Protein Synthesis in Isolated Nuclei and Nucleoli of HeLa Cells*

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ABSTRACT: Protein synthesis in isolated nuclei of HeLa cells was investigated since recent reports indicated that they lack ribosomes, and the origin of synthesis of ribosomal proteins was sought. The synthesis of proteins observed in HeLa cell nuclei was not due to cytoplasmic contamination, since the perinuclear cytoplasmic ribosomes were removed by treatment of the nuclear preparation with a detergent mixture and added cytoplasm could not account for the protein synthesis measured. Bacterial contamination was not responsible for the observed protein synthesis since chloramphenicol (an inhibitor of bacterial protein synthesis) had no effect on the protein synthesis, and nuclear preparations, fractionated with sterile technique, produced no bacterial colonies. Isolated nuclei, which had been incubated with radioactive amino acids to measure this protein synthesis, were fractionated into nucleoli and nucleoplasm. The labeled protein distributed in the nucleoli, and little was found in the nucleoplasm. It appears that the observed protein synthesis was apparently nucleolar because isolated nucleoli were shown to incorporate radioactive amino acids into protein. Analysis of this nucleolar protein by acrylamide gel electrophoresis revealed one major and one minor radioactive peak. The appearance of only two peaks suggests that bacterial contamination was absent since more radioactive peaks would be expected, and no ribosomal proteins were synthesized in the nucleoli, since at least 20–30 ribosomal proteins would be expected to be found in this organelle. The minor radioactive peak was found to be acid soluble, and therefore probably represents a histone. As yet, little information is known about the major radioactive peak. Little radioactive protein was extracted from the nucleoli with dilute salt solutions. When labeled nucleolar preparations were fixed with formaldehyde and subjected to cesium chloride equilibrium centrifugation, labeled protein was associated with particles having the same density as cytoplasmic ribosomes

Isolated nuclei of both animal (Allfrey *et al.*, 1957) and plant cells (Birnstiel *et al.*, 1962) were shown to carry out protein synthesis; however the possibility existed in this earlier work that ribosomes arising from cytoplasmic contamination were responsible for the synthesis of the proteins seen. On the other hand, evidence is presented by Allfrey (1963), that the protein synthesis observed in isolated nuclei from calf thymus differs from that of the cytoplasm in many important respects: inability of RNase to block nuclear amino acid uptake, sensitivity to DNase, and lack of CO inhibition. Furthermore, isolated nucleoli from nuclei of plant (Birnstiel and Hyde, 1963) and animal cells (Hnilica *et al.*, 1966; Maggio, 1966) were also shown to carry out protein synthesis.

In addition, ribosomes were claimed to be found in both the nuclei and nucleoli of plant cells such as tobacco leaves (Birnstiel *et al.*, 1961, 1963) and in nuclei of animal cells such as calf thymus. Allfrey (1963) reported that the protein synthesis seen in calf thymus was mediated by nuclear ribosomes. Recently several reports indicated the importance of removing polysomes from the perinuclear membrane, since these polysomes carry out protein synthesis (Bach and Johnson, 1966; Sadowski and Howdon, 1968). In nuclear preparations from rat liver, which were freed of these "outer membrane" polysomes, polysomes were also found inside the nuclei (McCarty *et al.*, 1966; Sadowski and Howden, 1968). However, Penman *et al.* (1966), after washing HeLa cell nuclei with detergents, presented evidence that no cytoplasmic ribosomes remained associated with these nuclei; and the nuclei contained little completed ribosomes, since only 0.2% of cellular 18S RNA was present.

Therefore investigations were carried out to determine if HeLa cell nuclei which supposedly contain no completed 78S ribosomes are capable of carrying out protein synthesis; and, if so, are ribosomal proteins synthesized in these nuclei. By employing the detergent washing of HeLa cell nuclei to remove cytoplasmic contamination and incubating these nuclei with radioactive amino acids, it was found that the nuclear preparation was synthesizing protein. Subsequent experiments showed that under the conditions of the experiments only nucleolar protein synthesis was predominant. Little if any synthesis was occurring in the nucleoplasm. Nucleolar preparations probably synthesized protein on nucleolar ribosomes, and analysis of the proteins by polyacrylamide gel electrophoresis revealed only one major and one minor peak.

Experimental Section

Materials

[^{14}C]Algal hydrolysate, [^{14}C]amino acid mixture, L-[4,5-^{3}H]leucine, and [5-^{3}H]uridine were purchased from the New England Nuclear Corp. The following were gifts: actinomycin D (Merck Sharpe & Dohme), cycloheximide (The Upjohn Co.), chloramphenicol (Parke Davis & Co.), and BRIJ®58 (polyoxythylene cetyl ether) (Atlas Chemical Industries, Inc.).

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Deoxyribonuclease and ribonuclease (Worthington Biochemical Corp.), dipotassium ATP (Pabst Brewing Co.), Tween 40 (Mann Research Laboratories, Inc.), dinitrophenol (Eastman), puromycin (Nutritional Biochemical Corp.), and sodium cyanide (J. T. Baker Chemical Co.) were commercial preparations.

Methods

Cell Propagation. HeLa S3 growing exponentially was used in all experiments (Zimmerman and Greenberg, 1965). The cells were grown in the medium described by Eagle (1959) containing 5–10% calf serum.

Isolation of Nuclei. The cells were harvested (600*g* for 5 min), washed with cold Earle's saline, and recentrifuged. The cell pellet was usually resuspended at 2×10^7 cells/ml in a hypotonic buffer containing 0.01 M Tris (pH 7.4), 0.01 M NaCl, and 1.5 mM $MgCl_2$ (RSB) unless otherwise indicated. After 10 min at 0°, the swollen cells were ruptured by ten strokes of a Dounce homogenizer using a tight-fitting pestle and the nuclei were centrifuged at 600*g* for 5 min. The nuclei were washed once with RSB at the same cell concentration, centrifuged, and resuspended in RSB. A detergent mixture containing 10% sodium deoxycholate–10% Tween 40 (1:2, v/v) was added to the nuclear suspension at a concentration of 0.15 ml/ml. The nuclei were shaken in a Vortex mixer for 3 sec and centrifuged at 600*g* for 5 min as described by Penman (1966). The detergent-washed nuclei were resuspended in the incubation buffer (0.05 M sodium phosphate (pH 7.3), 0.1 M NaCl, 0.1 M glucose, and 4 mM mercaptoethanol), modified from Allfrey *et al.* (1955). Usually the nuclear suspensions were subdivided into 0.5-ml aliquot portions (1–20 $\times 10^6$ cell equiv) that were to be used for the incubation with the radioactive amino acids before adding the detergent mixture. This was necessary because after addition of the detergent mixture, the nuclear suspension would gel and accurate aliquot portions could not be removed.

Isolation of Nucleoli. After addition of the detergent mixture to the total nuclear suspension and shaking on a Vortex mixer, the nuclei were centrifuged at 600*g* for 10 min and resuspended in 5 ml of RSB buffer. DNase was added to a concentration of 200 μg/ml and incubated at 37° with agitation until all the DNA clumps were digested (usually 3 min). The suspension was layered on 6 ml of 0.88 M sucrose in RSB and centrifuged at 20,000*g* for 10 min in a Sorvall HB-4 swinging-bucket rotor. The nucleolar pellet was resuspended in incubation buffer and aliquot portions were removed for incubation.

Incubation System. To 0.5–1.0 ml of the nuclear or nucleolar suspension was added an ATP-generating system (final concentration of 1 mM ATP, 10 mM phosphoenolpyruvate, and 40 μg/ml of phosphokinase) and 0.05 M $MgCl_2$. After addition of the radioactive amino acids, the reaction mixture was shaken at 37° as indicated. The final volume was 1.5–2 ml.

Radioactivity Measurements. Radioactive amino acid incorporation into polypeptide was determined as follows. The reaction of triplicate samples was stopped by addition of 2 ml of 10% trichloroacetic acid. Samples were heated to boiling for 30 min, chilled in ice for 30 min, and precipitates were collected on Whatman glass filters (GF/C) and washed five times with 5 ml of 5% trichloroacetic acid. Radioactivity was measured in a scintillation counter using a toluene, 2,5-diphenyloxazole, and 1,4-bis[2-(5-phenyloxazolyl)]benzene mixture. Alternatively the precipitate on the filter was dissolved in 1 ml of 1 N NaOH with shaking overnight. Aliquot portions were taken for the determination of protein by the method of Lowry *et al.* (1951) and the radioactivity in the remainder added to 10 ml of Bray's solution (Bray, 1960) and measured.

Acrylamide Gel Electrophoresis. After incubation of the nucleolar preparation, the reaction was stopped as indicated by addition of 2 ml of ice-cold 0.01 M Tris (pH 7.4). The chilled suspension was immediately centrifuged at 12,000*g* for 10 min and washed twice with the Tris buffer. Protein in a nucleolar pellet was solubilized in 0.5% sodium dodecyl sulfate, 0.5 M deionized urea, and 0.01 M sodium phosphate, final pH 7.2 (buffer A), plus 10% w/w sucrose, and 0.3 ml was layered on the polyacrylamide gel. The gel consisted of 15% acrylamide, 1% ethylene diacrylate, 0.2% ammonium persulfate (freshly prepared), and 2 μl/ml of *N,N,N′,N′*-tetramethylethylenediamine in buffer E (buffer A containing 0.1 M sodium phosphate, pH 7.2). The gels were poured into Plexiglas tubes, 0.25-in to a height of 16 cm, layered with water, and allowed to polymerize. After at least 30 min, the water layer was removed, the radioactive sample was applied, and buffer E was carefully added above the sample and into the reservoir. The gels were electrophoresed at room temperature for 16 hr at a current of 7 mA/gel. After the run, the polyacrylamide became soft and was, therefore, frozen in a hexane–Dry-Ice mixture, extruded out of the tube, and sliced in a screw-driven apparatus on Dry Ice to keep the gel frozen. The gel slices were solubilized in 0.5 ml of concentrated NH_4OH for 1 hr. Bray's solution (10 ml) was added and the radioactivity was measured.

CsCl Centrifugation of Isolated Nucleoli and Ribosomes. Nucleoli were isolated as described above except that TEA buffer (0.01 M triethanolamine–0.05 M KCl–1 mM $MgCl_2$, pH 7.2) was substituted for RSB buffer. After incubation with [^{3}H]leucine, nucleoli were centrifuged and washed twice with cold TEA buffer. Polysomes (6×10^7 cell equiv) and monosomes (2×10^7 cell equiv) were each separated by centrifuging the 10,000*g* supernatant fractions (prepared in TEA buffer) on 5–30% sucrose gradients in TEA buffer at 19,000 rpm for 2 and 16 hr, respectively. Absorbance at 260 mμ was monitored and fractions containing polysomes and monosomes (78 S) were each pooled. Ribosomes and nucleoli were each mixed with 36% formaldehyde to yield a final concentration of 6% and incubated at 0° for 12 hr. Formaldehyde was removed by dialysis against TEA buffer and solid CsCl was added to give a density of 1.486. After centrifugation in an International B-60 ultracentrifuge for 60 hr at 35,000 rpm, 10-drop fractions were collected. Absorbancy at 260 mμ, density in a refractometer, and radioactivity (hot trichloroacetic acid precipitates, as described above) were then monitored.

Results

Nuclear Protein Synthesis. In order to determine whether HeLa cell nuclei could carry out protein synthesis, a modified incubation system of Allfrey *et al.* (1955) was employed. The incorporation of a mixture of [^{14}C]amino acids into 5% trichloroacetic acid precipitates after boiling was used as a measure of protein synthesis. Table I shows that nuclei washed twice with RSB buffer and incubated for 30 min at 37° incorporated 20,463 cpm into protein after the zero time control (1455 cpm) was subtracted. However, since Penman (1966) showed that HeLa cell nuclei isolated under these conditions contained cytoplasmic ribosomes attached to the perinuclear

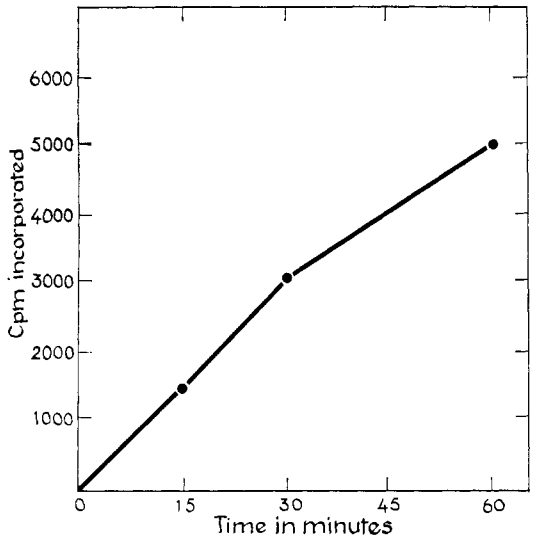

FIGURE 1: Incorporation of [^{14}C]amino acids into detergent-treated nuclei of HeLa cells with time. Cells were swollen in RSB and nuclei isolated and washed with sodium deoxycholate–Tween mixture as described in Methods section. [^{14}C]Amino acids (2.5μCi) were added to each assay mixture containing nuclei from 1 $\times$ 10^7 cells and incubated at 37° for indicated period of time. Zero time control of 960 cpm was subtracted from each time point.

membrane, various detergent treatments were tested in order to remove these contaminants. When HeLa cells were homogenized in hypotonic buffer (RSB) containing 0.5% BRIJ, only net 3877 cpm was incorporated (3). Alternatively, when a mixture of sodium deoxycholate and Tween was used to strip off the cytoplasmic ribosomes from nuclei, about 2900 cpm was incorporated (2); approximately 2500 cpm was incorporated in those cells homogenized in BRIJ in which the nuclei were washed with the sodium deoxycholate–Tween mixture (4). Although the detergents may have been directly inhibiting the synthesis of nuclear proteins, since synthesis was significant, hereafter nuclei were washed with detergent in order to remove any cytoplasmic contamination. Figure 1 shows the nuclear protein synthesis observed with time, when nuclei had been washed with sodium deoxycholate and Tween. Incorporation in this experiment is linear for the first 30 min and diminishes only slightly in the next 30 min.

In spite of the detergent wash of the nuclei, it was still possible that the protein synthesis observed in the nuclear preparation was due to cytoplasmic contamination. In order to rigorously exclude this possibility, increasing amounts of cytoplasm containing incubation buffer were added to detergent-washed nuclei and total protein synthesis was measured (Figure 2). Nuclei alone incorporated 1,900 cpm. The 15,000g supernatant fraction of cytoplasm alone synthesized protein which increased as more cytoplasm was added. When the 15,000g supernatant fraction was added to nuclei, total incorporation increased; the slope of the increase being similar to increasing synthesis observed with the cytoplasmic fraction alone. In fact, 2 $\times$ 10^6 cell equiv of cytoplasm alone, equal to the number of nuclei employed, incorporated only 600 cpm. When increasing amounts of a 600g supernatant fraction were added to nuclei, protein synthesis increased greater than the 16,000g fraction plus nuclei, probably due to the presence of mitochondria known to synthesize protein (Roodyn *et al.*,

TABLE I: [^{14}C]Amino Acid Incorporation of HeLa Cell Nuclei in Presence and Absence of Detergent.[a]

Treatment	cpm	Protein (mg)	cpm/mg of Protein
1.	20,463	1.827	10,500
2. Sodium deoxycholate–Tween nuclei	2,912	0.970	3,270
3. BRIJ cells	3,877	1.343	3,180
4. BRIJ cells, Sodium deoxycholate–Tween nuclei	2,497	1.120	2,170

[a] HeLa cells were resuspended in RSB buffer (1 and 2) or in RSB buffer containing 0.5% BRIJ®58 (3 and 4). After nuclei were washed once with RSB, nuclei were resuspended in RSB to which a sodium deoxycholate–Tween 40 mixture was added as indicated. Nuclei were isolated and assayed for incorporation of radioactive amino acids into hot trichloroacetic acid precipitates as described in Methods section. [^{14}C]Amino acid mixture (2.5 μCi) was added to each nuclear preparation (1 $\times$ 10^7 cell equiv) containing an ATP-generating system and 0.05 M $MgCl_2$ and incubated at 37° for 30 min. Nonspecific adsorption of radioactive amino acids to protein was measured by adding trichloroacetic acid to triplicate samples at 0°, then adding labeled amino acids (zero time control). Average value represents 1455 cpm which was subtracted from each incubated sample.

1961). Since there was only an approximately 20% increase in protein synthesis when an equal number of cell equivalents of cytoplasm was added to the nuclear preparation, it is concluded that detergent-washed nuclei are carrying out protein synthesis, and cytoplasmic contamination containing either ribosomes or mitochondria is not responsible for the protein synthesis seen. The very inefficient synthesis of protein by cytoplasm is probably a reflection of the incubation system, *e.g.*, the lack of K^+ or NH_4^+ and the 0.05 M $MgCl_2$ employed.[1]

Characteristics of the nuclear protein-synthesizing system were next investigated. Figure 3 shows the synthesis of nuclear protein with increasing concentration of nuclei. The curve is not linear, with incorporation being proportionately less as nuclear concentrations were increased. The requirements of the assay system were next investigated. The ATP-generating system, $MgCl_2$, mercaptoethanol, and glucose were separately deleted from the incubation mixture. Also, 0.05 M Tris (pH 7.3) was substituted for sodium phosphate alone, or sodium phosphate and NaCl. When nuclei were incubated in the appropriate buffer with [^{14}C]amino acids for 30 min, no significant effects were found due to deletion of components; values were increased or decreased up to 20% when compared with the complete incubation system. The lack of inhibition seen when components were removed indicate that either some of them were not necessary or that they were present in sufficient quan-

[1] Protein synthesis in HeLa cell cytoplasm, using an incubation system as described by Zimmerman (1968), in the presence of 18 mM $MgCl_2$ was only 18% of that containing 6 mM $MgCl_2$.

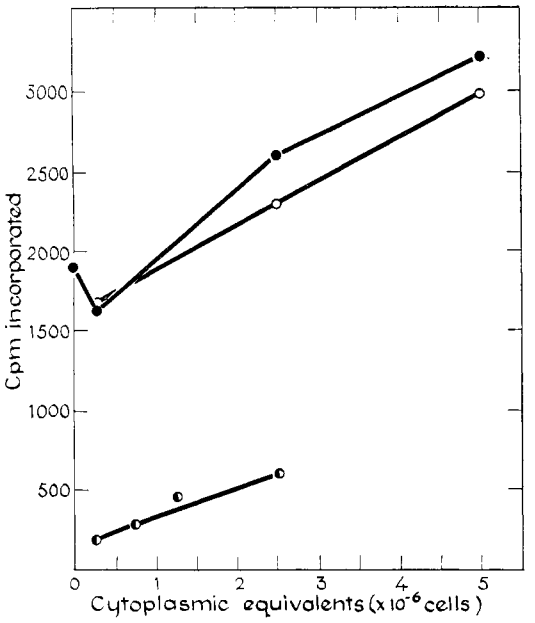

FIGURE 2: Effect of adding cytoplasm to detergent-treated nuclei. Cells were suspended in 0.01 M Tris (pH 7.4), 1.5 mM $MgCl_2$, and 0.5% BRIJ, homogenized, and nuclei were prepared in the above buffer lacking BRIJ but containing sodium deoxychlolate–Tween mixture. Nuclei were suspended in 0.05 M potassium phosphate (pH 7.3), 0.05 M $MgCl_2$, 0.175 M sucrose, 0.05 M NaCl, 0.1 M glucose, and 4 mM mercaptoethanol. The 600*g* supernatant fraction was divided and one part centrifuged at 15,000*g* for 30 min. Concentrated buffer was added to the 600*g* and 15,000*g* cytoplasmic fractions to yield the same concentrations as in the nuclear buffer. Nuclei derived from 2.5×10^6 cells were employed where indicated. Reaction mixtures contained an ATP-generating system as described in Methods section. (●) Nuclei plus 600*g* cytoplasmic fraction; (○) nuclei plus 15,000*g* cytoplasmic fraction; (◑) 15,000*g* cytoplasmic fraction alone. Reaction mixtures were incubated at 37° for 30 min and assayed for radioactivity. Appropriate zero time controls were carried out and their values were subtracted.

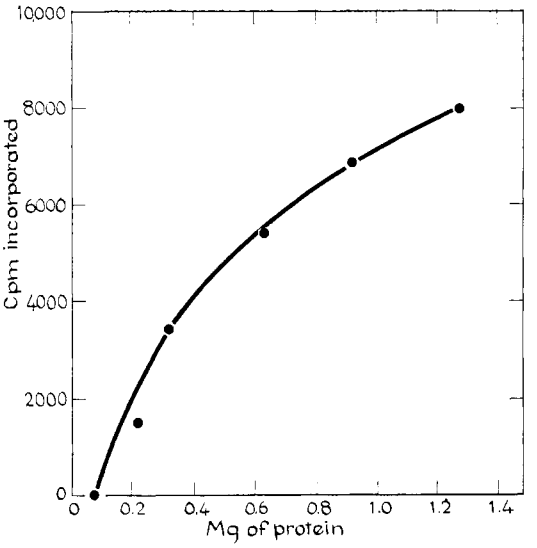

FIGURE 3: Protein synthesis per increasing concentration of detergent-treated nuclei. Nuclei were isolated and each sample was incubated with 2 μCi of [^{14}C]amino acid mixture (30 min) as described in Figure 1. Cell equivalents of 0.25–4 $\times 10^7$ cells were employed and protein was proportional to the number of cell equivalents in each sample. Appropriate zero time controls (1242–1948 cpm) were subtracted.

tity in the internal pool of the nuclei to sustain the reaction. In fact, it seems likely that the energy necessary to carry out protein synthesis is generated by nuclear oxidative phosphorylation. DNP, an inhibitor of oxidative phosphorylation, at 2×10^{-4} M markedly depressed nuclear protein synthesis; the incorporation of amino acids was 32% of control at 30 min (Figure 4a). Similarly, incubating the isolated nuclei for 30 min under anaerobic conditions, using N_2 atmosphere, allowed protein synthesis to 32% of control (Figure 4b). Finally, NaCN at 1×10^{-4} M allowed synthesis to 46% of control (Table II). These findings that nuclear oxidative phosphorylation is necessary to sustain nuclear protein synthesis in HeLa cells are in accord with those of Allfrey *et al.* (1957) who studied protein synthesis in isolated calf thymus nuclei.

Table II also shows the effects of inhibitors of protein synthesis on HeLa cell nuclei. Puromycin at 1.2×10^{-3} M almost completely suppressed protein synthesis (6% of control). On the other hand, cycloheximide at 300 μg/ml had no effect on protein synthesis; the average of four experiments was 102% of control. Even in the presence of 1000 μg/ml of cycloheximide, nuclear protein synthesis was 100% of control after 30-min incubation. The inhibition observed with puromycin would imply that aminoacyl-tRNA and ribosomes are involved in the process. The lack of inhibition by cycloheximide might be due to the drug not entering the nuclear-synthesizing system, since cycloheximide also does not inhibit mitochondrial protein synthesis (Clark-Walker and Linnane, 1966). When actinomycin D (10 μg/ml) was added to the nuclear system, protein synthesis was not suppressed (76 and 117% of control, Table II). Again, either the drug was not able to enter the system or the mRNA coding for the protein synthesized is

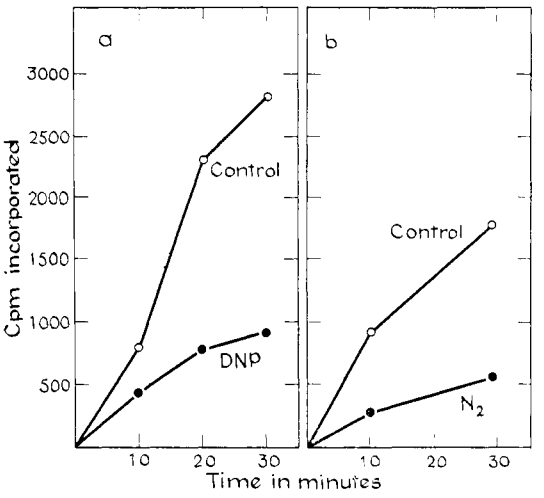

FIGURE 4: Protein synthesis in detergent-treated nuclei in presence of dinitrophenol and N_2 atmosphere. Nuclei were isolated and each sample was incubated with 10 μCi of [^{14}C]amino acid mixture for 30 min at 37° as described in Figure 1. (a) 2×10^{-4} M DNP was added as indicated; 1×10^7 cell equiv was used and zero time incorporation of 1450 cpm was subtracted from control and DNP nuclei. (b) Nuclei were incubated with shaking in N_2 atmosphere as indicated; 1×10^7 cell equiv was used and zero time incorporation of 1580 cpm was subtracted from control and N_2-incubated nuclei.

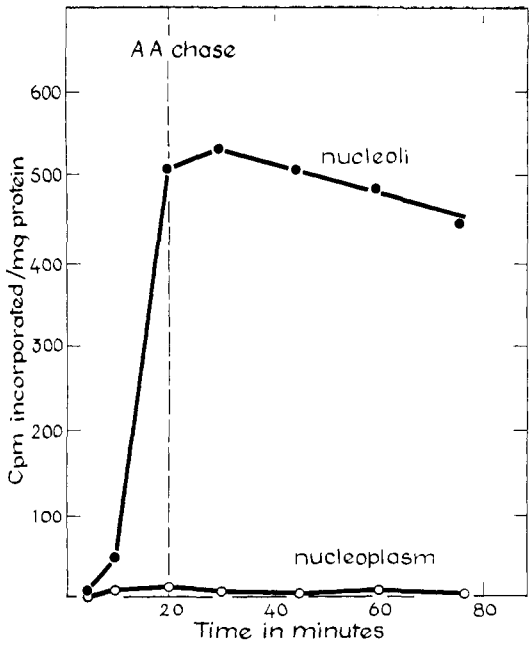

FIGURE 5: Distribution of radioactivity in nucleoli and nucleoplasm during nuclear protein synthesis; distribution after amino acid chase. Detergent-treated nuclei were isolated and each sample (1×10^7 cell equiv) was incubated with 10 μCi of [^{14}C]amino acid mixture. After 20-min incubation 50 μg each of 20 nonradioactive amino acids was added and incubation at 37° was continued. At indicated times, reaction in samples were stopped and nucleoli and nucleoplasm were separated as described in Methods.

TABLE II: Effect of Various Drugs on Protein Synthesis in Detergent-Washed Nuclei.[a]

Inhibitor (M)	Expt 1 cpm Incorpd	Expt 1 % Control	Expt 2 cpm Incorpd	Expt 2 % Control
	29,800	(100)	38,470	(100)
NaCN (1×10^{-4})	14,900	46		
Puromycin (4×10^{-5})			41,370	107
Puromycin (1.2×10^{-3})			2,122	6
Cycloheximide (300 μg/ml)	31,400	105	38,266	100
Actinomycin (10 μg/ml)	22,500	76	43,042	117
Chloramphenicol (1×10^{-4})			46,742	121

[a] Nuclei were isolated and reaction mixture (2×10^7 cell equiv) was incubated with 2.5 μCi of [^{14}C]amino acids and the appropriate drug as indicated for 30 min at 37°. Appropriate zero time controls were subtracted.

not rapidly turning over. Chloramphenicol at 1×10^{-4} M did not depress nuclear protein synthesis (121% of control). This lack of inhibition by a drug which inhibits protein synthesis in bacteria, makes it unlikely that bacterial contamination of the cultures had occurred.

In a previous experiment, when $MgCl_2$ was deleted from the assay system, the nuclei synthesized protein to 80% of the control, about equal to the variability of the system. In order to remove magnesium ion from the internal pool of nuclei, EDTA was added to the nuclear preparations. It was found that 0.01 and 0.1 M EDTA allowed protein synthesis to 22 and 11% of their controls, respectively (Table III). There was no greater inhibition of protein synthesis when 0.1 M EDTA was preincubated with nuclei 15 min before addition of labeled amino acids. Although EDTA chelates magnesium ion, the addition of EDTA might be inhibiting the reaction by binding to other necessary cations.

When nuclei were incubated in the presence of either RNase or DNase or preincubated for 15 min with the nucleases before addition of the labeled amino acids, there was no inhibition of nuclear protein synthesis (Table III). These results are in agreement with those of Flamm *et al.* (1963) who investigated nuclear protein synthesis in plant cells and would indicate that any nucleic acid involved in protein synthesis is probably inaccessible to the nuclease added.

In order to confirm that the observed protein synthesis was not due to bacterial contamination of the cell cultures, nuclei were prepared using sterile technique and tested for appearance of bacterial colonies. When 1×10^7 nuclei in 0.1 ml of RSB were plated on tryptone–yeast extract agar and incubated at 37° for 4 days, no bacterial colonies were observed.

Nucleolar Protein Synthesis. Next investigated was the distribution of labeled proteins into nucleoli and nucleoplasm after incubation of the isolated nuclei with the radioactive amino acid mixture. After incubation at different times, the reaction was stopped and nucleoli and nucleoplasm were separated (Penman *et al.*, 1966). It can be seen in Figure 5 that greater than 95% of the protein synthesized was associated with the nucleoli. Radioactivity in the nucleoplasm was in the background range. In order to see if protein was synthesized in one fraction and transported into another, nonradioactive amino acids were added to the incubation mixture after 20 min, and samples were subsequently removed and assayed for incorporation of radioactivity. The amino acid chase was effective 10 min later and thereafter radioactivity in either the nucleoli or nucleoplasm did not increase (compare with Figure 1). Hence no transport of proteins from one fraction to another was found.

Since nuclear proteins were apparently synthesized in the nucleolus of HeLa cells and DNase did not depress this synthesis, then nucleoli were isolated by digesting the chromatin with DNase and centrifuging them through 0.88 M sucrose. Figure 6 shows that the isolated nucleolar preparation was capable of synthesizing protein. The reaction was not linear and was decreasing with time.

Polyacrylamide gel electrophoresis was employed to analyze the proteins synthesized in the nucleolar preparation. Cold 0.01 M Tris (pH 7.4) buffer was used to stop the reaction, since only about 5% of the radioactive protein was solubilized in a preliminary experiment. Sodium dodecyl sulfate and urea were then used to solubilize the synthesized protein and electrophoresis was carried out in 15% acrylamide. When the products were analyzed (Figure 7a), surprisingly only two distinct radioactive peaks were observed. A major peak was

TABLE III: Effect of RNase, DNase, and EDTA on Protein Synthesis in Detergent-Treated Nuclei.[a]

Expt	Addition	cpm Incorpd	% Control	Preincubated cpm Incorpd	Preincubated % Control
1		14,283	(100)	10,739	(100)
	RNase (200 μg/ml)	16,276	114	14,936	139
	DNase (200 μg/ml)	10,804	76	12,634	118
	EDTA (0.1 M)	1,609	11	1,844	17
2		12,323	(100)		
	EDTA (0.01 M)	2,758	22		
3		17,865	(100)		
	RNase 200 (μg/ml)	14,595	82		
	DNase 200 (μg/ml)	20,526	115		

[a] Nuclei were isolated and reaction mixture (2×10^7 cell equiv) was incubated with 2.5 μCi of [^{14}C]amino acids and appropriate enzyme or EDTA as indicated for 30 min at 37°. Alternatively reaction mixtures were incubated with appropriate enzyme or EDTA for 15 min at 37°, at which time labeled amino acids were added and incubated for 30 min longer. Appropriate zero time controls were subtracted. $MgCl_2$ was deleted from incubation mixture in those samples where EDTA was added.

found at gel slice 29 and a slower moving, minor peak was seen at slice 26. In order to rule out nonspecific adsorption of radioactive amino acids onto nucleolar protein, a zero time control was carried out. There were no comparable peaks in this control. When [^{3}H]leucine was substituted for the [^{14}C]amino acid mixture, the same radioactive profile was observed (Figure 7c). When the reaction was stopped after 30 min using 5% trichloroacetic acid instead of cold Tris ([^{14}C]amino acid mixture), the minor, slower moving radioactive peak was not present (Figure 7b). Also, when nucleoli were incubated in the presence of 1×10^{-4} M chloramphenicol, the radioactivity in these two peaks was not depressed (data not presented).

In order to further characterize the two radioactive peaks, labeled nucleolar protein was first extracted with salt and then acid (Piha *et al.*, 1966), and the solubilized proteins were analyzed by electrophoresis on polyacrylamide gels, as before. Table IV summarizes the amount of radioactivity in each peak that was extracted and recovered. When the labeled nucleoli were extracted with 0.14 M NaCl and 0.01 M sodium acetate (pH 7.2), only 4% of the major peak and none of the minor peak were recovered. However, when the salt-extracted nucleoli were further extracted with 0.25 N HCl, all of the radioactivity in the minor peak was recovered agreeing with the results seen in Figure 7b, namely, the minor peak is acid soluble. As was also seen in Figure 7b, acid did not solubilize the major peak (Table IV).

Synthesis of Proteins on Nucleolar Ribosomes. The observation that isolated nuclei and nucleoli from HeLa cells could carry out protein synthesis seemed to contradict the findings of Penman, who showed little 18S rRNA in nuclei (Penman *et al.*, 1966) and no 18S rRNA (but 20S rRNA precursor) in nucleoli (Weinberg *et al.*, 1967). These findings would imply that nucleolar protein synthesis occurred without ribosomes. In order to look for nascent proteins on ribosomal particles in the nucleolar preparation, isolated nucleoli were incubated with a [^{14}C]amino acid mixture at 37° for 30 min. Nucleoli were washed twice with cold Tris, as before, and the nucleolar pellet was suspended in RSB and 0.5% sodium deoxycholate. The suspension was layered over a 15–30% sucrose gradient in RSB and centrifuged at 22,000 rpm for 17 hr to separate any 78S, 60S, and 40S ribosomal particles present. No radioactive peaks were found in these regions of the gradient. The nucleolar pellet contained 65% of the radioactivity and the majority of the remainder was in the 4S region of the gradient. Since it was possible that the labeled nascent protein in the nucleolar preparation was attached to polyribosomes, and therefore heavier than 78 S and present in the pellet fraction, another labeled nucleolar preparation was incubated with

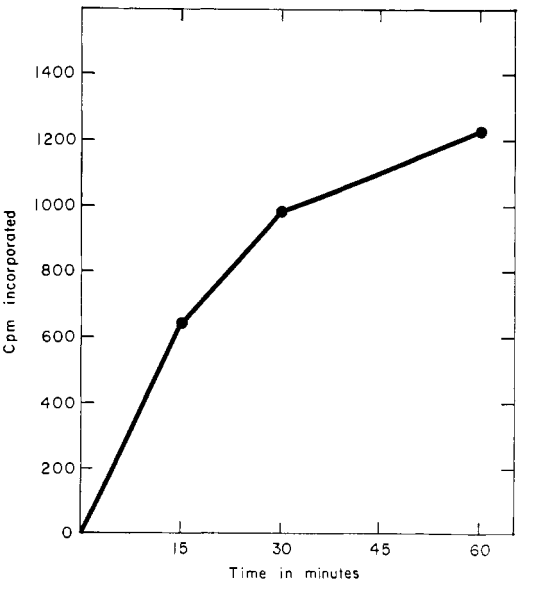

FIGURE 6: Incorporation of [^{14}C]amino acids into isolated nucleoli with time. Nucleoli were isolated as described in Methods. [^{14}C]-Amino acids (2.5 μCi) were incubated with nucleoli (2×10^7 cell equiv), incubation buffer, ATP-regenerating system, and 0.05 M $MgCl_2$. Zero time incorporation of 848 cpm was subtracted from each value.

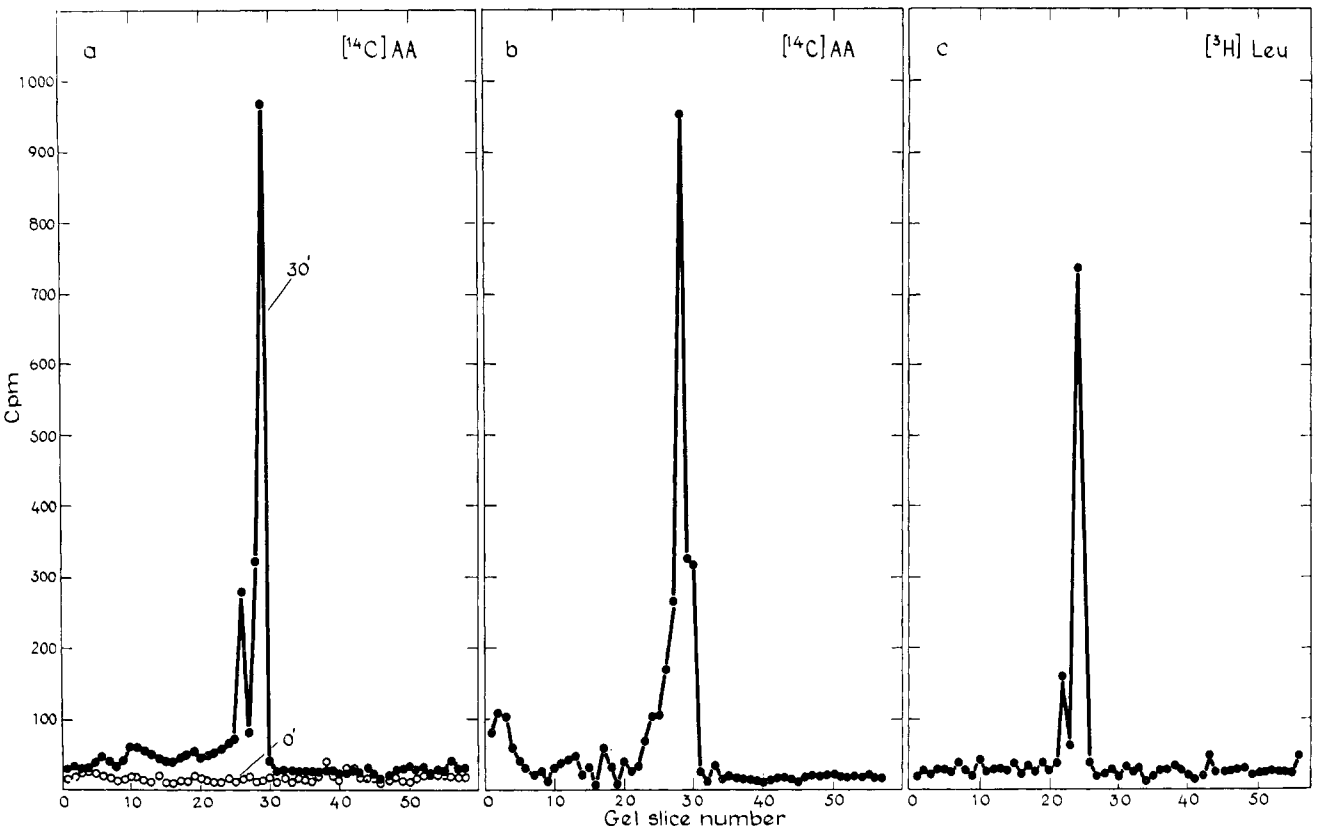

FIGURE 7: Acrylamide gel electrophoresis of protein synthesized in isolated nucleoli. (a) After 30-min incubation with 10 μCi of [^{14}C]amino acids, protein synthesis in nucleoli (2 × 10^7 cell equiv) was stopped by addition of 2 ml of cold 0.01 M Tris (pH 7.4). To zero time control, 2 ml of cold Tris was added before labeled amino acids. Suspensions were centrifuged at 10,000g for 10 min and nucleolar pellets were washed twice with 2 ml of cold 0.01 M Tris. Final pellets were resuspended in buffer A and electrophoresed in polyacrylamide gels as described in Methods. (b) Same as a except that reaction in 30-min incorporation was stopped by addition of 2 ml of 5% trichloroacetic acid and nucleoli were washed twice in trichloroacetic acid, which was removed by resuspending the nucleolar precipitate in 2 ml of cold acetone and recentrifuging to remove the acetone. (c) Nucleoli (4 × 10^7 cell equiv) incubated with 10 μCi of [^{3}H]leucine (500 μCi/μmole) for 30 min at 37°.

0.25 μg/ml of RNase at 0° for 30 min. This mild RNase treatment will degrade the mRNA in cytoplasmic polysomes, leaving nascent protein associated with 78S ribosomes (Zimmerman, 1963). Analysis by sucrose gradient centrifugation again revealed no radioactivity in ribosomal particles. The inability to find nascent proteins attached to nucleolar ribosomes could mean that: (1) nucleolar proteins were not synthesized on ribosomes, or (2) nucleolar ribosomes containing nascent protein were imbedded in the insoluble matrix of the organelle and therefore not extractable with dilute salt solutions. Therefore, concentrated CsCl extraction of nucleoli and centrifugation to equilibrium was next attempted in order to isolate nucleolar ribosomes. Formaldehyde fixation of the nucleoli was employed to prevent dissociation of ribosomal proteins from RNA in CsCl (Perry and Kelley, 1966). Markers for the density of ribosomes were prepared with cytoplasmic polysomes containing labeled nascent protein and monosomes with labeled RNA. The results are presented in Figure 8. When nucleoli were incubated with [^{3}H]leucine for 5 min, radioactivity was found in a peak at tube 4, and 39% of the radioactivity was lighter than this peak. Absorption at 260 mμ was also optimal in this peak. After the 30-min incubation of nucleoli, radioactivity and absorbance at 260 mμ were also found in the same position in the gradient (tube 4). Now, however, 57% of the radioactivity was lighter than this peak. The density of the labeled proteins was in both instances (1.575) heavier than free protein (1.250) and approximately the same as 78S cytoplasmic ribosomes, as reported by Perry and Kelley (1966). Furthermore, the density of these nucleoli was compared with that of cytoplasmic polysomes and monosomes. As indicated in Figure 8, the buoyant densities of all these particles were identical (compare dotted lines). It would therefore appear that protein synthesis in nucleoli of HeLa cells is carried out on nucleolar ribosomes.

Discussion

When isolated nuclei or nucleoli of HeLa cells were incubated with labeled amino acids, incorporation of radioactivity into 5% hot trichloroacetic acid precipitates was shown to occur. The evidence reported suggests that this represents protein synthesis in both nuclei and nucleoli and not cytoplasmic ribosome contamination, bacterial contamination, or nonspecific adsorption of amino acids to protein. Cytoplasmic contamination containing ribosomes or mitochondria is unlikely because: (1) detergents were employed to lyse any broken cells and remove cytoplasmic ribosomes from the perinuclear membrane (Penman, 1966); (2) addition of an equal number of cytoplasmic cell equivalents to the detergent-treated nuclear preparation resulted in an only 20%

TABLE IV: Salt and Acid Extraction of Proteins Synthesized in Isolated Nucleoli.[a]

Treatment	Peak on Acrylamide Gel	cpm	% Recov
1.	Major	7811	(100)
	Minor	200	(100)
2. 0.14 M NaCl–0.01 M sodium citrate	Major	319	4
	Minor	0	0
3. 0.25 N HCl extraction of 2	Major	0	0
	Minor	232	116

[a] Three aliquot portions of isolated nucleoli (5×10^7 cell equiv) were each incubated with 10 μCi of [^{3}H]leucine for 30 min at 37°. Each reaction was stopped by addition of 2 ml of cold 0.01 M Tris (pH 7.4), suspensions were centrifuged at 10,000g for 10 min and nucleolar pellets were washed twice with 2 ml of cold 0.01 M Tris. In treatments 2 and 3, nucleoli were washed twice with 2 ml of cold acetone, three times with 2 ml of cold 0.14 M NaCl and 0.01 M sodium citrate (pH 7.2), and three times with cold 70% ethanol. In treatment 2, the salt and ethanol washings were combined, the proteins were precipitated with cold trichloroacetic acid (20% final concentration), and the precipitate was washed once with 2 ml of cold acetone. In treatment 3, the salt-extracted nucleolar pellet was further extracted with 5 ml of 0.25 N HCl, stirred for 1 hr at 0°, and the acid-soluble protein precipitated with 20% trichloroacetic acid and washed with acetone, as above. Protein from treatments 1, 2, and 3 was solubilized in buffer A, electrophoresed in polyacrylamide gels, and counted as described in Methods. Background radioactivity was subtracted from each tube comprising the major and the minor peak.

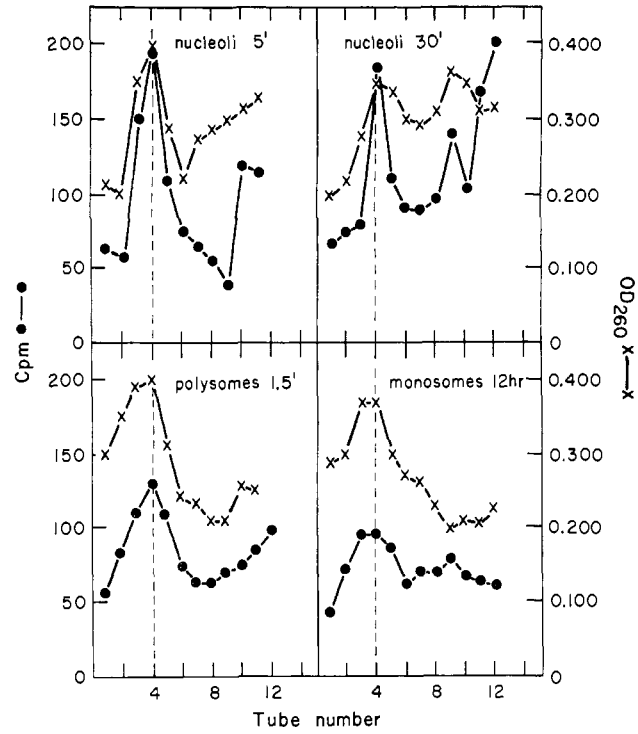

FIGURE 8: CsCl density centrifugation of labeled nucleoli and ribosomes previously fixed with formaldehyde. Nucleoli (1×10^8 cell equiv) isolated in TEA buffer were incubated with 10 μCi each of [^{3}H]leucine for 5 and 30 min. Polysomes from HeLa cells, labeled for 1.5 min at 25° with 20 μCi of [^{3}H]leucine (Speer and Zimmerman, 1968), and monosomes, labeled for 12 hr at 37° with 100 μCi of [^{3}H]uridine, were isolated; nucleoli and both ribosome preparations were fixed in formaldehyde and centrifuged to equilibrium in CsCl, as described in Methods.

increase in incorporation of radioactivity; (3) nucleoli that were subsequently isolated could nevertheless incorporate radioactivity; and, (4) only two nucleolar proteins were isolated. Many more proteins would be expected to be found if proteins were labeled from cytoplasmic ribosomes (Warner, 1966). Bacterial contamination is not likely because: (1) when nuclei were isolated with sterile technique, no bacterial colonies could be found; (2) chloramphenicol, a broad spectrum inhibitor of bacterial protein synthesis, did not depress the reaction; (3) many more than two proteins would be synthesized; and (4) synthesis of the two nucleolar proteins was not depressed with chloramphenicol. Nonspecific adsorption is also not responsible for the incorporation since (1) the incorporation in both nuclei and nucleoli increased with time of incubation at 37° and (2) no radioactivity was associated with either the major or the minor peak when labeled amino acids were mixed with nucleoli at 0° (Figure 7a).

In agreement with the results of Allfrey *et al.* (1957), inhibitors of oxidative phosphorylation block this mammalian nuclear protein synthesis. However, these authors showed a marked sodium ion dependence which could not be found in our experiments with nuclei from HeLa cells. The explanation offered by Allfrey (1963) was that the sodium ion is responsible for the uptake of amino acids into the nuclei. However, after detergent treatment and resuspension of the nuclei, the organization of the remaining nuclear membrane is lost since it was observed that the DNA gels and the nuclear preparation aggregates.

Analysis by polyacrylamide gel electrophoresis of the nucleolar protein synthesized revealed a major and minor peak. There are at least 20–30 ribosomal proteins in HeLa cells (Warner, 1966). Therefore, if nuclear protein synthesis had indeed occurred, then it is not likely that ribosomal proteins are synthesized in either the nucleoplasm or the nucleolus, the organelle where rRNA is synthesized (Perry, 1962) and the ribosomal particles are packaged (Warner and Soeiro, 1967). It would seem more plausible to expect that they were synthesized in the cytoplasm and were transported into the nucleolus to be associated with rRNA and thus form ribonucleoprotein particles. HeLa cells can transport cytoplasmic proteins into the nucleus (Speer and Zimmerman, 1968; Robbins and Borun, 1967). The minor protein peak has been shown to be acid soluble (Figure 7b and Table IV) and therefore probably represents a histone. Although Robbins and Borun (1967) present evidence that nuclear histones originate in the cytoplasm, their data do not rule out simultaneous

histone synthesis in the nucleus. What the major protein peak represents is not yet known.

It would appear that these nucleolar proteins are synthesized on nucleolar ribosomes because puromycin inhibited the synthesis, and labeled nucleolar proteins were found on particles having the same buoyant density as cytoplasmic ribosomes. The amount of radioactivity associated with these particles was approximately the same after incubation for 5 and 30 min, and the per cent of radioactivity lighter than these particles increased from 39 (5 min) to 57 (30 min). These data are in accord with the particles being precursor to the lighter areas of the gradient. The lack of proportional increase of radioactivity in the light region of the gradient with time of incubation is probably due to the nonlinear incorporation (see Figure 6) and an unknown loss of soluble protein during formaldehyde fixation and dialysis.

The observation that ribosomes are found in the nucleolus conflicts with the results of Weinberg *et al.* (1967) who showed no 18S rRNA present in their nucleolar preparation although our methods are similar to theirs. Since the association of labeled proteins to nucleolar ribosomes was not observed in dilute salt solutions (RSB) containing 0.5% sodium deoxycholate but in concentrated CsCl solution, it would appear that these ribosomes are imbedded in the insoluble matrix of the nucleolus.

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