2 are values taken from SRINIVASAN *et al.*[10] in runs where the cells were incubated for 10 min in [^{14}C]adenine and transferred for 10 and 20 min to non-labelled medium. Figures in Column 3 are values computed graphically from the radioactivity sedimenting in the heavy (integral radioactivity in Fractions 1–35) and lighter (integral radioactivity in Fractions 36–50) parts of the gradient. Fraction 35 is in the region where ribosome dimers are presumed to sediment.

	Radioautography % *in nucleus*	*Ultracentrifugation* % *in heavy fraction*
10	< 97	40
20	approx. 97	47
30	approx. 95	63–73

5 and 6, really represents their rate of formation inside the cell, this comparison would indicate that most of the newly formed polysomes are in the cell nucleus. However, this would not be a proof that cytoplasmic ribosomes or polysomes are formed inside the nucleus.

CONCLUSIONS

The RNA formed during a short pulse of [^{14}C]adenine in a dividing cellular population, where a great variety of proteins and other constituents are duplicated at each generation, can be expected to be complex. In the present density-gradient analysis, RNA species sedimenting slowly in both types of gradients probably consisted of a variety of s-RNA's, and a heavier species of RNA sedimenting up to about 20 S, which could contain unbound "polysomal" RNA's with DNA-like base ratios, as found recently by PENMAN *et al.*[13]. But, as stated earlier, a more refined analysis of the lighter part of the gradient as well as base analysis are still required to check these points. In the heavier fractions of a 5–20 % sucrose gradient (Fraction 1–40) a heterogeneous sedimentation pattern was found, and treatment of the homogenate with ribonuclease resulted in an important loss of this radioactivity. From the phenol-purified RNA analysis of PERRY[14] and of SCHERRER *et al.*[15], the labelling of HeLa cells by RNA precursors appears rapidly in high molecular RNA's sedimenting around 45 S (ref. 15), presumably of molecular weights above $2 \cdot 10^6$. Afterwards a 35-S species began to appear followed by the 16- and 28-S ribosomal RNA's. The 45-S and 35-S species might well be in the free state in our analysis, on account of the maximal radioactivity found in these regions of the gradients. Electron-microscopical analysis of fractions taken in this area showed the existence of rather pure approx. 50-S and approx. 30-S ribosomes[17] which are not labelled (preliminary data). The absence of other morphologically identifiable components and the non-parallelism between ribosomal absorbancy and radioactivity supports the view that free RNA might sediment in these regions.

In the denser regions (1–20 of the light gradient or 1–35 of the heavier), electron microscopy[17], as well as amino acid uptake, indicate the presence of polysomes; the uptake of RNA precursors in these fractions as well as the absence of uptake in the ribosome monomers after such short times (preliminary experiments of this laboratory) or in ribosomal RNA[15], indicate that the rapid sedimentation of these newly formed RNA's was chiefly due to polysomal formations from new RNA combining with older unlabelled ribosomes. Ribosomal dimers and heavier polysomes becoming labelled only after 5 min incubation, it cannot be excluded that after these short times of incubation some RNA is bound to other cell structures. A search for DNA–RNA hybrids was inconclusive, but our preliminary evidence indicates that further investigation is needed.

It is difficult at present to see how these various RNA species are related to each other; the only certain common denominator is that they are all synthesized in the cell nucleus and probably all are DNA primed. The heterogeneity of the newly labelled RNA gives the impression either that DNA-primed synthesis leads to the detachment of long RNA chains which break up afterwards, or that a variety of RNA's of heterogeneous chain lengths are produced. In the case of m-RNA it is not at all certain if the 6 to 22-S polysomal RNA's discovered by PENMAN *et al.*[13] are the original m-RNA's or if these arise from natural or artificial breakdown of original components of higher molecular weights. From the actinomycin D-inhibition studies of PERRY[14,16], ribosomal RNA's seem to come from heterogeneous heavy RNA's which are probably remodelled in the nucleoli. When total cell homogenates are similarly studied with low concentrations of actinomycin D, no particular inhibition pattern is found, and it is not possible to localize the possible r-RNA precursors in

any particular region of the sedimentation gradient. s-RNA probably also has its origin in the nucleus and could also be DNA primed[8], although the methylation of its rare bases occurs presumably as an independent step[23]. One of the many questions remaining open is the possibility of some of the RNA's being synthesized away from the chromosomes with RNA primers. This would not be astonishing on account of the RNA-primed replication of viral RNA[24], and of the evidence for natural RNA-primed RNA polymerases[25]. The primers necessary for these RNA dependent RNA polymerases might themselves be formed on DNA: these polymerases could perhaps remodel some of the RNA's formed initially on the chromatin, as suggested by PERRY for r-RNA's which could undergo such a transformation in the nucleoli. Such remodelling of RNA's might also occur in other cell sites, as in the cytoplasm, where some incorporation of RNA precursors might take place[10]. It is not impossible therefore that "early RNA" characterised by its high molecular weight, a part of which would be m-RNA, would correspond to one operon; this would be in line with present concepts on the regulation of protein synthesis. The exact nature of the polysome complexes as well as their mode of functioning will probably throw some light one these problems.

After completing this paper, results of GOODMAN AND RICH[26] were published showing the formation *in vitro* of HeLa cell polysomes from labelled ribosomes and cytoplasmic preparations, and giving further confirmation of their model of the protein synthetic machinery.

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REFERENCES

1 J. BRACHET, *Biochemical Cytology*, Academic Press, New York, 1957 p. 297.
2 M. GRUNBERG-MANAGO, *Ann. Rev. Biochem.*, 31 (1962) 301.
3 R. P. PERRY, *Exptl. Cell Res.*, 20 (1960) 216.
4 R. P. PERRY, M. ERRERA AND A. HELL, *Biochim. Biophys. Acta*, 49 (1961) 47.
5 R. P. PERRY, M. ERRERA, A. HELL AND S. DURWALD, *J. Biophys. Biochem. Cytol.*, 11 (1961) 1.
6 J. H. RHO AND J. BONNER, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1611.
7 J. A. YANOFSKY AND S. SPIEGELMAN, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1069.
8 I. H. GOLDBERG AND R. RABINOWITZ, *Biochim. Biophys. Acta*, 54 (1961) 202.
9 S. SPIEGELMAN, *Federation Proc.*, 22 (1963) 36.
10 P. R. SRINIVASAN, A. MILLER-FAURÈS, M. BRUNFAUT AND M. ERRERA, *Biochim. Biophys. Acta*, 72 (1963) 209.
11 H. FRAENKEL-CONRAT, B. SINGER AND A. TSUGITA, *Virology*, 14 (1961) 54.
12 R. J. BRITTEN AND R. B. ROBERTS, *Science*, 131 (1960) 32.
13 S. PENMAN, K. SCHERRER, Y. BECKER AND J. E. DARNELL, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 654.
14 R. P. PERRY, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 2179.
15 K. SCHERRER, H. LATHAM AND J. E. DARNELL, *Proc. Natl. Acad. Sci. U.S.*, 40 (1963) 240.
16 R. P. PERRY, *Exptl. Cell Res.*, 29 (1963) 400.
17 D. WINCKELMANS, M. HILL AND M. ERRERA, *Biochim. Biophys. Acta*, 80 (1964) 52.
18 J. R. WARNER, S. KNOLL AND A. RICH, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 122.
19 H. NOLL, T. STAEHELIN AND F. O. WETTSTEIN, *Nature*, 198 (1963) 632.
20 F. O. WETTSTEIN, T. STAEHELIN AND H. NOLL, *Nature*, 197 (1963) 430.
21 E. F. ZIMMERMAN, *Biochem. Biophys. Res. Commun.*, 11 (1963) 301.
22 T. Y. WANG, *Arch. Biochem. Biophys.*, 97 (1962) 387.
23 L. R. MANDEL AND E. BOREK, *Biochemistry*, 2 (1963) 555.
24 J. S. HOLLAND, *Biochem. Biophys. Res. Commun.*, 9 (1962) 556.
25 R. H. BURDON AND R. M. S. SMELLIE, *Biochim. Biophys. Acta*, 61 (1962) 633.
26 H. M. GOODMAN AND A. RICH, *Nature*, 199 (1963) 318.