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The Origin of the Polydispersity in Sedimentation Patterns of Rapidly Labelled Nuclear Ribonucleic Acid

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1. A study was made of the sedimentation properties of purified preparations of the rapidly labelled RNA in the nucleus and the cytoplasm of the HeLa cell. The sedimentation of the rapidly labelled nuclear RNA was very sensitive to changes in ionic strength and bivalent cation concentration. Under the conditions usually used in sucrose-density-gradient centrifugation the rapidly labelled nuclear RNA showed extreme polydispersity, and much of it sedimented more rapidly than the 28s RNA. At low ionic strength and after removal of Mg^{2+}, however, the rapidly labelled nuclear RNA sedimented as a single peak at about 16s. The conversion of the polydisperse material into the 16s form did not involve degradation of the RNA, since the effect could be reversed by increasing the ionic strength of the solution. 2. The cytoplasm did not contain any RNA that showed polydisperse sedimentation under the usual conditions of sucrose-density-gradient centrifugation, or that had the same sensitivity as the rapidly labelled nuclear RNA to changes in ionic strength. All the radioactivity in the cytoplasmic RNA sedimented with the 28s, 16s and 4s components over a wide range of physical conditions, but these components did contain a labelled fraction with some of the features of the rapidly labelled nuclear RNA on columns of methylated albumin on kieselguhr. 3. In both nucleus and cytoplasm the RNA detected by ultraviolet absorption could also be converted into a 16s form by removal of bivalent cations at low ionic strength; this effect was again, within certain limits, reversible. The nuclear RNA as a whole was more susceptible to changes in ionic strength than the cytoplasmic RNA. 4. It thus appears that all the RNA in the cell, except the 4s RNA, can be prepared, without degradation, as a single peak sedimenting at about 16s. The relationship of these various 16s components to each other is discussed.

The experiments of Brenner, Jacob & Meselson (1961) and Gros *et al.* (1961) gave rise to the idea that rapidity of labelling and polydispersity of sedimentation delineated a special class of short-lived RNA molecules that were responsible for the transfer of information from the genes to the cytoplasm of the cell ('messenger' RNA). A series of papers has since appeared dealing with the physical and biological characteristics of rl-RNA* in both bacterial and animal cells. The outstanding feature of rl-RNA is the extreme variability of the properties ascribed to it. In the original work by Brenner *et al.* (1961) and Gros *et al.* (1961) the rapidly labelled material in *Escherichia coli* and *E. coli* infected with phage T_2 sedimented as a broad peak at about 12s, a lower coefficient than those of the main u.v.-absorbing RNA components (28s and 16s). Later work, however, showed that the rl-RNA in *E. coli* and phage-infected *E. coli* was apparently much more heterogeneous: it was found to contain components that sedimented together with the main u.v.-absorbing fractions and also components that sedimented more rapidly than these (Monier, Naono, Hayes, Hayes & Gros, 1962; Ishihama, Mizuno, Takai, Otaka & Osawa, 1962; Asano, 1965). In animal cells the following sedimentation patterns have been described, among others, for the rl-RNA: heterogeneous material sedimenting in the same areas as the main u.v.-absorbing RNA components (Harris, Fisher, Rodgers, Spencer & Watts, 1963); heterogeneous material sedimenting partly with the main u.v.-absorbing components and partly less rapidly than these components (Cheng, 1961); heterogeneous material sedimenting partly with the main u.v.-absorbing components and partly more rapidly than these components (Hiatt, 1962; Perry, 1962;

* Abbreviations: rl-RNA, rapidly labelled RNA; n-RNA, nuclear RNA; r-RNA, ribosomal RNA; MAK, methylated albumin on kieselguhr.

Kidson, Kirby & Ralph, 1963; Fenwick, 1964; Roberts, 1965); two broad but relatively homogeneous components separating at approx. 45s and 35s (Scherrer & Darnell, 1962); extremely polydisperse material having a continuous range of sedimentation coefficients from about 80s to 10s (Attardi, Parnas, Hwang & Attardi, 1966).

When the rl-RNA from both bacteria and animal cells is chromatographed on MAK columns (Mandell & Hershey, 1960), it can be resolved as two major components eluted from the column after the main u.v.-absorbing RNA fractions (Takai, Kondo & Osawa, 1962; Yoshikawa, Fukada & Kawade, 1964). Under other conditions, the rl-RNA adheres very tightly to the MAK columns and can only be eluted by very drastic procedures (Ellem & Sheridan, 1964).

Measurements of the base composition of the rl-RNA have been frustrated by the failure, until very recently, to obtain enough of the material for direct base analysis. Attempts have been made to measure the base composition of the rl-RNA indirectly by exposing cells for short periods to [^{32}P]phosphate and then estimating the distribution of radioactivity in the 2′,3′-nucleotides released by alkaline hydrolysis of the RNA. With this method it was found, originally in *E. coli* and T_2-phage-infected *E. coli*, that the rl-RNA had an 'apparent' base composition that resembled that of the corresponding DNA (Volkin, Astrachan & Countryman, 1958; Brenner *et al.* 1961; Gros *et al.* 1961). Numerous authors have since found 'DNA-like' RNA in both bacteria and animal cells, but many others have failed to find it. Midgley & McCarthy (1962) found that in *E. coli* the 'apparent' base composition of the rl-RNA was intermediate between the base composition of the DNA and that of the ribosomal RNA. Scherrer, Latham & Darnell (1963) found that in HeLa cells the 'apparent' base compositions of both the 45s and the 35s rl-RNA components resembled that of the ribosomal RNA. Soeiro, Birnboim & Darnell (1966) subsequently found that the rl-RNA in HeLa cells also contained some 'DNA-like' RNA.

Speculations about the biological role of rl-RNA are no less confusing than the observations on its physical and chemical properties. The idea that this RNA was some special form of genetic 'messenger' was challenged by Kitazume, Yčas & Vincent (1962), who considered that the rl-RNA was simply a precursor of the ribosomal RNA. A compromise position was adopted by Midgley & McCarthy (1962), who concluded, from measurements of the 'apparent' base composition of the rl-RNA in *E. coli,* that about one-third of this RNA was 'messenger' and two-thirds ribosomal precursor. Scherrer *et al.* (1963), on the strength of evidence obtained by the use of actinomycin D and from estimates of 'apparent' base composition, concluded that in HeLa cells the 45s and 35s components of the rl-RNA were ribosomal precursors. Rake & Graham (1964) considered that the kinetic behaviour of the 45s RNA indicated that it contained both ribosomal precursor and an unstable RNA that might be a 'messenger', presumably 'polycistronic' on account of its size. Warner, Soeiro, Birnboim, Girard & Darnell (1966) have also taken the view that the 45s RNA contains an unstable component.

In animal cells, the idea that the rl-RNA was a messenger passing from the genes to the cytoplasm of the cell met a serious difficulty in the general failure to detect in the cytoplasm any RNA having the physical or kinetic properties of the rl-RNA (Harris, 1962; Harris *et al.* 1963; Hiatt, 1962; Roberts, 1965; Tsanev, Markov & Dessev, 1966; Attardi *et al.* 1966). It now appears to be agreed that much of the rl-RNA does have a short life (Harris, 1959; Watts & Harris, 1959; Harris & Watts, 1962; Scott, Taft & Letourneau, 1962; Harris *et al.* 1963; Watts, 1964; Roberts, 1965; Adams, 1966; Attardi *et al.* 1966), and evidence begins to accumulate in support of the view that at least some of the rl-RNA undergoes turnover within the nucleus (Harris *et al.* 1963; Scott, Kaltreider, Boeker & Taft, 1964; Harris, 1965; Roberts, 1965; Edström, 1965; Bruns, Fischer & Lowy, 1965; Attardi *et al.* 1966; Soeiro *et al.* 1966). These observations have naturally given rise to speculations on a possible intranuclear role for the rl-RNA (Harris, 1965; Pontecorvo, 1966; Attardi *et al.* 1966).

One possibility is that the variability in the sedimentation of the rl-RNA might be the result of variations in the ionic strength, bivalent cation concentration and temperature of the solutions in which this RNA is extracted, stored and assayed. Petermann & Pavlovec (1963), for example, have shown that changes in ionic strength, Mg^{2+} concentration and temperature may produce interconversion of 28s and 16s r-RNA and aggregation of these components to form more rapidly sedimenting material. Rodgers (1966) has shown that 28s r-RNA can be reversibly converted into 16s RNA by exhaustive removal of Mg^{2+}, and similar changes can be produced in r-RNA by treatment at pH 9 (Midgley, 1965). Ishihama *et al.* (1962) and Monier *et al.* (1962) have shown that rl-RNA from *E. coli* may form complexes with itself and with r-RNA under certain physical conditions, and similar effects have been shown for the rl-RNA formed in *E. coli* after infection with phage T_2 (Ishihama *et al.* 1962; Asano, 1965). Bishop (1966) has shown that the sedimentation of the RNA from coliphage MS_2 is markedly affected by changes in temperature and ionic strength. These observations, coupled with

the generally unsatisfactory state of our understanding of the biological role of rl-RNA, led us to examine in some detail the effect of physical conditions on the sedimentation behaviour of this material.

EXPERIMENTAL

Cultivation of cells. HeLa cells were grown in suspension culture as described by Harris & Watts (1962).

'Pulse-labelling' of RNA. The cells were spun out of 1l. of suspension culture at low speed, and resuspended in 50ml. of a modified Eagle's medium (Eagle, Oyama, Levy, Horton & Fleishman, 1956). The cells were stirred at 37° for 15min. and [^{3}H]uridine or [^{3}H]cytidine was then added to make a final concentration of 2μc/ml. The cells were harvested after 15min. exposure to the radioactive precursors.

Isolation of cell nuclei. The nuclei were isolated from the cells by a refinement of the Tween method of Fisher & Harris (1962). This method yields a quantitative recovery of unbroken nuclei, which are free from cytoplasmic contamination (Crawley & Harris, 1963), and which retain the rl-RNA (La Cour, 1964). Under controlled conditions the nuclei can be isolated within 4min. Each preparation of nuclei was examined microscopically before use.

Extraction of nuclear RNA. Some preliminary attempts were made to recover n-RNA from the precipitate obtained by treating the cells with phenol-saturated water (Harris & Watts, 1962). With this method, however, the DNA, n-RNA, most of the protein and much of the polysaccharide of the cell are precipitated together. This makes it difficult to isolate a relatively uncontaminated sample of n-RNA in good yield. With the Tween method the nuclei are separated from most of the cellular protein and polysaccharide and satisfactory recoveries of uncontaminated n-RNA can be obtained from the isolated nuclei. The n-RNA was extracted from the nuclei with phenol and sodium dodecyl sulphate as described by Harris *et al.* (1963), except that the treatment with phenol and detergent was carried out twice. The n-RNA was separated from DNA fragments and glycogen by extracting the ethanol precipitate with 2M-NaCl or -LiCl in tris buffer, pH7, at 0°. The precipitate was then taken up in 0·15M-NaCl and reprecipitated with 2vol. of ethanol at 4°. For experiments involving very low ionic strengths, this precipitate was dissolved in the smallest practicable volume of distilled water and again precipitated with ethanol (3vol.) to remove traces of NaCl. In some instances, the preparation was used immediately and in others stored up to 1 week at −17°. Storage produced no change in the sedimentation pattern of the RNA.

Recovery of rapidly labelled RNA. To check that the final RNA preparation contained a representative sample of the rl-RNA, each stage of the purification procedure was monitored and the loss of radioactivity determined. The following values represent average recoveries of radioactive RNA at each stage of the purification procedure from cells exposed to 100μc of [^{3}H]cytidine for 15min. If the total amount of radioactive RNA in a trichloroacetic acid precipitate of the whole cells is taken as the initial point of reference, the isolated nuclei contain 96% of this amount and the cytoplasmic fractions 4%. The supernatant obtained by extracting the isolated nuclei with phenol and sodium dodecyl sulphate contained 91% of the radioactivity present in the isolated nuclei, and 82% of the radioactivity in this supernatant was precipitated by ethanol. After extraction with 2M-NaCl the precipitate retained 92% of the radioactivity that it had before extraction. The greatest loss thus occurs during ethanol precipitation, largely owing to the relatively dilute solutions of RNA used. It is, however, clear that even after extensive purification, the bulk of the rl-RNA has been recovered.

Extraction of ribosomal RNA. The cytoplasmic fraction remaining after isolation of the nuclei was made 2% (w/v) with respect to sodium dodecyl sulphate and an equal volume of water-saturated phenol added. The mixture was rotated on a turntable for 30min. at 4°. After centrifugation, the supernatant was re-extracted with aqueous phenol for 15min., and the RNA was then precipitated with 2vol. of ethanol at 4°. Soluble RNA and glycogen were removed by extraction of the precipitate with 2M-NaCl at 0°. The precipitate was dissolved in distilled water and reprecipitated with ethanol to free it from salt.

Sucrose-density-gradient centrifugation. Linear sucrose gradients from 20% (w/v) to 0% were prepared in appropriate buffers by the method of Bock & Ling (1954). Samples containing 0·1–0·4ml. of solution were layered on to the top of the gradients and centrifugation was carried out in the SW39 rotor of a Spinco model L ultracentrifuge at 5° for 5hr. at 37500 rev./min., unless otherwise stated.

The bottom of the centrifuge tube was pierced with a hypodermic needle, and fractions, each containing 4 drops, were collected on an L.K.B. fraction collector (L.K.B. Instruments Ltd., London, S.E. 20). The fractions were diluted appropriately for measurement of extinction and radioactivity.

Determination of radioactivity. The Nuclear–Chicago liquid-scintillation system 725 (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) was used. Samples (0·5ml.) were introduced into counting bottles each containing 15ml. of scintillation fluid. The scintillation fluid was made up by adding 600ml. of ethanol to 1l. of toluene containing 0·1g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0·6g. of 2,5-diphenyloxazole.

Chromatography with columns of methylated albumin on kieselguhr. MAK chromatography was carried out as described by Mandell & Hershey (1960), except that only one layer of MAK was used. Each column contained 5g. of kieselguhr. Not more than 30 *E* units of RNA were applied to the column. Fractionation at different temperatures revealed that complete elution of r-RNA could only be achieved at 35° with a fast flow rate (4ml./min.).

Spectrophotometry. The effect of temperature on the extinction of the solution of RNA was measured in a Unicam SP.500 spectrophotometer fitted with an electrically heated cell compartment (Adkins and Co., Leicester). A thermistor thermometer (Grant Instrument Co., Cambridge), with the probe inserted into a blank spectrophotometer cell, was used to measure the temperature.

Reagents. EDTA (reagent grade), phenol (analytical reagent grade), sucrose (micro-analytical reagent grade) and α-amylase were obtained from British Drug Houses Ltd., Poole, Dorset. The phenol was redistilled before use. The amylase, in solution, was freed from ribonuclease by extraction with bentonite. Sodium dodecyl sulphate was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and tris buffer from Sigma Chemical Co. Ltd.,

London, S.W. 6. Hyflo Super-Cel (Johns–Manville, New York, N.Y., U.S.A.) was used for the MAK columns. Bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was methylated as described by Mandell & Hershey (1960). Pronase (grade B) was obtained from Calbiochem Ltd., London, W. 1. Deoxyribonuclease I was an electrophoretically purified enzyme obtained from Worthington Biochemical Co., Freehold, N.J., U.S.A. [5-^{3}H]Cytidine at a specific activity of 1830mc/m-mole and [5-^{3}H]uridine at a specific activity of 3540mc/m-mole were obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Purification of rapidly labelled RNA. (*a*) Contamination with protein. A preliminary examination of the behaviour of rl-RNA, on chromatographic columns of various types, revealed that this RNA adhered firmly not only to MAK columns but also to columns of kieselguhr alone. When samples of n-RNA from cells exposed to [^{3}H]uridine for 15min. were passed through columns of kieselguhr, virtually all the u.v.-absorbing material, but only about 30% of the radioactivity could be eluted from the columns with 0·15M-sodium chloride. However, if, before being applied to the columns of kieselguhr, the sample of n-RNA was treated for 30min. at 37° with Pronase at a concentration of 1mg./ml., all the radioactivity in the sample could be eluted with 0·15M-sodium chloride. This finding suggested that preparations of n-RNA obtained by extraction of cells with phenol and detergent still contained protein, and that the presence of this protein could influence the physical properties of the rl-RNA. That some cellular proteins are not denatured by phenol is, of course, well known (Kickhöfen & Bürger, 1962; Denborough & Ogston, 1965).

It was further found that the rl-RNA would bind to proteins that were added to the preparation. Thus, if Pronase were added in excess to an aqueous solution of labelled n-RNA, a precipitate was formed that contained most of the rapidly labelled component. Similarly, if, during the process of extraction of the n-RNA, deoxyribonuclease was added to the preparation and the solution then shaken with aqueous phenol in the absence of detergent, the rl-RNA was found in the interface material on centrifugation, while the bulk of the n-RNA remained in the supernatant.

(*b*) Contamination with polysaccharide. In experiments in which n-RNA was extracted from the precipitate obtained by treating whole cells with phenol-saturated water (Harris & Watts, 1962) it was found that the rl-RNA was grossly contaminated by polysaccharide. [This finding confirms the observation by Segovia, Sokol, Graves & Ackermann (1965).] When the labelled n-RNA prepared in this way was subjected to sucrose-density-gradient centrifugation, a pellet of material containing a high proportion of the radioactivity was deposited at the bottom of the tube. This pellet was insoluble in 0·15M-sodium chloride or in aqueous solutions containing Pronase, but it was soluble in aqueous solutions of α-amylase at a concentration of 0·5mg./ml. This pellet probably represented a complex of rl-RNA with glycogen and protein, since glycogen alone is soluble in aqueous solution.

Behaviour of purified rapidly labelled RNA on columns of methylated albumin on kieselguhr. Cells were exposed for 15min. to [^{3}H]uridine and then treated with phenol-saturated water. The n-RNA was extracted from the precipitate with phenol and sodium dodecyl sulphate. Deoxyribonuclease at a concentration of 0·1mg./ml. was then added to an aqueous solution of the labelled n-RNA. As described above, the rapidly labelled components form complexes with the added protein and can then be precipitated by the addition of phenol. This precipitate was freed of protein and polysaccharide by treatment first with Pronase and then with α-amylase. After this treatment the rl-RNA remained in the aqueous phase on re-extraction with phenol. The phenol was removed by repeated extraction with ether and the solution applied to the MAK column. The result is shown in Fig. 1. About 90% of the rl-RNA resisted

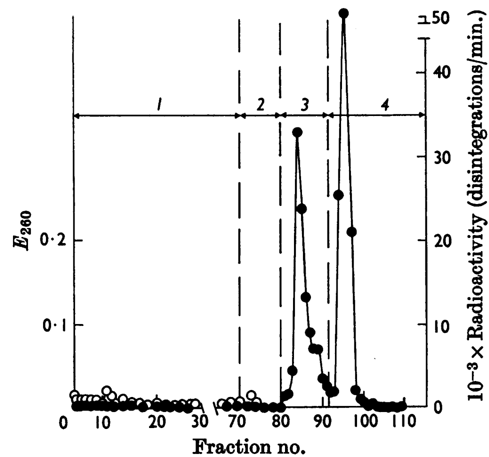

Fig. 1. Chromatography of rl-RNA on MAK at 35°. A gradient of 0·15–1·5M-NaCl in 10mM-tris buffer, pH7·4, was used (*1*), followed by 1·5M-NaCl in 10mM-tris buffer, pH7·4 (*2*), 0·04% sodium dodecyl sulphate (*3*) and then N-NaOH (*4*). The n-RNA was separated from the cytoplasmic RNA by extracting the cells with aqueous phenol. The rl-RNA was separated from the bulk of the n-RNA by precipitation with deoxyribonuclease. ○, E_{260}; ●, radioactivity.

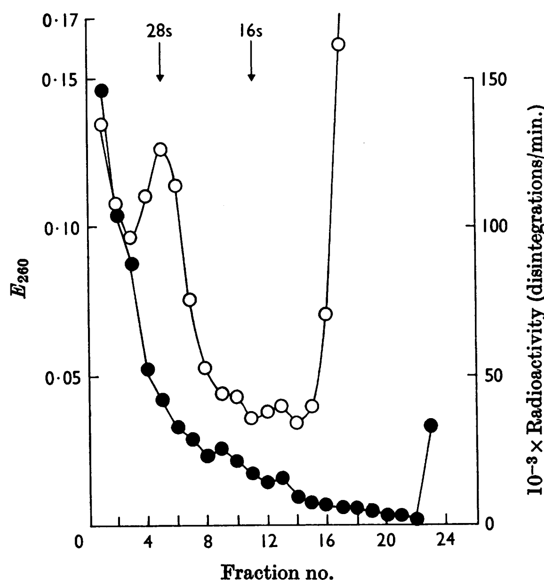

Fig. 2. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA containing DNA fragments and contaminating protein and polysaccharide. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 6hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

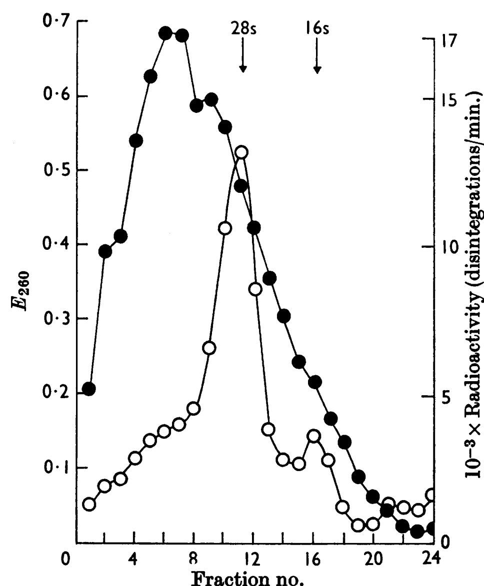

Fig. 3. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after extraction with 2M-NaCl. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 3·5hr. at 37500 rev./min. at 19°. ○, E_{260}; ●, radioactivity.

elution with 1·5M-sodium chloride, and only part could be removed with 0·04% sodium dodecyl sulphate. N-Sodium hydroxide was required to remove the rl-RNA quantitatively from the MAK column. This finding emphasizes the unusual binding properties of the rl-RNA and confirms the work of Ellem & Sheridan (1964).

Behaviour of nuclear RNA in sucrose density gradients. The sedimentation properties of both the rl-RNA and the main u.v.-absorbing n-RNA components in sucrose density gradients is affected by the method of preparation of the n-RNA and the physical conditions operative during centrifugation. Fig. 2 shows the sedimentation behaviour of pulse-labelled n-RNA from a preparation in which no special steps were taken to remove DNA fragments, protein or polysaccharide. Although there is a well-defined u.v.-absorbing component sedimenting at 28S, the usual 16S component is present in very small amount, if at all. However, a large u.v.-absorbing component is present that sediments more rapidly than the 28S component. Most of the radioactivity is associated with this rapidly sedimenting component, but some is also present in the pellet at the bottom of the tube. The large u.v.-absorbing component at the top of the tube is fragmented DNA. Fig. 3 shows the sedimentation behaviour of a similar preparation of n-RNA after extraction with 2M-sodium chloride to remove DNA fragments. A well-defined 16S component is now present, and the u.v.-absorbing material that, in Fig. 2, sedimented ahead of the 28S component has been reduced to a shoulder on the 28S peak. Much of the radioactivity has also been released from the pellet at the bottom of the tube. These observations indicate that the 16S n-RNA may form complexes that sediment more rapidly in sucrose density gradients, and that these complexes may be disaggregated by appropriate treatment. The formation of heavier complexes from the 16S RNA derived from cytoplasmic ribosomes has been described by Petermann & Pavlovec (1963).

Recently Penman (1966) concluded that n-RNA does not contain 16S RNA and that the presence of this component in preparations of n-RNA made by other authors was due to contamination with cytoplasmic RNA. However, Penman (1966) did find large amounts of u.v.-absorbing material sedimenting more rapidly than the 28S component. This indicates that the absence of a 16S component from the n-RNA preparations made by Penman (1966) might be due to the fact that this component has formed complexes that sediment more rapidly. Further evidence in support of this view is presented below.

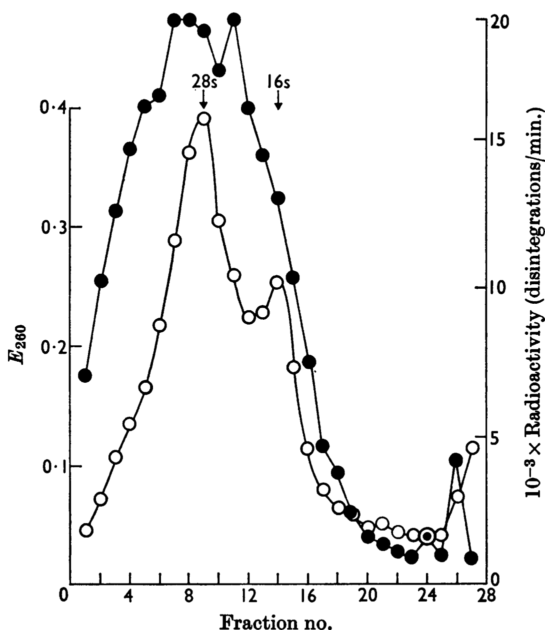

Fig. 4. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after heating for 2min. at 100° in a solution containing 50mM-NaCl, 10mM-EDTA and 0·4% sodium dodecyl sulphate at pH 7·4. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 3·5hr. at 37500 rev./min. at 19°. ○, E_{260}; ●, radioactivity.

Effect of heat on the distribution of rapidly labelled RNA in sucrose density gradients. A sample of pulse-labelled n-RNA, freed from DNA fragments and having sedimentation characteristics similar to those shown in Fig. 3, was heated for 2min. at 100° in 50mM-sodium chloride solution containing 10mM-EDTA and 0·4% sodium dodecyl sulphate. Fig. 4 shows the sedimentation pattern of the n-RNA after this treatment. The 16s u.v.-absorbing component is now much more pronounced, and the sedimentation of the rl-RNA is substantially decreased. Much of the rl-RNA now sediments with the u.v.-absorbing components. This treatment also produced a 50-fold decrease in the amount of radioactivity associated with the pellet at the bottom of the tube.

The effect of heating the n-RNA in higher concentrations of detergent was examined, and, to remove any heat-denatured protein that might be formed during the process, the RNA was heated before the final extraction of the preparation with phenol. The RNA precipitated by ethanol from the first phenol supernatant was dissolved in 2% (w/v) sodium dodecyl sulphate in water and then heated at 100° for 4min. The solution was cooled in ice and an equal volume of aqueous phenol added; the RNA was then extracted from the supernatant as described above. The sedimentation behaviour of this RNA is shown in Fig. 5. The distribution of radioactivity is now almost coincident with the distribution of u.v.-absorbing material, and both sediment as a single peak with

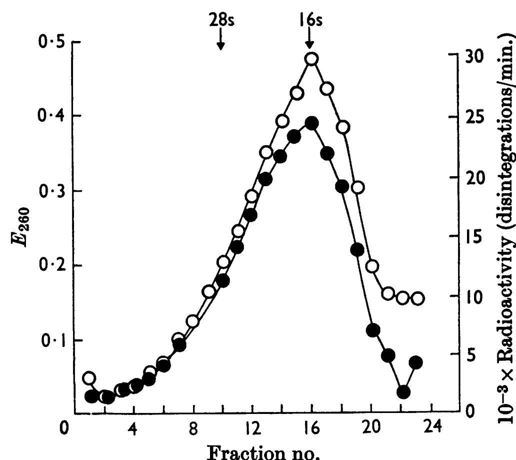

Fig. 5. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after heating in a solution containing 2% (w/v) sodium dodecyl sulphate in water for 4min. at 100°. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 3·5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

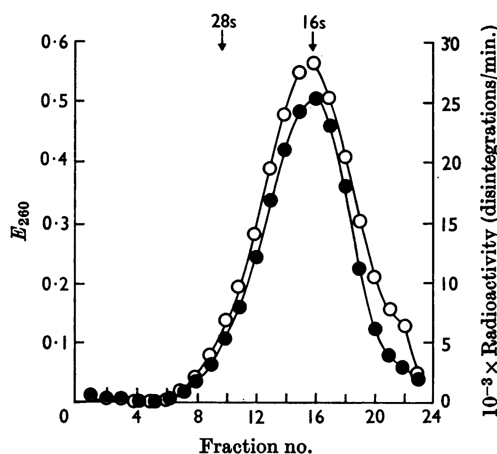

Fig. 6. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA treated first as described in Fig. 5, and then heated for a further 2min. at 100° in water alone. Conditions of centrifugation were as given in Fig. 5. ○, E_{260}; ●, radioactivity.

a sedimentation coefficient of about 16s. This peak shows a shoulder that trails forward into the 28s region. Fig. 6 shows the same material after a further brief heating in water alone (2min. at 100°). There is a noticeable sharpening of the peak and a marked reduction of the shoulder in the 28s region. It has been suggested that the conversion of 28s RNA into 16s RNA on heating is a form of 'thermal degradation' but other interpretations are possible (see Spirin, 1963). On heating, all the n-RNA components, including the rapidly labelled fractions, eventually sediment together as a single entity at about 16s.

Effect of EDTA on the sedimentation behaviour of nuclear RNA. Rodgers (1966) has shown that the 28s component of *E. coli* ribosomal RNA can be converted into 16s RNA by removal of Mg^{2+} at low ionic strength. A sample of pulse-labelled n-RNA similar to that shown in Fig. 3 was therefore treated with 5mM-EDTA in water (the acid form neutralized with tris to pH7·0) for 45min. at room temperature. The sedimentation behaviour of this EDTA-treated n-RNA is shown in Fig. 7. The effect is similar to that produced by heat. The rl-RNA and the u.v.-absorbing components sediment together as a single peak at about 16s. If the n-RNA is taken up in 5mM-EDTA in water immediately before sucrose-density-gradient centrifugation, most of the u.v.-absorbing material is present as a single peak sedimenting in a region intermediate between 28s and 16s, and there is a shoulder in the 16s position (Fig. 8). The rl-RNA, which in the absence of EDTA appeared mainly as polydisperse material sedimenting more rapidly than the 28s component, is now coincident with the u.v.-absorbing material. This indicates that the polydisperse rl-RNA, which sediments more rapidly than the 28s component, is converted into a form that sediments together with the u.v.-absorbing

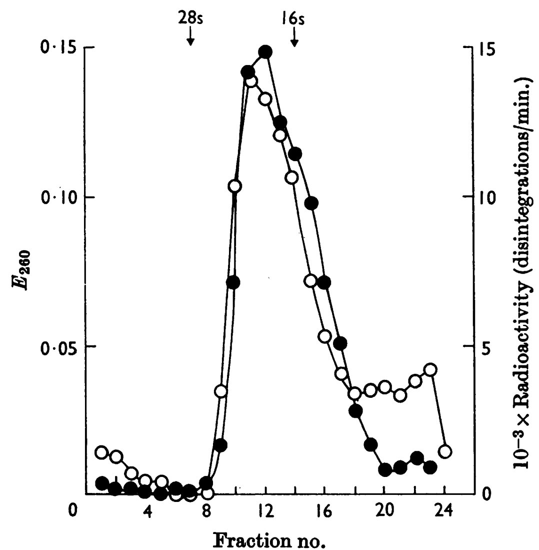

Fig. 8. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA dissolved in a solution containing 5mM-EDTA–tris buffer, pH7·0, and then subjected immediately to centrifugation in a gradient containing 5mM-EDTA–tris. Centrifugation was for 6hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

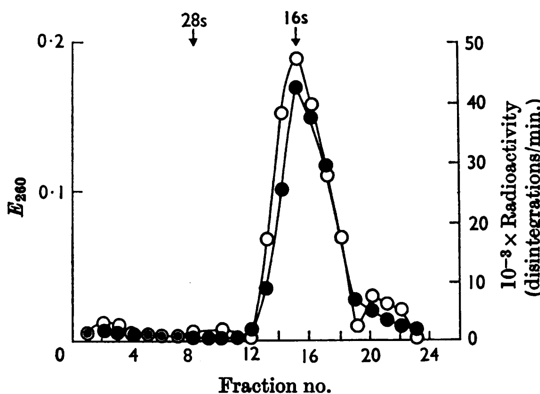

Fig. 7. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after treatment for 45min. at 21° with 5mM-EDTA in water (acid form neutralized with M-tris to pH7·0). The gradient also contained 5mM-EDTA–tris buffer, pH7·0. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

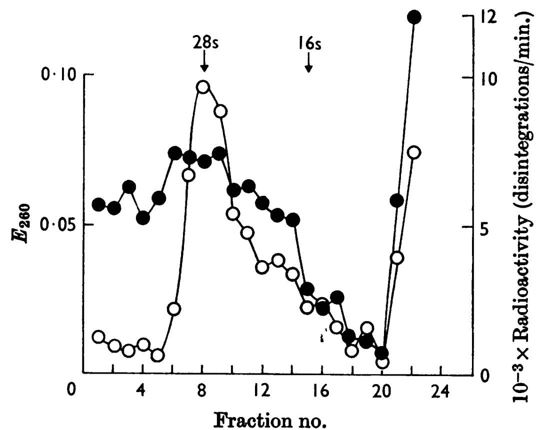

Fig. 9. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after treatment with 5mM-EDTA–tris buffer, pH7·0, for 45min. at 21°. The gradient contained 50mM-NaCl and 10mM-EDTA at pH7·4. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

material before all the latter is converted into the 16s form.

As shown by Rodgers (1966) for r-RNA, this effect of EDTA is, within certain limits, reversible. If a sample of pulse-labelled n-RNA is treated with EDTA for 45min. at room temperature and then spun in a gradient containing 50mM-sodium chloride, the sedimentation pattern shown in Fig. 9 is obtained. The u.v.-absorbing material has reverted mainly to the 28s form, and there are several intermediate components between 28s and 16s. The rl-RNA has again become polydisperse, and much of it sediments more rapidly than the 28s component. Even a brief exposure to EDTA before centrifugation causes the rl-RNA to sediment together with the u.v.-absorbing components (Fig. 8). These findings demonstrate that the conversion of the u.v.-absorbing components and the rl-RNA into a 16s form is not due to degradation. This transformation may represent a reversible disaggregation of complexes of smaller units (and this seems likely for at least those components that sediment to the bottom of the centrifuge tube), or it may simply represent a change in shape (Rodgers, 1966). If, as has been suggested, the rl-RNA is grossly heterogeneous with respect to molecular weight, it is remarkable that all these molecules of widely different sizes should have the same S value at low ionic strength.

If the n-RNA is treated with EDTA for periods longer than about 45min. at 5°, the effect becomes progressively less reversible. Fig. 10 shows the sedimentation pattern of a sample of pulse-labelled n-RNA treated with EDTA for 75min. at room temperature and then subjected to centrifugation in a gradient containing 50mM-sodium chloride. Most of the u.v.-absorbing material has reverted to the 28s form, but the rl-RNA remains coincident with this 28s peak and has not reverted to the more rapidly sedimenting polydisperse form. If the labelled n-RNA is dissolved in water and allowed to stand in solution before being treated with EDTA, the conversion of the rl-RNA into the 16s form takes place more rapidly. As suggested by Rodgers (1966), the time required to effect the conversion of n-RNA into the 16s form may reflect the progressive removal of Mg^{2+} from the RNA molecule. Cytoplasmic r-RNA, in our hands, is completely converted into the 16s form in about ½hr. at room temperature (Fig. 11). The effect is almost completely reversed when the EDTA-treated ribosomal RNA is centrifuged in a gradient containing 50mM-sodium chloride (Fig. 12).

After prolonged treatment with EDTA (18hr.

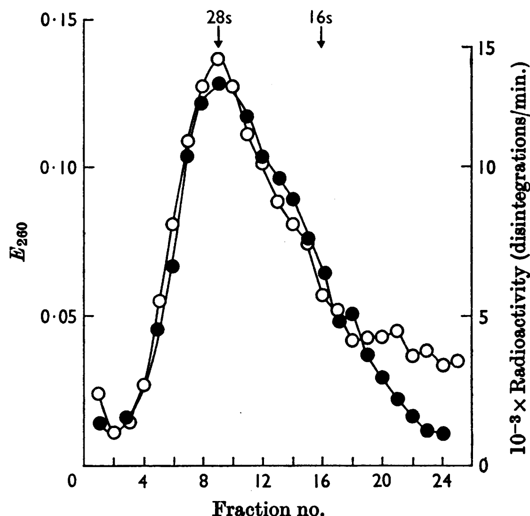

Fig. 10. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after treatment with 5mM-EDTA–tris buffer, pH 7·0, for 75min. at 21°. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 3·5hr. at 37500 rev./min. at 26°. ○, E_{260}; ●, radioactivity.

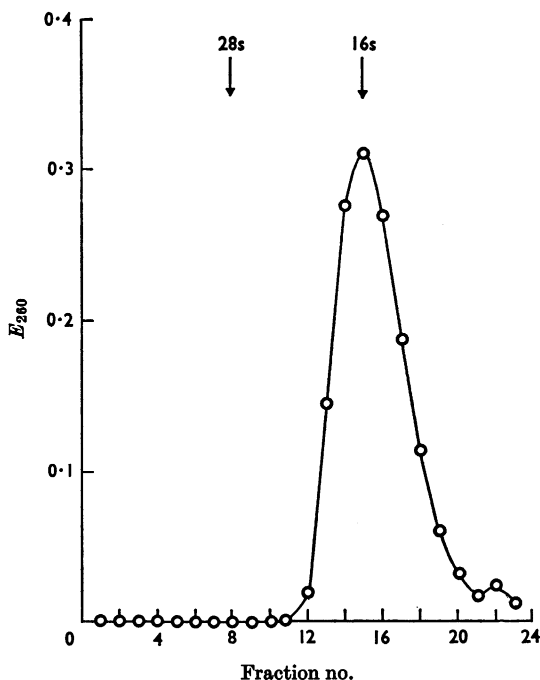

Fig. 11. Sucrose-density-gradient sedimentation pattern of ribosomal RNA after treatment with 5mM-EDTA–tris buffer, pH 7·0, for 30min. at 21°. The gradient contained 5mM-EDTA–tris. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}.

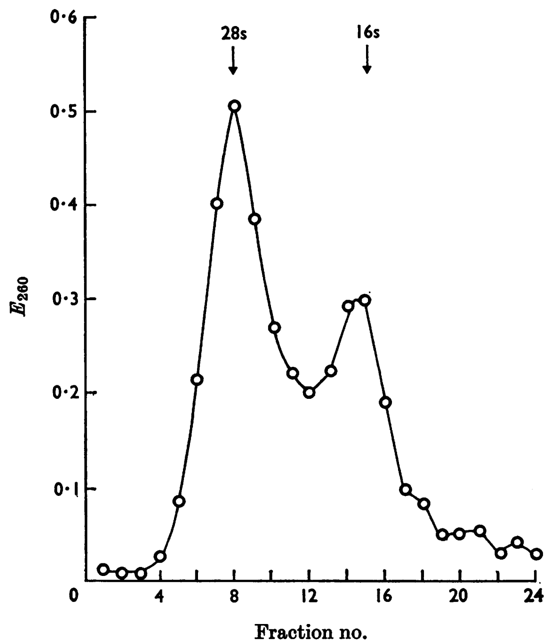

Fig. 12. Sucrose-density-gradient sedimentation pattern of ribosomal RNA after treatment with 5mM-EDTA–tris buffer, pH 7·0, for 30 min. at 21°. The gradient contained 5mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}.

at 5°) the labelled n-RNA that has been converted into the 16s form will not revert to 28s or heavier forms on centrifugation in sodium chloride. Fig. 13 shows the sedimentation pattern of pulse-labelled n-RNA after prolonged treatment with EDTA, in a sucrose density gradient containing 50mM-sodium chloride; both the rl-RNA and the u.v.-absorbing components still sediment at 16s. There is some broadening of the peak but no further decrease in the sedimentation of the RNA.

Effect of changes in ionic strength on the sedimentation of nuclear and ribosomal RNA. Studies on the sedimentation behaviour of n-RNA at higher ionic strengths revealed that it was more sensitive to changes in ionic strength than cytoplasmic r-RNA. Fig. 14 shows the sedimentation pattern of pulse-labelled n-RNA in a gradient containing 50mM-sodium chloride and 10mM-EDTA at pH 7·0. Fig. 15 shows the sedimentation pattern of the same n-RNA in a gradient containing 0·35M-sodium chloride and 10mM-EDTA, and Fig. 16 the sedimentation pattern of cytoplasmic r-RNA under the same conditions. Fig. 15 shows that at higher ionic strength much of the u.v.-absorbing material in the n-RNA has been converted into components that sediment more rapidly than the 28s component, and at least 30% of the u.v.-absorbing

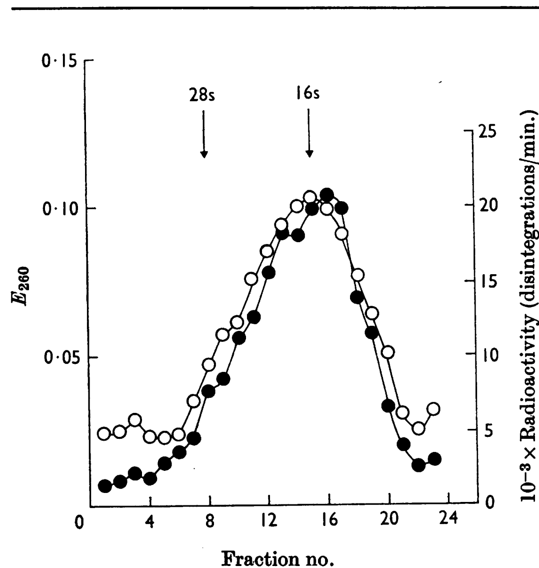

Fig. 13. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA after treatment with 5mM-EDTA–tris buffer, pH 7·0, for 18hr. at 5°. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·4. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

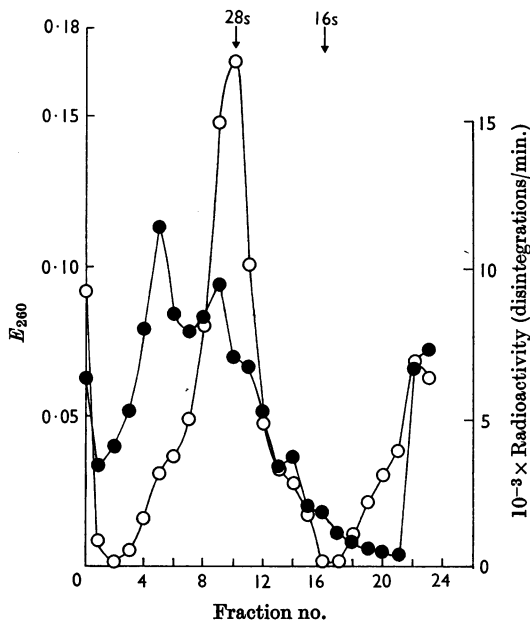

Fig. 14. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA in a gradient containing 50mM-NaCl and 10mM-EDTA at pH 7·0. Centrifugation was for 4·5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

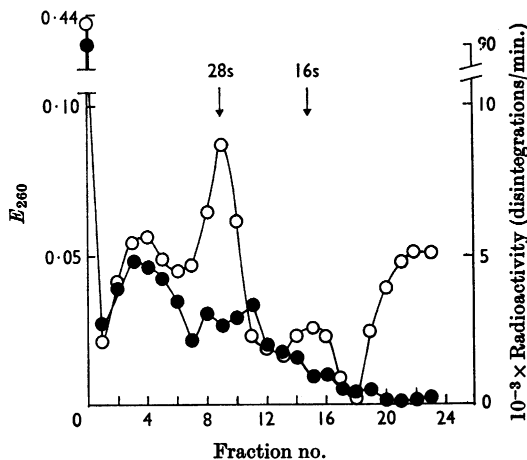

Fig. 15. Sucrose-density-gradient sedimentation pattern of pulse-labelled n-RNA in a gradient containing 0·35M-NaCl and 10mM-EDTA at pH7·0. Centrifugation was for 4·5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

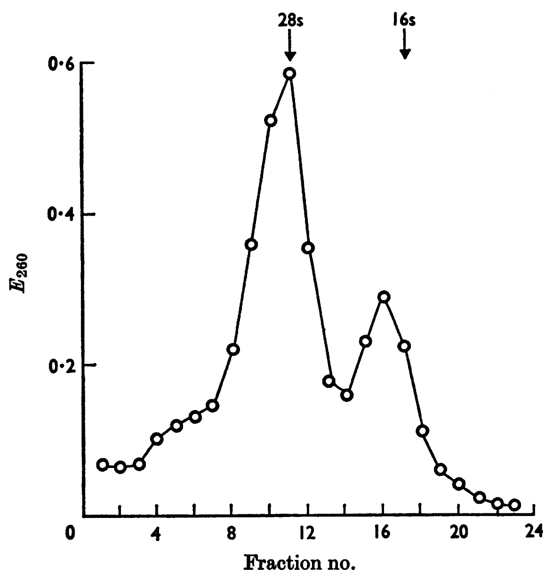

Fig. 16. Sucrose-density-gradient sedimentation pattern of r-RNA in a gradient containing 0·35M-NaCl and 10mM-EDTA at pH7·0. Centrifugation was for 5hr. at 37500 rev./min. at 5°. ○, E_{260}.

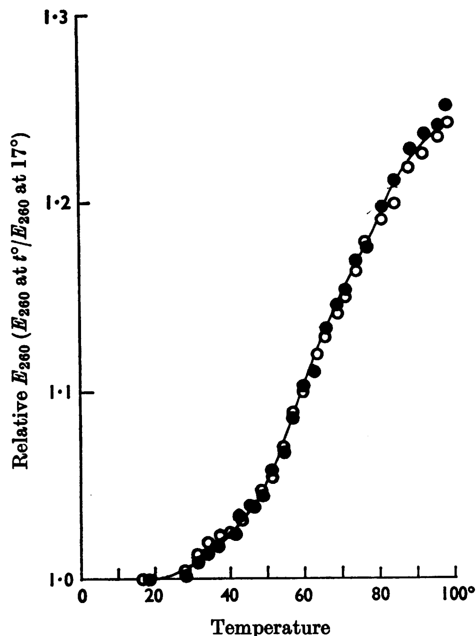

Fig. 17. Heating profiles of n-RNA (●) and r-RNA (○) in 0·15M-NaCl and 10mM-KH_2PO_4 adjusted to pH7·4 with N-NaOH.

material and 60% of the rl-RNA were deposited in the pellet at the bottom of the tube. As shown in Fig. 16, the effect of this ionic strength on cytoplasmic r-RNA was limited to the formation of a small shoulder sedimenting ahead of the 28s component. This result resembles the observations by Mitsui, Ishihama & Osawa (1963), who showed that, at certain concentrations of Mg^{2+}, the rl-RNA, but not the r-RNA, in *E. coli* underwent aggregation.

The finding that a large proportion of the n-RNA is more easily aggregated than the cytoplasmic r-RNA suggests that n-RNA may have a lower order of secondary or tertiary structure than r-RNA. Since the degree of hypochromicity of RNA is thought to be a measure of its secondary structure (Doty, Boedtker, Fresco, Hall & Haselkorn, 1959), the u.v. absorptions of n-RNA and r-RNA were measured over the temperature range 17–98°. As shown in Fig. 17, no difference was found between the heating profile of n-RNA and that of r-RNA. This suggests that the greater susceptibility of n-RNA to aggregation might reflect differences in tertiary rather than secondary structure.

Behaviour of EDTA-treated rapidly labelled RNA on columns of methylated albumin on kieselguhr. Despite prolonged treatment with EDTA (20hr. at 5°) only 42% of the radioactivity in pulse-labelled n-RNA could be eluted from the MAK columns with 1·5M-sodium chloride, whereas virtually 100% of the u.v.-absorbing material was eluted. This value may be a little higher than that

usually obtained with pulse-labelled n-RNA, but it is clear that despite exhaustive treatment with EDTA much of the rl-RNA retains its adhesiveness to MAK and can only be removed by extreme methods. This suggests that, even when their sedimentation coefficients are identical, the rl-RNA still has a lower order of secondary structure than the bulk of the n-RNA. This view is supported by the observation that polyuridylic acid (which has no secondary structure except at very low temperatures) and denatured DNA (which has a highly disorganized structure) bind strongly to MAK columns (Asano, 1965; Hershey, Goldberg, Burgi & Ingraham, 1963). Since the rl-RNA is apparently still attached to the chromosome when its extraction from the cell is undertaken (Harris, 1963; La Cour, 1964), much of it may still be aligned along the DNA in an extended configuration. When the DNA–protein backbone is disrupted by the extraction procedure it is possible that the rl-RNA collapses in a disorganized way, and, under appropriate physical conditions, forms non-specific complexes with itself and with contiguous proteins.

Rapidly labelled RNA fractions in the cell cytoplasm. Cells were grown for 3hr. in [^{3}H]cytidine and then enucleated. Precisely the same technique was used to prepare the labelled cytoplasmic RNA as had been used to prepare the pulse-labelled n-RNA. The cytoplasmic fraction was treated with phenol and sodium dodecyl sulphate, and the ethanol precipitate was extracted with 2M-sodium chloride. The RNA was then subjected to sucrose-density-gradient centrifugation. The result is shown in Fig. 18. As has been found previously, no polydisperse RNA can be detected in the cytoplasmic fraction of the cells either by its radioactive content or by u.v. absorption.

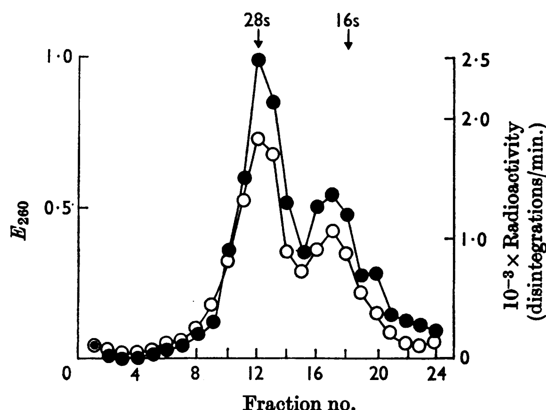

Fig. 18. Sucrose-density-gradient sedimentation pattern of cytoplasmic RNA extracted from cells labelled for 3hr. with [^{3}H]cytidine. The gradient contained 50mM-NaCl and 10mM-EDTA at pH 7·0. Centrifugation was for 4·5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

The finding that the rl-RNA retains its characteristic adhesiveness to MAK, whether it is in the 16s or polydisperse form, suggested the possibility that MAK columns might reveal physical heterogeneity in the labelled cytoplasmic RNA. This proved to be the case. Fig. 19 shows the behaviour of the labelled cytoplasmic 16s and 28s RNA on an MAK column. About 20% of the radioactivity resists elution from the column with 1·5M-sodium chloride, but can be removed with N-sodium hydroxide. Fig. 20 shows the sedimentation pattern of the labelled cytoplasmic RNA in a sucrose density gradient containing 0·35M-sodium chloride. The radioactivity still sediments with the u.v.-absorbing components, and there is no evidence of polydispersity. In 0·35M-sodium chloride the sedimentation of the rapidly labelled n-RNA is grossly polydisperse and more than half of it forms large aggregates that sediment to the bottom of the centrifuge tube (Fig. 15). Some of the labelled RNA in the cell cytoplasm thus resembles the rl-RNA in that it adheres tightly to MAK, but differs from it in that it does not form polydisperse material under the usual conditions of sucrose-density-gradient centrifugation, and in being more resistant to aggregation by solutions of high ionic strength. Its physical properties might be described as being intermediate between those of the rl-RNA and those of the bulk of the cytoplasmic r-RNA. Perry & Kelley (1966) have shown that the most rapidly labelled RNA in the cell cytoplasm, which has a sedimentation coefficient of 16s, is already within particles. These authors present evidence in support of the view that these particles are immature forms of ribosomal sub-unit.

Flow of radioactivity through RNA in the presence of actinomycin D. Scherrer *et al.* (1963) observed that, when cells that had been exposed for a short period of time to a radioactive RNA precursor were incubated further in the presence of actinomycin D, radioactivity disappeared from the polydisperse rl-RNA and appeared in the main u.v.-absorbing RNA components of the cell. This finding was originally thought to indicate the conversion of the rl-RNA into r-RNA, but, since radioactivity failed to appear in the cytoplasmic RNA in the presence of actinomycin D (Harris, 1963; Levy, 1963; Paul & Struthers, 1963; Lieberman, Abrams & Ove, 1963), the experiment was reinterpreted by Girard, Penman & Darnell (1964), and is now thought to show that much of the rl-RNA is converted into the main u.v.-absorbing RNA components of the cell nucleus. It has, however, been pointed out that, under the conditions used by Scherrer *et al.* (1963), incorporation of radioactivity from the RNA precursor pools is not necessarily abolished

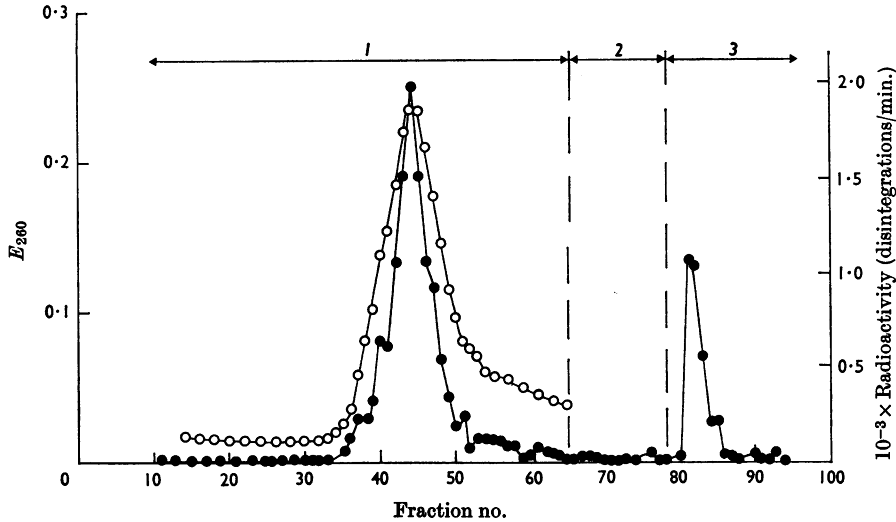

Fig. 19. Chromatography of cytoplasmic RNA on MAK at 35°. A gradient of 0·15–1·5M-NaCl in 10mM-tris buffer, pH 7·4, was used (*1*), followed by 1·5M-NaCl in 10mM-tris buffer, pH 7·4 (*2*), and then N-NaOH (*3*). The cells had been labelled for 3hr. with [^{3}H]cytidine. ○, E_{260}; ●, radioactivity.

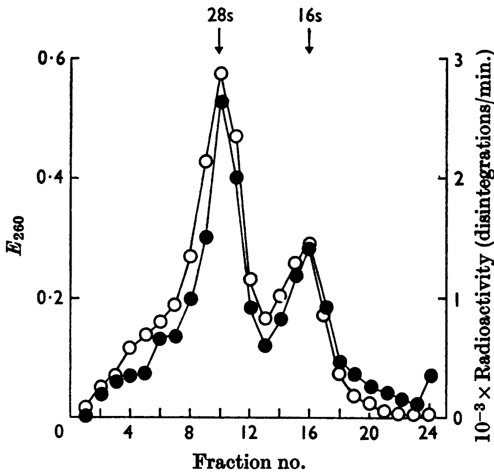

Fig. 20. Sucrose-density-gradient sedimentation pattern of the labelled cytoplasmic RNA in a gradient containing 0·35M-NaCl and 10mM-EDTA at pH 7·0. Centrifugation was for 4·5hr. at 37500 rev./min. at 5°. ○, E_{260}; ●, radioactivity.

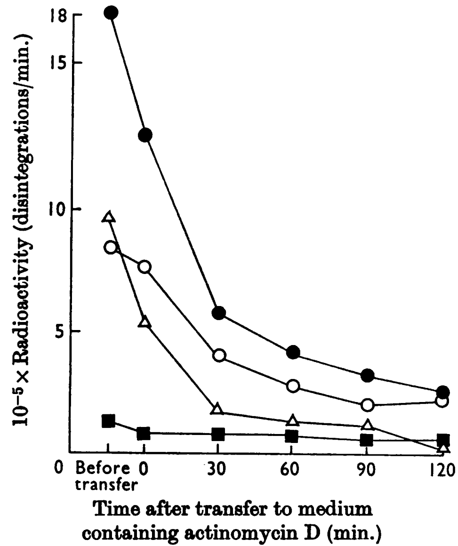

Fig. 21. Breakdown of n-RNA components in the presence of actinomycin D. The cells were labelled for 15min. with [^{3}H]uridine and then incubated in non-radioactive medium containing actinomycin D at a concentration of 7·5μg./ml. ●, Total n-RNA; △, radioactivity that binds strongly to MAK; ○, radioactivity associated with the u.v.-absorbing material; ■, cytoplasmic RNA.

by the concentration of actinomycin used (Harris, 1964), so that the interpretation of this experiment is subject to some uncertainty.

An examination was made of the effect of actinomycin concentration on the extent of this residual incorporation of radioactive precursors into RNA. It was found that, with the suspension cultures of HeLa cells used in the present experiments, the actinomycin had to be added at a concentration of 7·5μg./ml. before all residual

incorporation of precursors into RNA was abolished. This, then, was the lowest concentration of actinomycin that would permit an unambiguous interpretation of the flow of radioactivity through the RNA of the cell. Cells were therefore labelled for 15min. with [^{3}H]uridine and then transferred to non-radioactive medium containing actinomycin D at a concentration of 7·5μg./ml. Samples were taken at intervals over 2hr. and the nuclei were isolated from each sample. The n-RNA and the cytoplasmic RNA were extracted, and the rl-RNA was separated from the bulk of the n-RNA by adsorption to MAK. The total amounts of radioactivity in rl-RNA, the main u.v.-absorbing components of the n-RNA and the ribosomal RNA are shown in Fig. 21. Both the rl-RNA and the n-RNA as a whole undergo degradation, and there is no measurable passage of radioactivity from the rl-RNA to the main u.v.-absorbing components of the n-RNA, or from the n-RNA to the cytoplasmic RNA. This experiment does not, of course, show that the rl-RNA is not a precursor of the bulk of the n-RNA or of the r-RNA; it merely shows that the evidence in support of this view is inconclusive. Passage of radioactivity from rl-RNA to the bulk of the n-RNA only takes place at concentrations of actinomycin that permit some residual incorporation of radioactivity into RNA from the precursor pools; when concentrations of actinomycin are used that abolish this residual incorporation, passage of radioactivity from rl-RNA to the main u.v.-absorbing n-RNA components cannot be demonstrated.

DISCUSSION

The outstanding feature of the present study is the finding that all the RNA in the cell, except the 4s RNA, can be prepared, without degradation, as material sedimenting at 16s. But these 16s components display a marked heterogeneity that can be revealed by differences in their affinity for MAK and in their susceptibility to changes in ionic strength and bivalent cation concentration. It is obvious that sucrose-density-gradient sedimentation is a poor method for determining the size, the homogeneity or the identity of RNA molecules: molecules that sediment in the same region may have widely different conformations or molecular weights, and molecules that sediment in different regions may, under other physical conditions, sediment together.

We do not yet have any decisive information about the interrelationships of these various families of RNA molecules. It is possible that they simply represent different stages in the production and maturation of r-RNA. The difficulty posed by the apparent difference in size between the rl-RNA and the r-RNA is removed by this study: we do not at present have any clear idea of the size of the rl-RNA. The argument based on differences in 'apparent' base composition loses its force in the light of the experiments of Marbaix, Burny, Huez & Chantrenne (1966) and of Hadjiolov, Venkov, Dolapchiev & Genchev (1967), which confirm the long-standing suspicion that the [^{32}P]phosphate 'pulse' method does not provide a reliable estimate of the base composition of the rl-RNA (Spencer, 1962). And the significance of differences between the rl-RNA and r-RNA detected by DNA–RNA hybridization techniques is at least open to question in the light of the finding that the rl-RNA adheres tightly, and not, of course, by base-pairing, to other macromolecules in solution, whereas r-RNA does not.

The conversion of the rl-RNA into the main u.v.-absorbing 16s n-RNA component may therefore not require drastic and improbable processes such as the specific asymmetrical scission of giant molecules, as proposed by Scherrer *et al.* (1963), or major changes in base composition. Since the true base composition of the rl-RNA has not yet been ascertained, the only firm experimental distinction that can, at the moment, be made between the rl-RNA and the u.v.-absorbing 16s n-RNA component is the greater susceptibility of the former to the physical changes induced by variations in ionic strength and bivalent cation concentration. The stabilization of secondary structure necessary to convert the rl-RNA into the form assumed by the main 16s n-RNA component might be achieved by methylation, or other secondary modification, of certain bases, as suggested by the experiments of Saponara & Enger (1966) and Ludlum (1966), or by specific configurational changes imposed by attachment of the rl-RNA to protein. A similar relationship may well hold between the n-RNA and the r-RNA in the cytoplasm. Again, the only difference between the two is the greater stability of the cytoplasmic r-RNA to changes in ionic strength and bivalent cation concentration. One may thus envisage a sequence of events in which the initially loose structure of the rl-RNA is tightened and stabilized as it passes from its original extended form to the more ordered form assumed by the 16s n-RNA and finally to the still more ordered form that is found in complexes with protein in the 16s r-RNA. The precise relationship between 16s and 28s RNA is still not clear. The transformation of one into the other may represent a special, and perhaps selective, form of dimerization (Midgley, 1965), or a change in shape (Rodgers, 1966), or both.

This sequence of events must, however, take into account the fact that both *in vitro* and *in vivo* much of the rl-RNA is known to be involved in

rapid turnover and, apparently, in intranuclear turnover. The biological significance of this phenomenon readily becomes understandable if the rate-limiting step in the assembly of the ribosome is not the synthesis of RNA, but its stabilization and incorporation into the mature particle. If, under the usual experimental conditions, more of the rl-RNA is made than is incorporated into mature ribosomes, the turnover of rl-RNA could be accounted for by intranuclear elimination of the excess. This view agrees well with the numerous observations that have been made on the rate of turnover of the rl-RNA in various types of cell. Although this turnover has been demonstrated in cells growing exponentially (Scott *et al.* 1962; Watts, 1964; Roberts, 1965; Houssais & Attardi, 1966), as well as in more slowly growing or stationary cells (Harris, 1959; Watts & Harris, 1959; Adams, 1966; Owen, 1967), it does appear to be more pronounced in the latter situation and under 'step-down' conditions in which growing cells are transferred to poorer medium or subjected to other forms of physiological 'shock' (Kubinski & Koch, 1966; R. Ralph & J. D. Watson, personal communication; Bellamy, 1966). One may envisage the possibility that the processes that impose a stable secondary structure on the rl-RNA and eventually incorporate it into the ribosomes represent the essential regulatory mechanisms that 'engage' the production of RNA and thus determine the rate of assembly of ribosomes, and hence the rate of growth of the cell (Maaløe & Kjeldgaard, 1966).

If, however, the rl-RNA is not simply an early form of n-RNA, but an independent entity, we must seek some biological role for an RNA that appears to be confined to the nucleus, which, under certain conditions, has a sedimentation coefficient of 16s, which exhibits a loose and unstable secondary structure and which, compared with cytoplasmic r-RNA, has a short life.

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