ISOLATION OF TWO DISTINCT CLASSES OF POLYSOMES FROM A NUCLEAR FRACTION OF RAT LIVER

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ABSTRACT

Isolated rat liver nuclei were washed with Triton-X-100 in the presence of liver cell sap. This treatment liberated a fraction of polysomes which were isolated by differential centrifugation and were designated "outer membrane polysomes." The outer membrane polysomes synthesized protein in vivo. Shortly after injection of orotic acid-^{14}C, the RNA of outer membrane polysomes had a higher specific activity than that of cytoplasmic polysomes. It was postulated that outer membrane polysomes may be an intermediate in the transfer of newly synthesized RNA from the nucleus to the cytoplasm. In other experiments, Triton-washed rat liver nuclei were lysed in the presence of deoxycholate and deoxyribonuclease. A ribonucleoprotein fraction was isolated from the lysate by differential centrifugation. This fraction contained "intranuclear ribosomes," which sedimented like partially degraded polysomes in sucrose gradients. This degradation could be partially prevented if intranuclear ribosomes were purified by sedimentation through heavy sucrose. The resulting pellets were termed "intranuclear polysomes" because they contained some undergraded polysomes. Intranuclear polysomes were highly radioactive after a brief pulse with orotic acid-^{14}C, but did not appear to synthesize protein rapidly in vivo. Intranuclear polysomes may represent the initial stage of assembly of polyribosomes in the nucleus.

INTRODUCTION

Several reports have described the isolation of ribonucleoprotein particles from nuclear fractions. Ribosomes have been liberated from the nuclei by extraction with Tris buffer (1, 2), detergent treatments (3–6) or ultrasound (5, 7), and from nucleoli by deoxycholate (8). Although the nuclear fractions frequently contained considerable contamination by cytoplasmic components and whole cells (1–5), the nuclear ribosomes were, in many cases, functionally different from cytoplasmic ribosomes (1, 2, 4–6, 8).

An initial attempt to demonstrate polysomes in HeLa cell nuclei was unsuccessful (9). However, Bach and Johnson (10) reported that DNA[1] extracted a polyribosome-like fraction from HeLa cell nuclei. After a brief pulse with uridine-^{3}H, these polysomal particles were more radioactive than cytoplasmic polysomes. It was postulated that the nuclear polysomes might play a role in the transfer of newly synthesized RNA from the nucleus to the cytoplasm.

[1] Abbreviations: DNA = deoxyribonucleic acid; RNA = ribonucleic acid; DOC = deoxycholate; Tris = tris hydroxymethyl amino methane; DNase = deoxyribonuclease; RNase = ribonuclease; TCA = trichloroacetic acid; cpm = counts per minute; OD_{260} = optical density at 260 mμ.

Previous attempts to isolate polyribosomes from a crude nuclear fraction (11) and from purified nuclei (12) of rat liver were unsuccessful. The ribosomes sedimented as monomers and dimers in sucrose density gradients. In each case, the ribosomes were liberated by the suspension of the nuclei in buffer and the addition of deoxycholate. In the conventional method for the isolation of polyribosomes from the cytoplasm (13), deoxycholate was added to the postmitochondrial supernatant. Thus, membranes of the endoplasmic reticulum were disrupted in the presence of cell sap. Cell sap of rat liver is known to contain an inhibitor of ribonuclease (14–16). This inhibitor appears to protect polyribosomes from destruction by ribonucleases, which are activated in the presence of DOC (17, 18). Hence, it appears likely that earlier attempts (11, 12) to isolate polysomes from nuclear fractions failed because of activation of ribonuclease(s) following treatment with DOC. The nucleus is known to contain at least two nucleases (19) capable of hydrolyzing RNA. Lawford et al. (20) recently used liver cell sap, or a ribonuclease inhibitor purified from liver cell sap, to isolate a polyribosome-like fraction from rat liver nuclei. The polyribosome-like particles had sedimentation characteristics and electron microscopic appearance similar to those of cytoplasmic polysomes and were labeled more rapidly than cytoplasmic polysomes after a short pulse with orotic acid-^{14}C. Since the nuclei used in these experiments retained their outer membranes with attached ribosomes, it was uncertain whether these polysome-like particles were located inside the nucleus or on its surface.

In the present study, we have washed isolated liver nuclei in rat liver cell sap containing Triton-X-100. This detergent has been found to remove the outer nuclear membrane and its attached ribosomes, leaving the interior of the nucleus morphologically intact (21). This treatment liberated a fraction of undegraded polysomes which we have called "outer membrane polysomes." It is postulated that these polysomes may be an intermediate in the transfer of newly synthesized RNA from the nucleus to the cytoplasm. In addition, we have isolated from Triton-washed nuclei a ribosomal fraction which was highly radioactive after a brief pulse with orotic acid-^{14}C. These ribosomal particles have been called "intranuclear ribosomes." They appear to be partially degraded or partially formed polysomes and to be located in in the nonnucleolar regions of the nucleus.

MATERIALS AND METHODS

ANIMALS: Male Wistar rats, weighing 175-225 g weres ued (21).

RADIOACTIVE COMPOUNDS: Uniformly labeled amino acid-^{14}C mixture and orotic-6-^{14}C acid hydrate (3.8 and 5 mc/mole) were obtained from New England Nuclear Corp., Boston. Reconstituted protein-^{14}C hydrolysate was from Schwarz Bioresearch, Orangeburg, N.Y.

ENZYMES: Crystalline pancreatic RNase was obtained from Nutritional Biochemicals, Cleveland, and electrophoretically purified DNase from Worthington Biochemicals, Harrison, N.J. The enzymes were dissolved in water and the RNase was heated at 100°C for 10 min before use, as a means of inactivating any contaminating DNase.

MEDIA: Medium H (homogenizing medium) was 0.25 M sucrose containing 0.005 M $MgSO_4$, pH 5.2–5.5.

TMK buffer contained 0.05 M Tris-HCl, 0.005 M $MgSO_4$, 0.025 M KCl, pH 7.6.

CHEMICAL DETERMINATIONS: The determinations of RNA, DNA, and protein have been described previously (21).

ELECTRON MICROSCOPY: The procedures for fixation, embedding, sectioning and staining have been described (21). Ribosomal preparations were placed directly on Formvar grids for examination with the electron microscope. All operations were carried out at 4°. The ribosomes were suspended in TMK buffer, and an equal volume of phosphate-buffered formalin was added. Fixation was allowed to continue for 30 min before drops of the fixed suspension were placed on copper grids coated with Formvar. The suspension was allowed to settle for at least 1 min, and the grid was rinsed with distilled water. The preparation was stained by inverting the grid over saturated aqueous uranyl acetate for 1 hr. The grid was then rinsed with water and dried. A similar procedure was used with ribosomal fractions taken directly from sucrose gradients.

DETERGENTS: Triton-X-100 was the gift of Rohm and Haas, Toronto. Sodium deoxycholate was purchased from Fisher Scientific Co., Pittsburgh.

ISOLATION OF NUCLEI: Because we wished to isolate polysomes from the outer membrane of the nucleus, a fraction of nuclei, in which the outer membrane was preserved, was prepared by a modified Chauveau (22) procedure. All fractionation procedures were performed at 4°C, and centrifuges were refrigerated at 2°C. The livers were excised, passed through a tissue press into about four volumes of medium H, and homogenized (21). The homogenate was filtered through four layers of cheesecloth and

centrifuged at 750 g[2] for 10 min in the Sorvall HB-4 rotor. The supernatant was used to isolate cytoplasmic polysomes, as described below. The pellet was resuspended directly in 2.2 M sucrose containing 0.5 mM $CaCl_2$ and centrifuged at 90,000 g for 1 hr in an SW 25.1 or SW 25.2 Spinco rotor. The thick pellicle at the top of the centrifuge tube was removed with a spatula, the supernatant fluid was decanted, and the walls of the tube were wiped dry with a swab. The nuclei formed a pellet at the bottom of the tube.

PREPARATION OF CELL SAP: To prevent breakdown of the outer membrane polysomes, it was necessary to treat the nuclei with Triton-X-100 in the presence of liver "cell sap." This was prepared by passing the liver through the tissue press into two volumes of medium H and homogenizing the suspension with ten up-and-down strokes of the Potter homogenizer. The homogenate was centrifuged at 20,000 g for 10 min in the Sorvall SS 34 rotor. The resulting supernatant was centrifuged at 368,000 g for 1 hr in the Spinco 65 rotor. The supernatant was made 0.5% (v/v) with Triton-X-100 and was referred to as "Triton cell sap." The degree to which cell sap prevented the breakdown of polysomes depended on the amount of cell sap used. In a typical experiment with the nuclei isolated from six livers, cell sap was prepared from four livers, and the total volume of cell sap was about 42 ml.

ISOLATION OF OUTER MEMBRANE POLYSOMES: In order to remove the outer membrane polysomes, the nuclei were resuspended in one-half of the total volume of Triton cell sap, and gently homogenized. The nuclei were sedimented by centrifugation at 750 g for 10 min. The supernatant (containing outer membrane polysomes) was decanted and set aside. The nuclei were rewashed in the remaining Triton cell sap and sedimented by centrifugation at 750 g for 10 min. The supernatants from these two centrifugations were pooled, made 1.3% with DOC, and centrifuged through a discontinuous sucrose gradient, similar to that described by Wettstein et al. (13), at 144,000 g for 2 hr in the Spinco 40 rotor. The gradient consisted of 3 ml of 1.8 M sucrose in TMK buffer and 3 ml of 0.5 M sucrose in TMK buffer. The supernatant was decanted, and the wall of the tube was wiped dry with a cotton swab. The pellets were resuspended in TMK buffer. The nuclei which remained after being washed with Triton cell sap were free of contaminating cytoplasmic or outer membrane ribosomes and could be used for the purpose of isolating "intranuclear ribosomes."

ISOLATION OF CYTOPLASMIC POLYSOMES: The 750 g supernatant which resulted from the initial centrifugation during the isolation of nuclei was further centrifuged at 20,000 g for 20 min. This pellet corresponded to the "D pellet" of Lawford et al. (17). The supernatant was centrifuged again at 20,000 g for 20 min. The supernatant was made 1.3% with DOC and centrifuged through the discontinuous sucrose gradient (13) at 144,000 g for 2 hr. The supernatant was decanted, the wall of the tube was wiped dry with a cotton swab, and the pellets were resuspended in TMK buffer.

ISOLATION OF INTRANUCLEAR RIBOSOMES AND POLYSOMES (SEE FIG. 1): In order to be reasonably certain that any ribosomes isolated from nuclei were not due to the presence of cytoplasmic or outer membrane ribosomes, nuclei were isolated with Triton-X-100. For the isolation of intranuclear ribosomes, the nuclei prepared by the method described previously (21) or the nuclei which remained after the isolation of outer membrane polysomes (see previously) could be used equally well. The nuclei were suspended in 0.25 M sucrose in TMK buffer. The suspension was made 1.3% with deoxycholate and stirred with a magnetic stirrer in the cold room until no intact nuclei were visible with the phase-contrast microscope. This required 5–10 min. The suspension became very viscous because of the release of DNA. Then 20 μg of electrophoretically purified DNase were added per ml of suspension, and the stirring was continued until the viscosity disappeared (an additional 10 min). In a typical experiment, the nuclei isolated from six livers were resuspended in 50 ml of 0.25 M sucrose in TMK buffer. After treatment with DOC and DNase, the suspension was centrifuged at 20,000 g for 10 min in the SS 34 Sorvall rotor, yielding a white pellet. The 20,000 g supernatant was centrifuged directly at 290,000 g for 1 hr in the Spinco 65 rotor so as to yield a tenacious white pellet referred to as the "intranuclear ribosomal fraction." The supernatant was discarded. The intranuclear ribosomal pellets were resuspended in TMK buffer with the aid of a homogenizer. However, resuspension of the pellets was incomplete, and the bulk of the pellets remained aggregated, sedimenting after centrifugation at 1640 g (International Clinical centrifuge) for 5 min. (This was done to remove aggregated material prior to density gradient analysis). As will be discussed in the Results, the intranuclear ribosomal fraction was not homogeneous, but contained a large amount of aggregated ribonucleoprotein which was not in the form of ribosomes and which sedimented after centrifugation at 1640 g for 5 min.

In an attempt to remove this aggregated material, the 20,000 g supernatant, obtained following lysis of the nuclei with DOC and DNase, was centrifuged at 290,000 g for 1 hr through the same discontinuous gradient of sucrose used for the isolation of outer membrane and cytoplasmic polysomes (see Fig. 1). The tiny pellets obtained could be easily

[2] Gravitational fields refer to the maximum centrifugal force exerted at the bottom of the centrifuge tube.

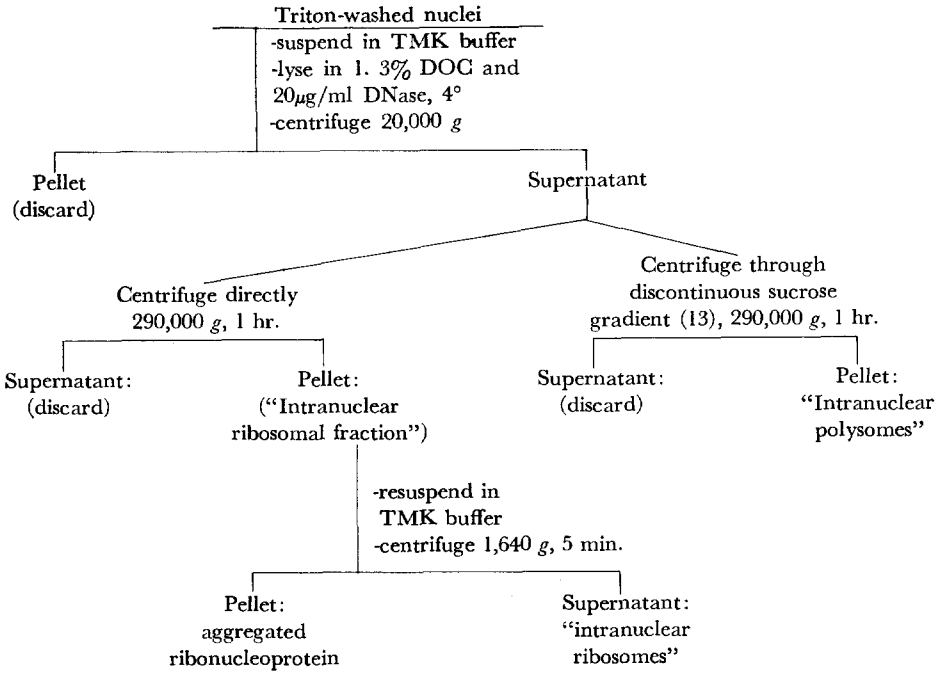

FIGURE 1 Isolation of intranuclear ribosomes and polysomes.

resuspended in TMK buffer with a glass rod and were tentatively designated "intranuclear polysomes," for reasons which are explained in the results.

DENSITY GRADIENT CENTRIFUGATION: Ribosome and polysome preparations were suspended in TMK buffer and centrifuged at 1640 *g* for 5 min so that aggregated material could be removed. The supernatant was layered carefully over a 27-ml linear sucrose gradient (0.3–1.0 M sucrose in TMK buffer) and centrifuged at 90,000 *g* for 2 or 3 hr in the SW 25 Spinco rotor (17). Gradients were analyzed by puncturing the bottom of the tube and recording the optical density at 260 mμ in a Gilford Model 2000 spectrophotometer equipped with a 1-cm flow cell (20). 1- or 2-ml fractions were collected for measurement of radioactivity (see below).

INJECTION OF RADIOACTIVE COMPOUNDS: Orotic acid-^{14}C was dissolved in dilute NaOH (20 μc/ml) and injected into the jugular vein of rats anesthetized with ether. Usually, each rat received 10 μc. In kinetic experiments, each rat received 50 μc per kilogram body weight. Amino acids-^{14}C were supplied in dilute HCl. The solution was neutralized with 0.1 N NaOH and diluted with physiological saline to 10 μc/ml. Under ether anesthesia, each animal was given 15 μc via the portal vein. 3 min following the injection, we terminated incorporation by perfusing the liver through the portal vein with cold medium H. The liver was immediately excised and placed in ice-cold medium H.

MEASUREMENT OF RADIOACTIVITY: To each fraction from sucrose gradients was added 100 μg per ml of bovine serum albumin as carrier. TCA (10%) was added to a final concentration of 5%. The fractions were allowed to precipitate at 4° overnight and deposited on Millipore filters (25 mm diameter, type HAWP). The filters were washed with cold 5% TCA, glued to disposable aluminum planchets, dried, and counted in a low-background, gas-flow counter (Nuclear Chicago, Model 4342). In some cases, aliquots of the RNA fraction or protein fraction obtained during chemical determinations (21) were plated in duplicate, dried, and counted. Counts were corrected for background. Self-absorption was negligible within the range of RNA or protein plated.

RESULTS

Characteristics of Nuclei Used for Isolation of Outer Membrane Polysomes

The nuclei used for the isolation of outer membrane polysomes contained less than 0.04% whole cells as determined by direct counts under phase contrast. Electron micrographs showed that the outer membranes of these nuclei were preserved (Fig. 2 *a*) and the interior of the nucleus was similar to that already described (21). These preparations contained occasional membranous

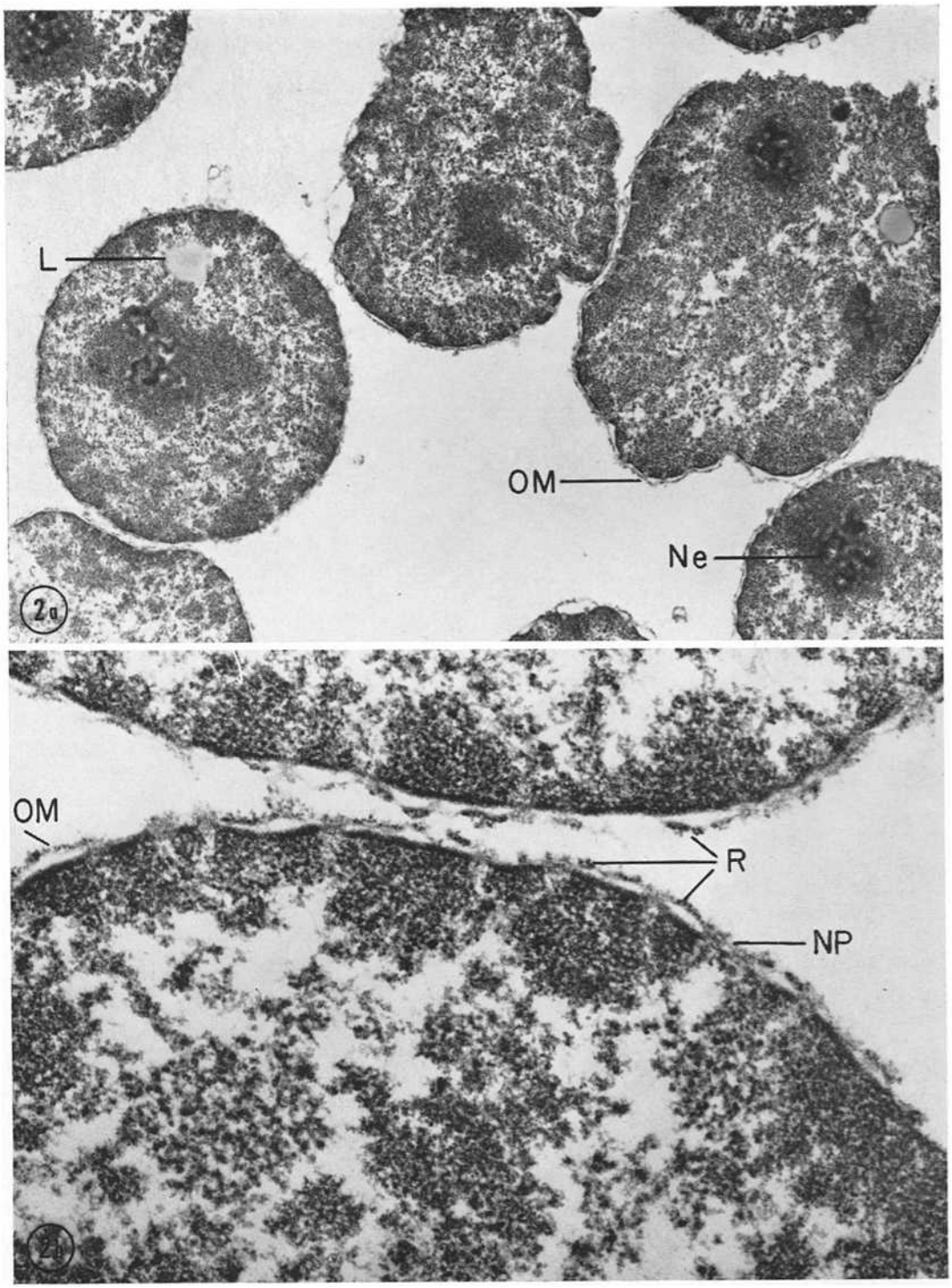

FIGURE 2 Electron microscopic appearance of isolated nuclei used to isolate outer membrane polysomes. 2 *a*, Low power electron micrograph. The outer membrane (*OM*) is preserved. The nucleoli (*Ne*) are well visualized. An intranuclear lipid inclusion (*L*) is present. OsO_4 fixation; uranyl acetate— lead hydroxide stain. 2 *b*, High power electron micrograph showing the presence of electron-opaque particles, probably ribosomes (*R*), on the outer nuclear membrane (*OM*). The chromatin outlines the nuclear pores (*NP*). OsO_4 fixation; uranyl acetate — lead hydroxide stain.
Fig. 2 *a*, × 13,500; Fig. 2 *b*, × 37,500.

components of the endoplasmic reticulum. A high-power micrograph (Fig. 2 *b*) shows the presence of ribosome-like particles on the outer membrane. The chemical composition was similar to that of nuclei isolated by a slightly different procedure (21).

Outer Membrane Polysomes

Our attempts to isolate outer membrane polysomes were based on two observations. In tangential sections of the nucleus, coils or chains of ribosomes are frequently seen with the electron microscope. These aggregates are probably polysomes and appear to be attached to the outer membrane of the nucleus (Fig. 3). Furthermore, nonionic detergents such as Triton-X-100 have been observed to remove the outer membrane of the nucleus with its attached ribosomes, leaving the interior of the nucleus apparently intact (21, 23, 24).

When the whole pellet of outer membrane polysomes was embedded and thin sections were examined with the electron microscope, the preparation was found to be essentially homogeneous and to consist of clusters of electron-opaque particles 200–230 A in diameter (Fig. 4 *a*). The polysomal nature of the aggregates is shown in Fig. 4 *b* in which the polysomes were placed directly on Formvar grids and stained positively with uranyl acetate. A similar preparation of cytoplasmic polysomes is shown in Fig. 4 *c*. The outer membrane polysomal fraction contained more protein relative to RNA than the cytoplasmic polysome fraction. Outer membrane polysomes had a mean RNA/protein ratio of 0.92, whereas the ratio for cytoplasmic polysomes was 1.58. DNA was not detectable in the outer membrane polysome fraction.

It is believed that virtually all cellular RNA is synthesized in the nucleus (25), but functions primarily in the cytoplasm (26). Because of the close proximity of outer membrane polysomes to the nucleus, it was thought that they might contain a greater quantity of newly synthesized RNA than the general pool of cytoplasmic polysomes. 30 min following injection of orotic acid-^{14}C, outer membrane polysomes had a specific activity (cpm/mg RNA) more than four times higher than that of cytoplasmic polysomes. In four experiments, the mean specific activity of outer membrane polysomes was 1333 cpm/mg RNA, while the mean specific activity of cytoplasmic polysomes was 315 cpm/mg RNA. This difference was consistent and was statistically significant ($P < 0.02$).

The ribosomal fractions were characterized by sucrose density gradient centrifugation. Outer membrane polysomes prepared in the presence of cell sap and Triton-X-100 showed a large amount of OD_{260}-absorbing material in the polysome region (Fig. 5 *a*). Outer membrane ribosomes prepared in the presence of homogenizing medium and Triton-X-100 but in the *absence* of cell sap had undergone degradation to monomers and dimers (Fig. 5 *b*) as anticipated (17). Furthermore, in this experiment, the yield of outer membrane polysomes (prepared with cell sap) was more than three times that of outer membrane ribosomes (prepared without cell sap) as determined by measurements of OD_{260}. The explanation for this result probably is that many of the monomers and dimers deriving from the degradation of polysomes failed to pass through the 1.8 M sucrose layer of the discontinuous sucrose gradient. The acid-insoluble radioactivity, at 30 min following injection of orotic acid-^{14}C, in the outer membrane polysomes was much higher (Fig. 5 *a*) than over the outer membrane ribosomes (Fig. 5 *b*), a fact which probably reflected breakdown of radioactive RNA to a nonsedimentable form by endogenous ribonucleases when no cell sap was present. The distribution of cytoplasmic polysomes is shown in Fig 4 *c* which shows that the radioactivity was lower relative to optical density than in outer membrane polysomes (Fig. 5 *a*). The treatment of cytoplasmic polysomes with a minute amount of RNase (1 μg/ml, 4° for 15 min) degraded the polysomes to monomers and dimers and the radioactivity was reduced (Fig. 5 *d*).

The kinetics of uptake of orotic acid-^{14}C into the RNA of the outer membrane and cytoplasmic polysomes were studied (Fig. 6). 10 min after the injection, there was virtually no radioactivity in cytoplasmic polysomes whereas the outer membrane polysomes already showed appreciable radioactivity. The specific activity of outer membrane polysomes rose more rapidly than that of cytoplasmic polysomes. These curves suggested that outer membrane polysomes might be precursors of cytoplasmic polysomes. However, this is still uncertain, for the specific activities of both fractions continued to rise with time. 24 hr after the injection of orotic acid, the specific activity of outer membrane polysomes was 3570 cpm/mg RNA, while that of cytoplasmic polysomes was 2675

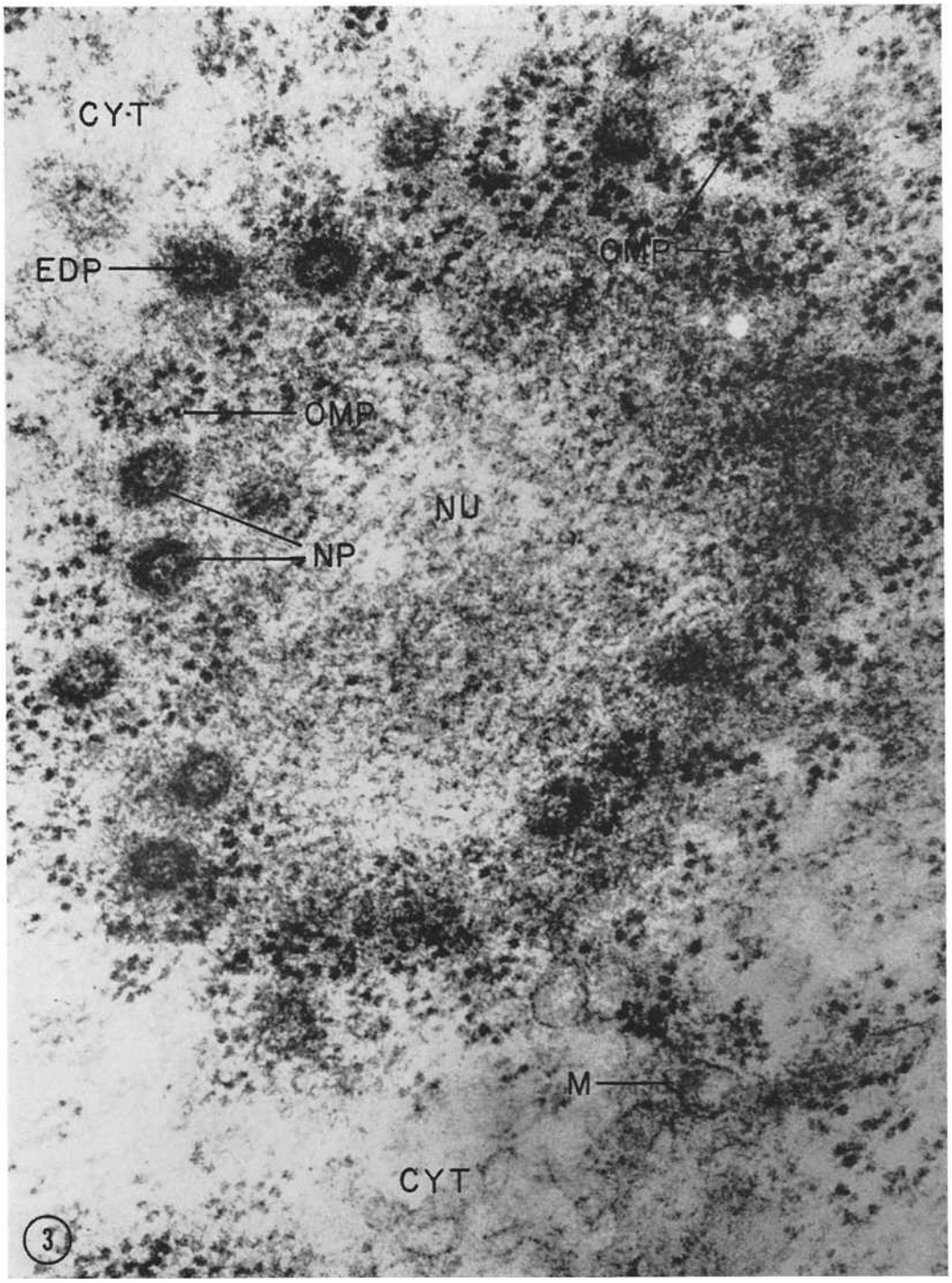

FIGURE 3 Tangential section of hepatic nucleus. The nuclear pores (*NP*) are seen in cross-section and are occasionally closed by an electron-dense particle (*EDP*). Coils of ribosomes are probably polysomes on the outer nuclear membrane (*OMP*). *CYT*, cytoplasm; *M*, membrane: *Nu*, nucleus. OsO_4 fixation; lead hydroxide stain. × 100,000.
(Micrograph, courtesy of Dr. Anne-Marie Jézéquel).

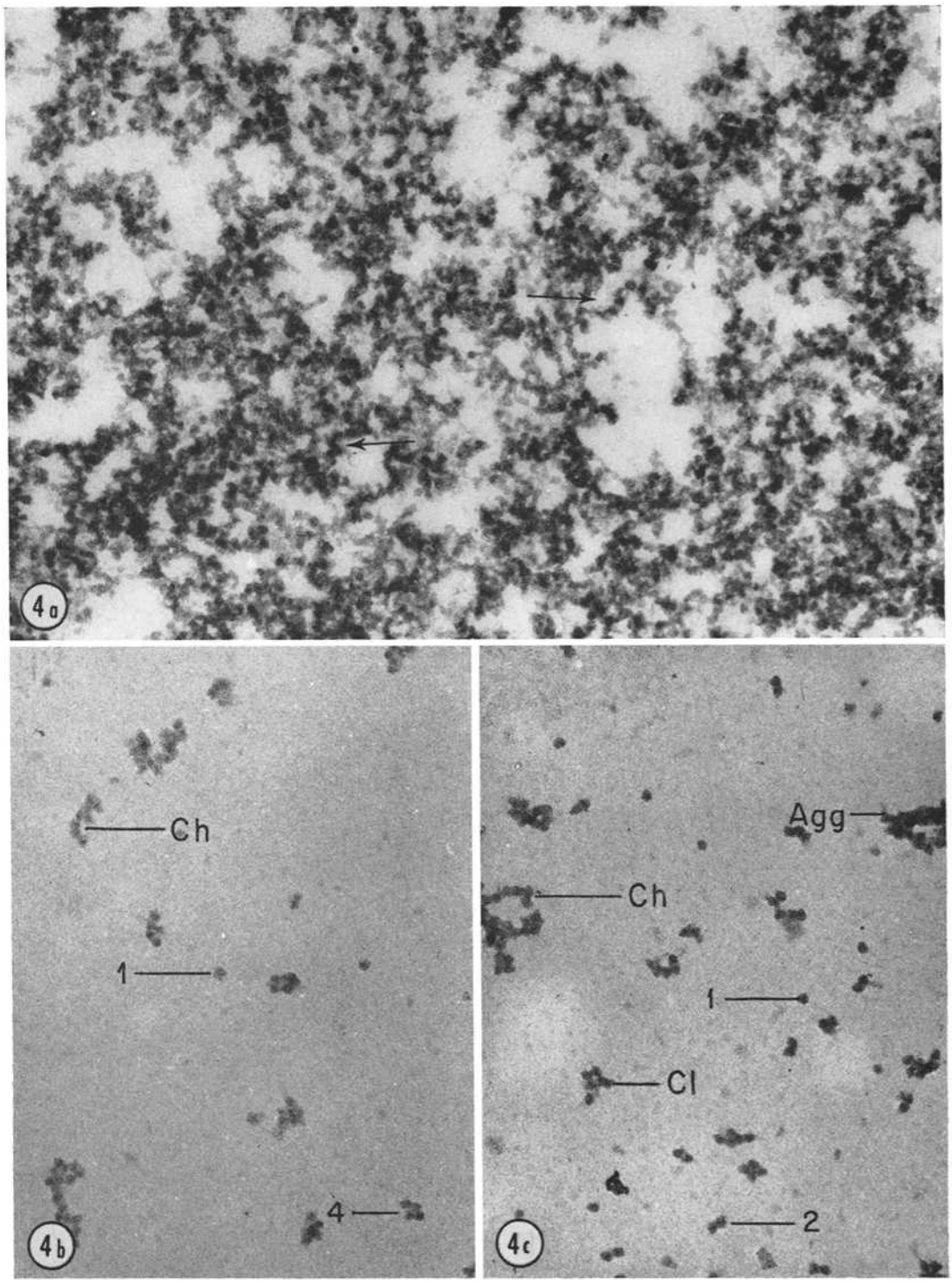

FIGURE 4 *a* Electron microscopic appearance of pellet of outer membrane polysomes. The preparation consists of aggregates of electron-opaque spherical particles which measure 200–230 A in diameter. Sometimes the particles are arranged in chains or clusters suggestive of polysomes (arrows). OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 76,000. 4 *b* Electron microscopic appearance of outer membrane polysomes. The polysomes are present as closed clusters (*4*) or open chains (*Ch*). A monomer is indicated at *1*. Formalin fixation; positive staining with uranyl acetate. × 76,000. 4 *c* Electron microscopic appearance of cytoplasmic polysomes. The individual ribosomes are arranged in clusters (*Cl*) or chains (*Ch*), and some large aggregates (*Agg*), monomers (*1*) and dimers (*2*) are also present. Formalin fixation; positive staining with uranyl acetate. × 75,000.

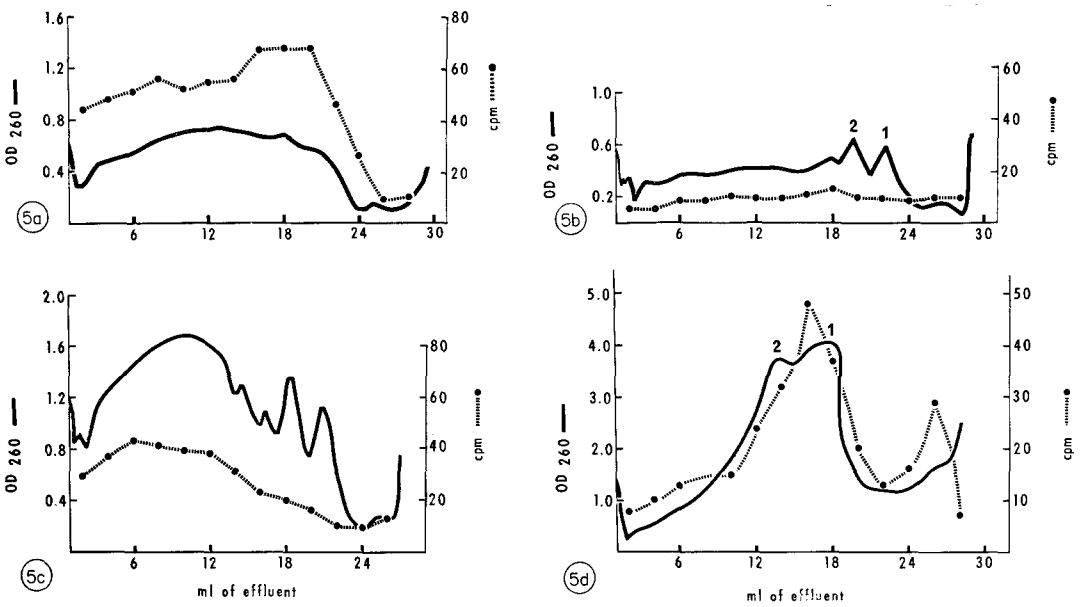

FIGURE 5 Sucrose gradient analyses of outer membrane and cytoplasmic ribosomes 30 min after injection of orotic acid-^{14}C. Outer membrane polysomes (*5 a*) were undegraded when prepared in the presence of cell sap. Outer membrane ribosomes (*5 b*) underwent degradation to monomers (*1*) and dimers (*2*) when prepared in the absence of cell sap. This degradation was accompanied by a loss of acid-insoluble radioactivity so that outer membrane ribosomes (*5 b*) were less radioactive than outer membrane polysomes. Cytoplasmic polysomes (*5 c*) were also less radioactive than outer membrane polysomes (*5 a*). Treatment of cytoplasmic polysomes with RNase (1 μg/ml, 4°, 15 min) caused degradation to monomers (*1*) and dimers (*2*) as shown in Fig. *5 d*. The gradients in *a*, *b*, and *c* were centrifuged for 2 hr, and that in *d* for 3 hr.

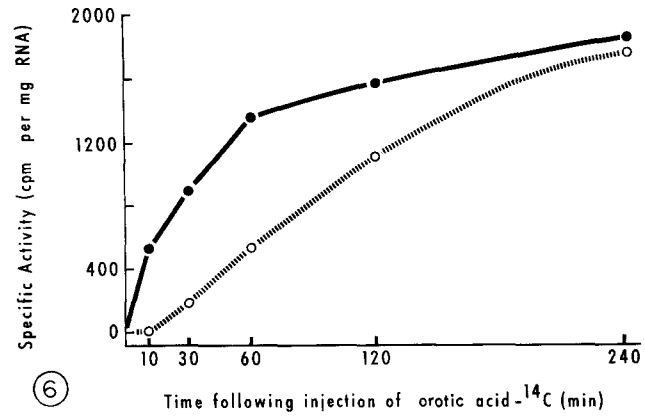

FIGURE 6 Kinetics of uptake of orotic acid-^{14}C into RNA of outer membrane polysomes and cytoplasmic polysomes. After 10 min of labeling, the outer membrane polysomes contain appreciable radioactivity. The specific activity of outer membrane polysomes rises faster than that of cytoplasmic polysomes, although by 4 hr the specific activities are similar. Solid line, outer membrane polysomes; broken line, cytoplasmic polysomes.

cpm/mg RNA. The possibility that cytoplasmic and outer membrane polysomes represent separate pools of ribosomes, each receiving newly synthesized RNA at a different rate, cannot be excluded.

For the purpose of excluding the possibility that outer membrane polysomes were derived from "heavy" microsomes which had co-sedimented with the nuclei, the incorporation of orotic acid-^{14}C into the polysomes of the "D pellet" of Lawford et al. (17) was studied. This 20,000 *g* pellet is known to contain a high proportion of ribosomes of the cell (11). At all times following the injection of orotic acid, the specific activity of the "D-polysomes" was nearly identical with that of the cytoplasmic polysomes.

The synthesis of protein by cytoplasmic and

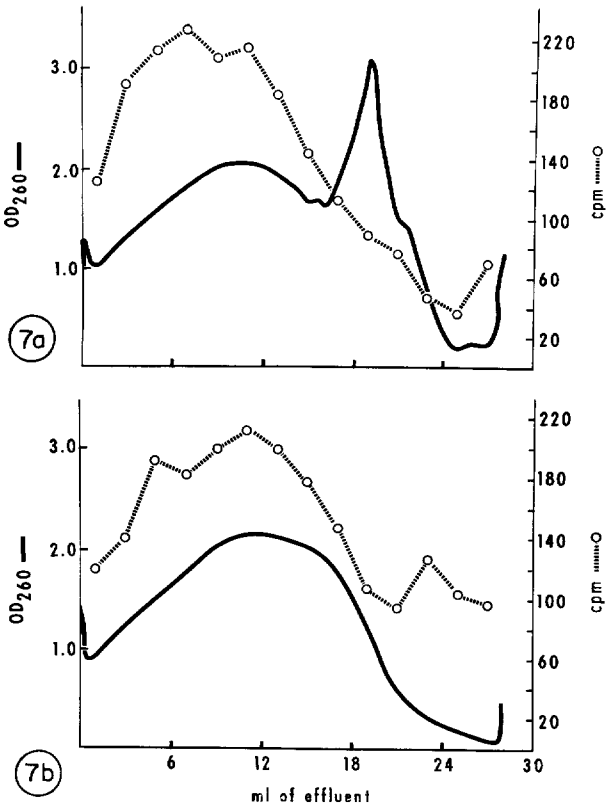

FIGURE 7 Distribution of newly synthesized protein on cytoplasmic polysomes and outer membrane polysomes in sucrose gradients. Labeling after 3-min pulse with amino acids-^{14}C. There is radioactivity located on both cytoplasmic polysomes (*7 a*) and outer membrane polysomes (*7 b*). The specific activities, cpm relative to optical density, are about equal for both gradients. The resolution of individual polysome peaks was poor since the gradients were deliberately overloaded to obtain maximum radioactivity. Centrifugation time, 2 hr.

outer membrane polysomes was studied after a 3-min pulse with amino acids-^{14}C. Fig. 7 shows that TCA-insoluble radioactivity was located on both cytoplasmic polysomes (7 *a*) and outer membrane polysomes (7 *b*) when they were sedimented in sucrose gradients. Furthermore, the specific activity (cpm/OD_{260}) was nearly identical for both classes of polysomes. These data showed that the outer membrane polysomes were fully capable of synthesizing protein in the cell.

Intranuclear Ribosomes

When the 20,000 *g* supernatant, obtained after lysing Triton-washed nuclei with DOC and DNase, was centrifuged directly at 290,000 *g* for 1 hr, a tenacious whitish pellet was obtained. This pellet was designated the "intranuclear ribosomal fraction." This fraction contained 56.3% (by weight) protein, 31.5% RNA, and less than 4.0% DNA (protein + RNA + DNA = 100%). It contained 32.6% of the RNA originally present in the nuclei and approximately 1% of total cellular RNA.

After electron microscopic examination of this fraction, it became apparent that the fraction was not a homogeneous preparation of ribosomes. It was composed predominantly of fibrillar material with occasional granules scattered throughout the fibrils (Fig. 8 *a*). The granules seemed to be of two distinct sizes. The larger granules measured 300–350 A in diameter. The smaller particles measured 200–230 A in diameter and were assumed to be "intranuclear ribosomes." The intranuclear ribosomal pellets were suspended in a small volume of TMK buffer. Because of the tenacious consistency of the pellets, it was necessary to use a homogenizer. Nevertheless, when the suspension was centrifuged at 1640 *g* for 5 min, the majority of the material formed a pellet. It was estimated that about 20% (by weight) of the material remained in the supernatant. This supernatant contained "intranuclear ribosomes" (see Fig. 1). It was found to consist of single ribosome-like particles, 200 A in diameter, as well as fine fibrillar material 40–100 A in diameter (Fig. 8 *b*). The fibrillar material was sometimes seen protruding from the particles. The nature of these fibrils is not known,

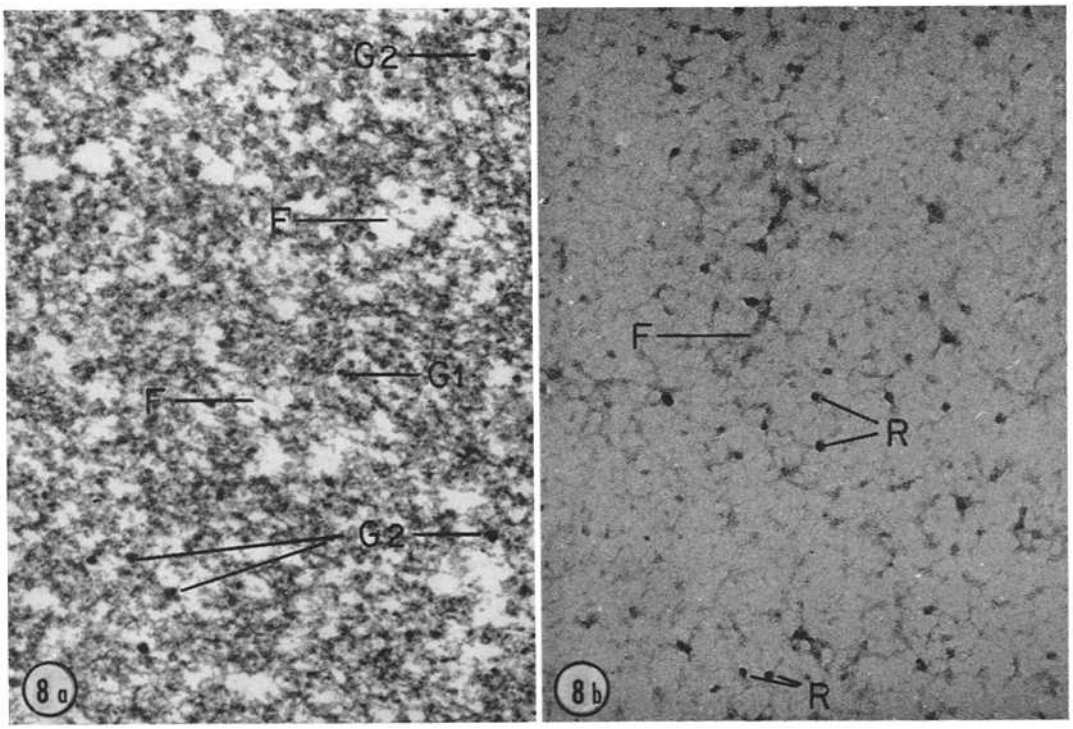

FIGURE 8 *a* Electron microscopic appearance of whole intranuclear ribosomal pellet. The pellet is composed of predominantly fibrillar material (*F*); the individual fibrils meausre 50–100 A in thickness. Interspersed among the fibrils are occasional granules. Those labeled *G1* measure about 200 A in diameter and those labeled *G2* about 300–350 A in diameter. OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 50,000. 8 *b* Electron microscopic appearance of intranuclear ribosomes placed directly on Formvar grids. Ribosomal particles (*R*) are present, as well as fine fibrillar material (*F*), 40–100 A in diameter. These fibrils occasionally protrude from ribosomal particles. Formol fixation; positive staining with uranyl acetate. × 76,000.

although they could represent partially degraded ribosomes.

The distribution of intranuclear ribosomes after sedimentation in sucrose gradients is shown in Fig. 9 *a*. The optical density pattern was suggestive of partially degraded polysomes, in that it showed prominent monomer and dimer peaks, with small trimer and tetramer peaks. The radioactivity 30 min after the injection of orotic acid-^{14}C showed that most of the radioactive material remained near the top of the gradient. However, there was some radioactive material throughout the gradient, even down into the polysome region. The effect of RNase (1 μg/ml, 4°, 15 min) upon these patterns is shown in Fig. 9 *b*. The optical density in the tetramer and trimer regions was eliminated, a finding which showed that the particles in these areas were sensitive to limited digestion by RNase. There were still prominent monomer and dimer peaks, however. The radioactive material over most of the gradient was rendered acid soluble, and only that at the top of the gradient remained. This experiment suggested that the radioactivity in the lower regions of the gradient might be in polysomes.

Several attempts were made to use cell sap to inhibit endogenous RNases and permit the isolation of undegraded intranuclear polysomes. If nuclei were suspended in cell sap and lysed with DOC, DNase failed to reduce the viscosity, as cell sap appeared to inhibit the action of DNase. In the presence of such high viscosity, it was impossible to sediment the ribosomes. When the nuclei were digested with DNase *prior* to their disruption with DOC in the presence of cell sap, no ribonucleoprotein particles were released.

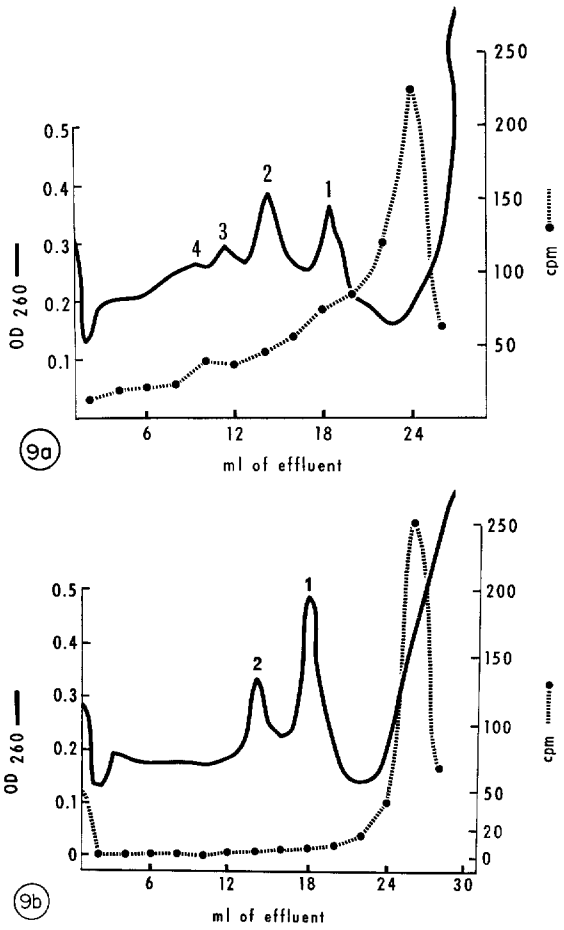

FIGURE 9 Sucrose gradient analyses of intranuclear ribosomes, labeling with orotic acid-^{14}C, 30 min. 9 *a*, Intranuclear ribosomes show prominent monomer (*1*) and dimer (*2*) peaks, as well as small trimer (*3*) and tetramer (*4*) peaks by optical density. The bulk of radioactivity remains near the top of the gradient but there are some counts in the polysome region as well. Centrifugation, 3 hr. 9 *b*, Distribution of intranuclear ribosomes treated with RNase (1 μg/ml, 15 min, 4°C). The optical density peaks of the trimers and tetramers have disappeared (the optical density in this region is not significantly greater than background), but prominent dimer (*2*) and monomer (*1*) peaks remain. Most of the radioactivity across the gradient has been removed except for the large amount of slowly sedimenting radioactivity near the top of the gradient. Centrifugation, 3 hr.

A disturbing feature of the gradients like that shown in Fig. 9 *a* was that most of the radioactive material remained at the top of the gradient rather than conforming to the distribution of optical density. This indicated that radioactive RNA had become degraded *after* the formation of the intranuclear ribosomal pellet, since it was unlikely that material which would not enter the sucrose gradient would have sedimented at 290,000 *g* in 1 hr. This peak of radioactivity did not represent a rapidly labeled ribonucleoprotein particle sedimenting at 45 s (27), since, even if centrifugation was prolonged to 6 hr, the radioactivity still remained near the top of the gradient.

The causes of the breakdown of RNA may have been the presence of a ribonuclease in the intranuclear ribosomal pellet or mechanical breakdown of the RNA during the homogenization necessary for the resuspension of the tenacious pellets. Therefore, we made attempts to remove the tenacious aggregated ribonucleoprotein from this pellet by sedimenting the 20,000 *g* supernatant through the same discontinuous sucrose gradient which we used to purify cytoplasmic and outer membrane polysomes (13). Indeed, the aggregated material was removed by this procedure. The tiny transparent pellets now could be readily resuspended and no aggregated material sedimented at 1640 *g*. This preparation was designated "intranuclear polysomes" so that it could be distinguished from the intranuclear ribosomes we have been discussing previously. The intranuclear polysomes contained 1.5% of the nuclear RNA and 0.1% of total cellular RNA.

The distribution of intranuclear polysomes after density gradient centrifugation (Fig. 10 *a*) was compared with that of intranuclear ribosomes isolated in the same experiment (Fig. 10 *b*). The optical density tracings were similar except for the large amount of slowly sedimenting material at the

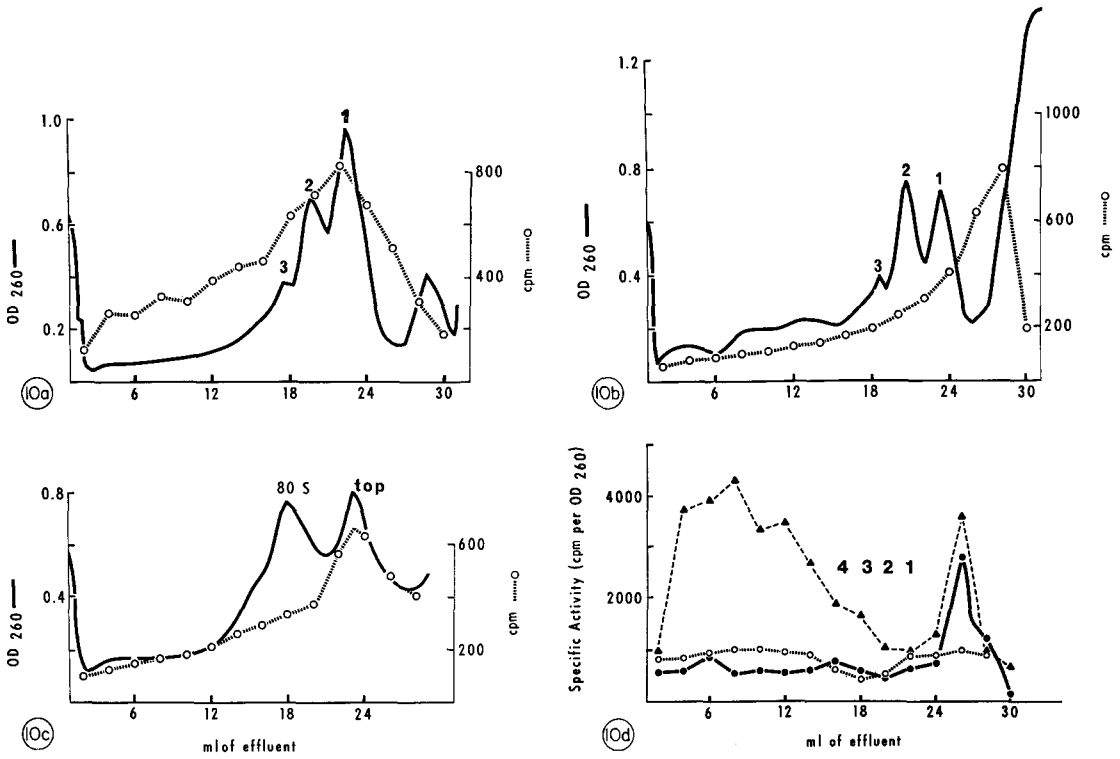

FIGURE 10 Sucrose gradient analyses of intranuclear polysomes and ribosomes, labeling with orotic acid-^{14}C, 30 min. 10 *a*, Intranuclear polysomes show a prominent monomer (*1*), dimer (*2*), and small trimer (*3*) peak with little OD_{260}-absorbing material in the polysome region or near the top of the gradient. The radioactivity conforms to the optical density tracing, with significant radioactivity over the polysome region and little near the top of the gradient. Centrifugation, 2 hr. 10 *b*, Intranuclear ribosomes show an optical density tracing similar to that of intranuclear polysomes except that there is a large amount of slowly sedimenting material near the top of the gradient. The radioactivity does not conform to the optical density pattern, and there is less radioactivity in the polysome region and more at the top of the gradient than in the case of intranuclear polysomes. Centrifugation, 2 hr. 10 *c*, Treatment of intranuclear polysomes with RNase (1 μg/ml, 15 min, 4°C) removed much of the radioactivity from the polysome region. The radioactivity no longer conformed to the optical density pattern. A large amount of OD_{260}-absorbing material and radioactivity now remained close to the top of the gradient. Centrifugation, 2 hr. 10 *d*, The specific activities (cpm/OD_{260}) for the gradients *a-c* are plotted in this figure. The numbers *1-4* indicate the monomer to tetramer region of the gradient. Intranuclear polysomes possess some particles with very high specific activity in the polysome region, whereas intranuclear ribosomes or intranuclear polysomes treated with RNase do not. Intranuclear polysomes,– – –▲; intranuclear ribosomes,——●; intranuclear polysomes + RNase ·····○.

top of the gradient for intranuclear polysomes. There was a distinct difference when the distributions of radioactivity 30 min after injection of orotic acid-^{14}C were compared. There was significantly more acid-insoluble radioactivity over the polysome region in the case of "intranuclear polysomes" than of "intranuclear ribosomes." Furthermore, the radioactivity of "intranuclear polysomes" now conformed to the optical density pattern, and there was no large amount of radioactive material at the top of the gradient. That the distribution of radioactivity seen in intranuclear ribosomes might have been due to breakdown of radioactive RNA by RNase was shown by the treatment of intranuclear polysomes with RNase (Fig. 10 *c*). A greater amount of radioactivity now sedimented near the top of the gradient, and the distribution of radioactivity did not conform to the pattern of optical density.

The data from Fig. 10 *a*, *b*, and *c* have been replot-

TABLE I

*Specific Activities of Intranuclear Polysomes, Intranuclear Ribosomes, Intranuclear Polysomes + RNase**

	Fraction	Total cpm (TCA-insoluble radioactivity)	Total OD_{260}	Specific activity (cpm/OD_{260})
Experiment 1	Intranuclear polysomes	18,000	9.0	2010
	Intranuclear ribosomes	10,793	9.5	1136
Experiment 2	Intranuclear polysomes	28,200	10.4	2710
	Intranuclear polysomes + RNase (1 μg/ml, 4°, 15 min)	9,240	8.8	1050

* Each rat was given 10 μc orotic acid-^{14}C intravenously and sacrificed 30 min after injection.

ted in Fig. 10 *d* for the purpose of displaying specific activities (cpm/OD_{260}) vs. the volume of effluent of the gradients. The prominent feature of this plot was that the highest specific activity was in the polysome region of the intranuclear polysomes. This high specific activity conformed closely to the distribution of optical density for cytoplasmic polysomes (e.g., Fig. 5 *c*). The specific activities for intranuclear ribosomes or intranuclear polysomes treated with RNase were relatively even throughout the gradient and showed that no such material of high specific activity was present in the polysome region. This suggested that, although they were there in small numbers (as evidenced by low OD_{260}), some highly radioactive particles were present in the polysome region of the gradient for "intranuclear polysomes." (It should be emphasized that all of these specific activities are much higher than those of either cytoplasmic or outer membrane polysomes isolated 30 min after injection of orotic acid-^{14}C. This clearly demonstrates that the intranuclear ribosomes or polysomes did not arise from cytoplasmic ribosomes). A comparison of specific activities (cpm/OD_{260}) 30 min after orotic acid-^{14}C for intranuclear polysomes, intranuclear ribosomes, and intranuclear polysomes treated with RNase is shown in Table I.

Since the presence of polysomes was suggested by gradient analyses, we examined fractions of intranuclear polysomes with the electron microscope. The appearance of the whole intranuclear polysome fraction placed directly on Formvar grids (Fig. 11 *a*) was similar to that already described for intranuclear ribosomes (Fig. 8 *a*), although intranuclear polysomes showed more ribosomes and even occasional polysomal aggregates. A sample from the monomer region of a sucrose gradient analysis of intranuclear polysomes (Fig. 11 *b*) showed a predominance of single particles. Some dimers and larger aggregates were also present. As expected from the very low optical density, only occasional polysomal aggregates were seen in samples taken from the polysome region of the sucrose gradient. In an attempt to concentrate these polysomes for electron microscopy, we isolated "intranuclear polysomes" from 48 g of liver and sedimented them in a sucrose gradient. The entire polysome region from the gradient (everything from the trimer region to the bottom of the gradient) was pooled, together with the washings from the bottom of the gradient tube. One-third of a volume of phosphate-buffered formalin, pH 7.4, was added, and fixation was allowed to continue for 15 min at 4°C. The polysomes were sedimented in two tubes of the Spinco 65 rotor at 290,000 *g* for 1 hr. The tiny pellets were resuspended in a small volume of TMK buffer, and droplets were placed directly on Formvar grids. The specimens were stained with uranyl acetate. Electron micrographs of such preparations (Fig. 11 *c*) showed an abundance of polysomal aggregates. Single ribosomes also occurred with surprising frequency. These may

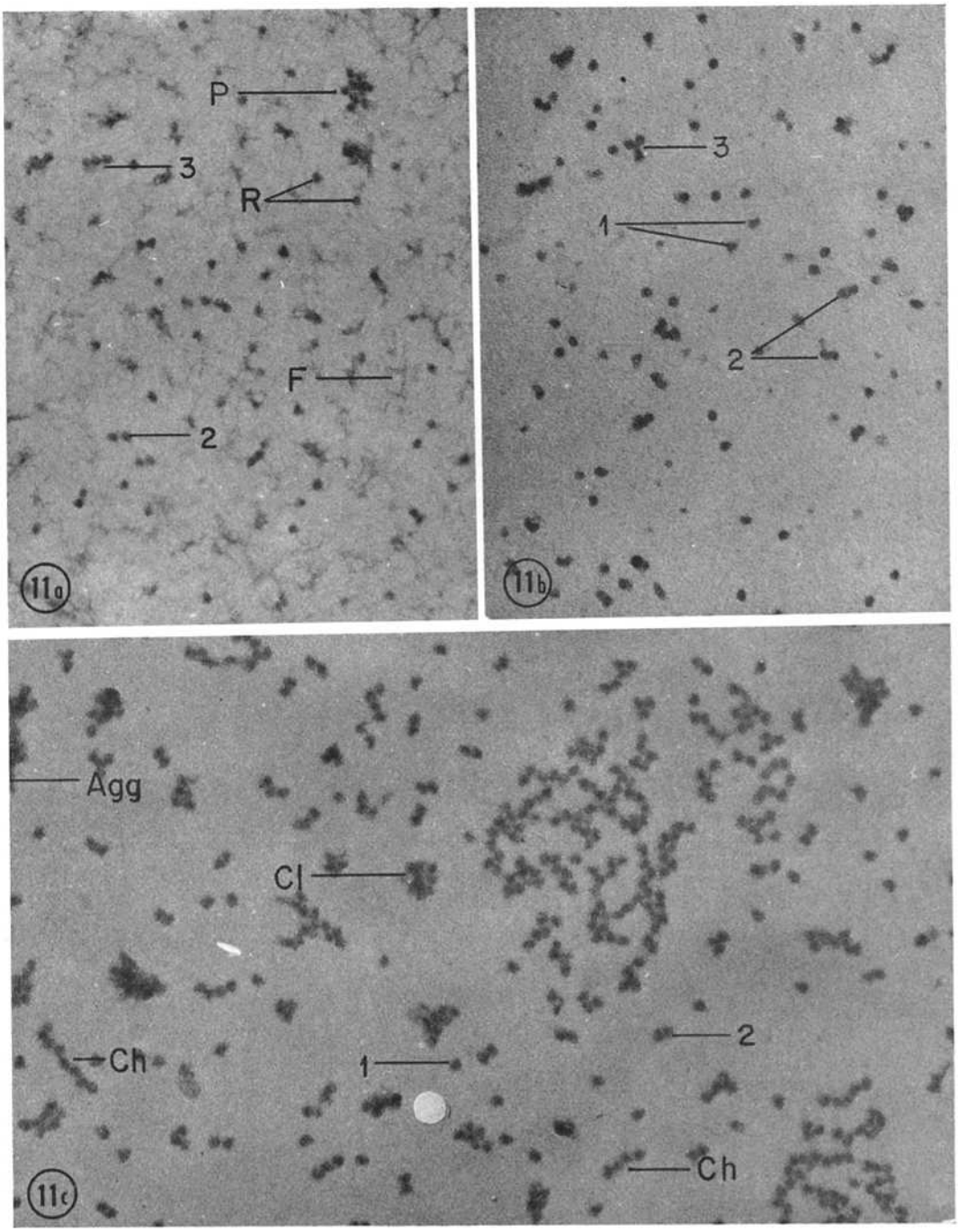

FIGURE 11 Electron microscopic appearance of intranuclear polysomes. 11*a*, Whole intranuclear polysome preparation contained ribosomal particles (*R*), occasional dimers (*2*), trimers (*3*), or even polysomal aggregates (*P*). The fibrillar material (*F*) noted previously in intranuclear ribosomes was also present. Formol fixation; positive staining with uranyl acetate. × 76,000. 11 *b*, Sample taken from monomer-dimer region of sucrose gradient analysis of intranuclear polysomes. Monomers (*1*), dimers (*2*), as well as occasional trimers (*3*) are present. Formol fixation; positive staining with uranyl acetate. × 81,000. 11 *c*, A concentrated sample of particles taken from the polysome region of a gradient analysis of intranuclear polysomes. Polysomal aggregates are abundant. The polysomes are present as open chains (*Ch*) or closed clusters (*Cl*), as well as occasional large aggregates (*Agg*). Some monomers (*1*) and dimers (*2*) are also present. Formol fixation; positive staining with uranyl acetate. × 75,000.

have represented quickly sedimenting monosomes or they may have become detached from polysomal aggregates during fixation and resedimentation of the polysomes.

We performed experiments to determine whether intranuclear ribosomes and polysomes contained newly synthesized protein 3 min after injection of amino acids-^{14}C. Table II shows that the intranuclear ribosomes and polysomes had a specific activity (cpm/OD_{260}) lower than cytoplasmic polysomes. This suggested that these intranuclear particles were less active in synthesizing proteins than cytoplasmic polysomes. However, the possibility that the radioactive amino acids did not penetrate into the nucleus during the brief labeling time could not be excluded. Nevertheless, this functional distinction showed that the intranuclear ribosomes were clearly different from cytoplasmic polysomes.

TABLE II

Incorporation of Amino-^{14}C Acids into Intranuclear Ribosomes and Polysomes

	Fraction	Specific activity* (cpm/OD_{260})	Specific activity* (cpm/mg protein)
Experiment 1	Intranuclear ribosomes	21	—
	Cytoplasmic polysomes	63	—
Experiment 2	Intranuclear polysomes	54	2170
	Cytoplasmic polysomes	88	3450

* 3 min after intraportal injection of amino-^{14}C acids.

Experiments for determining the intranuclear location of intranuclear ribosomes were performed. Nucleoli were isolated (21), and the nucleolar supernatant from the first 2000 *g* centrifugation (21) constituted the "nucleoplasmic fraction." Whole nuclear, nucleoplasmic, and nucleolar fractions were each lysed with DOC and DNase, and the lysates were subjected to differential centrifugation as described for intranuclear ribosomes. The resulting "ribosomal" fractions were each analyzed in sucrose gradients. The pattern obtained for the ribosomal fraction from "nucleoplasm" was identical with that obtained for intranuclear ribosomes from whole nuclei. No ribosomes were detectable in the "ribosomal" fraction from nucleoli. These results suggested that the intranuclear ribosomes were located in the nonnucleolar regions of the nucleus and may have corresponded to the nucleoplasmic granules described previously (21). The experiments do not prove that there are no ribonucleoprotein particles in the nucleolus. It could be aruged that sonication released such particles from the nucleoli and that the presence of intranuclear ribosomes in the nucleoplasmic fraction was an artifact produced during the isolation of nucleoli.

DISCUSSION

This study again points out the value of the use of cell sap from rat liver for preventing the breakdown of polyribosomes (17, 20). We isolated a fraction of polysomes by washing the nuclei of rat liver with the detergent Triton-X-100, in the presence of liver cell sap. These polysomes were named "outer membrane polysomes," as a means of designating their tentative site of origin within the cell. The outer membrane polysomes were able to synthesize protein in vivo to the same extent as the cytoplasmic polysomes. In addition, the outer membrane polysomes incorporated orotic acid-^{14}C into RNA in vivo more rapidly than did cytoplasmic polysomes, an observation which suggested that outer membrane polysomes might be an intermediate in the transfer of newly synthesized RNA from the nucleus to the cytoplasm. Preliminary studies suggest that this RNA is rapidly sedimenting RNA (28s and greater); but base determinations, hybridization studies, and assays of its in vitro stimulation of amino acid incorporation into a cell-free system would be needed before its identity as messenger RNA

could be suggested (28). The precise localization of these polysomes to the outer membrane of the nucleus remains tentative, since it could be argued that they represent a mixture of cytoplasmic polysomes with a small amount of intranuclear polysomes. It has been emphasized that the specific activity of intranuclear polysomes following injection of orotic acid-^{14}C was much higher than that of cytoplasmic polysomes. Hence, it is possible that a small amount of intranuclear polysomes may have been extracted from the nuclei during treatment with Triton cell sap and could have accounted for the outer membrane polysomes' having a higher specific activity than the cytoplasmic polysomes. The extraction of such particles from the nucleus may have escaped detection with the electron microscope.

We were unable to use cell sap as an inhibitor of ribonuclease in our attempts to isolate undegraded polysomes from nuclei which had been lysed in the presence of DOC. This was because cell sap inhibited the action of DNase in reducing the viscosity of the lysate. It is possible that the ribonuclease inhibitor purified from liver cell sap would be useful in this regard, since the purified inhibitor does not appear to inhibit DNase (15).

The distribution of "intranuclear ribosomes" in sucrose gradients was suggestive of partially degraded polysomes. This was confirmed by the distribution of acid-insoluble radioactivity in the particles shortly after injection of orotic acid-^{14}C. The degradation of radioactive material could be prevented if the intranuclear ribosomes were purified by sedimentation through a discontinuous sucrose gradient (13) which we used to purify cytoplasmic polysomes. The fraction obtained by this procedure was designated "intranuclear polysomes." Although the distribution of these particles in sucrose gradients as determined by OD_{260} was similar to that of intranuclear ribosomes, there were some highly radioactive particles in the polysome region of the sucrose gradients when orotic acid-^{14}C was used for labeling the ribosomes. The suspicion that these particles represented polysomes was confirmed by electron microscopy.

The intranuclear polysomes contained a small proportion of total cellular RNA (0.1%). It could, therefore, be argued that they represented simply contaminating polysomes from the cytoplasm or the outer nuclear membrane. We believe this to be unlikely because the intranuclear polysomes possessed two properties which clearly distinguished them from cytoplasmic or outer membrane polysomes. Firstly, the intranuclear polysomes were much more radioactive than outer membrane or cytoplasmic polysomes after a short pulse with orotic acid-^{14}C. Secondly, the intranuclear polysomes contained less newly synthesized protein than cytoplasmic or outer membrane polysomes 3 min after injection of amino acids-^{14}C.

The function of intranuclear polysomes within the intact cell is uncertain. It has already been pointed out that kinetic studies (Fig. 6) suggested that outer membrane polysomes might be precursors to cytoplasmic polysomes, although this could not be established conclusively. Similar studies of the kinetics of labeling of intranuclear ribosomes and polysomes were even less conclusive, since, even after 24 hr of labeling with orotic acid-^{14}C, intranuclear polysomes still had a sixfold higher specific activity than cytoplasmic polysomes. This may mean that intranuclear polysomes leave the nucleus slowly. Further kinetic analyses, including "pulse-chase" experiments (29) and "actinomycin chase" experiments (30), will be needed to clarify this point.

Ribosomes may have a role in regulating the synthesis of RNA and its release from DNA template (31–34). Hence, it would be attractive to postulate that polysomes are formed in the nucleus. While the distribution of intranuclear polysomes in sucrose gradients was suggestive of degraded polysomes, it might also represent polysomes in an early stage of assembly. If messenger RNA were present in excess, there might be insufficient ribosomes in the nucleus to form large polysomes containing several ribosomes on one strand of messenger. Large polysomes might form only when messenger RNA, with its few ribosomes, passed from the nucleus into the cytoplasm and began to synthesize protein rapidly.

The intranuclear ribosomes and polysomes did not incorporate amino acids-^{14}C to so great an extent as cytoplasmic polysomes. This feature showed that the intranuclear particles were functionally distinct from cytoplasmic polysomes. It could not be concluded, however, that intranuclear polysomes do not synthesize protein rapidly in vivo, since slow penetration of the amino acids into the nucleus could not be excluded. Nuclear ribosomes have been said to be the site of intranuclear protein synthesis (1–3, 35).

Such findings have been obtained by the incubation of isolated nuclei with radioactive amino acids in vitro. The recent finding that histones of HeLa cells were synthesized in the cytoplasm (36) suggests that caution is warranted in extrapolation of results obtained in vitro to the situation that exists in the intact cell.

The intranuclear ribosomes do not appear to originate in the nucleolus, in spite of electron microscopic (37, 38) and biochemical (8) evidence that the nucleolus contains ribosome-like particles or ribosomal subunits. We observed "beaded" filaments in the fraction sedimenting at 20,000 *g* after treatment of isolated nucleoli with DOC and DNase. This suggested that if particles were present in the nucleolus they were not liberated by deoxycholate. Another possibility was that the appearance of particles could have resulted from "knotting" of fibers or sectioning of the fibers normally (21). Finally, it is possible that nucleolar ribosomes are released during the isolation of the nuclei or nucleoli.

The intranuclear ribosomes and polysomes might correspond to the nucleoplasmic granules previously described in isolated nuclei (21), to the ribonucleoprotein particles of the nuclear sap and nucleonemes (39), or to the interchromatinic granules (37) which have recently been claimed to contain both RNA and protein (40). We have not been able to observe polysome-like aggregates in the chromatin or interchromatinic regions of the nucleus with the electron microscope.

A final comment is warranted about the claim (41) that the nucleus contains no mature ribosomes. This conclusion was drawn on the basis of the fact that HeLa cell nuclei purified by a mixture of a nonionic and an anionic detergent contained no appreciable 16s ribosomal RNA. A similar mixture of detergents was used by other authors (6) to *extract* ribosomes from the nucleus. The ribosomes may have been extracted from the nuclei in Penman's attempts (41) to rid the nuclei of cytoplasmic ribosomes. Finally, there could be differences between the nuclei of HeLa cells and those of normal liver. The present studies provide support for the existence of ribosomes within the nucleus. Although these ribosomes represent a small proportion of the total ribosomes of the cell, they may, nevertheless, be a class of ribosomes with an important role in the regulation of the synthesis and intracellular transport of RNA.

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