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Independent Protein Synthesis in Isolated Rat Tumor Nucleoli. Aminoacylation of Endogenous Transfer Ribonucleic Acid†

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ABSTRACT: Nucleoli isolated from the Novikoff ascites tumor of the rat incorporate all the commonly labeled amino acids, added singly without other factors, into protein *in vitro*, and are capable of forming hydroxamates from most of the amino acids when hydroxylamine and ATP are added. The present work shows that the nucleoli are able to transfer each of the common amino acids into a complex with endogenous transfer RNAs. The activated complex was extractable in phenol–water, precipitable in cold trichloroacetic acid, labile to mild alkali and hot trichloroacetic acid, nondialyzable, and reactive chemically with hydroxylamine to yield hydroxamates. The complex was isolated and shown to contain RNA and to transfer its amino acids to protein in a ribosomal system purified to require added aminoacyl-tRNAs. Endogenous ATP has been detected in the isolated nucleoli by exchange with [^{32}P]pyrophosphate. The results show that these nucleoli contain an integrated, self-sufficient system capable of genuine protein synthesis and that this system resembles the mammalian cytoplasmic ribosomal system in that amino acids are activated and transferred by means of tRNAs.

In recent years the possibility that cell nucleoli contain a separate capacity for the synthesis of protein has been examined by a number of laboratories. Early reports were directed to the intense labeling of the nucleolus observed in autoradiography studies (Sirlin and Waddington, 1956; Waddington and Sirlin, 1959; Errera *et al.*, 1961) and, in addition, Birnstiel *et al.* (1961, 1962) and Zimmerman *et al.* (1969) showed that amino acid incorporation by pea seedling nuclei and HeLa cell nuclei, respectively, was primarily into a nucleolar fraction. Several other investigators, in addition to the ones cited here, have shown uptake of several single amino acids into protein of isolated nucleoli. Although incorporations proven to represent complete and genuine protein synthesis endogenous to the nucleolar organelle were not fully demonstrated, the existence of such a system has become an important consideration because of the possibility that the proteins synthesized therein serve specialized functions, such as initiators or repressors of RNA synthesis, or as structural or enzymatic components of nucleolar activities.

Lamkin and Hurlbert (1972) showed that nucleoli isolated from the Novikoff ascites tumor of the rat were able to utilize essentially all of the common amino acids for protein synthesis, and that the nucleoli contained a complete complement of activating enzymes. The purpose of the present paper is to extend these observations with proof of the existence and formation of aminoacyl-tRNAs, and demonstration of the presence of another essential reaction component, ATP, in isolated Novikoff nucleoli. A detailed comparison of the nucleolar and cytoplasmic systems with respect to amino acid incorporation and the effect of various known inhibitors of cytoplasmic protein synthesis upon the nucleolar system are also presented.

Experimental Procedure

Materials

Reconstituted protein hydrolysates containing 13 uniformly ^{14}C-labeled L-amino acids (average specific activities, 123 and 283 mCi/mmol) were obtained in separate shipments from Schwarz BioResearch. The amino acids contained were: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. The ^{3}H-reconstituted protein hydrolysate (Schwarz BioResearch) contained glycine and histidine in addition to the amino acids cited above and had an average specific activity of 16.13 Ci/mmol. Individual ^{14}C-labeled L-amino acids, each possessing a specific activity of 50 mCi/mmol, were also purchased from Schwarz BioResearch. $NaH_2{}^{32}PO_4$ in water (500 mCi/mmol) and $Na_4{}^{32}P_2O_7$ in water (4580 mCi/mmol) came from New England Nuclear Corp. Crystalline RNase and electrophoretically pure DNase I were products of Worthington Biochemical Corp. Puromycin (dihydrochloride) was obtained from Nutritional Biochemicals Corp.; chloramphenicol was obtained from Parke, Davis and Co.

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Methods

Preparation of Nucleoli. Novikoff ascites nucleoli were prepared as described elsewhere (Lamkin and Hurlbert, 1972). Detergent-washed nucleoli were prepared by treatment for 5 min at 0–4° with 1 vol of 0.33% deoxycholate–0.66% Triton X-100 in 9 vol of standard buffer (0.25 M sucrose–25 mM potassium chloride–5 mM magnesium acetate–50 mM Tris-HCl (pH 7.6)), and sedimented as 225*g* for 5 min. The detergent wash was repeated, the suspension was centrifuged immediately, and the nucleolar pellets were then washed once with standard buffer before final suspension in a minimal volume of this buffer. The detergent washing of nucleoli was not performed routinely after it was established that incorporation of amino acids into protein and activated complex was not affected. Protein was determined by the method of Lowry *et al.* (1951). DNA and RNA were measured by the diphenylamine (Burton, 1956) and orcinol (Hurlbert *et al.*, 1954) reactions, respectively.

Preparation of Cytoplasmic Protein Synthesizing Components. For the amino acid incorporation studies the procedure of Korner (1961) was used for the preparation of cytoplasmic microsomes and washed ribosomes (method 2). The "pH 5" activating enzymes were prepared according to O'Neal and Griffin (1963). A partially purified transfer enzyme fraction was prepared essentially as described by Griffin and Black (1971), except that the initial 22% ammonium sulfate fractionation step was omitted, and 10^{-3} M mercaptoethanol was substituted for dithiothreitol in the dialysis solution.

For the study of the incorporation of labeled cytoplasmic and nucleolar aminoacyl-tRNAs, a more refined system dependent upon addition of aminoacyl-tRNAs was used. Ribosomes were prepared from the Novikoff tumor according to O'Neal and Griffin (1963), but received only one wash with deoxycholate. Ribosomal pellets obtained from 50 ml of the packed ascites cells were homogenized in 32 ml of standard buffer and procedure (iv) (Griffin and Black, 1971) for the preparation of deoxycholate–DEAE-cellulose washed ribosomes was then followed. Transfer enzymes from Novikoff cells were prepared from the initial S-275,000*g* supernatant material according to Griffin and Black (1971) using essentially the procedure developed by Arlinghaus *et al.* (1968) for rabbit reticulocytes, with the exception that tumor T_1 and T_2 were not separated by calcium phosphate gel fractionation.

For the initial studies with the nucleolar incorporating system, the cytoplasmic [^{14}C]aminoacyl-tRNAs prepared from Novikoff cells were generously donated by Dr. A. Clark Griffin. Otherwise, the cytoplasmic labeled aminoacyl-tRNAs were prepared on a minor scale using the method of Griffin and Black (1971). Following ethanol precipitation, the pellets were dissolved in a solution containing 0.01 M magnesium acetate–0.001 M EDTA–0.01 M sodium acetate (pH 4.5), and dialyzed against the same solution to remove bound GTP and other nucleotide phosphates.

Incubation and Assay of Amino Acid Incorporation. The complete reaction mixtures and assay conditions for the incorporation of amino acids into protein are presented in the legends to Figures 1 and 2 and Table I. Following incubation, duplicate 0.05-ml samples were pipeted onto Whatman No. 3MM paper disks (2.3 cm size). The disks were immediately immersed in an ice-cold solution of 10% trichloroacetic acid, and successively treated with cold and hot trichloroacetic acid, ethanol–ether, and ether as described by Mans and Novelli (1961). Radioactivity was measured in a Nuclear-Chicago Mark I liquid scintillation counter using a modification of the naphthalene–*p*-dioxane solution (Langham *et al.*, 1956) consisting of 60 g of naphthalene (Recrystallized, Eastman Kodak Co.), 40 ml of Permafluor (Packard Instrument Co.), and *p*-dioxane (Spectroquality, Matheson Coleman and Bell) to 1 l., with corrections for efficiency. All of the values reported are also corrected for nonspecific, zero-time absorption of label to the filter disk.

Measurement of Acid-Precipitable Charged Complex. For measurement of the amount of the individual labeled amino acids incorporated into aminoacyl-tRNAs, a suspension of nucleolar protein (7.5 mg/1 ml of assay volume) in standard buffer and 0.04 mmol of each labeled amino acid, added singly and each possessing a specific activity of 50 μCi/μmol, were incubated for 10 min at 37°. An equal volume of 90% phenol was added to precipitate protein and the suspension was mechanically shaken for 1 hr at room temperature. After centrifugation at 27,000*g* for 20 min the aqueous layer was removed; 0.1-ml aliquots were precipitated with 1 ml of cold 12% trichloroacetic acid and filtered according to Favorova and Kisselev (1970) through nitrocellulose B-6 membrane filters (Schleicher and Schuell, Inc.). The disks were washed with cold 5% trichloroacetic acid to remove precursor free amino acids, air-dried, and counted in a liquid scintillation counter.

Chemical Conversion of Charged Complex to Hydroxamates. For assay of the amount and identity of activated amino acid in the complex, aliquots of the reaction mixture were also treated with hydroxylamine to cause chemical conversion to the hydroxamate. Salt-free hydroxylamine was prepared according to Loftfield and Eigner (1966) and standardized with hydroxyquinoline (Frear and Burrell, 1955). Maximum conversion of the labeled complex to hydroxamic acids occurred when 0.1-ml portions of the aqueous layer from the phenol extraction were immediately mixed with 3 mmol (0.1 ml) of pure hydroxylamine and heated. Hydroxylamine, which is a solid at room temperature, did not completely remain in solution at 37° when mixed with the aqueous samples. Samples were therefore placed in a boiling water bath and the rate of hydroxamic acid formation *vs.* time was measured.[1] Hydroxamic acid formation increased sharply for the first 20–30 min, reached a maximum by 60 min, and remained constant when heating was continued up to 3 hr. Samples were thereafter routinely heated for 90 min. Conversion to the corresponding hydroxamates and separation from the free amino acids was measured by paper chromatography as described previously (Lamkin and Hurlbert, 1972). Aliquots (0.025 ml) of the reaction mixture for each amino acid were chromatographed with the appropriate carrier hydroxamate, and the carrier spot and neighboring areas were counted separately to establish identity of the label with the hydroxamate.

Preparation of Nucleolar [^{14}C]Aminoacyl-tRNAs. For preparation of the nucleolar [^{14}C]aminoacyl-tRNAs, suspensions of nucleoli in standard buffer (containing 20–25

[1] The conditions used were more vigorous than those reported by others (Hirsh and Lipmann, 1968; Favorova and Kisselev, 1970) for reaction of an aminoacyl-tRNA complex with hydroxylamine, but were, however, dictated by the experimental difficulties encountered with the nucleolar system. The nucleolar aminoacyl complex was not sufficiently stable to allow complete removal of the phenol from the aqueous extracts. Addition of salt-free (pure) hydroxylamine to the aqueous extracts gave a final pH (near 7) in the proper range for hydroxamate formation, which was not obtained when hydroxylamine hydrochloride, pH 7, was employed.

mg of protein), 12 mmol of ATP (pH 7), and 60 nmol of ^{14}C-labeled L-amino acid mixture in a total volume of 3 ml were incubated for 10 min at 37°. Following incubation, an equal volume of 90% phenol was added; the solutions were mechanically shaken for 1 hr at room temperature and then centrifuged at 27,000*g* for 20 min. The aqueous upper layers were collected, adjusted to a final concentration of 0.01 M with magnesium acetate (pH 4.5) and 0.001 M with EDTA, and concentrated *in vacuo* to approximately one-fifth of their original volume. One-tenth milliliter of 20% potassium acetate (pH 4.5) was added for each 0.9 ml of solution. Maximum precipitation of the tRNAs was obtained with 2.5 vol of ethanol (or *n*-propyl alcohol) after 90 min at −15°. The ethanol (or *n*-propyl alcohol) mixtures were maintained at −15° also during centrifugation; at 0° the precipitate tended to go back into solution. The solutions were centrifuged at 27,000*g* for 20 min; the pellets were dissolved in a minimal volume of solution containing 0.01 M magnesium acetate–0.001 M EDTA–0.01 M sodium acetate (pH 4.5), and dialyzed against the same solution.

[^{32}P]*Pyrophosphate–ATP Exchange Studies.* The assay mixtures contained, in 4 ml: 20 mg of nucleolar protein suspended in standard buffer, 0.01 μmol of each of 18 ^{12}C-labeled L-amino acids, and either [^{32}P]phosphate (4 μmol, 4.44 × 10^9 dpm) or [^{32}P]pyrophosphate (0.32 μmol, 3.23 × 10^9 dpm) as indicated. The mixtures were incubated at 37° for 10 min, and the reaction was stopped by addition of 0.5 ml of 4.4 N perchloric acid. Following centrifugation, the supernatant was mixed with 2 ml of a suspension of acid-washed Norit A (25%), dry weight). After 10 min the Norit was centrifuged, washed repeatedly by suspension and centrifugation until all of the counts per minute in the water supernatant had dropped to background, and was then extracted with 2-ml portions of 50% ethanol containing 0.3 M ammonium hydroxide until all of the radioactivity had been eluted from the charcoal. The eluates were immediately adjusted to pH 4 with acetic acid and aliquots were spotted for descending paper chromatography with marker AMP, ADP, ATP, inorganic phosphate, and pyrophosphate. Samples spotted on Whatman No. 1 paper (57 cm × 19 cm) were developed for 14 hr with isobutyric acid–concentrated ammonium hydroxide–water (66:1:35). Inorganic phosphate and pyrophosphate migrated behind ATP which was the slowest moving of the three nucleotides. Chromatography was also carried out on DEAE-cellulose paper (57 cm × 19 cm) by overnight development with 5 M ammonium acetate (pH 9.5)–saturated $Na_2B_4O_7 \cdot 10H_2O$–0.4 M EDTA–ethanol (5:20:0.15:16.5 ml/chromatogram). In this system phosphate and pyrophosphate migrated well beyond the adenosine nucleotides. Nucleotides were detected by ultraviolet absorption. Inorganic phosphate and pyrophosphate were detected by first spraying the chromatograms with 0.4% (w/v) ammonium molybdate in 8% nitric acid and heated at 80° for 5–10 min to break down pyrophosphate to inorganic phosphate. The chromatograms were sprayed with 0.05% benzidene dihydrochloride in 10% acetic acid, and the spots were developed by exposure to ammonia vapor. The appropriate nucleotide, inorganic phosphate, and pyrophosphate areas were cut out and counted separately in a liquid scintillation counter. For these experiments Aquasol scintillation fluid (New England Nuclear) was used with corrections for decay of ^{32}P.

Results

Amino Acid Incorporation Studies. The *in vitro* incorporation of amino acids by the cytoplasmic ribosomal and microsomal systems is known to require a complex medium containing ribosomes or microsomes with attached messenger RNA, pH 5 (activating) enzymes and tRNA, transfer enzymes, ATP, an energy generating system consisting of GTP, phosphoenolpyruvate, and pyruvate kinase, and a full complement of amino acids. In contrast, incorporation by purified intact Novikoff ascites nucleoli occurred in a simple sucrose–salts buffer (standard buffer) and required only the addition of a single labeled amino acid or a protein–hydrolysate mixture. Figure 1 presents the patterns of incorporation *vs.* time for the cytoplasmic ribosomal and microsomal systems and the nucleolar system. The total amount of radioactive label incorporated per milligram of nucleolar protein represents approximately one-eighth and one-sixth of the levels of incorporation observed with the more highly fractionated ribosomal and microsomal systems respectively when synthesis is expressed per milligram of ribosomal or microsomal protein.

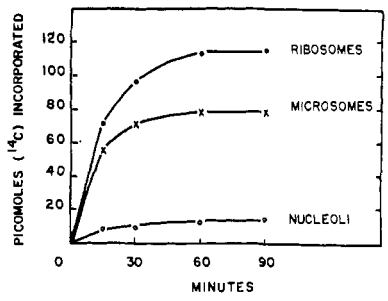

FIGURE 1: Comparison of the incorporation of ^{14}C-labeled L-amino acid mixture *vs.* time by the cytoplasmic ribosomal and microsomal systems and the nucleolar system. For the cytoplasmic systems the following incubation mixture was used: ribosomes or microsomes, 2 mg of protein; pH 5 enzyme fraction, 1 mg of protein; transfer enzyme fraction, 0.5 mg of protein; ^{14}C-labeled L-amino acid mixture, 8.7 nmol (1 μCi); ATP·Na (pH 7), 1 μmol; GTP, 15 × 10^{-3} μmol; phosphoenolpyruvate (Na), 5 μmol; phosphoenolpyruvate kinase, 10 μg; $MgCl_2$, 6 μmol; ammonium sulfate, 3.8 μmol; mercaptoethanol, 0.14 μmol; 0.1 ml of a tenfold concentrated solution of standard buffer; and sufficient water to bring the final volume to 1 ml. The incubation mixture for the nucleolar system contained 2 mg of nucleolar protein, 8.7 nmol of ^{14}C-labeled L-amino acid mixture, 0.1 ml of a tenfold concentrate of standard buffer, and water to a final volume of 1 ml. Zero-time samples were removed and the reaction mixtures were incubated at 37°. At the times indicated duplicate aliquots of 0.5 ml of the reaction mixtures were removed and washed with cold and hot trichloroacetic acid, ethanol, and ether prior to counting. Values represent the averages from three separate experiments. The picomoles of ^{14}C-labeled L-amino acid mixture incoporated by the cytoplasmic systems are expressed per milligram of ribosomal or microsomal protein; by the nucleolar system, per milligram of nucleolar protein.

In order to check the possibility that the nucleoli were contaminated by cytoplasmic or nuclear enzymes or ribosomal particles, comparisons of amino acid incorporation with buffer-washed *vs.* deoxycholate–Triton X-100-washed nucleoli (Methods) were carried out (Table I). Amounts of protein, DNA, and RNA in the supernatant solutions obtained by low-speed centrifugation after the detergent treatment and amount of protein in the nucleolar pellet were measured. Despite loss of 10% of the protein and 36% of the RNA by detergent treatment, the incorporating activity per milligram of nucleolar protein was essentially the same for the original and the detergent-washed nucleoli, indicating that the activity was associated with the nucleolar particles.

Figure 2 shows that when the nucleolar incorporation of a labeled amino acid mixture was allowed to proceed for 30 min and a 100-fold excess of each of 18 ^{12}C-labeled L-amino

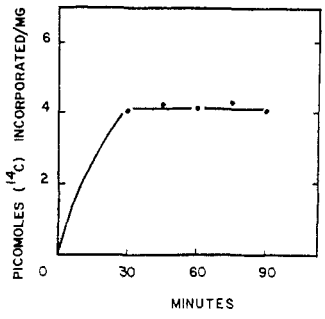

FIGURE 2: Measurement of ^{14}C-labeled L-amino acid incorporation following dilution with unlabeled L-amino acids. One-milliliter reaction systems containing 4 mg of nucleolar protein suspended in standard buffer and 4 nmol of ^{14}C-labeled L-amino acid mixture were incubated at 37° for 30 min. An excess of unlabeled L-amino acids (100 μmol of each of 18 amino acids) was added. Incubation was continued. Duplicate 0.05-ml aliquots were removed at the times indicated and spotted on Whatman No. 3MM paper disks. The disks were washed and assayed for amino acid incorporation.

acids was added to the system, followed by continued incubation and assay, the incorporated radioactivity remained at a constant level. Since the label in the protein cannot be thus diluted out, and since it is retained during rigorous washing of the protein prior to counting, the incorporation is judged to represent true covalent linkage and not mere binding of labeled amino acids onto nucleolar protein or other structures.

Varying degrees of inhibition of the cytoplasmic systems were observed when pH 5 enzymes, transfer enzymes, and the energy generating system were omitted from the reaction mixture; the more crude microsomal system was less affected by deletion of activating and transfer enzymes than the washed ribosomal system (Table II). These factors were not required by the nucleolar system. Addition of one or more of these factors to the nucleolar system did not cause further stimulation of amino acid incorporation. Addition of ATP over the range of 1–5 mM did not cause further stimulation of amino acid incorporation; with 5 mM ATP, 13% inhibition was observed after 60 min. Increased concentration of magnesium beyond that normally present in the standard buffer was not required for optimum incorporation by the nucleoli. [^{14}C]-Aminoacyl-tRNAs (80,000 dpm; specific activity, 100,000 dpm/mg of RNA) prepared from the cytoplasm of the Novikoff ascites cells were not utilized by the nucleoli; after 60 min of incubation, no increase in radioactivity was observed when compared with zero-time samples.

The effects of various known inhibitors of protein synthesis on the cytoplasmic ribosomal and microsomal systems and the nucleolar system were also compared (Table II). Hydroxylamine and puromycin were almost as inhibitory to the nucleolar system as they were to the cytoplasmic systems. Hydroxylamine traps activated amino acids; puromycin substitutes for aminoacyl-tRNA and thereby terminates peptide chain forma-

TABLE I: Comparison of Buffer-Washed *vs.* Detergent-Washed Nucleoli.[a]

	Supernatant			
	% Protein Lost	% DNA Lost	% RNA Lost	Pellet Protein Synthesis
Buffer-washed nucleoli				14.4
Detergent-washed nucleoli	10.1	2.1	36.3	13.8

[a] Nucleoli were washed as usual with standard buffer (pH 7.6) or additionally with deoxycholate–Triton X-100 in standard buffer (Methods). Loss of protein, DNA, and RNA was measured on the detergent-washed supernatant solution and expressed as a per cent of the amount in the buffer-washed nucleolar pellet. Nucleolar protein was determined after suspension of the pellet protein in standard buffer. For the amino acid incorporation studies 2 mg of nucleolar protein in standard buffer and 8.7 nmol of ^{14}C-labeled amino acid mixture in a final volume of 0.5 ml were incubated for 60 min at 37°. Duplicate samples of 0.05 ml were applied to Whatman No. 3MM disks, and the disks were washed with cold and hot trichloroacetic acid, ethanol, and ether, prior to counting. Values for protein synthesis are expressed as picomoles of ^{14}C-labeled amino acid mixture incorporated per milligram of pellet protein at 60 min.

TABLE II: Comparison of the Requirements for Protein Synthesis by the Cytoplasmic and Nucleolar Systems, and the Effects of Inhibitors of Protein Synthesis upon these Systems.[a]

System	Incorporation Expressed as % of Control (100%)		
	Nucleolar System	Microsomal System	Ribosomal System
Energy generating system			
Plus	100		
Minus		28	36
pH 5 enzymes			
Plus	105		
Minus		78	26
Transfer enzymes			
Plus	102		
Minus		75	39
Hydroxylamine (10^{-3} M)	32		1
Puromycin (10^{-3} M)	56	35	22
Chloroamphenicol (10^{-3} M)	100	93	94
RNase (0.1 mg)	104	21	17
DNase (0.1 mg)	101	100	
EDTA (10^{-2} M)	50	22	23
Dinitrophenol (10^{-3} M)	103	92	106
KCN (10^{-2} M)	93	90	85

[a] The assay systems used for the cytoplasmic and nucleolar systems were identical with those presented in the legend to Figure 1. The values reported were obtained after an assay period of 60 min; control values for these studies at 60 min were within the range specified in Figure 1. Where indicated, equivalent amounts of the various components of the cytoplasmic system were added to the nucleolar system. For the cytoplasmic systems, only the component specified was omitted from the reaction mixture. The concentrations of the different inhibitors used are presented in the table. Incorporation was measured by spotting duplicate 0.05-ml samples of the reaction mixtures on Whatman No. 3MM paper disks and treating the disks as described previously. Results represent the averages obtained from three–four separate experiments.

tion. Inhibition by hydroxylamine and puromycin provided the first indication that the cytoplasmic and nucleolar systems have at least some mechanisms for protein synthesis in common. Chloramphenicol, a drug which inhibits protein synthesis in bacteria, does not inhibit the mammalian-like protein synthesizing systems present in the cytoplasm of mammalian cells, rat liver, yeast, and *Neurospora*. These same cells, however, contain a bacterial-like protein synthesizing system in the mitochondrion (Scragg *et al.*, 1971) and inhibitors of bacterial protein synthesizing systems significantly decrease the rate of mitochondrial protein synthesis. The Novikoff nucleolar amino acid incorporating system was not inhibited by chloramphenicol.

Amino acid incorporation in mitochondria has been reported to be dependent upon the functioning of the electron transport chain (Reis *et al.*, 1959; Wintersberger, 1965). The nucleolar system was not affected by dinitrophenol. The slight inhibition noted when potassium cyanide, which is also an inhibitor of oxidative phosphorylation, was added to the nucleolar system was not further studied since similar degrees of inhibition were seen with the cytoplasmic systems where this reagent should not show any direct effect. The nucleolar system does not appear to resemble (or be contaminated by) the mitochondrial system.

DNase did not exert an effect on either the cytoplasmic or nucleolar systems. RNase was inhibitory to the cytoplasmic systems, but not to the nucleolar system. This latter finding is in agreement with results reported by Izawa and Kawashima (1969) for mouse ascites tumor nucleoli, and by Zimmerman *et al.* (1969) for HeLa cell nuclei where incorporation was shown to be primarily into a nucleolar fraction. Lack of inhibition by RNase would imply that, if RNA components are responsible for nucleolar protein synthesis, this RNA is not accessible to the nuclease in intact nucleoli. Lack of inhibition with RNase was exhibited both with freshly isolated Novikoff nucleolar suspensions and with frozen preparations whether RNase was added directly upon assay or the nucleoli were preincubated with RNase for 15 min at 37° prior to assay.

The amount of observed amino acid incorporation is not diminished by freezing the nucleolar suspensions. Frozen suspensions of nucleoli, stored individually in small amounts, were always thawed immediately prior to use, and, when once thawed, unused portions were discarded. When the effect of repeated freezing and thawing upon protein synthesis was studied, the effect of RNase upon these suspensions was also measured. RNase did not inhibit thawed nucleolar suspensions which were maintained at 0–4° for 4 hr prior to assay; the degree of protein synthesis was not lessened. When, however, these suspensions were refrozen and thawed, the amount of protein synthesis was 88% of that previously observed, and the systems now showed 20–25% inhibition (66% of the original incorporation) by RNase. After one more refreezing and thawing of these same suspensions, the synthetic activity was reduced to 76% of the original and inhibition by RNase remained at 25% (57% of the original incorporation).

Studies with the Activated Aminoacyl Complex Formed by Nucleoli. Lamkin and Hurlbert (1972) have reported that Novikoff ascites nucleoli were capable of activating most of the common amino acids tested and converting them to their respective hydroxamic acids and have compared the ability of the nucleolar enzymes to activate individual amino acids with that of the cytoplasmic enzymes from the Novikoff tumor. The cytoplasmic activating enzymes were quite active and determination of the relative amounts of the various hydroxamic acids formed did not present any problem. In comparison, the amounts of the various hydroxamates formed by the nucleoli were usually much lower. In the cases of alanine, aspartic acid, and serine, only 1 pmol or less of hydroxamate could be detected, proline hydroxamate could not be detected at all, and a suitable test system for glutamic acid was not available. The problem did not appear to be one of enzyme stability during storage as the same results were obtained using either freshly prepared or frozen samples. Various additions to the solutions used in the isolation procedure and/or in the assay procedures to stabilize the enzymes were without effect. The problem appeared to be inherently low activity of activating enzymes in the high concentrations of hydroxylamine necessary and different responses of the various enzymes to these conditions.

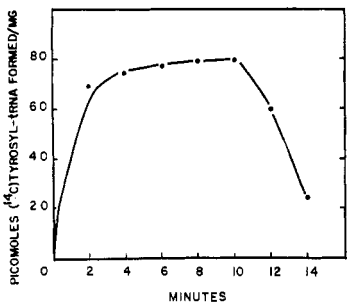

FIGURE 3: Formation of [^{14}C]tyrosyl-tRNA *vs.* time. Each 1-ml assay system contained 40 nmol of L-[^{14}C]tyrosine and 7.5 mg of nucleolar protein suspended in standard buffer. At the times indicated, tubes were removed from the 37° water bath and the reaction mixtures were extracted with an equal volume of 90% phenol. Aliquots (0.1 ml) of the aqueous layers were precipitated with cold 12% trichloroacetic acid and passed over nitrocellulose filters. The filters were washed with cold trichloroacetic acid, dried, and counted.

In order to show more clearly whether the nucleoli were capable of activating all of the common amino acids, and in order to examine the nature of the receptor group for the activated amino acids, the amount of active complex formed upon addition of individual labeled amino acids was studied. It had previously been noted that a significant amount of label was incorporated into a phenol-extractable, nondialyzable, cold trichloroacetic acid precipitable, hot trichloroacetic acid labile product. The rate of formation of labeled tyrosine complex by the nucleoli was first determined because of the high levels of incorporation previously observed with this amino acid. Figure 3 shows that incorporation of L-[U-^{14}C]tyrosine into a phenol-extractable, cold trichloroacetic acid precipitable product (Methods) increased rapidly for the first 4–6 min, remained constant until 10 min, and then sharply decreased between 10 and 14 min. Similar results were obtained with leucine. An incubation period of 8 min at 37° was used in subsequent studies. Formation of labeled complex for each amino acid added separately to the nucleoli is shown in Table III. Aliquots from the aqueous layers of these phenol extracts were simultaneously allowed to react with salt-free hydroxylamine (Methods) and the amount of label specifically in the hydroxamic acid corresponding to the amino acid tested was measured by paper chromatography. Labeled hydroxamates were not found in identical reaction mixtures in which the aqueous layer was first adjusted to pH 9–10 with sodium hydroxide to destroy the aminoacyl bond to tRNA before addition of hydroxylamine.

The picomoles of cold trichloroacetic acid precipitable aminoacyl complex trapped on the nitrocellulose filters agree closely in most cases with the picomoles of this material which

TABLE III: Comparison of ^{14}C-Labeled Amino Acid Incorporated into Protein and [^{14}C]Aminoacyl-tRNA Formation by the Nucleolar System.[a]

	pmol/mg of Nucleolar Protein		
		[^{14}C]Aminoacyl-tRNA Formed	
^{14}C-Labeled Amino Acid	^{14}C-Labeled Amino Acid Incorp into Protein	Nitrocellulose Filters	Converted to Hydroxamic Acid
Alanine	3.0	169.9	167.6
Arginine	6.5	39.2	28.9
Aspartic acid	12.1	159.4	
Glycine	2.8	109.4	85.6
Glutamic acid	3.9	162.6	
Histidine	7.5	88.1	131.4
Isoleucine	2.2	55.8	50.9
Leucine	2.3	76.8	84.3
Lysine	4.1	80.1	98.3
Phenylalanine	2.9	49.9	39.9
Proline	4.6	52.6	41.4
Serine	3.2	196.0	78.1
Threonine	4.8	28.0	33.2
Tyrosine	79.4	75.9	76.2
Valine	0.2	22.6	27.8

[a] Each assay system contained, in a volume of 1 ml: 40 nmol of the ^{14}C-labeled L-amino acid indicated and 7.5 mg of nucleolar protein suspended in standard buffer. The reaction systems were incubated for 10 min at 37° and then extracted with an equal volume of 90% phenol. Triplicate 0.1-ml aliquots of the aqueous layers were treated with cold 10% trichloroacetic acid and passed over nitrocellulose filters, and the filters were washed with additional cold trichloroacetic acid, dried, and counted. Triplicate 0.1-ml samples were simultaneously treated with 3 mmol of unneutralized, salt-free hydroxylamine, and the amount of labeled hydroxamic acid formed was measured by liquid scintillation counting of the hydroxamate regions of the paper chromatograms. The column labeled "^{14}C-labeled amino acid incorporated into protein" from a previous paper (Lamkin and Hurlbert, 1972) is included for comparison.

are chemically converted to specific hydroxamic acids. In most cases the values observed were considerably higher and less variable from one amino acid to another than those previously reported (Lamkin and Hurlbert, 1972) for hydroxamate formation when hydroxylamine was added directly to the assay system. Direct formation of methionine hydroxamate was obtained in the previous work. Because of the vigorous reaction conditions employed, the possibility that hydroxamates were being formed from an aminoacyl derivative other than aminoacyl-tRNA could not be excluded at this point (Raacke, 1958). However, the data presented in Table III proved certainly that all of the amino acids tested were activated and made it appear highly probable that the activated amino acids are indeed bound to tRNA by these isolated nucleoli. The existence in Novikoff nucleoli of a 4S RNA capable of accepting amino acids in the presence of *Escherichia coli* activating enzymes has been previously shown by Nakamura *et al.* (1968).

Isolation of Labeled Aminoacyl-tRNA from Nucleoli. It was necessary to determine whether an aminoacyl complex obtained by reaction of nucleolar suspension with a uniformly labeled protein hydrolysate mixture could substitute for labeled cytoplasmic aminoacyl-tRNA in a highly purified cytoplasmic incorporating system. In preliminary studies the amount of phenol-extractable, cold trichloroacetic acid precipitable material trapped on nitrocellulose filters was lower than anticipated from studies with individual amino acids. Addition of ATP enhanced formation of the complex; 4 mM ATP gave optimal results. Addition of magnesium beyond that present in the standard buffer, KCl, and mercaptoethanol (Yang and Novelli, 1968) did not further enhance formation of the complex. It had been determined previously that the maximum amount of complex was obtained after a phenol extraction period of 45 min. No detectable loss of the complex was observed when this extraction period was extended up to 2 hr. However, once the aqueous layer was separated from the phenol, samples maintained at 4° showed 90% degradation of the cold trichloroacetic acid precipitable complex at the end of 2 hr. Phenol treatment at room temperature without detergent is mild with regard to removal of nucleases and relatively specific with regard to extraction of low molecular weight RNAs. Adjustment of the aqueous layer to a final concentration of 0.01 M with magnesium acetate (pH 4.5) and 0.001 M EDTA prevented breakdown of the nucleolar complex. Therefore, glassware and dialysis tubing used for the isolation of the nucleolar complex were routinely washed first with 0.001 M EDTA followed by several washes with glass distilled water.

Labeled cytoplasmic aminoacyl-tRNA is precipitated by treatment of the aqueous extract with 0.1 vol of 20% potassium acetate and 2 vol of ethanol. At least 95% of the cytoplasmic complex was routinely recovered from this step. Under similar conditions, only about 30% of the nucleolar complex was precipitated. When the aqueous layers following phenol treatment were adjusted to 0.01 M magnesium acetate (pH 4.5)–0.001 M EDTA, extracted with ether, and concentrated *in vacuo* to approximately one-fifth of their original volume, approximately 60% of the complex was precipitated by potassium acetate and ethanol. Substitution of *n*-propyl alcohol for ethanol resulted in 70% recovery of the nucleolar complex. It has also been noted that increasing the amount of nucleolar protein up to 7.5 mg/ml of initial reaction mixture yields an increase in the amount of labeled complex formed.

[^{14}C]Aminoacyl-tRNA was prepared from the nucleoli and from the cytoplasmic pH 5 fraction and dialyzed to remove bound GTP and other nucleotide phosphates. The amounts of labeled complex before and after dialysis were measured by treating aliquots of the samples with cold trichloroacetic acid and passing them over nitrocellulose disks. Following dialysis, 92% of the cytoplasmic complex remained and possessed a specific activity of 1238 dpm/μg of RNA. In contrast, not more than 30–40% of the nucleolar complex remained following dialysis. Equal amounts of radioactivity from these two complexes were treated separately in the tumor amino acid incorporating system described by Griffin and Black (1971). As shown in Table IV, the percentage of the total number of counts incorporated from the nucleolar complex nearly equals the per cent of the total number of counts incorporated from the cytoplasmic aminoacyl-tRNA complex. RNAse was effectively inhibitory toward both the cytoplasmic and nucleolar complexes.

Figure 4 shows the sucrose density gradient centrifugation pattern of the nucleolar complex employed in the tumor amino acid incorporating system described above. A single radio-

TABLE IV: Utilization of Nucleolar Aminoacyl-tRNA for Protein Synthesis with Purified Cytoplasmic Ribosomes and Transfer Enzymes.[a]

[^{14}C]Aminoacyl-tRNA	Incorporation of Label into Protein: Zero-Time dpm	30-min (Corrected) dpm	Incorp (%)
Cytoplasmic	158	855	16.1
plus RNase		43	
minus transfer enzymes		0	
Nucleolar	227	753	13.8
plus RNase		27	
minus transfer enzymes		82	

[a] The system for assay of aminoacyl-tRNAs as described by Griffin and Black (1971) was used and contained, in 0.2 ml: 19 μmol of ammonium sulfate, 0.72 μmol of mercaptoethanol, 1 μg of phosphoenolpyruvate, 10 μg of pyruvate kinase, 0.12 μmol of GTP, 0.2 mg of transfer enzyme protein, 0.4 mg of ribosomal protein, and [^{14}C]aminoacyl-tRNA prepared from the cytoplasmic (5325 dpm) or nucleolar (5475 dpm) fractions by incubation with a ^{14}C-labeled L-amino acid mixture. Where indicated, 0.02 mg of RNase was added or transfer enzymes were omitted. The mixtures were incubated at 37° for 30 min and absorbed on Whatman No. 3MM paper disks (2-cm size), and the disks treated successively with cold and hot trichloroacetic acid, ethanol, and ether prior to counting. Zero-time absorption was determined for each system and subtracted from the reported 30-min values. Values are the averages obtained from two experiments using nucleolar tRNAs from two separate preparations.

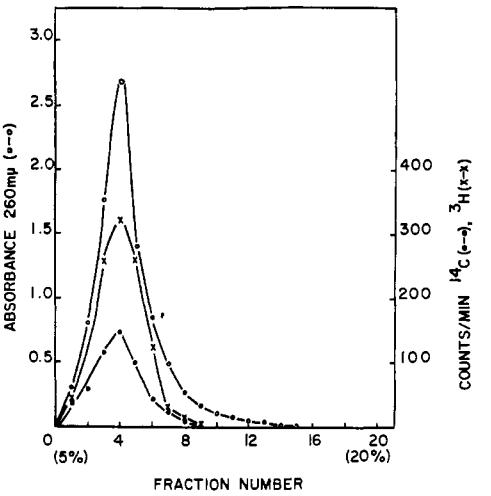

FIGURE 4: Sucrose density gradient centrifugation pattern of labeled cytoplasmic and nucleolar aminoacyl-tRNA. [^{3}H]Cytoplasmic aminoacyl-tRNA and ^{14}C-labeled nucleolar aminoacyl-tRNA, each suspended in minimal volumes of a solution containing 0.01 M magnesium acetate–0.001 M EDTA–0.01 M sodium acetate (pH 4.5), were combined. The mixture was layered upon 5-ml continuous gradients of 5–20% sucrose suspended in 0.1 M sodium chloride–0.01 M magnesium acetate–0.001 M EDTA (pH 4.5), and centrifuged in a Spinco SW 50L rotor for 3.5 hr at 274,000g. Fractions of 0.25 ml were collected using an ISCO Model D density-gradient fractionator with recorder. One milliliter of albumin solution (1 mg/ml) was added to each tube, and the material was precipitated and washed with cold 10% trichloroacetic acid, and radioactivity was determined by a standardized double-label technique.

active peak in the 4–6S region comigrated with a [^{3}H]aminoacyl tRNA marker prepared from the cytoplasmic fraction of Novikoff cells.

Since it was of interest to determine what fraction of the total nucleolar RNA was present as 4–6S RNA, nucleolar suspensions of known RNA and protein content were treated with phenol, and the orcinol reaction was run on the aqueous layers. Per milligram of total nucleolar RNA, 0.06 mg of this low molecular weight RNA was detected. Since the protein:DNA:RNA ratio of these nucleoli is 10:1:1, approximately 6 μg of tRNA is associated with each milligram of nucleolar protein. In comparison, from similar studies with the cytoplasmic fraction, a value of 20 μg of tRNA was obtained per milligram of pH 5 protein.

[^{32}P]*Pyrophosphate–ATP Exchange Studies.* The source of the biosynthetic energy necessary for the protein synthesis carried out by these isolated, intact nucleoli has thus far been conjectural. Unlike the cytoplasmic amino acid incorporating system, a source of exogenous ATP was not necessary for *in vitro* amino acid incorporation by the nucleoli (although it was needed for optimal formation of the aminoacyl complex). Addition of ATP to the assay system did not increase the amount of incorporation observed. The nucleolar protein synthesizing system must, therefore, be self-sufficient with respect to energy sources.

[^{32}P]Pyrophosphate–ATP exchange studies in which ATP is added to the reaction mixture have been of value in demonstrating amino acid activation by cytoplasmic enzymes (Hoagland *et al.*, 1956) and also fatty acid and acetate activation (Berg, 1956). It was considered that the exchange reaction would also enable detection of ATP in the nucleoli. Exchange studies were carried out in separate reaction systems using only [^{32}P]phosphate or [^{32}P]pyrophosphate as substrates, a mixture of 18 ^{12}C-labeled L-amino acids, and nucleoli suspended in standard buffer. Following incubation, the reaction mixtures were precipitated with perchloric acid, the acid-soluble extracts were treated with charcoal as described in Methods, and portions of the ethanol–ammonia eluates from the charcoal were chromatographed. A slight incorporation of [^{32}P]phosphate into ATP was noted. A tenfold greater number of counts was incorporated into ATP when [^{32}P]-pyrophosphate was used. Table V presents the total radioactivity from the ATP and ADP regions and the picomoles of ATP formed. The counts in the ADP region presumably arise from breakdown of ATP since subsequent chromatograms run using aliquots from these same samples following freezing and thawing showed a larger fraction of the total radioactivity in the ADP region. Radioactivity was not detected in the phosphate and pyrophosphate regions of the chromatograms. While these exchange studies do not enable an accurate estimation of the total ATP pool, they do show that a significant amount of endogenous ATP, able to undergo exchange with pyrophosphate, is present in isolated Novikoff nucleoli.[2]

[2] ^{3}H-Labeled adenosine nucleotides were also detected in nucleoli isolated from tumor-bearing rats which had received ^{3}H-labeled adenosine intraperitoneally 1 hr before death. The nucleoli were extracted with cold perchloric acid and the extracts were chromatographed (10) on Dowex-1 (formate) columns. The amount of labeled ATP estimated was of the same order of magnitude as determined by exchange with pyrophosphate, and labeled ADP predominated. We are indebted to Karin Cibula for these studies.

TABLE V: Exchange of [^{32}P]Phosphate and [^{32}P]Pyrophosphate with Endogenous Nucleolar ATP.[a]

Substrate	Total dpm Recovered		pmol of ATP Exchanged/mg of Nucleolar Protein
	ADP	ATP	
[^{32}P]Phosphate	11,266	69,856	0.344
[^{32}P]Pyrophosphate	47,609	6,137,674	3.320

[a] Twenty milligrams of nucleolar protein suspended in standard buffer, 0.01 μmol of each of 18 unlabeled L-amino acids, and either [^{32}P]phosphate (4.44×10^6 dpm) or [^{32}P]-pyrophosphate (3.23×10^9 dpm) as indicated, were incubated at 37° for 10 min. The reaction mixtures were treated with perchloric acid, and the nucleotides were adsorbed onto Norit A, eluted, and chromatographed as described in Methods. The picomoles of ATP were calculated from the specific activities of the [^{32}P]phosphate (500 mCi/mmol) and [^{32}P]pyrophosphate (4580 mCi/mmol).

Discussion

To date the most significant known function of the nucleolus is the synthesis and processing of ribosomal RNA and assembly of this ribosomal RNA with proteins. While most of the preribosomal proteins possessing rapid turnover appeared to be formed in the cytoplasm (Tsurugi *et al.*, 1972; Maisel and McConkey, 1971; Kumar and Warner, 1972), the origin of all classes of protein associated with preribosomal and other nucleolar RNA has not been elucidated. Reports indicating the incorporation of labeled amino acids into nucleolar protein were of interest to us in conjunction with other studies in this laboratory involving the processing of preribosomal RNA since nucleolar protein synthesis might be related to this function. It is conceivable that the protein synthesized by the nucleoli might enter the ribosomes or might be utilized in the nucleolus as one or more of the regulatory, enzymatic, or structural factors necessary for processing preribosomal RNA. It is also possible that the nucleolus might be responsible for synthesis of certain enzymes, *e.g.*, RNA polymerase or NAD^+ pyrophosphorylase, or synthesis of proteins involved in the structure of nucleoli or the structure or movement of chromosomes.

We believe that the results presented here show that isolated Novikoff tumor nucleoli contain a discretely localized, tightly integrated, self-sufficient system capable of carrying out protein synthesis, and that this system conducts activation of amino acids and transfer of the activated amino acids to tRNAs by the same mechanism as the cytoplasmic system. We believe these results explain certain aberrancies of the nucleolar system which led Kawashima *et al.* (1971) to the conclusion that the nucleolus has no function *in situ* as a site of protein biosynthesis through a typical ribosomal system. Considering the reports of others on amino acid incorporations into nucleolar proteins in other tissues and organisms, we believe that protein synthesis by nucleoli may prove to be a generally occurring function of cellular physiology. Our results do not, however, bear directly on the question of protein synthesis in the extranucleolar parts of the nucleus, a process which may occur in varying degrees in nuclei of various origins (*cf.* Ono and Terayama, 1968; Goldstein, 1970).

The discrete localization of the nucleolar system requires consideration of the question whether the various components are identical with those of the cytoplasmic system. The observation that nucleolar aminoacyl-tRNA gave nearly the same degree of incorporation of amino acid in the highly purified ribosomal system as did aminoacyl-tRNA of cytoplasmic origin indicates that the two components are at least similar enough to be interchangeable. Compared with the readily achieved preparation of the cytoplasmic aminoacyl-tRNA, the difficulties initially encountered in stabilizing, precipitating, and dialyzing the active nucleolar complex probably result more from the very small concentrations of nucleolar material rather than innate differences in stability of the two complexes. Structural and functional differences between nucleolar and cytoplasmic transfer RNAs may yet exist; differences between mitochondrial and cytoplasmic tRNAs and aminoacyl-tRNA synthetases have been reported by Buck and Nass (1968, 1969).

Knowledge of the existence of nucleolar amino acid activating enzymes and tRNAs does not of course complete the description of nucleolar protein synthesis; rather it raises questions about the nature and source of the presumably necessary messenger RNA, ribosomes, other enzymes of protein synthesis, and the type and function of protein formed. Three related questions have been explained to some extent and are discussed below.

It is in apparent contradiction to the proven participation of tRNA and postulated participation of messenger RNA that we and others (Zimmerman *et al.*, 1969; Izawa and Kawashima, 1969) have observed that exogenous RNase is not inhibitory to the nucleolar system. The long time period of amino acid incorporation also indicates that the endogenous nucleolar ribonucleases (Chakravorty and Busch, 1967) are not inhibitory. As described in the Results, we find that repeated freezing and thawing of nucleoli does render them partially susceptible to exogenous RNase. We also noted that a crude microsomal protein synthesizing system is less susceptible to RNase than a ribosomal system. From this we infer that the nucleolar protein synthesizing system is not accessible to the nucleases either because it is tightly organized and integrated within itself or is well protected by the nucleolar structure. The nucleolar structure is known to be highly compact and, although not apparently surrounded by a membrane structure, is resistant to mild attempts to lyse it by sonication, detergent, and change in pH or tonicity. The frequently observed sensitivity of nucleolar protein synthesis to DNase is probably a result of disruption of nucleolar structure with consequent effects on the integrated synthetic system, including increased susceptibility to endogenous ribonuclease.

Other problems posed by the nucleolar system are its apparent lack of need for a full complement of amino acids in the incorporation of a single amino acid and for added ATP or energy-generating components. With regard to the former, we can only state that we infer the presence of a low level of free amino acids and assume that some fraction of each of the endogenous tRNAs is charged with amino acid in the isolated nucleoli. With regard to the latter problem, we note that optimal rates for aminoacylation with a mixture of amino acids were shown to be clearly dependent upon added ATP. Furthermore, we have detected significant amounts of ATP in these isolated nucleoli and suspect that some source for regeneration of this "high-energy" phosphate exists. Negative experiments with an atmosphere of nitrogen, or addition of

KCN or dinitrophenol, appear to rule out oxidative phosphorylation as an energy source.

The nucleolus thus appears to be a more organized and complex organelle than was previously realized. At least in the case of Novikoff rat tumor, the nucleolus is now known to contain a full complement of amino acid activating enzymes, the corresponding tRNAs, and ATP. Presumably it also contains other enzymes and components of protein synthesis, and it has been shown by Liau *et al.* (1972) that these nucleoli contain a group of tRNA methylating enzymes separate from the cytoplasmic methylating enzymes. These components are in addition to the many other proteins, enzymes, and polynucleotides involved in the biosynthesis of ribosomes.

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