RA-HGH) was submitted to fractionation by gel filtration on Sephadex G-100 in 0.01*M* NH_4HCO_3 solution. While the HGH appeared to be homogeneous (Fig. 1, top), the treatment with urea introduced some heterogeneity (Fig. 1, middle), and further treatment during the reduction and alkylation reactions resulted in a heterogeneous mixture (Fig. 1, bottom). The various fractions from the gel filtration separations were pooled as shown in Fig. 1, and the materials were recovered by lyophylization. Samples of HGH-1 and U-HGH-2 were submitted for amino acid analyses (*3*). The composition of the two samples was identical and indistinguishable from that of the material used for the amino acid sequence studies (*1*). Moreover, the U-HGH-2 was fully active when assayed in the rat tibia test (*4*) or in the local crop-sac assay (*5*), as shown in Table 1.

Table 2. The amino acid composition of a sample of reduced alkylated human growth hormone.

Amino acid residues	Residues per mole (No.)	
	RA-HGH-4*	HGH†
Tryptophan	1	1
Lysine	8.8	9
Histidine	3.2	3
Arginine	10.3	10
S-Carboxymethyl-cysteine	4.2	
Aspartic acid	19.8	20
Threonine	10.0	10
Serine	18.2	18
Glutamic acid	26.1	26
Proline	7.7	8
Glycine	7.9	8
Alanine	7.1	7
Half cystine		4
Valine	7.3	7
Methionine	3.1	3
Isoleucine	7.8	8
Leucine	24.7	25
Tyrosine	7.8	8
Phenylalanine	12.9	13

* See Fig. 1. † From Li *et al.* (*1*).

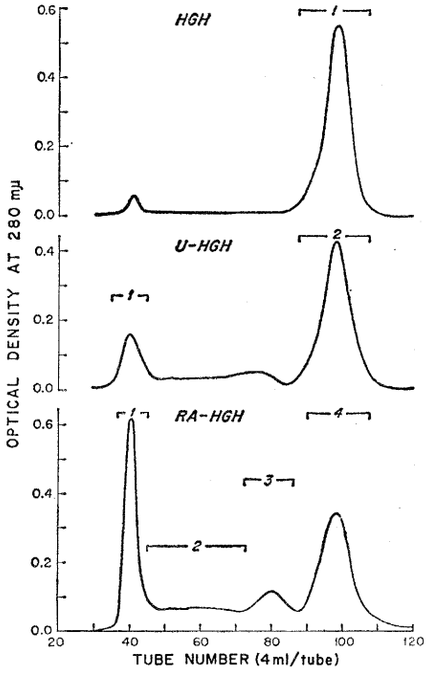

Fig. 1. Elution patterns obtained when 25 mg samples of HGH (top), U-HGH (middle), and RA-HGH (bottom) were submitted to gel filtration on a Sephadex G-100 column (75 by 3 cm) in 0.01*M* NH_4HCO_3; flow rate, 30 ml/hour. Fractions were pooled as indicated and recovered by lyophylization.

Samples of RA-HGH-1, RA-HGH-3, and RA-HGH-4 (see Fig. 1, bottom) were also submitted for amino acid analyses (*3*), and the results showed that their composition was identical with that of HGH with one exception: no free cystine was present in any of the samples, but from each a quantitative recovery (4 moles per mole of protein) of *S*-carboxymethylcysteine was obtained. Table 2 gives the amino acid composition of RA-HGH-4 compared with that of HGH (*1*). The RA-HGH-4 was further characterized by digestion with carboxypeptidase A (Worthington COA-DFP lot No. 6130); 1 mole of phenylalanine and about 0.2 mole of glycine per mole of protein (molecular weight 21,500) were liberated during 24 hours of the digestion with an enzyme-to-substrate ratio of 1/25 by weight in 0.5 percent $NaHCO_3$ solution at 37°C.

When RA-HGH-4 and U-HGH-2 were assayed for growth-promoting activity by the rat tibia test, the statistical evaluation of these results (Table 1) indicated parallelism of the slopes of the log-dose response plots and equal potency in the 95 percent confidence limit. Moreover, the results of the assay of RA-HGH-4 in the pigeon crop-sac test (as shown in Table 1) indicate full lactogenic potency in this derivative of HGH. Thus, the biological potency is retained in the reduced alkylated derivative of HGH even though it is completely devoid of —S—S— bridges.

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Ribosomal RNA Synthesis and Processing in a Particulate Site in the HeLa Cell Nucleus

Abstract. *A particulate fraction has been isolated from detergent-prepared HeLa cell nuclei. The fraction consists largely of organelles that resemble the nucleoli of intact cells. The 45S RNA that is precursor to 28S and 18S ribosomal RNA is associated with the fraction. The 32S RNA that is labeled after the 45S RNA and is the apparent precursor to 28S RNA is also associated with the fraction. The nucleoplasm contains 28S RNA that behaves as an intermediate between the 32S nucleolar RNA and the 28S cytoplasmic RNA.*

Advances in the knowledge of the RNA metabolism of mammalian cells have been extensive, particularly with respect to the synthesis and processing of ribosomal RNA. The initial event in the synthesis of the 18*S* and 28*S* ribosomal RNA species is the formation of a precursor RNA molecule with a sedimentation coefficient of 45*S* (*1*). Recent results indicate that approximately 25 minutes after formation, the 45*S* ribosomal precursor is cleaved to form 18*S* RNA and a species whose sedimentation coefficient is 35*S*. The 35*S* RNA is the precursor to the 28*S* RNA found in mature ribosomes (*2, 3*).

The location of the events in ribosomal RNA formation in subfractions of the HeLa cell has been studied (*3*). A nuclear preparation has been described which is substantially free of cytoplasmic contamination when viewed by either light or electron microscopy (*4*). This nuclear fraction contains over 90 percent of the cellular DNA. The mixed detergent used in preparing the

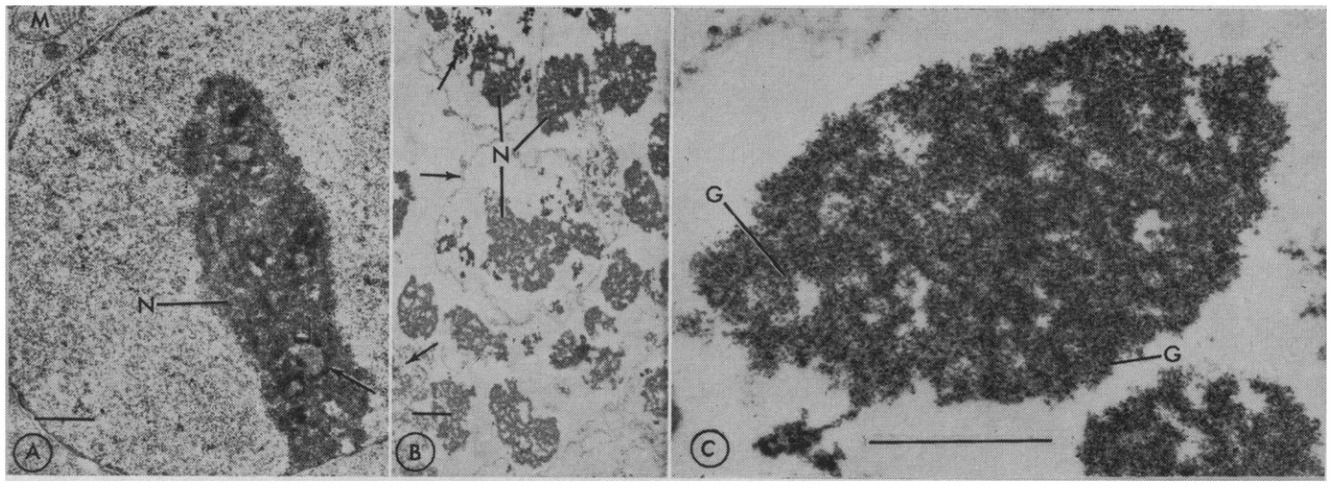

Fig. 1. Electron micrographs of HeLa nucleoli fixed with glutaraldehyde and then by osmic acid, embedded in Araldite and stained with uranyl and lead. Length of bars equivalent to 1 μ. (A) Nucleus of an intact untreated cell showing a nucleolus (N) with characteristic dense strands (arrow) surrounded by granules 10 to 20 mμ in size; M, a mitochondrion in the cytoplasm. (B) Portion of a pellet of the "nucleolar fraction" showing nucleoli (N) together with contaminating membranes and fragments of chromatin (arrows). (C) View at higher power of an isolated nucleolus, showing the numerous nucleolar granules (G).

nuclear fraction removes the outer nuclear membrane with its attached perinuclear ribosome-like granules (*4*). Virtually no 18*S* RNA is found in the nuclear fraction (0.2 percent of cellular 18*S*). Therefore few, if any, mature ribosomes are associated with nuclei prepared in this manner, and the absence of cytoplasmic contamination is further substantiated. The method of preparation may remove material such as mature ribosomes from the nucleus (*3*). While nuclei so prepared yield information about RNA metabolism, the relation of these nuclei to those of intact cells remains to be established.

Most of the newly synthesized RNA, including all of the 45*S* ribosomal precursor, is found in this nuclear fraction. When the 45*S* RNA is cleaved, the 18*S* RNA formed is rapidly exported to the cytoplasm, and the 35*S* RNA is retained in the nucleus. Furthermore, the transformation of 35*S* RNA to 28*S* takes place in the nucleus. The resulting 28*S* RNA remains in the nucleus and appears later in the cytoplasm (about 60 minutes after the beginning of incorporation). When the sedimentation of nuclear RNA is followed by optical density at 260 mμ, a peak corresponding to 45*S* RNA is found as well as a peak at 30*S* which is a composite of 35*S* and 28*S* species.

The nucleolus is apparently the most probable site of synthesis of ribosomal RNA (*5*, *6*). We now describe the further fractionation of the HeLa cell nucleus and the isolation of a particulate fraction which contains the cell nucleoli and all the early ribosomal

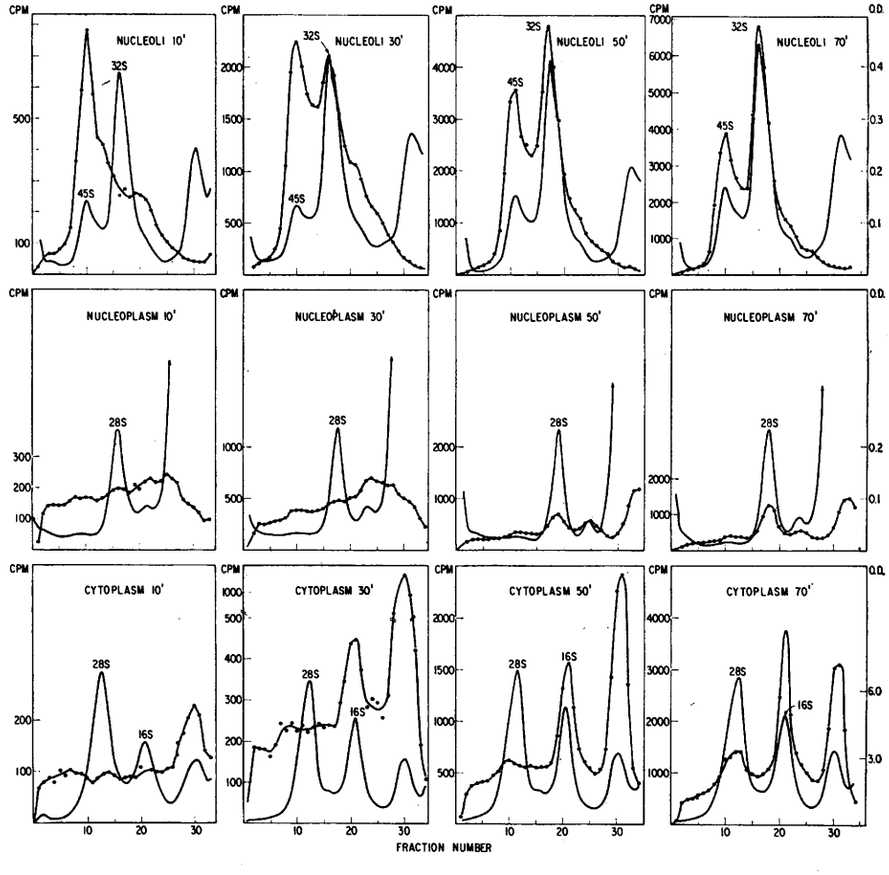

Fig. 2. Sucrose-gradient analysis of the RNA extracted from nucleolar, nuclear, and cytoplasmic fractions. HeLa cells growing at a concentration of 4×10^5 cells per milliliter were concentrated to 2×10^6 cells per milliliter. Results are unaffected for at least several hours by this procedure. C^{14}-uridine (20 μc/μmole specific activity) was added to a final concentration of 0.1 μc/ml. The incubation medium is made $10^{-5}M$ in thymidine and deoxycytidine. Samples containing 6×10^7 cells are removed at the indicated time. Sucrose gradients (15 to 30 percent) were made with SDB buffer ($10^{-1}M$ NaCl, $10^{-2}M$ tris, *p*H 7.4, $10^{-3}M$ EDTA, 0.5 percent sodium dodecylsulfate) and centrifuged (Spinco SW25.3 rotor) for 16 hours at 25°C. Centrifugation speed was 21×10^3 rev/min for the cytoplasm fractions. -●-●- radioactivity; ——— optical density, 260 μm.

RNA precursors. Other methods of obtaining nucleolar preparations have been described (*6*), but these preparations do not yield as clear a delineation of the events in ribosomal RNA formation as ours.

Analysis of sucrose-density gradients of the RNA obtained from our fractions has shown that a small correction is necessary in the assigned sedimentation coefficient for one of the ribosomal precursors. The initial product of the cleavage of 45*S*, formerly called 35*S*, sediments as approximately 32*S*. We now refer to this species by the reassigned sedimentation constant.

The method of cellular fractionation consists in first preparing the detergent-treated nuclei (*3*). Previously (*3*) reference was omitted to earlier work by Traub and co-workers who used the mixed detergent system to isolate nuclei (*7*). They first lysed cells in a low concentration of sodium dodecyl sulfate and then used a mixture of Tween 20 and sodium desoxycholate.

Briefly, our method consists of swelling 6×10^7 cells in 3 ml of hypotonic buffer ($10^{-2}M$ NaCl, $3 \times 10^{-3}M$ $MgCl_2$, $10^{-2}M$ tris, *p*H 7.4) and breaking them with a close-fitting stainless-steel ball homogenizer. After being washed by centrifugation, the nuclei are suspended again in the same buffer and stirred for 15 minutes in the presence of 0.5 percent Tween 40. This serves to open the few remaining unbroken cells (*8*). The nuclei are again washed and suspended in the buffer and treated with a mixture of the nonionic detergent Tween 40 and the ionic detergent sodium desoxycholate. The nuclei are then deposited by centrifugation and subsequently suspended in high-salt buffer ($5 \times 10^{-1}M$ NaCl, $5 \times 10^{-2}M$ $MgCl_2$, $10^{-2}M$ tris *p*H 7.4). The nuclei are lysed in this buffer, yielding a nucleohistone gel. Deoxyribonuclease (100 μg), electrophoretically purified, is then added and the mixture is incubated for 2 minutes at 37°C. The viscosity of the solution is greatly reduced by this treatment.

Polyribosomes are not degraded in this high ionic strength buffer (*9*). It seemed possible, then, that the structures involved in ribosome synthesis might also be stable. In fact, a particulate fraction, rich in newly synthesized RNA, could be obtained by low-speed centrifugation of the digested nuclei. If the initial treatment with Tween 40 alone is omitted, there is occasionally a small contamination of the nuclei with 18*S* RNA amounting at times to 1 or 2 percent of the cytoplasmic 18*S* RNA instead of the consistent 0.2 percent obtained in these experiments. The exposure of nuclei to Tween 40 has no effect on the RNA isolated from the nucleolar fraction. The nucleoli used for electron microscopy were prepared from nuclei from which the single detergent step was omitted and only the mixed detergent step used.

The digested nuclear preparation was centrifuged at 10,000*g* for 5 minutes. A fraction of the resulting pellet, examined in the electron microscope (Fig. 1), contains nucleoli together with some fragments of nuclei including membranes and bits of chromatin. Although some are partly disrupted, many of the nucleoli are comparable in size and shape to the nucleoli of isolated nuclei or of untreated intact cells (*4*). The nucleolar granules, 10 to 20 mμ in size, are still present. However, the dense intranucleolar strands and associated structures readily seen in intact nuclei are frequently no longer evident in the isolated nucleoli. This may reflect partial disruption and changes in distribution within the organelle as well as possible partial extraction of the components of the strands (*10*).

The location of newly synthesized RNA in the various cellular fractions was then investigated. For identification, the pellet obtained after centrifugation of the digested nuclei will here be called the nucleolar fraction, and the supernatant from the centrifugation will be termed the nucleoplasm. The supernatants resulting from homogenization of the cells, nuclear washing, and detergent treatment of the nuclei are termed cytoplasm.

HeLa cells were grown in suspension culture (*11*). The cells were exposed to a concentration of C^{14}-labeled uridine in excess of that necessary for the maximum rate of incorporation into RNA. The fractions obtained were then extracted by a modification of the technique of Sherrer and Darnell (*1*), which consisted of using phenol and chloroform (*3*). The modified technique resulted in nearly 100 percent recovery

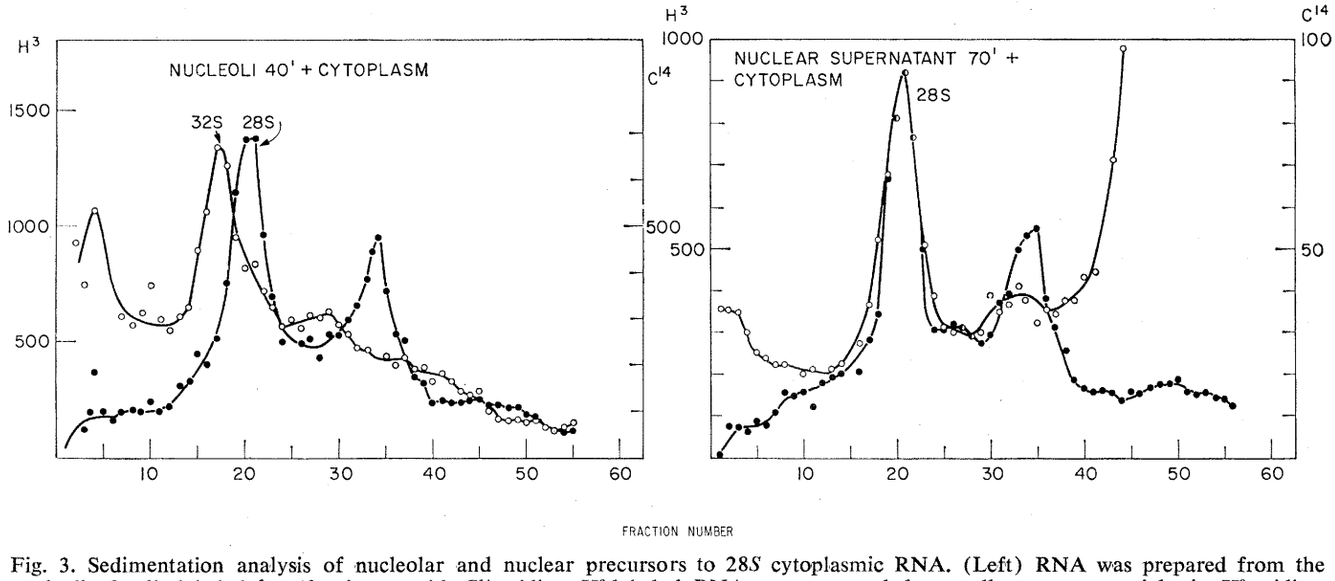

Fig. 3. Sedimentation analysis of nucleolar and nuclear precursors to 28*S* cytoplasmic RNA. (Left) RNA was prepared from the nucleoli of cells labeled for 40 minutes with C^{14}-uridine. H^3-labeled RNA was prepared from cells grown overnight in H^3-uridine. The conditions of labeling the nucleoli and sucrose-gradient analysis are described in Fig. 2. Centrifugation was performed in the Spinco SW25.1 rotor for 16 hours at 23×10^3 rev/min. -○-○, C^{14}, -●-●, H^3. (Right) RNA was prepared from nucleoplasm of cells labeled for 70 minutes with C^{14}-uridine. All other conditions are as at left. -○-○, C^{14}, -●-●, H^3.

of RNA from each fraction. The results of sucrose density-gradient sedimentation analysis are shown in Fig. 2.

The optical-density profiles of the sucrose gradients show that the principal components of the nucleolar fraction consist of 45*S* and 32*S* RNA. There is a small amount of 28*S* RNA in the nucleoplasm fraction and the usual 28*S* and 18*S* species of ribosomal RNA in the cytoplasm. Absorption at 260 $m\mu$ (fractions 30 to 35) of the nucleoplasm is due to DNA (*3*). Its molecular weight is very small since the preparation is treated with electrophoretically purified deoxyribonuclease after extraction.

The profile of the radioactivity in the acid precipitable material after a 10-minute incorporation period indicates that the 45*S* species of RNA is labeled in the nucleolar fraction very rapidly. Visible in this gradient there is a considerable polydisperse tail, which has been identified as a nonribosomal species of RNA (*12*). The nucleoplasm fraction is very rich in polydisperse RNA, which is rapidly labeled and appears throughout the density gradient. This polydisperse RNA has a DNA-like base composition (*13*).

In agreement with previous results, the incorporation of radioactive uridine for 30 minutes results in the labeling of the 32*S* species of RNA in the nucleolus. The 18*S* RNA is apparently transported rapidly to the cytoplasm, although a very small amount may be associated with the nucleoplasm fraction. The amount of radioactivity associated with 18*S* in the nucleoplasm is never very large and may represent the RNA in transition from the nucleolus to the cytoplasm. Its location was masked in earlier experiments by the eventual entrance of label into DNA. This labeling was suppressed in our experiments by the addition of unlabeled thymidine and deoxycytidine to the incubation medium.

By 50 minutes, the amount of radioactivity in the 32*S* RNA of the nucleoli has increased considerably, and there is the first appearance of radioactivity in the 28*S* RNA in the nucleoplasm. Finally, by 70 minutes, labeled RNA has appeared in the 28*S* RNA of the cytoplasm. The nuclear 28*S* RNA achieves nearly maximum specific activity by 70 minutes and does not increase thereafter; this suggests that the nuclear 28*S* RNA is a precursor to the cytoplasmic 28*S* RNA.

The difference in sedimentation constant between the 32*S* nucleolar RNA and the nucleoplasmic and cytoplasmic 28*S* RNA is small, and determinations of sedimentation constants from different sucrose gradients are not reliable. To establish the difference in sedimentation constants of these species of RNA unambiguously, a "double-label" experiment was performed (Fig. 3). The RNA from a nucleolar fraction prepared from cells labeled for 40 minutes with C^{14}-uridine was combined with cytoplasmic RNA from cells which had been labeled overnight with H^3-uridine. A significant difference in sedimentation between the nucleolar 32*S* RNA and the cytoplasmic 28*S* RNA can be seen. Since, after this length of labeling, the radioactivity in the 32*S* region of a sucrose gradient of nucleolar RNA is coincident with the optical density, this experiment serves to identify the peak in optical density of nucleolar RNA as 32*S*. When the nucleoplasmic 28*S* RNA from cells labeled for 70 minutes is combined with the cytoplasmic marker RNA, an absolute coincidence in the sedimentation of 28*S* species is observed.

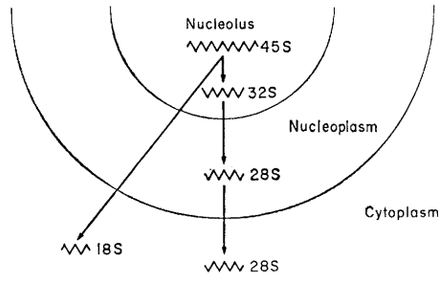

Fig. 4. Representation of the processing of ribosomal RNA in cellular substructures. The data presented do not indicate the site of transformation of 32*S* to 28*S* RNA.

Very little of the cellular DNA (less than 1 percent) is associated with the particulate nucleolar fraction. In this connection, deoxyribonuclease digestion of the nucleohistone gel necessary for preparing nucleoli renders only 20 to 30 percent of the cellular DNA soluble in acid, and very little of the remaining DNA is associated with the nucleolar fraction. The nucleolar fraction contains approximately 1.6 percent of the labeled cellular protein and about 10 percent of the nuclear protein as determined after 24 hours of incorporation of approximately equal amounts of radioactive leucine and arginine.

It is interesting to note the absence of a measurable amount of 18*S* RNA in the nucleolar fraction. The granular structures seen in the electronmicrophotographs of the nucleoli, therefore, cannot be identical with mature ribosomes.

From these and other experiments (*2, 3, 12*), a fairly clear picture of the location of the events in the formation of ribosomal RNA has emerged (Fig. 4). Our experiments do not demonstrate the site of transformation of 32*S* to 28*S* RNA, but merely indicate the predominant species in each cellular subfraction.

Some questions about ribosomal RNA formation remain. The nature of the possible transition of 32*S* to 28*S* RNA is not known. Whether the transition represents a change in molecular weight or a difference in conformation of the RNA is also unknown. The presence of 28*S* RNA in the nucleoplasm seems to be related to the formation of a ribosomal subunit (*14*). However, the possibility that this RNA is actually associated with the nucleolus and is removed by the high-salt treatment cannot be ruled out.

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