*ACRYLAMIDE GEL ELECTROPHORESIS OF HELA CELL NUCLEOLAR RNA**

By Robert A. Weinberg,† Ulrich Loening,‡ Margherita Willems, and Sheldon Penman§

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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The nucleolus of eucaryotic cells has been identified by Perry and others[1-4] as the site of ribosomal RNA synthesis. It has recently become possible to obtain from HeLa cells a nucleolar preparation which, as seen by electron microscopy, is relatively free of chromatin.[5, 6] Fractionation of C^{14}-uridine-labeled cells confirmed the hypothesis that the nucleolus is the site of synthesis of the 45*S* ribosomal RNA precursor.[5] Another species of RNA (32*S*) is present in relatively large amounts. The nucleolus appears to contain only ribosomal precursor RNA since if fractionation is performed carefully, very little of the nucleoplasmic heterodisperse RNA is associated with it.[7, 8] The following picture of the major events in ribosomal RNA formation has emerged. The initial event is the synthesis of a high-molecular-weight precursor molecule with a sedimentation constant of 45*S*.[9-11] After 15 to 20 minutes, this molecule is cleaved, yielding 18*S* ribosomal RNA and a species of RNA whose sedimentation constant is 32*S*.[12] The 18*S* RNA is quickly transported from the nucleolus and appears in the cytoplasm as part of the smaller ribosomal subunit. After additional processing time in the nucleolus, the 32*S* molecule is converted to 28*S* and eventually emerges into the cytoplasm as part of the larger ribosomal subunit.

With the development by Loening of a method of polyacrylamide gel electrophoresis for molecules as large as ribosomal RNA,[13] it has become possible to study in greater detail the events of nucleolar RNA processing. The gels used in these experiments have been modified by the addition of glycerol to facilitate freezing and slicing.

The following information has been obtained concerning nucleolar processing of ribosomal RNA. (1) The site of transformation of 32*S* to 28*S* RNA is the nucleolus. (2) Several additional short-lived intermediate species of ribosomal RNA have been identified with estimated sedimentation constants of 41*S*, 36*S*, and 20*S*. (3) Some short-lived intermediates increase in amount under conditions that disrupt normal nucleolar RNA processing, e.g., poliovirus infection. (4) The conversion of 45*S* RNA to mature ribosomal RNA is accompanied by a net loss of RNA. This is also shown for the transformation of 32*S* RNA to 28*S*.

Materials and Methods.—Cells: HeLa type 3 cells were grown and labeled in suspension culture as previously described.[14]

Radioisotopes: L-methionine-methyl-C^{14} (49 mc/mM) and uridine-2-C^{14} (27 mc/mM) were purchased from Schwarz BioResearch. L-methionine-methyl-H^3 (1400 mc/mM) was purchased from Nuclear Chicago. Methyl labeling was performed in Eagle's medium free of unlabeled methionine and containing adenosine and guanosine (2×10^{-6} *M*). When noted, unlabeled methionine was added back to this medium to prevent methionine starvation.

Cell fractionation: Cells were separated into nuclear and cytoplasmic fractions as previously described.[11] The cleaned nuclei from approximately 4×10^7 cells were suspended in 1 ml of high-ionic-strength buffer (HSB: 0.5 *M* NaCl, 0.05 *M* $MgCl_2$, 0.01 *M* Tris, pH 7.4), warmed briefly to 37°, and digested with 50 μg of Worthington electrophoretically purified DNase. The digest

was layered on a 16-ml sucrose gradient [15–30% (w/w) sucrose in HSB] and centrifuged 15 min at 22,000 rpm at 5°C in the SW 25.3 rotor of the Spinco model L-2 ultracentrifuge. The pellet was termed nucleoli and the supernatant fraction termed nucleoplasm.

RNA extraction: RNA was extracted with hot phenol-SDS (sodium dodecyl sulfate) as described before.[9, 11] The extracted RNA was precipitated with 2 to 3 volumes of 95% ethanol after addition of yeast tRNA (2 optical density units/ml) as carrier (Mann Research Co.). Precipitates were collected by centrifugation for 15 min at 15,000 rpm in the International model B-20. Pellets were suspended in 50 μl of electrophoresis buffer (0.04 *M* Tris, 0.02 *M* sodium acetate, 2 mM EDTA, 0.5% SDS, adjusted to pH 7.4 with acetic acid) made 15% (v/v) in glycerol. This solution was layered directly onto the polyacrylamide gel.

Polyacrylamide gels: Gels were polymerized[13] in a buffer identical to the above electrophoresis buffer except that the SDS was absent and the glycerol concentration was 10% (v/v). The polymerizing mixture was made 2.7% (w/v) in acrylamide (purchased from Eastman Chemical Co. and recrystallized from chloroform) and 0.25% (v/v) in the alkali-labile cross-linking agent ethylene diacrylate[15] (K & K laboratories). Acrylamide solutions and glycerol were shaken with hexane at room temperature to remove residual material absorbing at 2600 Å. Cylindrical gels of 0.6 cm diameter and 6 cm length were cast. Electrophoresis tanks were filled with electrophoresis buffer containing the SDS and 10% (v/v) glycerol. Electrophoresis was performed at 5 ma per gel for the period specified. The gels were scanned for absorbancy with a Gilford recording spectrophotometer adapted for this purpose. Gels containing radioactivity were subsequently frozen and sliced in 1-mm slices in a bath of hexane adjusted to about −30°C by the addition of dry ice. The glycerol contained in the gel prevents ice crystal formation upon freezing and therefore facilitates slicing. Gel slices to be counted by scintillation were hydrolyzed in vials with 0.5 ml of concentrated NH_4OH for 1 hr. Ten ml of Bray's[16] scintillation fluid were added. Slices to be counted in a gas-flow system were placed on planchettes adapted for this purpose and hydrolyzed with 1 ml of concentrated NH_4OH, which was subsequently evaporated by heating.

Results.—The greatly improved resolution possible with acrylamide gel electrophoresis compared to sucrose density gradients is demonstrated in Figure 1. RNA

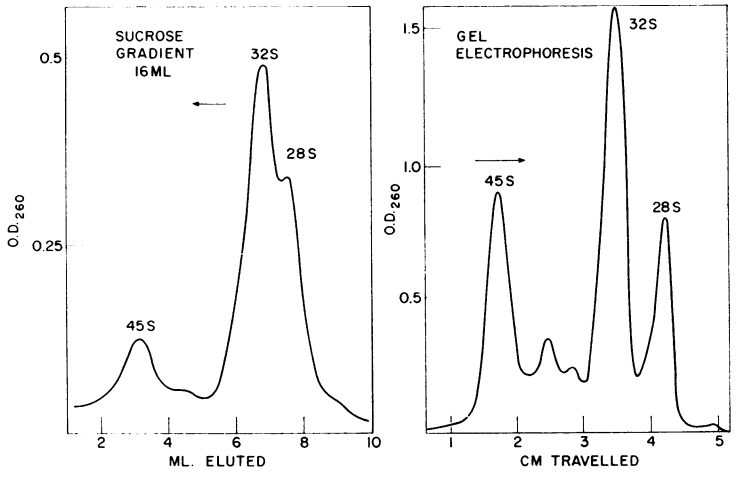

FIG. 1.—Comparison of sucrose gradient and gel electrophoresis analysis of nucleolar RNA. Each frame represents nucleolar RNA from approximately 4 × 10^7 cells. *Sucrose gradient:* Sample resuspended in 0.4 ml of SDS buffer and run for 14 hr at 23,000 rpm. The gradient was 15–30% (w/w) sucrose in SDS buffer (0.1 *M* NaCl, 0.01 *M* Tris pH 7.4, 0.001 *M* EDTA, 0.5% sodium dodecyl sulfate). The total volume was 16 ml. Centrifugation was in the SW 25.3 rotor of the L-2 Spinco ultracentrifuge. The bottom two-thirds of the elution pattern is displayed here. *Gel electrophoresis:* The sample was run for 6 hr. The top two-thirds of gel is displayed here.

from HeLa cell nucleolar preparations was analyzed on both a sucrose gradient and an acrylamide gel. The optical density profiles of both are shown. The principal components of nucleolar RNA are visible in the pattern obtained from the sucrose gradient. These are 45*S* and 32*S* RNA as well as a shoulder corresponding to a 28*S* RNA component and a suggestion of ultraviolet-absorbing material sedimenting between 45*S* and 32*S* RNA. These additional species of RNA are clearly resolved by gel electrophoresis. In addition to 45*S* and 32*S* RNA, there is a well-separated peak corresponding to 28*S* ribosomal RNA. There is an additional peak visible which corresponds to RNA which would sediment at approximately 41*S* as well as a very minor component at approximately 36*S*. The identification of the RNA species observed in the gels was confirmed by electrophoretic analysis of the RNA species isolated from conventional 15–30 per cent sucrose gradient zones.

When electrophoresis is carried out for a shorter period, two additional very small peaks of absorbing material are visible which correspond to 18*S* and 20*S*. The profile of nucleolar RNA obtained after four hours of electrophoretic migration is shown in Figure 2. The nucleolar RNA had been labeled with methionine-me-

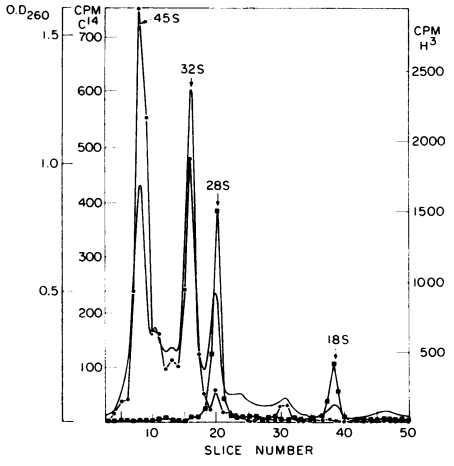

FIG. 2.—Electrophoretic analysis of nucleolar and ribosomal RNA. One hundred ml of cells at a concentration of 4×10^6 cells/ml were centrifuged and resuspended for 30 min in 20 ml of methionine-free medium containing 10 μc of C^{14}-methione. Nucleolar RNA was run for 4 hr with H^3-uridine-labeled cytoplasmic ribosomal RNA as marker. O.D., ——; C^{14}-methionine, ●–●–●; H^3-uridine, ■–■–■.

thyl-C^{14} for 30 minutes prior to fractionation. It has been shown previously that the only high-molecular-weight RNA that is methylated in short pulses is the 45*S* precursor and that methylation takes place shortly after synthesis.[17, 18] By 30 minutes, some of the labeled 45*S* has been processed and radioactivity has begun to enter the 32*S* RNA and 20*S* peaks. Tritium-labeled ribosomal RNA of high specific activity was added as a marker and served to identify the 18*S* and 28*S* nucleolar RNA.

After 30 minutes, all species of nucleolar RNA are labeled by methionine, with the exception of 28*S* and 18*S*. This finding is consistent with the view that the minor components are intermediates in the formation of mature ribosomal RNA. It is tentatively assumed that the 41*S* and 36*S* RNA are precursors of 32*S* and that 20*S* RNA is the precursor of 18*S*.

The very small amounts of 41*S*, 36*S*, and 20*S* RNA found in the nucleolus indicate that their lifetime is in the order of two minutes. This short lifetime, coupled with possible dispersion in the processing time of individual RNA molecules, prevents measurement of precise kinetics of formation of these minor components. The

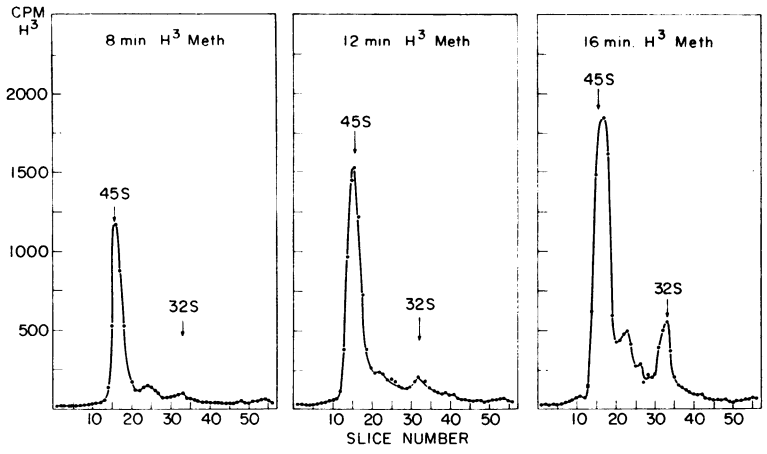

FIG. 3.—Kinetics of labeling of nucleolar RNA. Three hundred ml of cells were centrifuged and resuspended in 30 ml of methionine-free medium. Cells were labeled for 8, 12, and 16 min with 10 μc/ml of H^3-methionine. Nucleolar RNA was run for 5 hr.

following experiment indicates that 41*S* and 36*S* are labeled after the 45*S* RNA and, in the presence of actinomycin D, decay after the disappearance of 45*S*. The results of short periods of incorporation of methyl-labeled methionine are shown in Figure 3. It can be seen that even after 12 minutes of incorporation, only the 45*S* species has appreciable radioactivity. By 16 minutes, however, the 41*S* and 36*S* RNA are apparently nearly fully labeled and radioactivity has begun to appear in the 32*S* region.

The decay of the 41*S* and 36*S* species in actinomycin is shown in Figure 4. Cells were labeled for 16 minutes with methionine and then actinomycin was added to the culture. Nine minutes after the addition of actinomycin, the 45*S* RNA radioactivity has decayed to approximately one third of its initial value. However, the radioactivity remains associated with 41*S*-36*S* RNA. By 26 minutes, all the radioactivity in 45*S*, 41*S*, and 36*S* peaks has disappeared and has apparently largely migrated to the 32*S* peak in the nucleolus and 18*S* in the cytoplasm.[11] The pattern of radioactivity after 26 minutes in actinomycin also shows the first appearance of radioactivity in the nucleolar 28*S* species of RNA.

The minor components of nucleolar RNA are difficult to analyze because of their

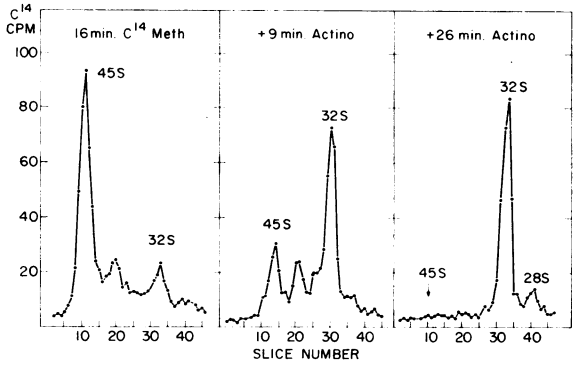

FIG. 4.—Effect of actinomycin on prelabeled nucleolar RNA. Three hundred ml of cells were centrifuged and resuspended in 25 ml of methionine-free medium. Cells were labeled for 16 min with 12 μc of C^{14}-methionine and a sample was taken. Actinomycin D was then added to a concentration of 10 μg/ml. Samples were taken at 9 and 26 min after the addition of actinomycin. Nucleolar RNA was run for 7 hr.

extremely low concentration. It is possible, however, to affect cell metabolism so that the content of the various species of RNA is altered and the concentration of some of the minor species is increased. One means of altering nucleolar RNA content is by infection with poliovirus. HeLa cells are unusual in that infection with small RNA viruses such as poliovirus does not result in the abrupt cessation of host RNA synthesis.[19] Rather, host-cell-directed RNA synthesis continues until quite late in infection but the processing of RNA, especially in the nucleolus, becomes aberrant shortly after infection. It was first noted by Willems that various intermediates apparently accumulate in the nucleolus.[20] In the experiment whose results are shown in Figure 5, HeLa cells were infected with poliovirus at a multiplicity of 500

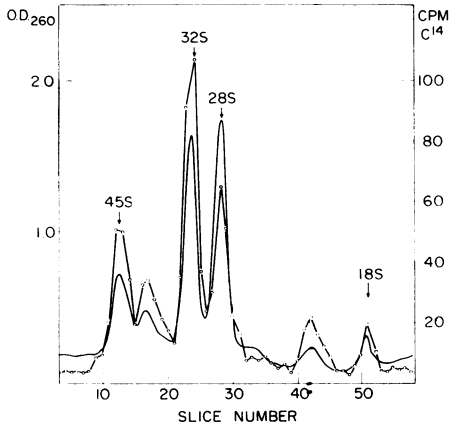

FIG. 5.—Effect of poliovirus infection on nucleolar contents. One hundred ml of cells at 4×10^5 cells/ml were centrifuged and resuspended in 3 ml of serum-free Eagle's medium containing 5×10^9 PFU/ml of poliovirus and 2 mM guanidine. After $^1/_2$ hr absorption, cells were centrifuged and resuspended in 20 ml of medium containing 3 μc of C^{14}-methyl methionine, 5×10^{-5} *M* cold methionine, and 2 mM guanidine. Cells were fractionated after 90 min of labeling. Nucleolar RNA was run for 5 hr. O.D., ——; C^{14}-methionine, –O–O.

PFU per cell. Thirty minutes after infection, C^{14}-methionine was added and incorporation allowed to continue for 90 minutes. The nucleolar content of the minor RNA species, 41*S*, 28*S*, 20*S*, and 18*S*, is seen to be significantly increased when compared to the profile seen in Figure 2. This increased content is apparently due to aberrant processing induced by poliovirus infection. It should be noted that these experiments were carried out in the presence of 2×10^{-3} *M* guanidine, which prevents viral RNA replication[21] but permits the expression of several virus-directed effects on host cell metabolism including the suppression of host cell protein synthesis[22] and the disarrangement of nucleolar processing shown here.[7] A detailed report on further experiments of this type will appear elsewhere.

The high resolution afforded by the acrylamide gel electrophoresis technique makes another type of measurement possible. Cells can be labeled with both methionine and uridine so that all species of RNA in the nucleolus have been uniformly labeled. It is then possible to measure the uridine and methyl group content of the various species of nucleolar RNA. This type of experiment could not be done previously because of the extensive cross-contamination of the various species of RNA obtained in sucrose gradients. It has been shown that the 45*S* ribosomal precursor RNA is methylated while it is being polymerized on the DNA template,[17] and that virtually all methylation takes place on the 45*S* molecule.[17, 18] Furthermore, the detailed pattern of methylation of the 45*S* precursor RNA is shown to be identical to that of 18*S* plus 28*S* RNA.[23] It appears, therefore, that virtually all methyl groups destined to be associated with 18*S* and 28*S* RNA are already attached to the 45*S*.

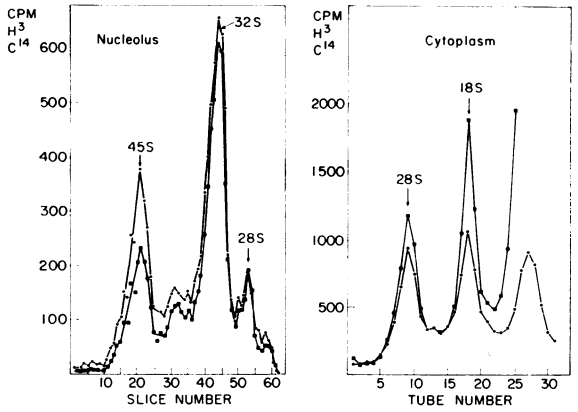

FIG. 6.—Comparison of uridine and methyl labeling of nucleolar and ribosomal RNA. One hundred ml of cells were centrifuged and resuspended in 50 ml of methionine-free medium to which were added 0.5 mg of cold methionine, 0.72 mg of cold uridine, 400 μc of H^3-methionine, and 1.5 μc of C^{14}-uridine. The cells were labeled for 2.5 hr and the nucleoli run for 7 hr. The cytoplasm was centrifuged for 15 hr at 24,000 rpm through a 28 ml 15–30% sucrose gradient in SDS buffer on the SW 25 rotor of the Spinco model L ultracentrifuge. C^{14}-uridine, —●—●—; H^3 - methionine, —■—■—.

Previous experimental results imply that there is little loss of methyl label as the 45*S* precursor is processed to form the mature 28*S* and 18*S* components of ribosomes.[23] The experimental results shown in Figure 6 show a uridine-to-methionine ratio which is highest for 45*S* and decreases for 32*S*, 28*S*, and 18*S*. This implies the loss of uridine- but not methionine-labeled RNA during processing. Cells were labeled for 2.5 hours with C^{14}-uridine and H^3-methionine. The specific activity and concentration of radioactive precursor was adjusted so that there was little change in the rate of uptake during the period of incorporation. RNA extracted from the nucleolar fraction was analyzed by gel electrophoresis. The total RNA from the cytoplasm was analyzed on a sucrose gradient. The label specifically associated with each peak was estimated and the results are shown in Table 1.

Discussion.—The electrophoretic separation of RNA on acrylamide gels makes possible a degree of resolution heretofore unobtainable and permits an extremely detailed analysis of the events in ribosomal RNA processing. Only part of the 45*S* precursor is converted to 28*S* and 18*S* end products. The fate of the remaining portion is unknown at this time. If the reduction in molecular weight proceeds stepwise, then it would be expected that short-lived intermediates would be observed. Several such intermediates have been identified in these experiments and their proposed role is shown in Figure 7.

It would be desirable to know whether the formation of 18*S* RNA or its possible 20*S* precursor accompanies the formation of 41*S* intermediate RNA, or whether they are products of the subsequent cleavage of this molecule. Because of the very short lifetime of the intermediates, it has not been possible to establish unambiguously whether 18*S* or 20*S* RNA appear coincident with or subsequent to the formation of 41*S* RNA.

TABLE 1

RATIO OF C^{14}-URIDINE LABEL TO H^3-METHYL METHIONINE LABEL FOR RNA OF VARIOUS TYPES

Nucleolus		Cytoplasm		Weighted av. of
45*S*	32*S*	28*S*	18*S*	28*S* and 18*S*
1.59	1.16	0.79	0.55	0.75

The radioactivity derived from C^{14}-uridine and H^3-methyl methionine associated with each species of RNA in Figure 6 was estimated and the ratio calculated. The weighted average of 18*S* and 28*S* was calculated on the assumption that they have molecular weights of 6×10^6 and 1.6×10^6 daltons, respectively.[26]

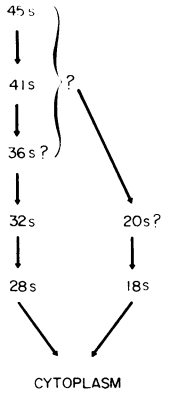

FIG. 7.—Proposed metabolic relation of the various species of nucleolar RNA.

Two separate kinds of evidence support the conclusion that there is a loss of molecular weight in the processing of the 45*S* ribosomal precursor into its final products. The first of these concerns the conversion of 32*S* into 28*S* RNA. It is observed that the electrophoretic mobility of 28*S* RNA is higher than that of 32*S* RNA. However, the 32*S* RNA sediments faster than 28*S* RNA. If 28*S* RNA represented a conformational change of 32*S* RNA, then it should be a more expanded conformation to account for its slower sedimentation constant. If 28*S* were more expanded, then its electrophoretic mobility should be lower than 32*S* since the separation in this type of acrylamide gel should be primarily on the basis of size. Since the 28*S*, in fact, migrates in gels more rapidly than 32*S*, it cannot be a more expanded form of that molecule and hence must represent a structure of smaller molecular weight.

Other evidence for nonconservative conversions is derived from comparison of C^{14}-uridine/H^3-methyl ratios as shown in Figure 6 and Table 1. On the assumption that methyl label is conserved during processing, the observed change in the ratio of uridine to methyl label is consistent with the loss of approximately half of the 45*S* precursor molecule during processing. Assuming that uridine labeling is roughly proportional to molecular weight, and that the molecular weights of the 18*S* and 28*S* are approximately 0.6×10^6 and 1.6×10^6,[26] we estimate that the molecular weights of the 45*S* and 32*S* RNA are 4.6×10^6 and 2.3×10^6 daltons, respectively. The molecular weights, estimated by assuming that $S \propto M^{0.5}$,[24] are 4.1×10^6 daltons for the 45*S* and 2.1×10^6 daltons for 32*S*. These determinations are extremely crude and only indicate the nonconservative nature of the transitions.

The nonconservative nature of the processing of 45*S* has also been indicated in experiments in which the base composition of 45*S* RNA has been compared to that of its various products. A change in base composition is observed which is consistent with the loss of a significant fraction of the 45*S* precursor molecule.[27]

The experiment in which nucleolar content is altered by poliovirus infection indicates that the various minor components found by gel electrophoresis are not artifacts since it is unlikely that alterations in cell metabolism could affect the physicochemical processes responsible for artifacts during the extraction procedure.

While precise kinetics on the formation and decay of the various intermediates are not at present measurable, it has been demonstrated that the 41*S* and 36*S* RNA are formed after 45*S* and before 32*S* RNA. Similarly, other experiments have shown that the 20*S* peak is labeled after 45*S* and, as shown in Figure 2, before the 18*S* nucleolar components. On the assumption that all these RNA species are, in fact, involved in the processing of ribosomal RNA and do not represent aberrant forms, a scheme incorporating the observations reported here is shown in Figure 7. The question marks indicate that the kinetic data on 41*S*, 36*S*, and 20*S* RNA are not at present sufficient to establish the unambiguous role of these species and, as mentioned previously, the stage at which the 20*S* precursor is formed is not known. The scheme in Figure 7 should then be taken as summarizing our knowledge as it now exists and as indicating the most probable steps in the processing of ribosomal precursor RNA.

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† Predoctoral fellow, grant F1-GM-23898 from the National Institutes of Health.

‡ Permanent address: Department of Botany, University of Edinburgh, Edinburgh, Scotland.

§ Career Development Awardee of U.S. Public Health Service.

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