PROTEIN SYNTHESIS BY ISOLATED PEA NUCLEOLI

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ABSTRACT

A new method is described for the preparation of active, nucleus-free nucleoli and chromatin in relatively high purity and in sufficient quantities to permit biochemical and electron microscopic investigation. This method consists of disintegrating previously isolated nuclei by grinding with glass beads in an isotonic medium thus liberating structurally intact nucleoli and chromatin threads. Nucleoli and chromatin are then purified by differential centrifugation in Ficoll solutions. A study of the chemical composition, submicroscopic structure, and biological activity of the nucleolar preparation has been made. An equivalent study of the chromatin material has also been carried out in order to assess the significance of chromosomal contamination in nucleolar protein synthesis. The isolated nucleoli rapidly incorporate leucine-C^{14} into acid and base stable compounds *in vitro*. Such incorporation lasts for 20 minutes at 37°C and is enhanced by the addition of an energy-regenerating system and a complete amino acid mixture. It is independent of the nuclear pH 5 enzymes. The bulk of the incorporated label is recovered in the residual, ribosome-like nucleolar protein fraction and a small percentage is found in the acid-extractable basic proteins. The rate of protein synthesis by isolated nucleoli is more rapid than that occurring in the chromatin fraction. This is taken as an additional proof that the nucleolus is the principal site of protein synthesis in the interphase pea nucleus.

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INTRODUCTION

In recent years intranuclear protein synthesis has attracted considerable attention. In particular, the role of the nucleolus and the chromatin has been under scrutiny. Thus Ficq (1) and others found the nucleolus to be the most active site for protein synthesis. This finding has been disputed, notably by Carneiro and Leblond (2), and has led to considerable controversy. A discussion of these results, obtained by the techniques of autoradiography, is found in recent reviews by Sirlin (3, 4).

From the work of Allfrey and his coworkers (5–10), Rendi (11), and Wang (12), who have studied isolated nuclei and extracts from such nuclei, much has been learned about the biochemical apparatus of nuclear protein synthesis and about its basic similarities to the cytoplasmic ribosomal apparatus. In many laboratories the combination of the cytological and biochemical approach has been achieved by isolating subnuclear components such as nucleoli (13–17), but these methods have not yet been extended to protein synthesis.

In previous publications we have described amino acid incorporation by isolated pea nuclei (18). Fractionation of such nuclei (19) has established the nucleolus as the principal site of nuclear protein synthesis (20, 21). We now wish to show that nucleus-free preparations of isolated nucleoli of relatively high purity are able to incorporate labeled amino acids into protein and that such in-

corporation differs in its properties from that carried on by the chromatin prepared from the same nuclei.

MATERIALS AND METHODS

CHEMICALS: Sugar (C & H "Bakers' Special" from California Hawaiian Sugar Refining, San Francisco) was used for the preparation of young pea seedlings. Ficoll was purchased from Pharmacia, Uppsala. ATP, phosphocreatine sodium hydrate, RNase (5 x recrystallized) and DNase (crystalline) was obtained from Sigma, St. Louis. Creatine kinase was purchased from California Corp. Biochemical Research, Los Angeles. L-leucine-C^{14} (24 μc/μM) was obtained from New England Corp., Boston. Puromycin was the generous gift of the American Cyanamid Corp. 62 μ glassbeads "superbright" were obtained from Minnesota Mining and Manufacturing Co., Minneapolis.

PREPARATION OF NUCLEI: The subnuclear fractions were prepared from previously isolated nuclear material. Nuclei were obtained from 2 to 4 kg of 48-hour-old axes of germinated peas. 100 to 150 lb of pea seeds (var. Alaska) were washed with detergent, soaked in cold running water for 12 hours, and sprayed continuously for an additional 36 hours in 30 gallon barrels (40 to 50 lb/barrel). The young seedlings were harvested by a semi-automatic 3-step process involving the liberation of the axes by crushing the pea seeds between grooved rollers, a flotation of the axes from the cotyledons in a sucrose solution (approximately 0.3 M), and a sieving in a mechanical shaker. From this the young seedlings were recovered in high purity and active state.

This material was sterilized with 20-fold diluted Clorox at room temperature for 3 minutes, vigorously rinsed with distilled water, soaked for 10 minutes in 0.05 M $CaCl_2$ at 0°C, and rinsed 4 times with ice-cold distilled water.

The nuclei were gently liberated from tissue by passing the young axes through the "pea popper" (22). The nuclei were sedimented from the roller mill–produced homogenate (containing a final concentration of 0.45 M sucrose, 0.002 M $CaCl_2$) at 350 g for 10 minutes and were used as a starting material for the isolation of nucleoli and chromatin.

The nuclei could be extensively purified, without marked loss of activity, by centrifugation through a Ficoll density gradient (Ficoll 25 to 5 per cent; 0.4 M sucrose; 0.002 M $CaCl_2$ and 0.001 M tris; final pH 7.2) in the Spinco No. 25 at 8000 RPM for 15 minutes.

PREPARATION OF NUCLEOLI: All operations were carried out at a temperature of 0 to 4°C unless otherwise specified. Immediately upon recovery of the nuclei the pellet was stirred vigorously together with an equal volume of 62-μ glassbeads for 75 seconds in a heavy-walled 12 ml Servall centrifuge tube with a plastic stirrer. By this glassbead technique the nuclei are quantitatively disintegrated to liberate nucleoli and chromatin threads in high yield. Rupture of nuclei was greatly reduced if the whole tissue or the isolated nuclei had been previously treated with an excess of Ca ions. The nuclear homogenate was next diluted 10-fold by the addition of medium A (0.4 M sucrose; 0.0005 M $CaCl_2$; 0.0001 M KPO_4; 0.0001 M $MgCl_2$; 5 per cent (w/v) Ficoll; final pH 7.2). The glassbeads were removed by centrifugation at 50 g for 5 minutes and the crude nucleolar preparation recovered at 1200 g for 6 minutes in a swinging bucket table model centrifuge. The supernatant was retained for the preparation of chromatin.

The nucleolar pellet was then resuspended in 15 ml of medium B (like medium A, but 25 per cent (w/v) Ficoll, final pH 7.2) and the suspension centrifuged in a Spinco SW39 rotor at 2000 g for 5 minutes. The pellet was discarded and the nucleoli then pelleted by centrifugation at 8000 g for 5 minutes. The pellet was resuspended in medium C containing 200 μg DNase/ml and incubated for 2 to 4 minutes at 37°C accompanied by gentle syringing. This treatment was sufficient to break up the contaminating chromatin threads to such an extent that they no longer sedimented at the same rate as nucleoli. The reaction mixture was chilled by addition of 5 to 8 volumes of ice-cold medium B and the suspension again recycled as described above. The fraction sedimenting between 1250 g and 6500 g was collected. Preparations which, after staining with methylene blue, showed significant chromosomal contamination, as determined by the light microscope, were discarded.

Starting from 2 to 4 kg of 48 hour old pea axes, we were able to obtain 5 to 10 mg nucleoli using the procedure noted above. This represents a 20 to 40 per cent recovery from the nuclei previously isolated from this tissue.

PREPARATION OF CHROMATIN: For the preparation of chromatin, the initial nucleolar supernatant was centrifuged in the Servall centrifuge at 1500 g for 5 minutes, to remove contaminating nucleoli, and the chromatin was then sedimented at 6000 g for 10 minutes. The pellet was resuspended in 0.5 ml medium A in thick-walled 12-ml centrifuge tubes and underlayered with 1.5 ml medium B and centrifuged at 12,000 g for 15 minutes. The supernatant was decanted and the chromatin pellet used for further studies. A few nucleoli are found in this fraction, as are some starch granules. Electron micrographs showed no identifiable cytoplasmic contamination in either the nucleolar preparation or the chromatin.

PREPARATION OF THE NUCLEAR PH 5 FRACTION: The initial nuclear preparation was

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ground vigorously with an equal amount of glass-beads as specified above. Grinding was continued until light microscope examination showed that most of the nucleoli had been disintegrated as well. The homogenate was then diluted with 5 volumes of 0.001 M tris + 0.0001 M $MgCl_2$ and the glassbeads were removed by low speed centrifugation. The turbid supernatant was then transferred to stainless steel Spinco tubes and the particulate matter pelleted at 105,000 *g* in a 40 Spinco rotor for 120 minutes. The supernatant was carefully removed and brought to pH 4.8 with 1 M acetic acid in the cold. The precipitation was recovered by sedimentation at 12,000 *g* for 20 minutes in the Servall centrifuge and the pellet dissolved in 0.005 M tris (pH 7.2) with gentle homogenization and readjusted to pH 7.2.

INCUBATION AND CHEMICAL EXTRACTION: The subnuclear fractions were incubated as specified in Table III at 37°C. Controls were kept at 0°C for the same time interval and treated later like incubated samples. Those proteins soluble at low ionic strength were extracted by bringing the incubation mixture to a concentration of 0.1 M tris; 0.003 M $CaCl_2$; pH 7.4. A 1000-fold excess of unlabeled amino acid was simultaneously added. After stirring at 0°C, the suspension was centrifuged at 14,000 *g* for 10 minutes in the Servall centrifuge, and the extraction repeated. Basic proteins were prepared from the residue by 0.2 N HCl extraction (twice at 0°C; 4 hours each), leaving the residual proteins. The basic proteins were precipitated by addition of $\frac{1}{20}$ volume of concentrated NH_3 and 2 volumes of ice-cold ethanol.

CHEMICAL DETERMINATIONS: RNA and DNA were usually measured by the methods of Ogur and Rosen (23). RNA determination was also carried out by the orcinol method (24), and DNA determined according to Burton (25). Protein was determined by the biuret reaction (26) and the Lowry procedure (27).

WASHING PROCEDURE: The extracted or total protein fractions were precipitated with 10 per cent perchloric acid containing 2 per cent unlabeled DL-leucine, kept at 100°C for 15 minutes and chilled in an ice bath. The solutions containing the basic protein fractions were titrated with NaOH to approximately pH 9, and two volumes of ice-cold ethanol were added to complete precipitation. The precipitates of all fractions were homogenized and washed with 2 ml washing medium (20 per cent perchloric acid; 0.5 per cent DL-leucine) at 0°C, dissolved twice in 1 M NaOH (1 per cent DL-leucine), and precipitated twice with 20 per cent perchloric acid (final). The pellets were then washed three times with 1 to 2 ml washing medium, followed by a hot ethanol and a hot ethanol-ether (1:1) wash. The pellets were dissolved in 0.4 ml 88 per cent formic acid, and aliquots were used for the determination of protein and of radioactivity. C^{14}-activity was measured in a Nuclear Chicago low background counter equipped with a micromil window.

TABLE I

Chemical Composition of Ficoll-Sucrose Isolated Nuclei, Nucleoli, and Chromatin

(Sum of RNA, DNA, and protein is taken as 100 per cent)

	RNA	DNA	Total protein	Weak saline extractable proteins	Basic proteins	Residual proteins
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Nuclei	8	14	78	12	25	41
Nucleoli	11	5	84	7	18	59
Chromatin	12	24	64	6	27	31

RESULTS

Nucleoli, prepared as described above, consist of three principal constituents: RNA, DNA, and protein. As shown in Table I, these nucleolar preparations contain 11 per cent RNA. This value is slightly lower than that obtained from nucleoli prepared by the sucrose-citrate fractionation procedure (19). The small amount of DNA persists even in fractions which appear by electron microscopic examination to be of high purity. The proteins of the nucleolus account for the bulk of the nucleolar mass. $\frac{1}{5}$ to $\frac{1}{4}$ of these proteins are acid extractable, base precipitable, and are, therefore, basic. The proportion of 0.1 N tris-extractable proteins is low (7 per cent), whereas the residual protein fraction amounts to 59 per cent of the nucleolar mass. The amino acid composition of this residual protein is given in Table II.

The chromatin fraction of nuclei possesses a protein:nucleic acid ratio of 1.7 to 2. This is in marked

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contrast to the corresponding ratio for nuclei (3.6) or nucleoli (5.0). The chromatin fraction is slightly richer in protein than that obtained from the same tissue by different methods as reported by Huang and Bonner (28), but is similar in composition to Chromatin I obtained by the sucrose-citrate fractionation from previously purified nuclei (19). Basic proteins occur in amounts approximately equal to the DNA. Chromatin contains also a considerable amount of RNA and residual proteins.

TABLE II

Amino Acid Composition of Nucleolar Residual Proteins

The nucleic acids were extracted from nucleolar preparations by the SDS (sodium dodecyl sulfate) method according to Wallace *et al.* (35) prior to the 24 hour hydrolysis in 6 N HCl. The hydrolysate was assayed in the automatic amino acid analyzer as described by Stein and Moore. Tryptophan was estimated by the method of Bencze *et al.* (36). Correction for threonine + 10 per cent; serine + 15 per cent; methionine + 3 per cent.

Amino acid	$\mu\mu$moles per 100 $\mu\mu$moles recovered amino acids	Amino acid	$\mu\mu$moles per 100 $\mu\mu$moles recovered amino acids
Asparagine	10.2	Isoleucine	4.9
Threonine	4.6	Leucine	8.6
Serine	6.5	Tyrosine	2.2
Proline	4.7	Phenylalanine	3.9
Glutamic acid	12.9	Lysine	9.2
Glycine	7.6	Histidine	2.0
Alanine	8.3	Arginine	6.4
Valine	6.7	(Tryptophan	
Methionine	1.3		approx. 1.5)

Figs. 1 to 6 show the subnuclear fractions as seen under the electron microscope. Fig. 1 shows a typical cross-section through our nucleolar preparation, and Fig. 2 represents a survey of dried-down chromatin. Figs. 3 to 6 are pictures at higher magnification and show more detailed structures as described in the legend.

The requirements for maximal incorporation of L-leucine-C^{14} into acid and base stable compounds by nucleoli are shown in Table III. The incorporation is energy dependent and is enhanced by the addition of a complete amino acid mixture. Nuclear pH 5 fraction has little effect and nuclear 105,000 *g* supernatant obtained from nuclear homogenates is inhibitory, possibly due to dilution of the C^{14}-leucine pool. RNase added at the beginning of the incubation produces only moderate inhibition. DNase is not effective. Puromycin reduced incorporation by half. Preincubation of the nucleoli in medium A with these same materials for 5 minutes at 37° doubled the effect of the RNase, but did not enhance the inhibition of amino acid incorporation by puromycin or DNase.

The characteristics of leucine incorporation into chromosomes are depicted in Table IV. Like incorporation into nucleoli, it is enhanced by the presence of an energy-regenerating system and of a complete amino acid mixture. The addition of the nuclear pH 5 fraction in this case causes a slight increase in incorporation. RNase, and to an even greater extent, DNase are both effective in reducing the incorporation rate. The effect is accentuated by preincubation in both cases.

A time course study of the L-leucine-C^{14} incorporation by Ficoll-purified nuclei, nucleoli, and chromatin is given in Table V which shows that initial incorporation rates are similar for all three fractions. However, as the incubation proceeds, incorporation into nucleoli and chromatin falls off more rapidly than incorporation into nuclei. While up to 50 $\mu\mu$moles of labeled leucine are incorporated per mg of nuclear protein, only about 20 $\mu\mu$moles per mg are incorporated by the nucleolar fractions, and chromatin incorporates to a somewhat lesser extent. Examination of the types of protein synthesized during *in vitro* incubation of nucleoli and chromatin showed that L-leucine-C^{14} is incorporated into all protein fractions. Residual proteins are the most highly labeled in both cases. Labeling of the acid-extractable fraction occurs mainly in the nucleolar fraction (Table VI).

DISCUSSION

We have now developed methods which enable us to obtain active nucleoli and chromatin of relatively high purity from pea nuclei and in sufficient quantities to permit biochemical and electron microscopic investigations. Thus we have obtained nucleoli of apparent structural integrity which are rich in protein and RNA, as well as DNA-rich chromatin whose appearance in the electron microscope seems largely due to laterally aggregating

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and anastomosing nucleohistone strands (Figs. 2 and 6).

The composition of our nucleoli compares favorably with that described by Vincent (13) in his extensive paper on isolated sea urchin nucleoli. Our nucleoli contain more RNA, perhaps due to our isotonic extraction method and the use of Ficoll which preserves nucleolar structure. As in Vincent's case, we also find that the bulk of the nucleolus is protein. The majority of this protein consists of the residual protein with an amino acid composition very similar to that of cytoplasmic

TABLE III

Characterization of L-Leucine-C^{14} Incorporation into Nucleoli

Incubation mixture (1 ml): 1.06 mg nucleolar proteins; 1 μM $CaCl_2$; 1 μM $MgCl_2$ 10 μM tris pH 7.4; 150 μM sucrose; 50 μg Ficoll; 9.6 μg L-leucine-C^{14} (24 μc/μM); 0.1 μM ATP; 10 μM phosphocreatine sodium salt; 100 μg kinase; 6.1 μg L-alanine; 13.2 μg L-arginine; 4.7 μg L-aspartic acid; 4.7 μg L-asparagine; 1.8 μg L-cysteine; 9.0 μg L-glutamic acid; 9.0 μg L-glutamine; 4.4 μg glycine; 2.0 μg L-histidine; 2.0 μg L-isoleucine; 10.2 μg L-lysine; 1.0 μg L-methionine; 3.1 μg L-phenylalanine; 2.9 μg L-proline; 3.4 μg L-serine; 5.5 μg L-threonine; 1.0 μg L-tryptophan; 3.5 μg L-tyrosine; 4.7 μg L-valine; 0.23 mg pH 5 enzymes; 0.17 mg 105,000 *g* supernatant proteins; Incubation at 37°C aerobically for 20 min. Preincubation was 5 min. in medium A. After 5 min., incubation mixture was completed and the samples were incubated for an additional 20 min.

Incubation system	μμmoles of L-leucine-C^{14} incorporated per mg protein
Nucleoli alone	7.6
" + ATP + energy-regenerating system	10.5
" " " + amino acid mixture	12.5
" " " " + nuclear pH 5 fraction	12.8 (complete)
" " " " + " 105,000 *g* supernatant	9.5
Nucleoli (complete) + 100 μg RNase	10.3
+ 100 μg DNase	11.8
+ 100 μg puromycin	6.5
Nucleoli (complete) preincubated in presence of 100 μg RNase	8.1
" " " " 100 μg DNase	13.2
" " " " 100 μg puromycin	7.1

TABLE IV

Characterization of L-Leucine-C^{14} Incorporation into Chromatin

Incubation mixture, incubation, and preincubation as specified in Table III

Incubation system	μμmoles of L-leucine-C^{14} incorporated per mg protein
Chromatin alone	6.5
" + ATP + energy-regenerating system	8.5
" " " + amino acid mixture	9.3
" " " " + nuclear pH 5 fraction	10.5 (complete)
Chromatin (complete) + 100 μg RNase	8.0
" + 100 μg DNase	6.3
Chromatin (complete) preincubated in presence of 100 μg RNase	7.1
" " " " 100 μg DNase	5.5

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ribosomes (21, 29). There is about 20 per cent basic, non-histone (13, 21) protein. This occurs in many-fold excess of the DNA. It is hard to decide whether the small but persistent content of DNA (also observed in other investigations (15, 30)) is due to a minor chromosomal contamination or is a part of the nucleolus proper. Electron micrographs of nucleoli and chromatin (Figs. 1 to 6) may provide some evidence concerning the DNA content of the nucleolar fraction. Thus occasionally in sections of nucleolar preparations we observe blebs attached to the nucleolar bodies. While their structure resembles that of the rest of the nucleolus, the size of their constituent granules when stained with lead is clearly different from that of the granules found in the nucleolus proper. In sections of isolated nucleoli prepared by other methods, such blebs show a character which appears to combine the granular structure of nucleoli with the stranded nature of chromatin. Quite similar structures have been described in thin sections of plant nuclei by Peveling (31) and Rossner (32) who call them chromocenters. We consider that these blebs may represent nucleolus-associated chromatin, *i.e.* nucleolar organizers, and may, therefore, account

FIGURES 1 TO 6

Electron micrographs of pea nucleoli and chromatin. Sections were made from material fixed in OsO_4, embedded in Epon, and post-stained as indicated. The chromatin suspension was dropped on a carbon-coated grid, stained briefly with 1 per cent uranyl acetate, rinsed in distilled water, and allowed to air dry. The dark line represents 1 micron.

FIGURE 1

A section through a pellet of isolated nucleoli. Uranyl acetate stain. The nucleoli appear as round, well defined objects and contain large vacuoles. Starch, the major contaminant, can be seen as large, white, undifferentiated granules. Some chromatin contamination is usually present. × 5000.

FIGURE 2

Isolated chromatin, showing the characteristic stranded structure. × 37000.

FIGURE 3

Portion of a nucleolus *in situ* in a root tip cell. Stained with uranyl acetate and lead perchlorate. The nucleolus is differentiated into an amorphous region and a largely peripheral particulate region. The diameter of these particles is 200 A. Large deposits of stain are associated with the amorphous region. × 52000.

FIGURE 4

Portion of an isolated nucleolus from the same tissue. Stained with uranyl acetate and lead perchlorate. The difference between the amorphous and the particulate regions is less clear. The dense deposits of stain are no longer present. × 41000.

FIGURE 5

An isolated nucleolus stained with lead perchlorate only. In this picture it is evident that the particulate material is embedded in an amorphous matrix. On one side a dark staining bleb can be seen. The particles contained within this bleb are larger and denser than those observed in the peripheral regions of the nucleolus proper. It is thought that this bleb may represent a nucleolar organizer. × 22,000.

FIGURE 6

Isolated chromatin. The mass of material forms a network. This may fray out into increasingly finer threads. The finest strands observed measure about 35 A in diameter and appear identical to molecularly dispersed nucleohistone (33). The structurally unresolved background material may be derived from chromosomal RNA and residual protein which occur in these preparations in considerable amounts. × 41000.

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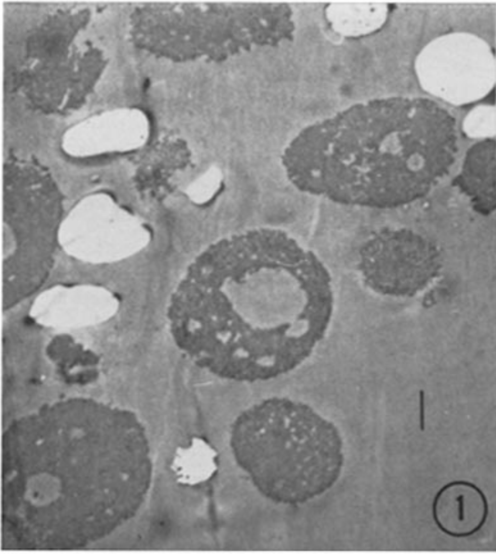

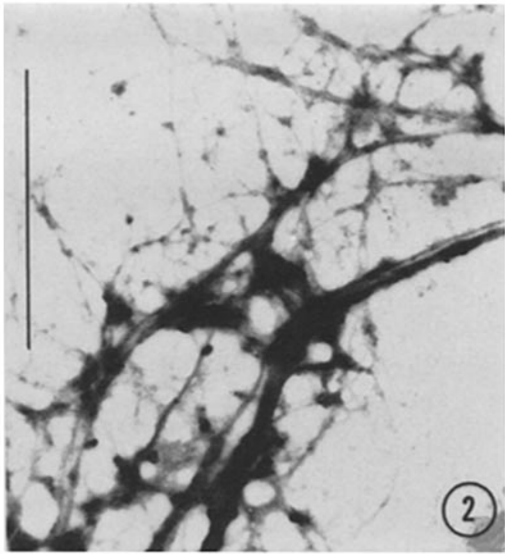

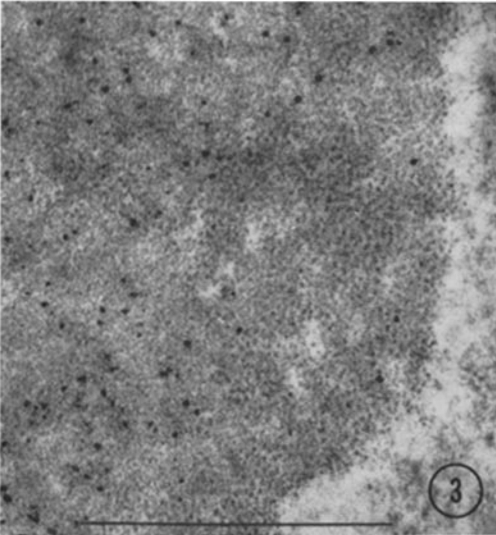

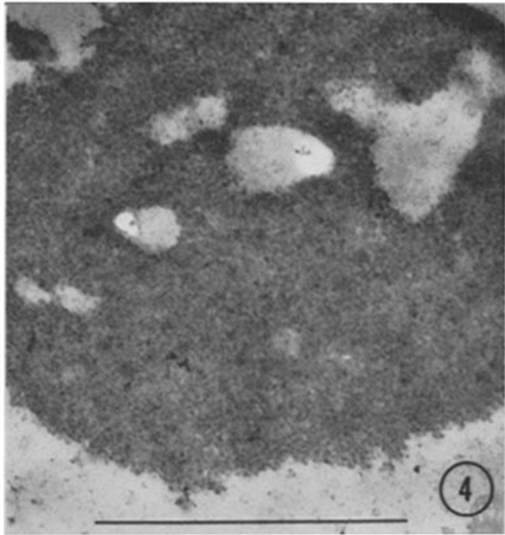

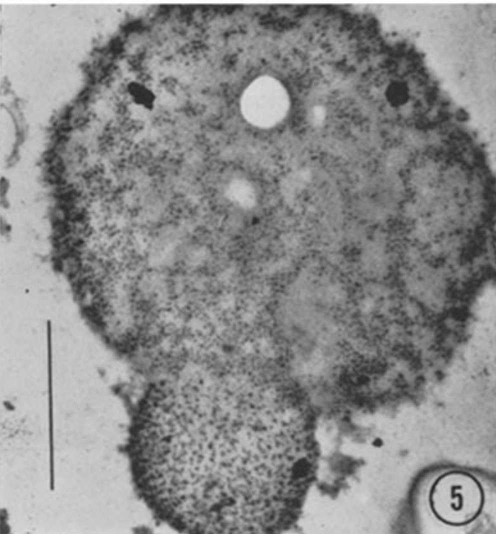

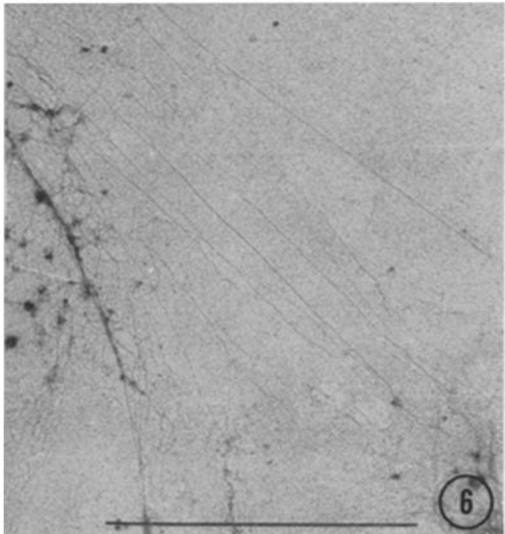

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in part for the DNA found in our nucleolar preparations. It will be noted that an isolated nucleolus (Fig. 4), like that in an intact cell (Fig. 3) is still surrounded by granules, although some of them have been lost during the isolation. These granules possess a diameter of 200 A and strongly resemble the cytoplasmic ribosomes found in sections through pea root cells.

In order to evaluate further the importance of contaminating chromatin, we have also made a study of the composition, structure, and activity of chromatin. The chromatin fraction prepared for this purpose is essentially composed of nucleoplasm fragments which have retained high organization and contain, in addition to nucleohistone (with a DNA:histone ratio close to 1), a considerable amount of RNA, 0.1 N tris-extractable proteins, and residual proteins.

Figs. 2 and 6 show electron micrographs of air-dried chromatin. Its structure consists of finely dispersed threads of considerable length (> 10,000 A). These may anastamose to form thicker strands or may ramify into a fine network. Strands as thin as 35 A can be found and must, therefore, represent molecularly dispersed nucleohistone (33). These are accompanied by a structurally unresolved background material, presumably constituted of the chromosomal residual protein and RNA. Both isolated subnuclear fractions were found to be active in amino acid incorporation, although at a low level. Since such incorporation is dependent on an energy-regenerating system and is enhanced by the addition of a complete amino acid mixture, and since the label is recovered in acid and base stable compounds, we consider that the C^{14}-leucine is incorporated intramolecularly into peptide linkages.

TABLE V

Kinetics of L-Leucine-C^{14} Incorporation into Ficoll-Purified Nuclei, Nucleoli, and Chromatin

The sum of RNA, DNA, and protein of Table I is taken as a measure of dry weight.

Incubation time	Nuclei* $\mu\mu$moles per mg protein	Nucleoli‡ $\mu\mu$moles per mg protein	Chromatin‡ $\mu\mu$moles per mg protein
5	4.3	5.8	4.4
10	12.0	10.4	7.9
20	25.7	15.3	13.6
30	35.3	16.6	13.7
40	43.0	—	—
	$\mu\mu$moles per mg dry weight	$\mu\mu$moles per mg dry weight	$\mu\mu$moles per mg dry weight
30	25.6	13.1	8.5

* Incubation mixture as in reference 3.
‡ Incubation as specified in Table III.

TABLE VI

L-Leucine-C^{14} Incorporation into the Protein Fractions of Nucleus-free Nucleolar and Chromatin Fraction

Preparation	Fraction	$\mu\mu$moles incorp. per mg protein fraction	$\mu\mu$moles incorp. per 10 mg [dry weight] nucleoli or chromatin
Nucleoli	Weak saline-extractable proteins	9.2	6.4
	Basic, non-histone proteins	7.2	10.8*
	Residual proteins	14.0	89.1
Chromatin	Weak saline-extractable proteins	5.2	3.1
	Histones	1.8	4.9*
	Residual proteins	11.2	34.7

* Corrected for loss of material caused by washing procedure.

Amino acid incorporation into nucleoli is not completely inhibited by puromycin. In this attribute it may be compared to the chloramphenicol insensitivity of protein synthesized by calf thymus nuclear ribosomes (12). In its reaction to ribonuclease the nucleolus stands midway between nuclear ribosomes, which are completely sensitive (10), and intact nuclei, which are insensitive (5, 11). In the case of DNase, a brief preliminary treatment with this enzyme is involved in the preparative procedure for nucleoli. The nucleolus remains active and further incubation or preincu-

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bation with this enzyme does not produce inhibition of C^{14}-leucine incorporation, but we appreciate that any system dependent on the presence of newly synthesized, DNA-primed RNA may have been inactivated. However, we conclude for this *in vitro* system that amino acid incorporation into nucleoli, in contrast to that into chromatin, is DNase insensitive. Because of this difference we can distinguish between the two types of incorporation. Since chromatin is less active in amino acid incorporation than is the nucleolar fraction, and is, in addition, DNase sensitive, we can exclude the possibility that amino acid incorporation by the nucleolar fraction is due to chromatin contamination.

The differences observed between the characteristics of incorporation by the two fractions are also apparent in the specific activities of the several protein fractions. Thus, in the nucleolar fractions all three types of proteins are labeled, whereas in the chromatin only the residual protein contains appreciable activity. The specific activity of the chromosomal histone fraction is low.

The presence of high activity in the residual fraction may indicate synthesis of residual protein or, since *in vitro* systems in general exhibit poor release of newly synthesized proteins, it may indicate that this fraction contains newly synthesized peptides still bound to the template. These peptides, of course, may or may not be of residual protein type.

While it is difficult to compare the activities of the two fractions in protein synthesis, since we can obviously measure only survivor activities, nevertheless the over-all picture is one indicating that the nucleolus is more active in protein synthesis per unit (protein or dry weight) than is the chromatin—in particular incorporates more C^{14}-leucine into acid-extractable proteins than does the chromatin. These results are remarkably similar to those obtained by *in vitro* incubation of whole nuclei (21) and by *in vivo* studies on subnuclear protein synthesis of nuclei in the post DNA synthetic phase (34).

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REFERENCES

1. Ficq, A., *Exp. Cell Research*, 1955, **9**, 286.
2. Carneiro, J., and Leblond, C. P., *Science*, **129**, 391 (1959).
3. Sirlin, J. L., The Cell Nucleus, New York, Academic Press, 1960.
4. Sirlin, J. L., *Progr. Biophys. and Biophys. Chem.*, 1962, **12**, 27.
5. Allfrey, V. G., *Proc. Nat. Acad. Sc.*, 1954, **40**, 881.
6. Allfrey, V. G., Mirsky, A. E., Osawa, S., *J. Gen. Physiol.*, 1957, **40**, 451.
7. Hopkins, J. W., *Proc. Nat. Acad. Sc.*, 1959, **45**, 1461.
8. Allfrey, V. G., and Mirsky, A. E., Symposium on Protein Synthesis, London, Academic Press, 1960.
9. Frenster, J. H., Allfrey, V. G., and Mirsky, A. E., *Proc. Nat. Acad. Sc.*, 1960, **46**, 432.
10. Frenster, J. H., Allfrey, V. G., and Mirsky, A. E., *Biochim. et Biophysica Acta*, 1961, **47**, 130.
11. Rendi, R., *Exp. Cell Research*, 1960, **19**, 498.
12. Wang, T. Y., *Biochim. et Biophysica Acta*, 1961, **49**, 108.
13. Vincent, W. S., *Proc. Nat. Acad. Sc.*, 1952, **38**, 139.
14. Baltus, E., *Biochim. et Biophysica Acta*, 1954, **15**, 263.
15. Monty, K. J., Litt, M., Kay, E. R. M., and Dounce, A. L., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 127.
16. Johnston, F. B., Setterfield, G., and Stern, H., *J. Biophysic. and Biochem. Cytol.*, 1958, **6**, 53.
17. Edström, J. E., Grapp, W., and Schor, N., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 549.
18. Birnstiel, M. L., Chipchase, M. I. H., and Hayes, R. J., *Biochim. et Biophysica Acta*, 1962, **55**, 728.
19. Birnstiel, M. L., Rho, J. H., and Chipchase, M. I. H., *Biochim. et Biophysica Acta*, 1962, **55**, 734.
20. Birnstiel, M. L., Chipchase, M. I. H., and Bonner, J., *Biochem. and Biophys. Res. Comm.*, 1961, **6**, 161.

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21. Birnstiel, M. L., and Chipchase, M. I. H., in preparation.
22. Rho, J. H., and Chipchase, M. I. H., *J. Cell Biol.*, 1962, **14,** 183.
23. Ogur, M., and Rosen, G., *Arch. Biochem.*, 1950, **25,** 262.
24. Militzer, W. E., *Arch. Biochem.*, 1946, **9,** 85.
25. Burton, K., *Biochem. J.*, 1956, **62,** 315.
26. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, 1949, **177,** 751.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, **193,** 265.
28. Huang, R. C., and Bonner, J., *Proc. Nat. Acad. Sc.*, 1962, **48,** 1216.
29. Ts'o, P. O. P., Bonner, J., and Dintzis, H., *Arch. Biochem. and Biophysics*, 1958, **76,** 225.
30. Maggio, R., Siekevitz, and Palade, G. E., *2nd Meeting Am. Soc. Cell Biol.*, 1962.
31. Peveling, E., *Planta*, 1961, **56,** 530.
32. Rossner, W., *Chromosoma*, 1961, **12,** 717.
33. Zubay, G., and Doty, P., *J. Molec. Biol.*, 1959, **1,** 1.
34. De, D. N., *Nucleus*, 1961, **4,** 1.
35. Wallace, J. M., Squires, R. F., and Ts'o, P. O. P., *Biochim. et Biophysica Acta*, 1961, **49,** 130.
36. Bencze, W. L., and Schmid, K., *Anal. Chem.*, 1957, **29,** 1193.

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