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Preparation, Molecular Weight, Base Composition, and Secondary Structure of Giant Nuclear Ribonucleic Acid†

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ABSTRACT: Previous methods of heterogeneous ribonucleic acid (HnRNA) extraction yield material which "disaggregates" into small molecules. This could be the fault of either ribonuclease knicks in the polymers sustained during the extraction procedure or disaggregation into real subunits. The present communication distinguishes between these possibilities by describing an RNA extraction procedure which does not yield subunits when HnRNA is denatured. By the criteria of sedimentation through sucrose, formaldehyde, and dimethyl sulfoxide, it is estimated that the majority of the radioactivity of giant HnRNA after a 30-min pulse of [^{3}H]uridine is associated with molecules in the range 5–10 $\times$ 10^6 daltons. In the electron microscope, under denaturing conditions, 84% (mass %) of giant HnRNA has a contour length of 4–9 μ corresponding to a molecular weight of about 5–10 $\times$ 10^6. Giant HnRNA has a "DNA-like" base composition (G + C = 46–54%) and has considerable secondary structure (*ca.* 60% helix conformation) as judged by its melting profile and reactivity with formaldehyde.

The nuclei of mammals contain a class of heterogeneous RNA (HnRNA) that is rapidly labeled, sediments heterogeneously in sucrose gradients, and has a DNA-like base composition (Scherrer *et al.*, 1963; Attardi *et al.*, 1966; Soeiro *et al.*, 1966; Schutz *et al.*, 1968; Soeiro and Darnell, 1970). A portion of HnRNA has a sedimentation coefficient greater than 45 S as judged by sedimentation through sucrose. This has been equated with a molecular weight in excess of 4 $\times$ 10^6 daltons, using the available equations to relate molecular weight and sedimentation velocity (Gierer, 1950; Spirin, 1961). However the dangers in using such equations have been pointed out by Gesteland and Boedtker (1964) and Strauss and Sinsheimer (1967). Estimates of the molecular weight of a variety of cellular and viral RNAs have been obtained by other methods such as sedimentation or electrophoresis in denaturing solvents (Boedtker, 1968; Fenwick, 1968; Strauss *et al.*, 1968; Staynov *et al.*, 1972), light scattering (Gesteland and Boedtker, 1964), viscosity (Mitra *et al.*, 1963), and electron microscopy (Gransboulan and Scherrer, 1969; Robberson *et al.*, 1971). However there are few reports in the literature in which such techniques have been applied to HnRNA. Granboulan and Scherrer (1969) describe a class of HnRNA molecules with a molecular weight in the range 5–10 $\times$ 10^6 daltons as judged by visualization in the electron microscope under partially denaturing conditions. On the other hand, Mayo and de Kloet (1971) using formaldehyde sucrose gradients, and Scott and Kuhns (1972) using electrophoresis in the presence of Me_2SO present evidence that giant HnRNA "disaggregates" into smaller molecules presumably because denaturation reveals hidden nicks in the RNA.

This paper describes a method for the isolation of giant HnRNA from rat ascites cells which does not disaggregate under denaturing conditions. Such RNA has low G + C content and is heterogeneous in size with a molecular weight in the range 5–10 $\times$ 10^6 daltons as judged by sedimentation and electron microscopy under denaturing conditions. The

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helix–coil transition and reactivity with formaldehyde indicate that this giant HnRNA has considerable secondary structure *in vitro*.

Methods

Isolation of Nuclei. Male albino Sprague-Dawley rats (Berkeley Pacific Laboratories) were injected 6 days prior to harvest with 0.5 ml of Novikoff ascites fluid per rat. For the preparation of [^{3}H]uridine-labeled HnRNA, rats were given an intraperitoneal injection of [^{3}H]uridine (New England Nuclear Corp., 180 Ci/mol) prior to harvesting the fluid. Details of the amount of isotope and labeling time appear in the descriptions of individual experiments. Generally one rat yielded approximately 20 ml of fluid with a concentration of $1–5 \times 10^7$ ascites cells/ml.

Except where stated all steps were carried out at 4°. Ascites fluid was centrifuged at 1000*g* for 30 sec, packed cells were suspended with the aid of a spatula in about 10 volumes of deionized water and then centrifuged at 1500*g* for 5 min. This yields a pellet of crude nuclei as judged by phase contrast microscopy.

Isolation of Total Nuclear RNA. (I) The pellet of crude nuclei was lysed at room temperature in approximately 25 volumes of lysis mixture (2% sodium dodecyl sulfate (Matheson, Coleman & Bell, recrystallized from ethanol)), 7 M urea (Schwarz-Mann Ultra Pure), 0.35 M NaCl, 1 mM EDTA, and 0.01 M Tris, pH 8). Reagent grade urea but not Mann Ultra Pure urea, degrades RNA in solution (Sedat *et al.*, 1969). An equal volume of phenol mixture (see Miscellaneous Methods) was added and the mixture was shaken at room temperature for 15 min. The phases were separated by low-speed centrifugation and the phenol phase and interface reextracted with one-half volume of lysis mixture. The combined aqueous phase was reextracted with one-half volume of phenol mixture and the final aqueous phase (II) precipitated by the addition of 25% sodium acetate (pH 6) to 2% followed by two volumes of cold 95% ethanol. Following storage at −18° for 2–4 hr the DNA was spooled out, the flocculent precipitate was pelleted at 12000*g* for 10 min, and the pellet was washed once with cold 95% ethanol. The DNA and the pellet were partially resuspended in TNM (0.01 M Tris-HCl (pH 8)–0.01 M NaCl–0.01 M $MgCl_2$) with the aid of a spatula, DNase I (Worthington Co., repurified, see below) was added to a final concentration of 100 μg/ml, and the mixture was incubated at 37° for 3 min using constant agitation with a spatula. The reaction was stopped by plunging in ice, followed by the addition of 25% sodium dodecyl sulfate to 2% and 0.1 M EDTA to 1 mM. An equal volume of phenol mixture was added and the mixture was extracted at 4° as described above. The combined aqueous phases (III) were precipitated as described above. This precipitate contains the majority (66%) of the nuclear RNA.

Sepharose B Chromatography. Total nuclear RNA was resuspended in 2 mM phosphate buffer (pH 6.8)–1 mM EDTA and subjected to chromatography on Sepharose 2B (Pharmacia; column 2.5 × 45 cm), in the same buffer at a flow rate of approximately 15 ml/hr (Baltimore, 1968; Oberg and Philipson, 1969; Clements and Martin, 1971). Aliquots (25 μl) of each fraction were counted in Aquasol and dilutions were made for absorbance readings. Up to 10 mg of RNA in 4 ml of buffer have successfully been fractionated as described. Fractions corresponding to the excluded volume of the column were pooled and precipitated as described above (IV–V).

Sucrose Gradients. (1) NONDENATURING. Preformed linear 5–20% sucrose (Schwarz-Mann Ultra Pure) gradients in 0.1 M NaCl–1 mM EDTA–0.01 M sodium acetate (pH 6)–0.2% sodium dodecyl sulfate were run at 39,000 rpm at 18° in the Spinco SW-39 rotor. RNA (20–100 μg) was layered on the gradients in 100 μl of the same buffer as the sucrose gradient. In some instances the RNA was heated at 80° in 1 mM EDTA (pH 8) for 2 min and cooled to about 20° in Dry Ice–ethanol prior to loading. Using a first-order rate constant of 5×10^{-8} sec^{-1} (Eigner *et al.*, 1961) and thee quation of Spirin (1961) to relate sedimentation coefficient to molecular weight, it is estimated that the sedimentation coefficient of RNA should fall by about 5% after such heat treatment. The gradients were dripped onto Whatman No. 3MM filter paper; washed in succession with two changes of cold 10% trichloroacetic acid and two changes of 95% ethanol. The filters were air-dried, vacuum oven-dried, and counted in a toluene-based scintillation cocktail.

(2) DENATURING FORMALDEHYDE GRADIENTS. RNA was sedimented in formaldehyde-containing sucrose gradients as described by Fenwick (1968). The gradients were as described above but without sodium dodecyl sulfate and including 6% formaldehyde (Mallinckrodt, reagent grade). The samples were heated at 55° for 5 min in the same bufter prior to loading.

Dimethyl Sulfoxide Gradients. Centrifugation of RNA in a linear dimethyl sulfoxide gradient was carried out essentially by the method of Strauss *et al.* (1968) and Sedat *et al.* (1969), with the following modification. RNA (20–25 μg) was resuspended in 100 μl of dimethyl sulfoxide (Matheson, Coleman & Bell, Spectrograde quality) and heated at 60° for 2 min prior to loading on the gradient. The gradients were assayed for radioactivity as described above.

Preparation of rRNA. Crude ribosomes were isolated from a cytoplasmic extract of rat ascites cells by the method of Moldave and Skogerson (1967). RNA was isolated from the ribosomes by phenol extraction and purified by centrifugation on a 5–20% linear sucrose gradient for 16 hr at 25,000 rpm at 18° in the Spinco SW-25.2 rotor as described above. Fractions corresponding to the peaks of 28S and 18S rRNA were precipitated and rerun separately in 5–20% linear sucrose gradient as described above.

E. coli rRNA was a gift from Lloyd Smith and wheat germ rRNA was purchased from Calbiochem.

Preparation of rRNA Precursor. Rats were labeled for 1 hr prior to the harvest of ascites cells by intraperitoneal injection of 1 mCi of [^{3}H]methylmethionine/rat (Schwarz). Crude nuclei were prepared from rat ascites cells as described. Nucleoli were prepared by the method of Jeanteur *et al.* (1968). Nucleic acids were extracted from the nucleoli by phenol extraction as described previously. Following precipitation the nucleic acids were suspended in TNM and subjected to DNase I treatment, followed by phenol extraction and precipitation as described above. The purified nucleolar RNA was layered on a 5–20% linear sucrose gradient as described above. Sedimentation was for 130 min at 39,000 rpm at 18° in the Spinco SW-39 rotor. The tubes corresponding to the peak 45S RNA were pooled, precipitated, and rerun on a 5–20% linear sucrose gradient as described above.

Purification of DNase. Electrophoretically pure DNase I was purchased from Worthington Biochemical Co. Different lots varied considerably in their ability to degrade HnRNA under standard conditions and it was found necessary to routinely repurify the DNase. DNase I (1 mg) was dissolved in 1 ml of 0.3 M ammonium acetate (pH 5.8) and chromatographed on Sephadex G50 (fine; column 20 × 3 cm). Fractions which corresponded to the peak activity were pooled and dialyzed

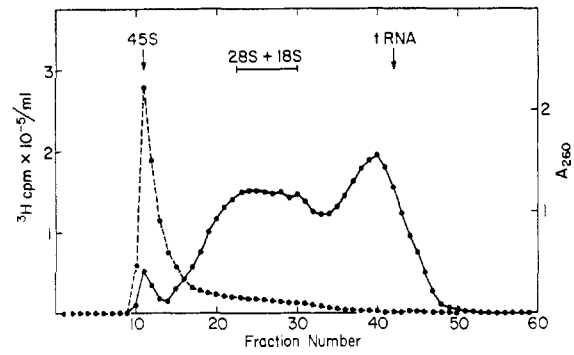

FIGURE 1: Chromatography of total nuclear RNA on Sepharose 2B: (●—●) A_{260} and (●- - - -●) ^{3}H radioactivity. Rat ascites cells were labeled for 0.5 hr with 2 mCi of [^{3}H]uridine as described in Methods. The positions of elution of 45S rRNA precursor, 28S + 18S rRNA, and tRNA standards are shown. The sample was applied in, and the column eluted with, 2 mM phosphate buffer (pH 6.8) + 1 mM EDTA. The column dimensions were 2.5 × 45 cm. The flow rate was about 15 ml/hr and the fraction volume was about 3.6 ml (125 drops).

against 0.01 M ammonium acetate (pH 5.8). The DNase was used immediately.

Determination of Sedimentation Coefficients. Estimation of RNA sedimentation coefficients in formaldehyde was performed by band sedimentation in the Model E analytical ultracentrifuge, essentially by the method of Boedtker (1968) with the following modification. RNA was heated at 55° for 5 min in 6% formaldehyde plus 0.01 M monosodium and 0.09 M disodium phosphate prior to loading onto 6% formaldehyde in the same buffer made with D_2O. We thank Robert Watson for running the Model E.

Electron Microscopy. RNA was prepared for electron microscopy by the method of Robberson *et al.* (1971) with the exception that Mann Ultra Pure urea was used in place of reagent grade urea; 45S rRNA precursor was used as a standard. We thank Douglas Ridder for his expert preparations of RNA for the electron microscope.

Nucleotide Analysis. ^{32}P-labeled RNA was prepared by intraperitoneal injection of 2 mCi of ^{32}P 1 hr prior to harvest of ascites fluid. RNA was incubated at 37° for 18 hr in 0.3 N KOH. At the end of incubation it was neutralized with 3 N $HClO_4$, allowed to flocculate at 4° for 30 min, and the insoluble salt pelleted by low-speed centrifugation. The supernatant was desalted by passage through activated charcoal as described by Sedat (1971). Ribonucleotides were separated on a Picker Nuclear LCS 100 automated nucleotide analyzer. The 2′,3′-ribonucleotides standards were purchased from Calbiochem.

Thermal Denaturation of RNA. Melting of RNA was carried out in a Gilford automatic recording spectrophotometer equipped with a thermostatistically controlled water bath. Correction was made for the thermal expansion of water.

Analytical Methods. Protein was assayed by the method of Lowry *et al.* (1951) using BSA as a standard. RNA was determined by the orcinol reaction of Dische and Schwartz (1937) using purified yeast tRNA as a standard. DNA was determined by the diphenylamine reaction described by Burton (1956), using rat ascites DNA as a standard.

Miscellaneous Methods. (1) Phenol distillation: 1 day before use, phenol (Fisher) was redistilled under N_2, immediately diluted with an equal volume of chloroform (Matheson, Coleman & Bell, Spectrograde quality), made 1% (v/v) isoamyl alcohol and 0.1% (w/v) 8-hydroxyquinoline, saturated with water, and stored under pressure in an N_2 atmosphere at −18°. (2) Water for making all solutions was distilled, deionized, and glass distilled. (3) All glassware and metal utensils were heated at 180° for at least 5 hr. (4) All plastic ware was stored in 0.1% sodium dodecyl sulfate plus 0.1% EDTA and washed in succession in water, methanol, water before use. (5) Gloves were used for handling all equipment.

TABLE I: Yield of Acid-Precipitable Counts at Various Steps in the Isolation of Giant HnRNA.

	Acid-Precipitable Counts of [^{3}H]Uridine	% Counts Remaining
I.[a] Crude nuclear lysate	8×10^7	100
II. Combined aqueous phases after first phenol extraction	7.4×10^7	93
III. Combined aqueous phases after DNase step and second phenol extraction	5.3×10^7	66
IV. Included volume of Sepharose 2B column (<45S RNA)	1.8×10^7	22
V. Excluded volume of Sepharose 2B column (≥ 45S RNA)	3.4×10^7	42

[a] Roman numerals refer to steps described in Methods. An aliquot from each step was precipitated with cold 10% trichloroacetic acid. The precipitate was collected onto a nitrocellulose filter, washed with cold 10% trichloroacetic acid followed by 60% ethanol. The filter was dried and dissolved in 1 ml of ethyl acetate and counted in a toluene-based scintillation cocktail.

Results

Yield. The yield of acid-precipitable counts at various steps in the isolation of nuclear RNA is given in Table I.

Sepharose 2B Chromatography. Figure 1 shows the pattern of separation of radioactivity associated with total nuclear RNA on a column of Sepharose 2B. The majority of the radioactivity (66%) after a 30-min pulse of [^{3}H]uridine, but very little of the mass, is eluted in the excluded volume together with a marker of 45S rRNA precursor. There are some residual DNA oligonucleotides in the included volume of the column even after the DNase digestion and it is therefore not possible to resolve other classes of RNA on the basis of absorbance.

Sucrose Density Gradient Sedimentation. Figure 2a shows the sedimentation pattern of Sepharose 2B excluded RNA on a linear 5–20% sucrose gradient. The majority of the radioactivity sediments faster than an *Escherichia coli* rRNA marker, and is polydisperse with some indication of peaks at 45 and 70 S. The inclusion of EDTA and sodium dodecyl sulfate in the gradients makes it unlikely that these high sedimentation coefficients are the result of aggregation of the RNA due to heavy metal ions or contamination with proteins. No DNA (detection limit 1%) could be detected in the RNA. Thermal denaturation of the RNA prior to centrifugation results in a very slight decrease in the sedimentation coefficients which can probably be attributed to thermal scission of phosphodiester bonds during heating (see Methods).

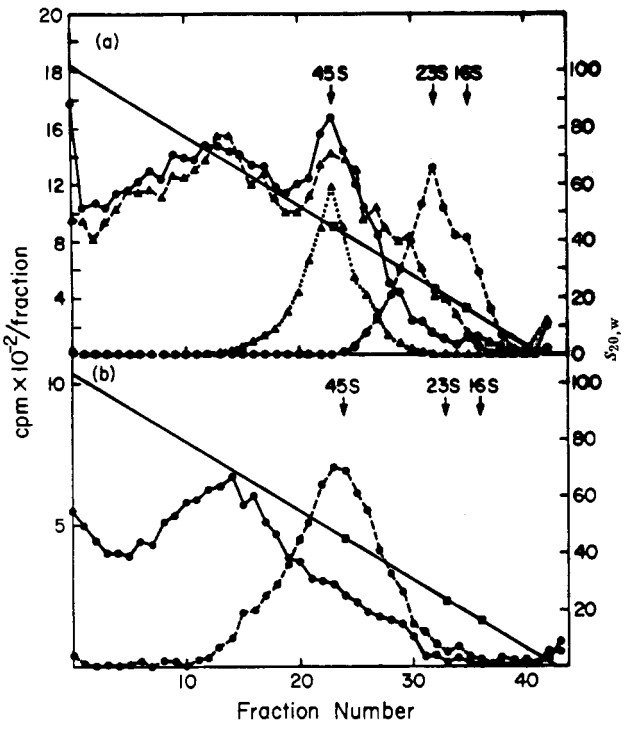

FIGURE 2: Sedimentation of RNA in a linear 5–20% sucrose gradient containing 0.1 M NaCl–1 mM EDTA–0.01 M sodium acetate (pH 6)–0.2% sodium dodecyl sulfate. The sample was applied in 100 μl of the same buffer. The gradients were centrifuged at 39,000 rpm at 18° in the Spinco SW-30 rotor for 90 min. (a) The radioactive profiles of three gradients have been superimposed in this figure, using *E. coli* 23S + 16S rRNA as a marker. ^{3}H excluded RNA (fractions 10–15, Sepharose 2B, Figure 1) before (●—●) and after (▲- - -▲) heat treatment. (△- - -△) ^{3}H-labeled 45S rRNA precursor from purified rat nucleoli. (●- - -●) ^{14}C-labeled *E. coli* 23S + 18S rRNA. Fraction 0 corresponds to RNA which has pelleted. (b) (●—●) Fractions 0–18 and (●- - -●) fractions 19–28 from a gradient similar to that depicted in Figure 2a. Two separate sucrose gradients have been superimposed in this figure and the position of sedimentation of ^{3}H-labeled 45S rRNA precursor from purified rat nucleoli and ^{14}C-labeled *E. coli* 23S + 16S rRNA have been indicated by ticks.

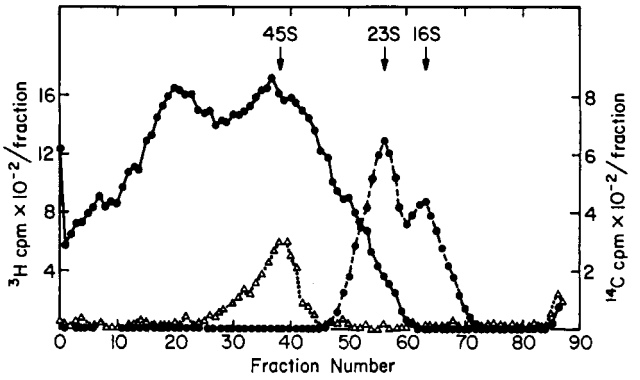

FIGURE 3: Sedimentation of RNA in a linear 5–20% sucrose gradient containing 0.1 M NaCl–1 mM EDTA–0.01 M sodium acetate (pH 6)–6% formaldehyde. The RNA was heated at 55° for 5 min in 100 μl of the same buffer prior to loading. The gradients were centrifuged at 39,000 rpm at 18° in the Spinco SW-39 rotor for 200 min. (●—●) ^{3}H-labeled excluded RNA (fractions 10–15 Sepharose 2B, Figure 1); (△- - -△) ^{3}H-labeled 45S rRNA precursor from purified rat nucleoli; (●- - -●) ^{14}C-labeled *E. coli* 23S + 18S rRNA. The radioactive profiles from two gradients have been superimposed in this figure.

Figure 2b shows the sedimentation profile of isolated 70S and 45S RNA in a second cycle of sucrose density gradient centrifugation. It is clear that the RNA maintains its integrity on a second passage through sucrose. Using values of 70 S and 45 S for the modal sedimentation coefficients of the RNA we obtain molecular weight estimates of 1.26×10^6 and 4.77×10^6 daltons, respectively, using the equation: $M = 1100\ S^{2.2}$ (Gierer, 1950), or 1.18×10^7 and 4.6×10^6 daltons, respectively, using the equation: $M = 1550\ S^{2.1}$ (Spirin, 1961).

However, the reliability of molecular weight estimates from sedimentation coefficients in sucrose has been criticized due to undefined conformational differences in RNA molecules (Gesteland and Boedtker, 1964; Strauss and Sinsheimer, 1967). We have therefore examined the sedimentation pattern of excluded RNA under conditions which minimize differences in conformation by elimination of base pairing.

Sucrose Density Gradient Centrifugation in the Presence of Formaldehyde. Low concentrations of formaldehyde efficiently denature RNA (Boedtker, 1967; Fenwick, 1968). As shown in Figure 3, excluded RNA treated with formaldehyde at 55° for 5 min and run on a sucrose gradient in the presence of 6% formaldehyde (Fenwick, 1968) maintains a position relative to marker RNAs which is comparable to that found in a non-denaturing sucrose gradient. This result differs from the report of Mayo and de Kloet (1971) who showed that HnRNA isolated from Ehrlich ascites cells "disaggregated" into smaller molecules after treatment with formaldehyde. During the initial stages of this investigation our preparations of giant HnRNA also had a tendency to "disaggregate" presumably because the denaturing action of the formaldehyde revealed breaks in the HnRNA which were not apparent on nondenaturing sucrose gradients. However, the cause of this breakage was traced to residual ribonuclease activity in the commercial DNase used.

Molecular Weight in Formaldehyde. Sedimentation coefficients of RNA in formaldehyde were obtained in the Model E analytical ultracentrifuge essentially by the method of Boedtker (1968) using *E. coli* 23S and 16S rRNA and rat ascites 45S, 28S, and 18S rRNA as standards. RNA eluted in the void volume of Sepharose 2B sedimented as two broad peaks with some tailing on the low molecular weight side. The modal sedimentation coefficients of the peaks were 31 ± 2.0 and 20.5 ● 1.5 which correspond to molecular weights of approximately 1.1×10^7 and 3.6×10^6 daltons, respectively (Boedtker, 1968). A decrease of about 1% in the $s_{20,w}$ values is expected from thermal degradation of the RNA during its preparation for centrifugation, which is well within the experimental error of ±7% for RNA from different preparations.

However, two lines of evidence suggest that the reaction of RNA with formaldehyde could yield spurious estimates of molecular weight. It is known that formaldehyde has no effect on single-stranded stacking interactions (Stevens and Rosenfeld, 1966) which have been shown to have an effect on the radius of gyration of synthetic RNA (Inners and Felsenfeld, 1970). Also, formaldehyde may alter the radius of gyration by formation of methylene bridges (Feldman, 1967).

Sedimentation on a Dimethyl Sulfoxide Gradient. Under defined conditions dimethyl sulfoxide is known to completely denature a number of single-stranded RNAs, to inhibit ribonuclease, and to have no effect on the biological activity of viral RNA (Strauss *et al.*, 1968; Sedat *et al.*, 1969). Figure 4 is a standard curve of RNA species of known molecular weight on Me_2SO gradients essentially by the method of Sedat *et al.* (1969). Log molecular weight has a linear relationship

CHART I

Species of RNA	Mol Wt of Ascites RNA (Daltons)	No. of Expt	Mol Wt (Daltons) from the Lit.	Ref
E. coli 16S rRNA			0.55×10^6	Stanley and Bock (1965)
E. coli 23S rRNA			1.07×10^6	Stanley and Bock (1965)
Rat 18S rRNA	$0.69 \times 10^6 \pm 0.03 \times 10^6$	2	0.70×10^6	Loening (1968)
Rat 28S rRNA	$1.74 \times 10^6 \pm 0.08 \times 10^6$	2	1.75×10^6	Loening (1968)
Rat 45S rRNA precursor	$4.32 \times 10^6 \pm 0.26 \times 10^6$	3	4.4×10^6	McConkey and Hopkins (1969)

with sedimentation distance in agreement with Sedat *et al.* (1969) and McGuire *et al.* (1972).

Figure 5a shows the Me_2SO gradient centrifugation pattern of radioactive RNA eluted in the excluded volume of Sepharose 2B. The majority of the radioactivity sediments more rapidly than *E. coli* rRNA corresponding to molecular weights between 1 and 10×10^6 daltons.

RNA from fractions 11–18 in Figure 5a was pooled, precipitated and rerun on a Me_2SO gradient (Figure 5b). The RNA maintains its integrity on a second passage through Me_2SO and has molecular weights between 5 and 10×10^6 daltons, as judged by the radioactivity profile.

It was found necessary to heat both giant HnRNA and 45S rRNA prior to centrifugation in Me_2SO. If the heating was omitted, 90–95% of the RNA pelleted. Although heat treatment was not necessary to solubilize *E. coli* 23S and 16S rRNA in Me_2SO, if such RNA was added to the HnRNA sample prior to centrifugation and the heating omitted, then the rRNA also pelleted with the HnRNA. (Control experiments in which *E. coli* 23S and 16S rRNA were heated as described showed no detectable decrease in molecular weight of these species.) Simmons and Strauss (1972) have observed a similar phenomenon using Sindbis 49S RNA.

We can offer no definite reason for the apparent insolubility of the RNA prior to heat treatment. Although 45S rRNA sediments in Me_2SO in a position consistent with its molecular weight, it is possible that large RNAs (>28S rRNA) are close to precipitation in Me_2SO under the conditions used, and, therefore, our estimate of the molecular weight of giant HnRNA in Me_2SO should be viewed with some reservation.

Electron Microscopy. Figure 6 shows electron micrographs of giant HnRNA purified through Me_2SO. Figure 7a shows a frequency distribution of the lengths, and Figure 7b shows the mass distribution of this RNA. Table II lists the parameters that describe these distributions. Giant HnRNA is heterodisperse with 84% of the mass of the RNA or 63% of the molecules having a contour length of 4–9 μ. 45S rRNA purified through Me_2SO has a more uniform distribution with 83% of the mass of the RNA or 53% of the molecules with a contour length between 3 and 4 μ. Using a value of 1.29×10^6 daltons/1 μ derived from the study of 18S rRNA (Robberson *et al.*, 1971) 63% of the giant HnRNA has a molecular weight in the range $5–11 \times 10^6$ daltons with a weight-average molecular weight of about 7.8×10^6 daltons in reasonable agreement with the results of Granboulan and Scherrer (1969). Similarly 53% of the 45S rRNA has a molecular weight of about $3.9–5.2 \times 10^6$ daltons with a weight-average molecular weight of about 4.3×10^6 daltons. This is consistent with the estimate of 4.3×10^6 daltons derived from the sedimentation velocity of 45S rRNA in Me_2SO presented above.

Two possible sources of error in the estimate of the length of 45S RNA and giant HnRNA in the electron microscope should be mentioned. First, a number of molecules of HnRNA could not be measured due to ambiguous contour lengths resulting from a lack of complete denaturation. Examples of these are shown in Figure 6d. Some of the molecules whose lengths were measured had small bushes, and regions of apparent secondary structure, which were assumed to be and were measured as duplex regions. Second, our data are uncorrected for the effect of base composition on the contour length of the RNA because the relationship is not well documented (Robberson *et al.*, 1971).

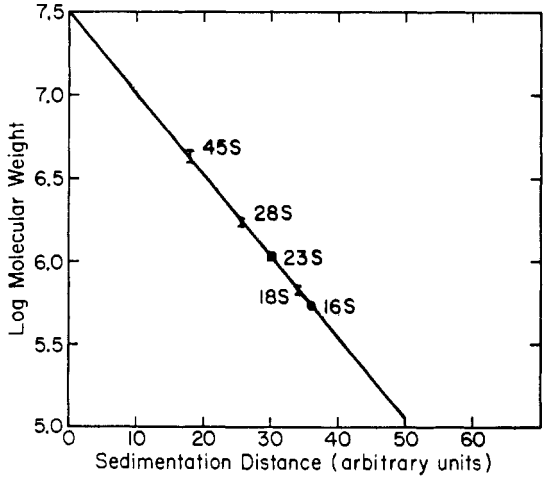

FIGURE 4: Dependence of log molecular weight on sedimentation distance for various RNA classes on Me_2SO. The Me_2SO gradients were run as described in Methods. The classes of RNA are tabulated in Chart I. The estimate of the molecular weight of the RNA species and its standard deviation refers to the appropriate peak fraction only.

TABLE II: Length Measurements and Distribution Parameters for RNA.[a]

	RNA	
	45 S	Giant Hn
Number-average length L_n (μ)	2.91	4.68
Weight-average length L_w (μ)	3.36	6.03
Model length (μ)	3.5	6.0
T_n (μ)[b]	1.2	2.48
T_w (μ)[c]	0.87	1.96
T_w/L_w	0.26	0.32
Molecular weight[d] (daltons) $\times 10^{-6}$	4.33	7.78

[a] The data presented in this table were obtained from Figure 6. [b] Standard deviation based on number-average length. [c] Standard deviation based on weight-average length. [d] Molecular weight of the weight-average length where 1 μ corresponds to 1.29×10^6 daltons based on an examination of rRNA under similar conditions (Robberson *et al.*, 1971).

TABLE III: Base Composition of Various Classes of RNA.

Species of RNA	Mol %					% ^{32}P				
	C	U	A	G	G + C	C	U	A	G	G + C
Giant HnRNA[a]	26.0	24.4	21.5	28.1	54.1	23.8	25.6	22.6	27.0	51.8
Giant HnRNA[b]	24.2	30.1	24.1	21.3	45.5	26.1	29.3	24.1	20.5	46.6
45S RNA[c]	28.1	18.4	18.6	34.9	63.0					
Precursor[d]	33.1	17.2	14.7	35.1	68.2					

[a] Sucrose purified giant HnRNA (fractions 0–18, Figure 2b). [b] Me_2SO purified giant HnRNA (fractions 11–18, Figure 5a). [c] Sucrose purified 45S RNA (fractions 19–28, Figure 2b). [d] 45S rRNA precursor from purified rat nucleoli.

Base Composition. Table III shows the base composition of both the giant HnRNA and 45S RNA isolated from either a sucrose or a Me_2SO gradient. The giant HnRNA has a "DNA-like" base composition (rat DNA = 41% G + C) and relatively high U content. These results are consistent with the findings of others (Attardi *et al.*, 1966; Scherrer *et al.*, 1966; Soeiro *et al.*, 1966, 1968). The slightly higher G + C content of sucrose purified giant HnRNA compared to that isolated on a Me_2SO gradient indicates that it is probably contaminated with 45 S rRNA precursor. The high G + C content of 45S RNA supports the view that a considerable proportion of it is 45S rRNA precursor (rat ascites 45S rRNA precursor = 68% G + C).

Assuming that 20% of HnRNA molecules have one poly(A) segment of about 200 A's/molecule (Greenberg and Perry, 1972) then the contribution of poly(A) to overall A content of HnRNA (about 4000 A's/HnRNA molecule) is negligible.

Secondary Structure. The relative absorbance–temperature profile of sucrose purified giant HnRNA in sodium phosphate buffer (pH 6.8) is shown in Figure 8a; wheat germ rRNA (51% G + C) is included in the figure for comparison. The relative increase in absorbance at 260 mμ of giant HnRNA is 24% (20% hypochromicity) and the T_m is 56.5°. Using an estimate of 29.5% hypochromicity for the helix–coil transition of a completely helical RNA (G + C = 50%), obtained by adding the hypochromic contributions of poly(A + I) and poly[d(G + C)] (Doty, 1962), it is estimated that 68% of giant HnRNA is in a helix conformation (uncorrected for the contribution of single-stranded stacked bases).

A Van't Hoff plot of the temperature dependence of absor-

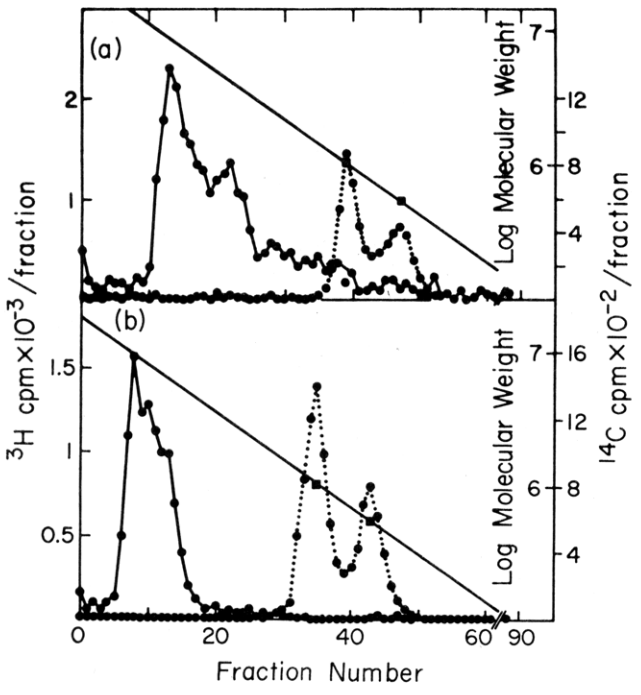

FIGURE 5: Sedimentation of RNA on Me_2SO gradients. The gradients were prepared as described in Methods. (a) Sedimentation of ^{3}H excluded RNA (fractions 10–15 Sepharose 2B, Figure 1) (●—●) and *E. coli* 23S + 16S rRNA (●- - -●). Centrifugation was for 8 hr at 27° at 65,000 rpm in the Spinco SW-65 rotor. (b) Sedimentation of giant HnRNA (fractions 11–18, Figure 5a) (●—●) and *E. coli* 23S + 16S rRNA (●- - -●). Centrifugation was for 10 hr as described above.

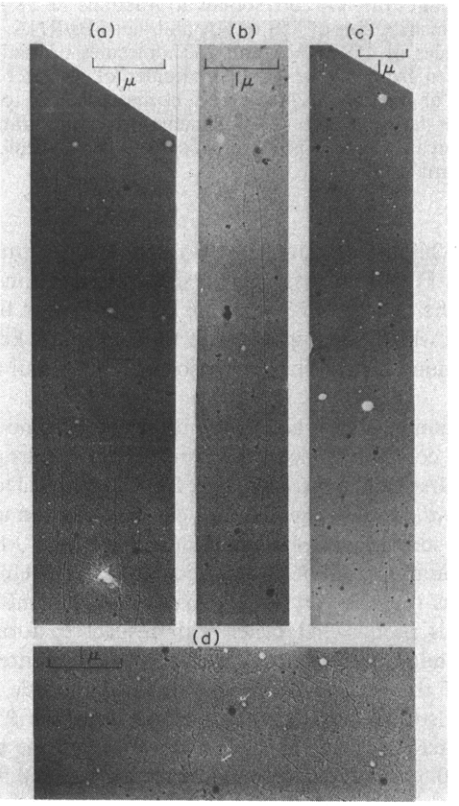

FIGURE 6: Electron micrographs of giant HnRNA prepared by Me_2SO gradient sedimentation velocity (fractions 11–18 Figure 5a). The RNA was prepared for visualization under the electron microscope essentially by the method of Robberson *et al.* (1971). The scale shown is 1 μ (about 1.29 × 10^6 daltons): (a,b,d) × 29,000; (c) × 16,000.

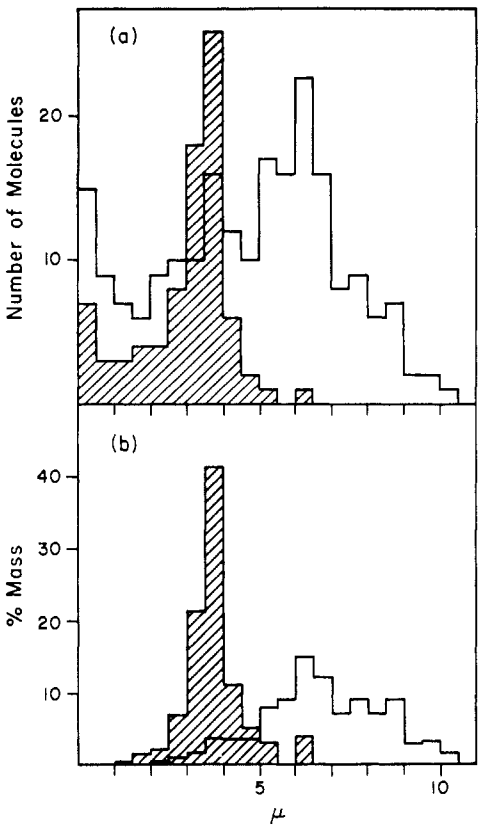

FIGURE 7: Frequency and mass distribution of RNA as visualized under the electron microscope. (a) Frequency distribution of 45S rRNA and giant HnRNA prepared by sedimentation through Me_2SO (giant HnRNA corresponds to fractions 11–18 Figure 5a). (b) Mass distribution of 45S rRNA and giant HnRNA. A total of 98 molecules of 45S rRNA and 252 molecules of giant HnRNA were scored from five grids. 15 molecules of 45S rRNA and 42 molecules of giant HnRNA could not be measured due to ambiguity in contour length resulting from incomplete denaturation. Cross hatched area = 45S rRNA prepared from rat nucleoli and open area = giant HnRNA.

bance at 260 mμ of giant HnRNA and wheat germ rRNA is shown in Figure 8b. Assuming a single equilibrium constant, ΔH for the change is 25 kcal/mol of linkages for both types of RNA, which is comparable to the ΔH = 20 kcal/mol of linkage found for calf liver microsomal RNA (Hall and Doty 1959).

The reaction of formaldehyde with the free amino groups of bases is a convenient measure of the extent of hydrogen bonding in RNA (Hall and Doty, 1959; Haselkorn and Doty, 1961; Mitra *et al.*, 1963). The reaction can be followed at 275 mμ in 0.12 M sodium phosphate buffer (pH 6.8) at 25°. The extent of denaturation can be monitored at 245 mμ. Under these conditions there is very little denaturation of HnRNA. The reaction is pseudo-first order with respect to formation of methylol adducts (Penniston and Doty, 1963). Figure 8c shows a plot of the extent of reaction of formaldehyde with free mononucleotides and sucrose purified giant HnRNA. The pseudo-first-order rate constants derived from the slopes are 2.25×10^{-2} min^{-1} for free mononucleotides and 0.98×10^{-2} min^{-1} for giant HnRNA. Assuming that formaldehyde reacts at the same rate and to the same extent with available bases in giant HnRNA as it does with free bases then a comparison of the rate constants indicates that about 57% of the bases of giant HnRNA are not available for reaction under the conditions used.

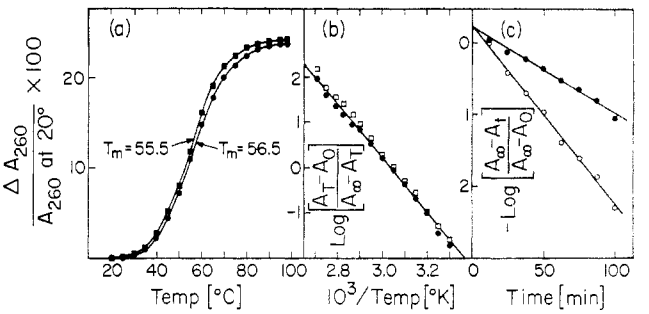

FIGURE 8: (a) Optical melting profile of RNA in 0.12 M phosphate buffer (pH 6.8) recorded in the Gilford automatic recording spectrophotometer equipped with a thermostatically controlled water bath. The temperature was raised automatically at the rate of 0.5°/min. (●—●) Sucrose purified giant HnRNA, fractions 0–18 from a gradient similar to that shown in Figure 2b; (■—■) wheat germ 25S + 17S rRNA. (b) A Van't Hoff plot of the data from Figure 8a. $A_0 = A_{260}$ at 20°. $A_\infty = A_{260}$ at 98°: (●) sucrose-purified giant HnRNA, (□) wheat germ 25S + 17S rRNA. (c) A first-order plot for the reaction of sucrose-purified giant HnRNA (see Figure 8a) (○) and a mixture of ribonucleotides (G = 22%, C = 24%, A = 24%, U = 30%), (●) with 1% formaldehyde (Mallinckrodt reagent grade) in 0.12 M phosphate buffer (pH 6.8) at 25°. The reaction was monitored at 275 mμ. $A_0 = A_{275}$ at the beginning of reaction. $A_t = A_{275}$ at the appropriate time and $A_\infty = A_{275}$ after equilibration.

Discussion

The preparation of HnRNA presents a number of technical probelms which, although probably not unique, are comparatively more serious than in the preparation of r- or tRNA. These are: (1) the tendency of HnRNA to aggregate with proteins and escape into the interface during phenol extraction (Parish and Kirby, 1966; Kidson *et al.*, 1964); (2) its susceptibility to nucleolytic digestion during isolation; and (3) the lack of an all-encompassing simple separation procedure due to the range of responses of HnRNA to the usual separation techniques, for example, its polydispersity in a centrifugal field.

The tendency of HnRNA to be found complexed with protein during isolation may reflect its *in vivo* association with protein in the form of ribonucleoprotein particles (Georgiev and Samarina, 1971), or it may be just the tendency of a large polyanion to electrostatistically bind some of the more basic proteins (Girard and Baltimore, 1966). We have attempted to overcome this problem by including 7 M urea in the mixture used to lyse the nuclei and in all subsequent phenol extractions. The observation that 93% of the radioactivity after a 30-min pulse of [^{3}H]uridine is associated with the aqueous phase in the first phenol extraction is a measure of the success of this procedure. One of the most frequently used techniques for the isolation of HnRNA from a nuclear lysate involves phenol extraction at elevated temperatures (Georgiev *et al.*, 1963). In our hands a phenol extraction and reextraction at 60° results in partially degraded HnRNA as judged by sedimentation on Me_2SO, although the RNA still appears to be "undegraded" on a nondenaturing sucrose gradient (unpublished data). Recently a procedure using "chaotrophic" agents such as the lithium salt of tricarboxylic acid to solubilize pulse-labeled RNA during phenol extraction has been published (Scott and Kuhns, 1972). The authors report good yields of RNA, although there is a tendency for the pulse-labeled RNA to disaggregate under denaturing conditions.

The use of urea throughout the extraction of HnRNA yields molecules which do not disaggregate in denaturing conditions; presumably by reducing ribonuclease activity, and by four

criteria, sedimentation through sucrose, formaldehyde, and Me_2SO and by electron microscopy we are able to isolate a population of heterogeneous rapidly labeled nuclear RNA molecules of very high molecular weight.

A variety of methods for following the processing of HnRNA have been developed. For example, the selection of a system where there is little rRNA synthesis (Attardi *et al.*, 1966) or where rRNA synthesis has been reduced by drugs (Scherrer *et al.*, 1966); by separation of particular classes of HnRNA, *e.g.*, that which is tenaciously bound on columns of methylated albumin kieselguhr (Billing and Barbiroli, 1970), or that which sediments as giant HnRNA on sucrose gradients (Attardi *et al.*, 1966; Warner *et al.*, 1966); or by following changes in the base ratio of nuclear RNA. We chose to use a separation procedure based on size because it yielded preparative amounts of HnRNA which were, by the criterion of size and base composition comparatively free from 45S rRNA precursor. Our procedure is essentially an adaptation of the frequently used separation of giant HnRNA by sucrose density gradient centrifugation. The novel aspects are the use of urea in the extraction of RNA from nuclei and the introduction of chromatography of whole nuclear RNA on Sepharose 2B. The Sepharose 2B column chromatography represents a convenient way of isolating comparatively large quantities of $\geqslant$45S RNA from bulk nuclear RNA without recourse to a considerable number of sucrose gradients. This then allows the separation of giant HnRNA from 45S RNA precursor in preparative quantities by centrifugation. For this step both nondenaturing sucrose gradients and Me_2SO gradients were used. The former allows more RNA to be handled but yields an RNA which is slightly degraded and which contains a small amount of 45S RNA contamination. The latter yields an RNA which is almost completely undegraded and has a very low (46%) G + C content.

Giant HnRNA, isolated from a sucrose gradient, has considerable secondary structure in solution as judged by its melting profile and reaction with formaldehyde. This may be related to the finding of Georgiev *et al.* (1972) and Jelinek and Darnell (1972) who have demonstrated the existence of ribonuclease-stable regions in HnRNA which are presumably base paired. It is not clear at present, whether the observed secondary structure *in vitro* has any relation to that *in vivo*, but it is tempting to speculate on the relationship of HnRNA secondary structure to its subsequent processing.

Our conclusion is that it is possible to isolate giant HnRNA which, by a variety of criteria, consists of molecules with a molecular weight in the range 5–10 $\times$ 10^6 daltons and that do not disaggregate under denaturing conditions. This RNA has a "DNA-like" base composition and considerable secondary structure in solution. Subsequent papers will examine the nature and distribution of the hybridizable sequences present in giant HnRNA.

Acknowledgments

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Time of Synthesis of Genes for Ribosomal Ribonucleic Acid in *Physarum*†

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ABSTRACT: The time in the cell cycle when the genes for ribosomal RNA are synthesized was determined in the plasmodial stage of *Physarum polycephalum*. Three approaches were used. (1) Plasmodia were exposed to [^{3}H]thymidine during the G2 phase of the cell cycle; nuclear satellite DNA, which is preferentially labeled under these conditions, was isolated and hybridized in solution with ribosomal RNA (rRNA). Analysis of the hybridization mixture revealed that 10–25% of the labeled DNA was in DNA–RNA hybrids, which demonstrates synthesis of rDNA during the G2 phase. (2) The per cent of nuclear DNA hybridizable with saturating amounts of rRNA was measured for DNA isolated from plasmodia at different times in the cell cycle. The per cent hybridization increased about 50% during the G2 phase, which also demonstrates synthesis of rDNA during this period. (3) Plasmodia were incubated with the DNA density label iododeoxyuridine, nuclear DNA was isolated, and the buoyant density profile of rDNA was determined. A fraction of the rDNA was shown to be of increased density when the density label was applied during either the S phase, the first half of the G2 phase, or the second half of the G2 phase (there is no G1 phase in *Physarum* plasmodia). Thus, rDNA synthesis occurs during all phases of the mitotic cycle in this organism.

Using density labeling techniques, Mueller and Kajiwara (1966) and Braun and Wili (1969) have shown that the DNA replicated during a given interval of one cell cycle is replicated during the same interval of subsequent cell cycles. In addition, Plaut *et al.* (1966) have shown that DNA in polytene chromosomes from *Drosophila* is synthesized in a defined temporal sequence. These lines of evidence suggest that most genes are synthesized at a particular time in the cell cycle. In order to determine whether this suggestion holds for particular genes, we have begun to study the timing of gene synthesis in *Physarum polycephalum*, an acellular slime mold which displays highly synchronous nuclear division (Rusch, 1970). Our approach is to identify genes by their hybridization to purified RNA species.

In this paper, we report on the time of synthesis of the genes coding for ribosomal RNA. Our results, which corroborate our preliminary report (Sonenshein *et al.*, 1970), and a recent report by others (Zellweger *et al.*, 1972), demonstrate that the genes for rRNA[1] are synthesized in both the S phase and the G2 phase of the mitotic cycle. Studies with other organisms show that rDNA is synthesized throughout the S phase (Amaldi *et al.*, 1969; Balazs and Schildkraut, 1971; Gimmler and Schweizer, 1972); however, these studies do not show whether rDNA is synthesized in the other parts of the cell cycle. In amphibian oocytes, it is clear that rDNA is synthesized in the absence of bulk DNA synthesis (Gall, 1968).

Nuclear division in the multinucleate *Physarum* plasmodium occurs about every 12 hr under the conditions of our experiments. Nuclear DNA synthesis follows metaphase without any measurable gap in time (Nygaard *et al.*, 1960; Sachsenmaier, 1964; Braun *et al.*, 1965); thus, the observation of rDNA synthesis in both the S phase (3–4 hr) and the G-2 phase (8–9 hr) implies an essentially continuous synthesis. However, a brief cessation of rDNA synthesis early in S phase has been reported (Zellweger *et al.*, 1972).

We have approached the problem of determining the time of synthesis of rDNA in three ways. First, we have studied the hybridization of rRNA with a nuclear satellite DNA, that can be preferentially labeled during G2 phase (Holt and Gurney, 1969). Second, we have measured the fraction of the total DNA coding for rRNA as a function of the phase of the mitotic cycle. Finally, we have incubated plasmodia with a density label for DNA during different periods of the mitotic

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[1] Abbreviations used are: rRNA, 19S and 25S ribosomal ribonucleic acid; rDNA, ribosomal deoxyribonucleic acid, the DNA that contains the sequences homologous to rRNA; nDNA, nuclear deoxyribonucleic acid; SSC, standard saline-citrate (0.15 M NaCl–0.015 M sodium citrate, pH 7.1–7.5); MAK, methylated albumin kieselguhr; MI, MII, etc., the first and second mitoses following preparation (fusion) of a surface culture.