STUDIES ON ISOLATED NUCLEI

II. Isolation and Chemical Characterization of Nucleolar and Nucleoplasmic Subfractions

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ABSTRACT

This paper describes the subfractionation of nuclei isolated from guinea pig liver by the procedure presented in the first article of the series (8). Centrifugation in a density gradient system of nuclear fractions disrupted by sonication permits the isolation of the following subfractions: (*a*) a nucleolar subfraction which consists mainly of nucleoli surrounded by a variable amount of nucleolus-associated chromatin and contaminated by chromatin blocks derived primarily from von Kupffer cell nuclei; (*b*) and (*c*), two nucleoplasmic subfractions (I and II) which consist mainly of chromatin threads in a coarser (I) or finer (II) degree of fragmentation. The protein, RNA, and DNA content of these subfractions was determined, and their RNA's characterized in terms of NaCl-solubility, nucleotide composition, and *in vivo* nucleotide turnover, using inorganic ^{32}P as a marker. The results indicate that there are at least three types of RNA in the nucleus (one in the nucleolus and two in the nucleoplasm or chromatin), which differ from one another in NaCl-solubility, nucleotide composition, turnover, and possibly sequence. Possible relations among these RNA's and those of the cytoplasm are discussed.

INTRODUCTION

In contrast with the large literature on nuclear fractions, there are relatively few reports on nuclear subfractions. Most of them deal with the isolation of nucleoli. The problems to be solved in such a project are: the rupture of the nuclear membranes, the prevention of gel formation by DNA,[1] a fine dispersion or solubilization of the nucleoplasm, and finally, the concomitant preservation of the nucleoli. Were these conditions met, the latter could be easily separated from the other nuclear materials by differential centrifugation, on account of their large size and great density.

A number of different approaches have been used so far to achieve these conditions. The first, proposed by Litt *et al.* (1) and by Monty *et al.* (2), relied on sonic vibrations to disrupt liver cell nuclei and disperse their nucleoplasm; it yielded a nucleolar fraction with a high content of DNA. In the second approach (3, 4), the "solubilization" of the nucleoplasm was obtained through Ca^{2+} removal effected by suspending the nuclei in a citrate-containing medium; the nuclear envelope

[1] The abbreviations used in this paper are: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; RNP, ribonucleoprotein; AMP, adenylic acid; CMP, cytidylic acid; GMP, guanylic acid; UMP, uridylic acid; ψ UMP, pseudouridylic acid; DOC, deoxycholate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate.

was subsequently ruptured and the nucleoplasm dispersed by stirring the suspension in a mixer. In a third approach (5), isolated nuclei were homogenized in a high density medium and the liberated nucleoli separated by differential centrifugation upon dilution of this homogenate. Finally, Vincent (6) and subsequently Baltus (7) broke cells and nuclei simultaneously, by forcing starfish oocytes through a small aperture. The freed nucleoli were subsequently isolated by differential centrifugation.

The present paper deals with a procedure for obtaining nucleolar and nucleoplasmic subfractions from nuclei isolated from guinea pig liver as previously described (8); it presents, in addition, data on the RNA's of these nuclear subfractions.

EXPERIMENTAL

General Procedures

1. Cell Fractionation

a. NUCLEAR AND SUBNUCLEAR FRACTIONS: Nuclear fractions were isolated either by our method (8) or by that of Chauveau *et al.* (9). In our procedure, the tissue is homogenized in 0.88 M sucrose–1.5 mM $CaCl_2$ and the nuclei isolated by differential centrifugation in a discontinuous density gradient (homogenate over 2.2 M sucrose–0.5 mM $CaCl_2$). In the procedure of Chauveau *et al.* (9), both tissue homogenization and nuclear isolation are carried out in 2.2 M sucrose without Ca^{2+}.

The isolation of nucleolar and nucleoplasmic subfractions will be described in detail under RESULTS.

b. CYTOPLASMIC FRACTIONS: Microsomal, postmicrosomal, and final supernate fractions were isolated from guinea pig liver following the procedures given in (10). Guinea pig hepatic and pancreatic ribosomes (RNP particles) were prepared from DOC-treated microsomal fractions as in (10) and (11).

Samples of *Escherichia coli* ribosomal RNA were obtained from Dr. D. Nathans. Samples of soluble RNA were kindly supplied by Dr. J. F. Kirsch (guinea pig liver) and Dr. G. Zubay (*E. coli*).

2. Chemical Procedures

The methods used for the determination of RNA, DNA, total N, and protein are given in (8).

3. Extraction Procedures

Phosphate extraction removes little RNA from nuclei isolated by our procedure (8); hence it was omitted from the extraction schedule of the nuclear subfractions. The 1 M NaCl extraction was extended to ~15 hours because after shorter periods large amounts of DNA remained unextracted. In each experiment nuclear subfractions isolated from 9 to 18 gm fresh liver were used, and each subfraction was extracted twice with 30 to 40 ml of 1 M NaCl, the rest of the procedure being the same as in the case of nuclear fractions (8).

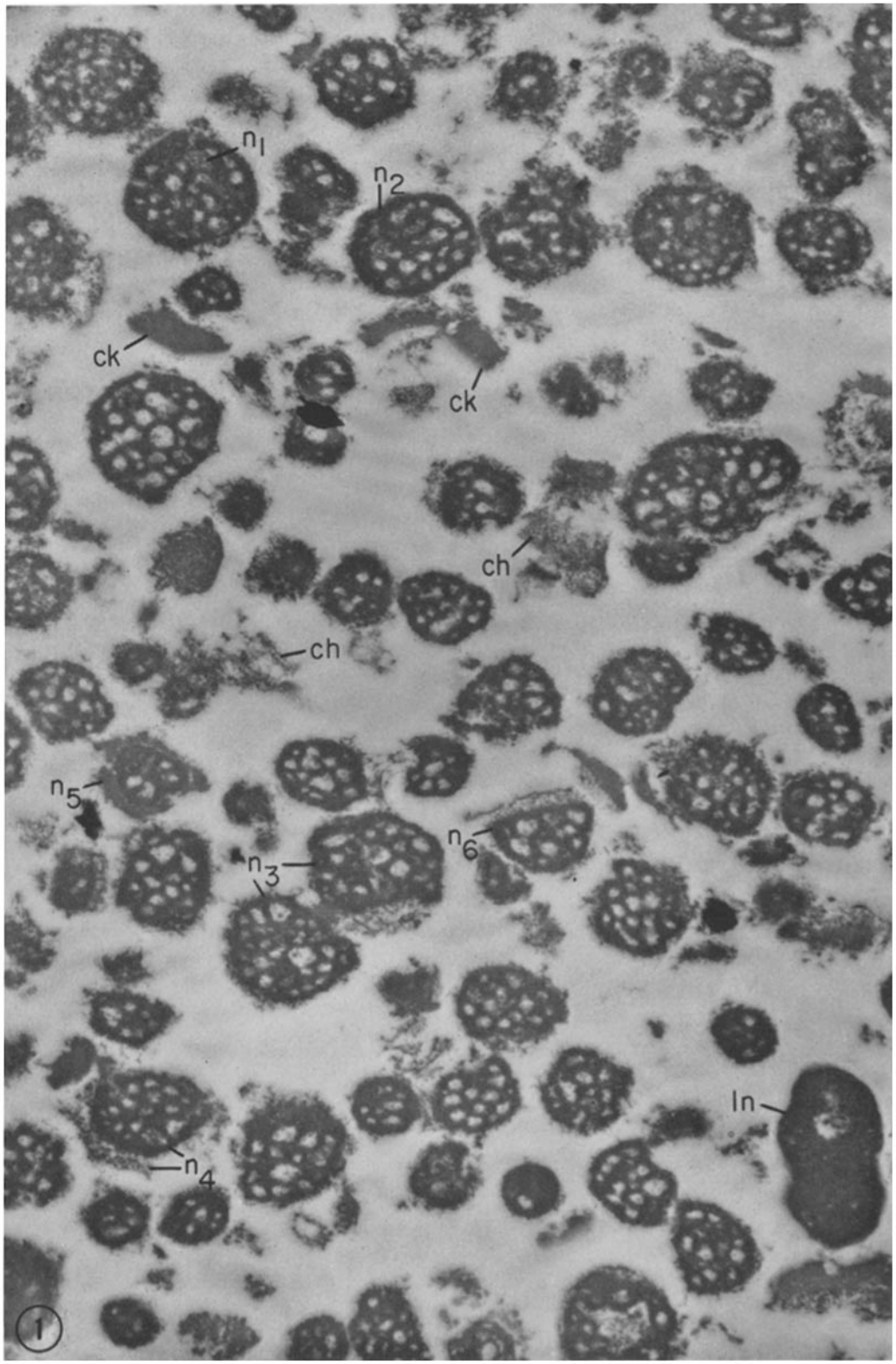
4. Radioactivity Measurements

^{32}P was administered intravenously as 1 ml inorganic phosphate solution (containing 100 μM P and 350 μC) to guinea pigs fasted for 1 day. The livers were removed under ether anesthesia at selected intervals after the injection of the label. The RNA's of the nuclear and subnuclear fractions were isolated, digested, and their mononucleotides chromatographically separated as in (8).

FIGURE 1 *Nucleolar subfraction* (middle layer). At this level, and throughout the upper ⅔ of the pellet, the preparation mainly consists of recognizable nucleoli (n_1 to n_6). Most of them contain variable amounts of chromatin either in their lacunae (n_1, n_2), or as a more or less continuous peripheral shell (n_3 to n_5) that probably corresponds to the "nucleolus-associated chromatin" described *in situ*. The nucleolus marked n_6 apparently broke away with the whole layer of chromatin that separated it *in situ* from the surface of the nucleus.

There are a few masses of recognizable chromatin derived from liver cell nuclei (*ch*); the most important contaminant of the preparation is represented by fragments of leucocytic nuclei (*ln*) and blocks of compact chromatin (*ck*) derived from von Kupffer cell nuclei.

Pellet fixed in 0.039 M OsO_4 in 0.88 M sucrose adjusted to pH 7.8 with Tris; embedded in methacrylate. Section stained with $Pb(OH)_2$. × 16,000.


n1
n2
ck
ck
ch
ch
n5
n6
n3
ln
n4
1

The final chromatograms were examined under ultraviolet light to localize the mononucleotide spots, and subsequently exposed for 3 days to Kodak "no screen" x-ray film to obtain their autoradiographs. Five autoradiographic spots were obtained, of which four were congruent in size and location with the mononucleotide spots detected in the ultraviolet. The fifth autoradiographic spot appeared well separated from those of the mononucleotides, did not absorb ultraviolet light, and was identified as inorganic phosphate by the spray reagent of Bandurski and Axelrod (12).

After the estimation of its nucleotide concentration (8), the entire extract of each spot was deposited on metal planchets, a drop of acetone added to give a uniform drying, and the solution evaporated under infrared light. Radioactivity was determined with a Nuclear Chicago gas flow counter provided with a "micromil" window. The radioactivity registered per sample varied from 500 to 3,000 CPM from one experiment to another. The specific radioactivity was obtained as CPM per mole of each nucleotide or as CPM per mg RNA. The latter value was obtained by dividing the sum of all counts recorded in the four nucleotides by the calculated amount of RNA.

For the determination of the recovery of nucleotides and radioactivity after the chromatography, total RNA and total radioactivity were measured in samples of the original alkaline RNA hydrolysate (8). (A control of radioactivity losses incurred at the extraction stage of the chromatograms was not carried out.) The results showed that nucleotide recovery amounted to 70 to 75 per cent, whereas the radioactivity recovered in nucleotides did not exceed 40 to 50 per cent of the total. Since a well separated radioactive spot was identified as inorganic phosphate, it is assumed that the lower radioactivity recovery is due to contamination of the alkaline hydrolysate by non-RNA ^{32}P. As repeatedly discussed in the past (*cf.* reference 13), contamination by non-nucleic acid ^{32}P is an important source of error when determining the radioactivity of RNA fractions isolated by the Schmidt-Thannhauser procedure. The isolated mononucleotides are apparently free of such contamination.

5. Pseudouridine Determinations

To find out whether ψ UMP is present in the RNA's of nuclear and subnuclear fractions, we used a modification of the methods of Yu and Allen (14) and Dunn (15) capable of detecting small amounts of ψ UMP, as for example that obtained from ~0.4 mg soluble RNA.

In a first step, the hydrolyzed RNA samples were submitted to electrophoresis in 0.05 M Na citrate buffer at 4° on Whatman 3 MM filter paper which caused the UMP and ψ UMP to migrate together as a spot well separated from the spots of the other mononucleotides. After electrophoresis, the paper was dried and the spot containing UMP and ψ UMP cut out and extracted with a large volume of H_2O. The extract was concentrated *in vacuo* and finally dissolved in 1 to 2 ml of 0.05 M Na acetate buffer, pH 5.3. In the second step, the mononucleotides of the concentrated extract were dephosphorylated by incubation with prostatic phosphomonoesterase at 37° for 8 to 10 hours according to the method of Schmidt (16), the reaction mixture being subsequently concentrated *in vacuo*. In the final step pseudouridine was separated from uridine by the descending chromatography of this concentrate in the *n*-butanol/water (86/14; v/v) solvent of Markham and Smith (17). In this solvent the R_f of the ψ uridine is half that of uridine (14). For adequate separation, it was found necessary to run the chromatogram for ~4 days at room temperature. ψ uridine was identified by its ultraviolet absorption spectra at pH 1 and 12 (18) and by its reaction with orcinol (18). For its quantitative determinations, an E_{260} value of 8.5×10^3 in 0.01 N HCl was used (18).

The method described was worked out with a sample of ψ UMP kindly supplied by Dr. W. E. Cohn, and was checked by determining the amounts of ψ UMP present in soluble RNA fractions obtained from various sources. By ultraviolet scanning, we were able to detect amounts of ψuridine (ψ UMP) as small as 0.025 to 0.03 μmole. When no spot was visible, the region of the paper to which ψuridine was expected to migrate was cut out and extracted (with 0.01 N HCl); spectra of these extracts were routinely recorded.

RESULTS

A. *Nuclear Subfractionation*

1. Preliminary Experiments

a. Ca^{2+} chelation: This approach was tried in a few experiments by suspending isolated nuclei in a 0.02 M citrate–0.5 mM EDTA–0.88 M or 2.2 M

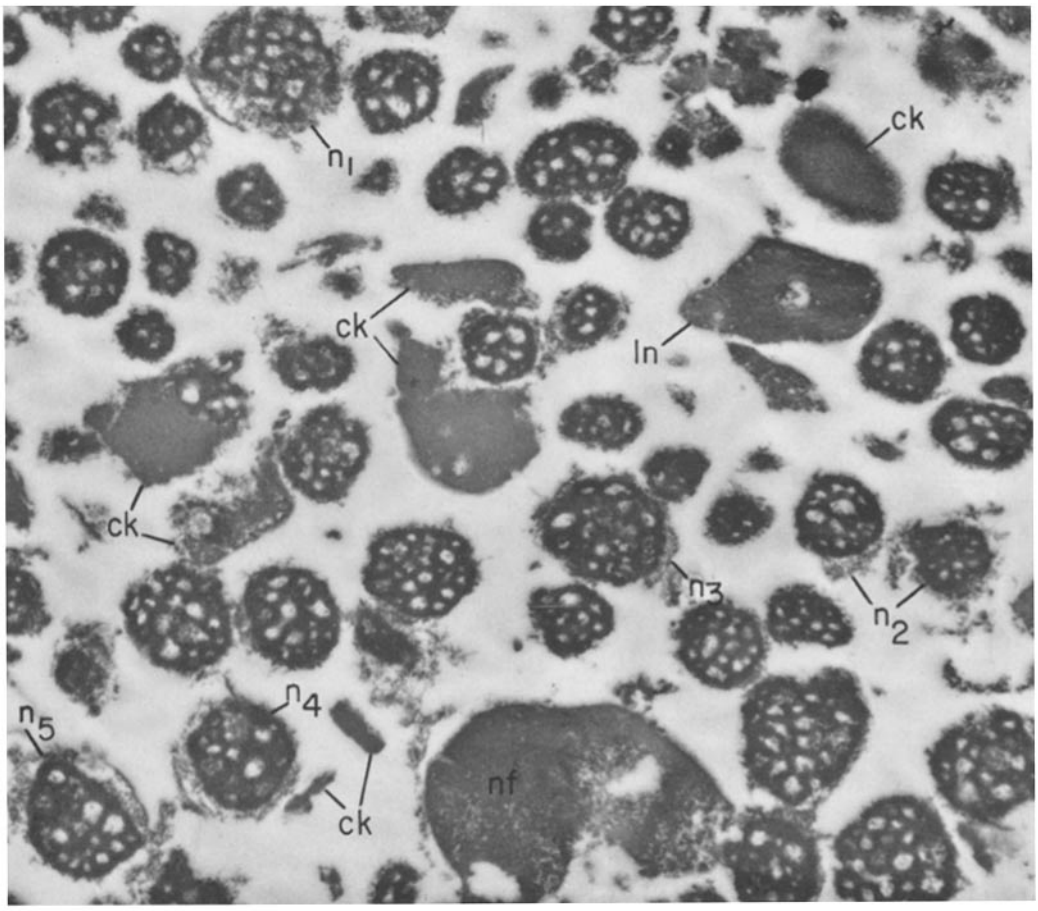


FIGURE 2 *Nucleolar subfraction* (bottom layer). Nucleoli (n_1 to n_5) are still the main component at this level in the pellet, but the contamination by chromatin blocks and larger fragments (*nf*) of compact nuclei originating from leucocytes (*ln*) and von Kupffer cells (*ck*) is noticeably increased.

Many nucleoli show a more or less continuous corona of associated chromatin (n_1 to n_4) and some came out with the whole layer of chromatin interposed between them and the surface of the nucleus (n_5).

Preparation for electron microscopy as for Fig. 1. × 16,000.

sucrose followed by 30 minutes of stirring at 4°. The suspensions were subsequently fractionated by differential centrifugation with or without a discontinuous density gradient (*cf.* reference 8). The ensuing fractions were monitored by electron microscopy and biochemical determinations. After this treatment only a few nuclei remained intact; the nucleoli did not disperse, but they did not separate from the nucleoplasm, which formed a gel. Similar RNA/DNA ratios were found in all fractions. Attempts to reduce the viscosity of the common nucleoplasm-nucleolar fractions by further dilution with citrate-EDTA or by deoxyribonuclease digestion failed.

b. DISRUPTION BY SONIC VIBRATIONS: Nuclei were resuspended in various media and subjected for varied periods to sonic vibrations in a Raytheon magnetostriction oscillator operating at 10,000 cycles/sec. the results being monitored by phase contrast microscopy. Sonication in unbuffered 0.88 M sucrose (pH ~6.5) disrupted a relatively small percentage of nuclei; the nucleoli liberated in the process appeared intact and free of coarse nucleoplasm tabs; moreover they retained their size and refractivity over long periods of storage at 4°. For this reason 0.88 M sucrose was chosen as a basal medium for further experiments carried out to increase the efficiency of nuclear disruption.

pH and ionic strength effects: The addition of 0.1 M phosphate buffer, pH 7.1, to the basal sucrose medium

caused a marked decrease in the frequency of unbroken nuclei. The freed nucleoli appeared undamaged, but their isolation was precluded by a general precipitation of the nucleoplasm which occurred shortly after sonication. A further rise in pH was tried without concomitant increase in ionic strength (*cf.* reference 19) by adjusting the pH of the sucrose solution with Tris just before use. At pH 7.5 to 8.0 only occasional nuclei remained intact; the freed nucleoli retained their size and refractivity; the nucleoplasm was finely dispersed and did not agglutinate even after prolonged storage. At pH 8.5 general solubilization of all nuclear structures occurred.

Other media, recommended for maintaining nucleohistones in solution, *e.g.* saline-EDTA (20), were also tried with or without sucrose, but found unsatisfactory, for the liberated nucleoli swelled and finally disintegrated.

Duration of sonic treatment: This variable was investigated by subjecting aliquots of a nuclear suspension to sonic vibrations for 15, 20, 25, and 27 minutes and by following by phase microscopy the frequency of intact nuclei in the treated suspensions. Intact nuclei were common in the 15 minute sample and only occasionally encountered in those treated for 20 and 27 minutes. Nuclear disruption was found to be influenced also by the concentration of nuclei in the original suspensions, which must therefore be controlled.

C. FRACTIONATION OF SONICALLY TREATED SUSPENSIONS: After trying various systems and centrifugal fields, we succeeded in separating nucleoplasm from nucleoli by using a discontinuous density gradient in which the lower phase was brought up to 2.2 M sucrose. A two (0.88 M/2.2 M sucrose) and a three (0.88 M/1.5 M/2.2 M sucrose) layer system were tried and found to be equally effective in the separation of nucleoli from nucleoplasm.

The required centrifugal field was determined by monitoring for the absence of nucleoli in the band formed at the interface between 0.88 M/2.2 M sucrose in a two layer system. Nucleoli were only occasionally encountered when the field was set at 20,000 *g* for 60 minutes. Nucleolar recovery was improved when crowding at this interface was avoided by dilution of the sonicated suspension and by using the larger interface provided by the SW 25 rotor.

2. Final Method for Subfractionating Nuclei

The best results obtained in these preliminary experiments determined the parameters of the final procedure that follows:

Nuclei isolated from 9 gm fresh liver tissue were resuspended by careful stirring with a glass rod in 0.88 M sucrose adjusted to pH 7.8 with a few drops of Tris just before use. The suspension was made up to 12 ml and divided into 4 ml aliquots, using plastic tubes for the Spinco 40.3 rotor. The tubes were capped, introduced into the water-filled chamber of the magnetostriction oscillator and submitted to vibrations of 10,000 cycles/sec.

FIGURE 3 *Part of an isolated nucleus in a nuclear fraction prepared from guinea pig liver.* The nucleus shows a large nucleolus surrounded and penetrated by chromatin threads (ch_1 and ch_2 respectively). The nucleolar mass (*nm*) has a distinct fine granular texture. The nucleolar lacunae (*l*) appear distended and "empty," presumably extracted, except for those which contain some chromatin threads. The nuclear envelope marked *ne* bears attached ribosomes (*r*) on its outer membrane.

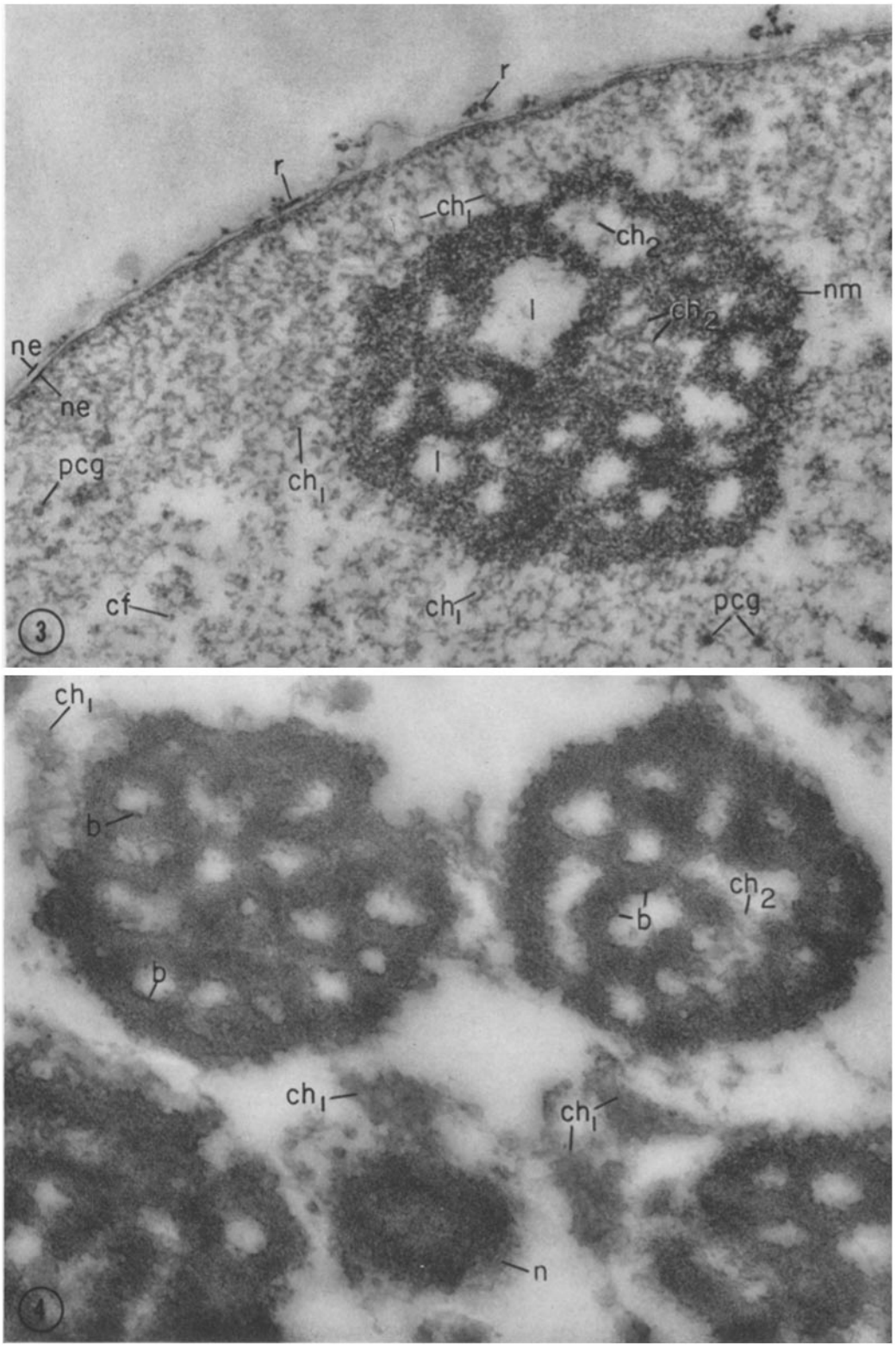
The nucleoplasm contains, in addition to chromatin threads, some large, dense granules (*pcg*) and clusters of fibrillar and/or granular material of finer texture (*cf*). The former may correspond to Watson's perichromatin granules (51).

Pellet fixed in 3.3 M HCHO in 0.1 M phosphate buffer, pH 7.6, followed by 0.039 M OsO_4 in the same buffer; embedded in methacrylate. Section stained with $Pb(OH)_2$. × 47,000.

FIGURE 4 *Nucleolar subfraction.* A comparison with Fig. 3 shows that isolated nucleoli retain the size and general morphology they have in intact nuclei; the nucleolar mass appears, however, more compact and as a result its granular texture is less clearly visible. Most lacunae are "empty" and seem to be limited by a relatively sharp boundary (*b*).

The chromatin threads have aggregated into coarser strands (ch_1, ch_2) than in intact nuclei and appear as pale, relatively homogeneous masses either in the lacunae (ch_2) or at the periphery of the nucleoli (ch_1). The nucleolus marked *n*, just grazed by the section, shows no cut open lacuna.

Pellet fixed in 0.039 M OsO_4 in 0.88 M sucrose adjusted to pH 7.8 with Tris; methacrylate embedded. Section stained with $Pb(OH)_2$. × 47,000.


r
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ch1
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b
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1

for 5 minutes, followed by 2 to 3 minutes of cooling of the tubes in an ice bath. This cycle was repeated 4 times to give a total of 20 minute sonic treatment. In a few experiments the operation was repeated until a total of 27 minute sonication was reached. After sonication, the suspension was diluted 1:3 with 0.88 M sucrose, adjusted to pH 7.8 with Tris, and 16 to 18 ml aliquots thereof were layered on 10 ml of 2.2 M sucrose adjusted in the same way to the same pH. The two layer gradients were subsequently centrifuged at 20,000 *g* for 60 minutes in an SW 25 rotor. At the end of the centrifugation the tubes showed: (*a*) A transparent supernate (*nucleoplasmic subfraction II*) in the upper layer which was collected by pipetting. It was optically empty in the phase contrast microscope, but upon centrifugation at 105,000 *g* for 120 minutes (rotor No. 40) gave a visible pellet and a supernate, hereafter referred to as *final supernate*. Both contained TCA precipitable nucleoproteins or proteins. (*b*) A band of white material (*nucleoplasmic subfraction I*) at the 0.88 M/2.2 M interface, which was collected by cutting the tube below it. In the phase contrast microscope it contained filamentous elements and occasionally a few nucleoli. Upon dilution with H_2O to a final sucrose concentration of ~0.88 M and centrifugation at 60,000 *g* for 60 minutes (rotor No. 40) it yielded a supernate and a white pellet. The latter contained all the TCA precipitable material of the band. (*c*) A pellet at the bottom (*nucleolar subfraction*) which in the phase contrast microscope appeared to consist mainly of nucleoli with a few contaminating filamentous structures, presumably chromatin threads. The electron microscopy of these nuclear subfractions is described in detail in the next section.

3. Electron Microscopy

a. METHODS Preliminary experiments showed that the final appearance of isolated nucleoli was markedly influenced by the fixation procedure. By comparison with controls maintained in the sonication medium, nucleoli swelled and lost density when fixed in 3.3 M formaldehyde in 0.88 M sucrose with acetate-Veronal buffer (final pH ~8.3); or in 0.039 M OsO_4 in 0.88 M sucrose buffered with acetate-Veronal (final pH 7.8), or with 0.1 M phosphate (final pH 7.1). Similar results were obtained when the isolated nucleoli were fixed first in HCHO and then in OsO_4 following procedures (*b*) and (*c*) described in (8). There was no detectable swelling even after 15-hour suspension in 0.039 M OsO_4 in 0.88 M sucrose when the concentration of the phosphate buffer was reduced to 0.05 M and the pH lowered to 6.0 or 5.5 Equally satisfactory results were obtained with 0.039 M OsO_4 in 0.88 M unbuffered sucrose or in 0.88 M sucrose adjusted to pH 7.8 with Tris.

The last two mixtures were used to fix *in situ* pellets of nuclear subfractions obtained as described, the other conditions for fixation, embedding, and microscopy being the same as given in (8) for nuclear pellets.

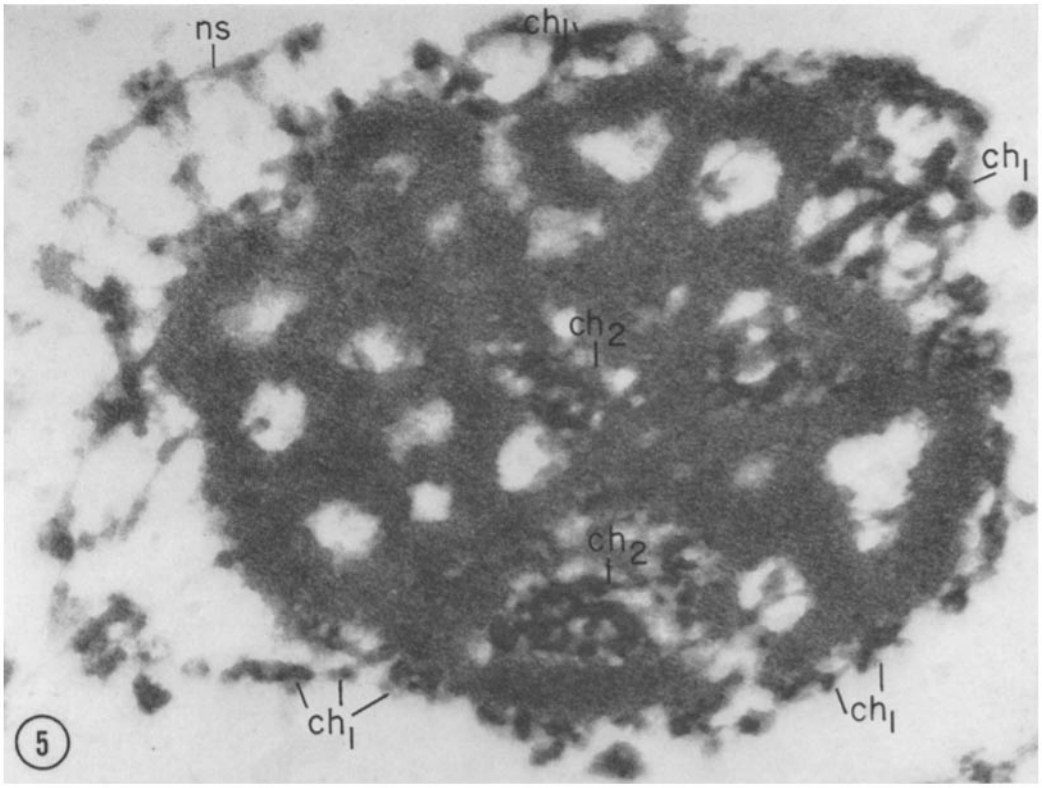
b. RESULTS: *Nucleolar subfractions:* The pellet was mainly comprised of recognizable nucleoli which generally retained the size and shape they had in intact liver cells and in isolated nuclei (Fig. 1). The nucleolar lacunae were, however, frequently enlarged and their content diluted or extracted. Conversely the material surrounding the lacunae appeared more condensed than *in situ* and its granular texture was less evident (*cf.* Figs. 3 and 4). In many places the dense nucleolar material was sharply delineated towards the outside of the lacunae, giving the impression that a definite boundary existed at that level. In many other places the nucleoli appeared in continuity with recognizable chromatin material located either within the lacunae or at the periphery of the nucleolar masses (Figs. 5 and 6). This association was clearly demonstrated in sections stained

FIGURES 5 and 6 These micrographs show isolated nucleoli surrounded by a shell of nucleolus-associated chromatin (ch_1) which appears thin and more or less continuous in Fig. 5, and discontinuous but thicker in Fig. 6. The chromatin located in some nucleolar lacunae is marked ch_2. Remnants of nuclear surface are visible in Fig. 5 at *ns*.

The nucleolar mass, which in this preparation appears less dense than chromatin, is condensed to a point that obscures its granular texture.

Differences in the size of the nucleolar profiles and in the number and size of their lacunae are mainly due to the position of the sections: medial in Fig. 5, lateral in Fig. 6.

Pellet fixed in 0.039 M OsO_4 in 0.88 M sucrose adjusted to pH 7.8 with Tris; embedded in methacrylate. Sections stained with uranyl acetate followed by $Pb(OH)_2$. Fig. 5, × 78,000; Fig. 6, × 66,000.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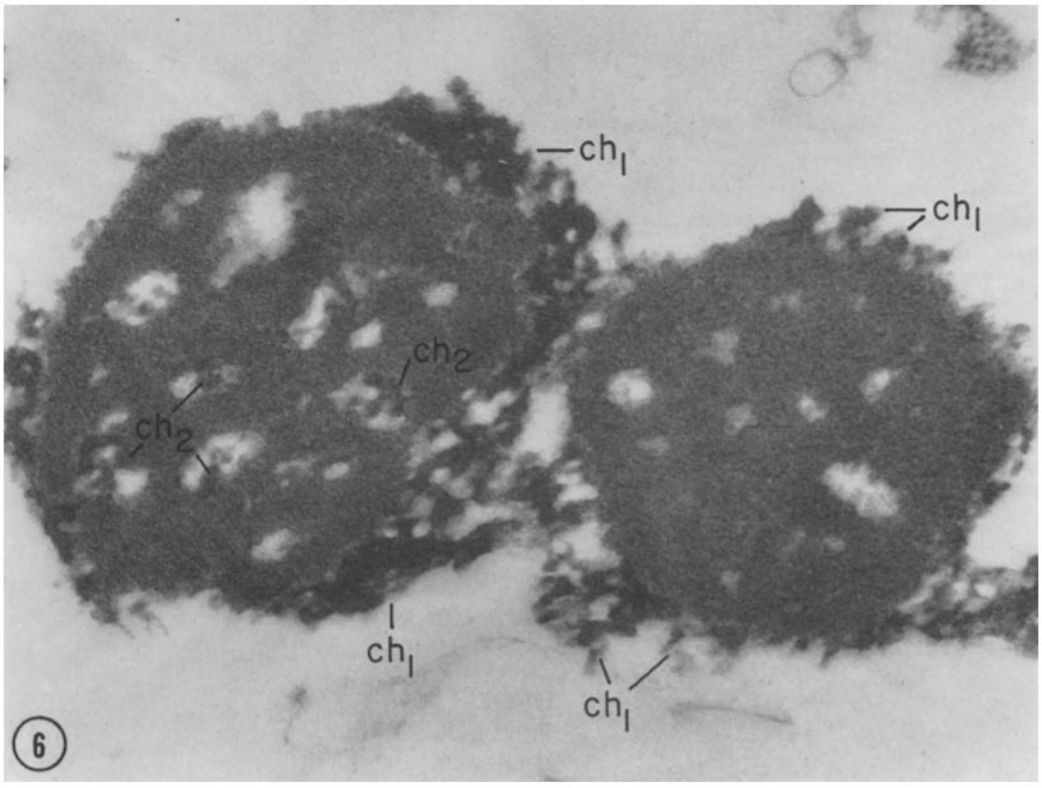
ch1
ch1
ch2
ch2
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ch1
6

with both uranyl acetate and lead hydroxide (*cf.* reference 8) in which the density of chromatin was markedly increased. This "nucleolus-associated chromatin" (*cf.* reference 21) seemed to resist sonic disruption more successfully when located in a relatively thin layer between the nuclear surface and the nucleolar mass. In addition to being contaminated by this type of chromatin derived from liver cell nuclei, the preparation contained blocks of dense chromatin (Figs. 1 and 2) which, on account of their shape, density, and texture, could be traced back to the nuclei of von Kupffer cells and leucocytes. As already mentioned, these nuclei are characterized by a peripheral concentration of condensed chromatin; although they are in minority in our nuclear fractions, their chromatin is fragmented into blocks rather than being finely dispersed by sonic treatment, and these blocks are large and heavy enough to become the most important contaminant in the nucleolar pellets. Counts in a representative preparation gave 525 chromatin blocks for 861 nucleoli. On a volume basis this contamination is estimated at ~30 per cent.

Nucleoplasmic subfractions: The two fractions consist of the same material: dense, kinky, filamentous or ribbon-like structures of variable length and ~40 to 50 mμ in diameter (Figs. 7 and 8). These filaments, which are shorter in the second, and longer in the first fraction, derive from the fine textured regions of the nucleoplasm visible in intracellular as well as in isolated nuclei, and hence represent the nuclear chromatin. This chromatin apparently undergoes aggregation or reorganization into coarser filaments upon rupture of the nuclear envelope, an assumption supported by the existence of intermediate appearances in partially ruptured nuclei, and by the demonstration of finer fibrils (2.5 to 5.0 mμ) within the isolated filaments (Fig. 9). The bottom layer of the first fraction contains fragmented nuclei in which the reorganization mentioned is evident (Fig. 8) and in which the coarsened chromatin threads frequently form elaborate networks. The same layer is contaminated by a few intact nucleoli. Both fractions are free of recognizable nucleolar fragments. We were not able to trace the fate of the other nuclear components (coarse nucleoplasmic granules and filaments) during the fractionation procedure because, if still present, they do not differ enough in their general appearance from the main component of the nucleoplasmic fractions, *i.e.*, the fragments of coarsened chromatin filaments. The fate of the nuclear envelope is also unknown.

c. COMMENTS: It should be clear that the nuclear subfractionation achieved represents only the first approximation to the solution of a difficult problem. The nucleolar subfraction is recognizably contaminated by chromatin from two different sources (perinucleolar chromatin and chromatin blocks). The nucleoplasmic fractions are apparently more homogeneous, but this probably reflects the insufficiency of our morphological criteria rather than a truly successful fractionation. On the one hand, knowledge about

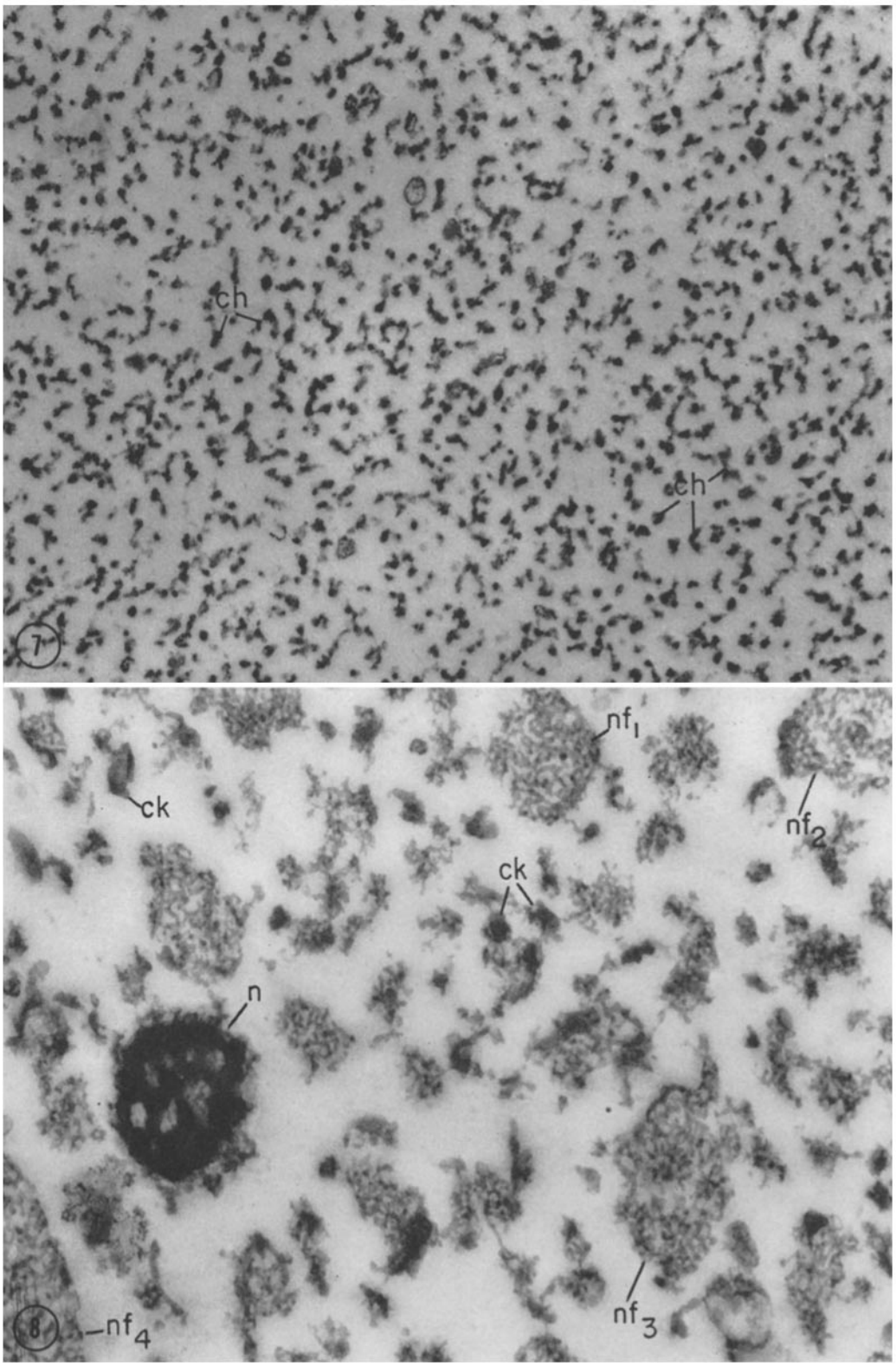
FIGURE 7 *Second nucleoplasmic subfraction.* The preparation consists of small fragments of chromatin threads (*ch*) which appear less uniform and generally thicker than those seen in isolated nuclei (see Fig. 3). For this reason, it can not be ascertained whether or not the pellet contains other nucleoplasmic components known to exist *in situ* (large, dense granules and clusters of fine granules or filaments).

Pellet fixed in 0.039 M OsO_4 in 0.88 M sucrose adjusted to pH 7.8 with Tris; embedded in methacrylate. Section stained with $Pb(OH)_2$. × 31,000.

FIGURE 8 *First nucleoplasmic subfraction* (bottom of the pellet). At this level the preparation consists mainly of fragments of nuclear chromatin, each appearing as a network of recognizable chromatin threads (nf_1 to nf_4). Some of the fragments comefrom the surface of the nucleus as indicated by the curvature of one of their sides (nf_1, nf_4). Denser and coarser fragments (*ck*) probably represent von Kupffer cell chromatin.

Nucleoli (*n*) and recognizable nucleolar fragments are only occasionally encountered at the very bottom of the pellet. The upper $\frac{4}{5}$ of the pellet is similar in composition to the second nucleoplasmic subfraction (Fig. 7).

Preparation for electron microscopy as for Fig. 5. × 31,000.


ch
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7
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nf1
nf2
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nf4
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the organization of interphase nuclei at this dimensional level is still very limited, and on the other hand we can not reliably trace through the fractionation procedure a number of nucleoplasmic components recognizable *in situ*. For this reason, we prefer to refer to these fractions as nucleoplasmic, although their main component is definitely chromatin. It should also be noted that among our nuclear subfractions there is no equivalent of the "nuclear sap" of the cytologists.

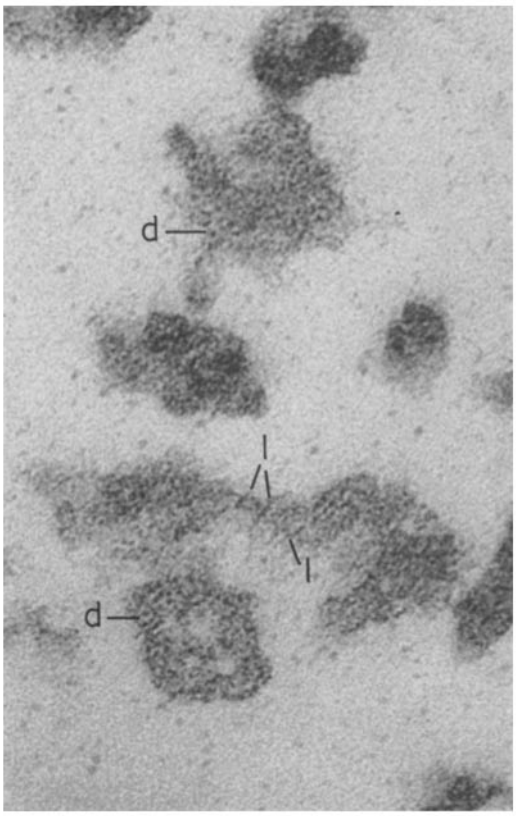

FIGURE 9 *Chromatin strands in the 2nd nucleoplasmic subfraction.* The strands show dense dots (*d*) and lines (*l*) on their sectioned surface, presumably profiles of sectioned fibrils 25 to 30 A in diameter.

Preparation for electron microscopy as for Fig. 7 except that the section was doubly stained in uranyl acetate followed by $Pb(OH)_2$. × 250,000.

B. *Chemistry*

1. Gross Chemical Composition

Like the parental nuclear fraction (8), the nuclear subfractions (Table I) showed considerable variations in the amounts of RNA, DNA and protein recovered per gram fresh tissue. The relative concentrations expressed as percentages or ratios were noticeably more stable.

The nucleolar fraction represents 6 to 11 per cent of the original nuclear mass and consists of ~4 per cent RNA, 9 to 10 per cent DNA, and 86 to 87 per cent protein (Table I), the corresponding figures for the nucleoplasmic fractions being ~2 per cent, 10 to 14 per cent, and 84 to 88 per cent, respectively. (For these calculations the amount of RNA + DNA + protein was equated to 100.) The nucleolar subfractions accounted for 11 to 17 per cent of the RNA, 5 to 7 per cent of the DNA and 6 to 10 per cent of the protein of the nuclear fraction (Table II). In terms of DNA and protein, RNA was 2 times more concentrated in the nucleolar than in the nucleoplasmic fractions. Since the nucleolar fraction is heavily contaminated by perinuclear chromatin and peripheral chromatin blocks, an attempt was made to reduce this contamination by more extended sonic treatment. The results obtained after 27 minutes of sonic nuclear disruption did not show, however, any striking improvement over the usual preparations (Tables I and II).

Since by morphological criteria available at present the nucleoplasmic fractions appear homogeneous, it may be tentatively concluded that their RNA represents chromatin or chromosome-associated RNA *in situ*. The conclusion is based on the assumption that sonic treatment does not result in extensive nucleolar disruption and contamination of the nucleoplasm fractions by unrecognizable nucleolar fragments. The RNA of the nucleolar fraction, however, is clearly a mixture of nucleolar and chromosomal RNA. If all the nucleolar DNA is considered to represent chromatin contamination, then ~40 per cent of the nucleolar RNA is also of chromatin origin.

2. NaCl Extractions

RESULTS: Table III gives the results of two experiments in which the RNA, DNA, and protein contents were determined in nucleoprotein fractions obtained by NaCl extraction from nucleolar and nucleoplasmic subfractions. In the case of nucleolar subfractions, figures are given only for the salt residue, for the extraction removes little material and not more than 5 to 10 per cent of the total nucleolar RNA. Since the nucleolar subfraction is known to be contaminated by

TABLE I

Chemical Composition of Nuclear Subfractions

Nucleoplasmic subfraction I is the material at the 0.88 M/2.2 M sucrose interface; nucleoplasmic subfraction II is the entire material in the 0.88 M sucrose supernate.

RNA, DNA, and protein were determined or calculated as in (8).

No. of experiments	Subfraction	RNA		DNA		Protein		RNA/ Protein	RNA/ DNA
		*mg/gm**	*per cent‖*	*mg/gm**	*per cent‖*	*mg/gm**	*per cent‖*		
1	Nucleolar‡	0.009	4.0	0.022	10.0	0.19	86.0	0.047	0.64
	Nucleoplasmic I	0.014	2.0	0.100	13.4	0.63	86.6	0.024	0.26
	Nucleoplasmic II	0.029	2.2	0.173	13.5	1.09	84.3	0.026	0.28
3¶	Nucleolar§	0.009	4.1	0.020	8.9	0.19	87.0	0.047	0.45
	Nucleoplasmic I	0.032	2.2	0.186	12.0	1.23	85.0	0.026	0.17
	Nucleoplasmic II	0.039	2.0	0.200	10.7	1.64	87.3	0.024	0.19
3¶	Nucleolar‡	0.011							
	Nucleoplasmic I	0.018							
	Nucleoplasmic II	0.032							

* per gram original tissue.

‡ subfractionation after 20-minute sonic treatment.

§ subfractionation after 27-minute sonic treatment.

‖ the sum of RNA + DNA + protein in each subfraction being considered 100

¶ the values given are the averages of the three experiments. The values in the 27-minute sonication experiments were within a few per cent of the average, while those in the 20-minute sonication experiments were within 20 per cent of the average.

TABLE II

Distribution of RNA, DNA and Protein in Hepatic Nuclear Subfractions (Guinea Pig)

The data in Table I were recalculated to give the percentage distribution of RNA, DNA and protein among nuclear subfractions.

No. of experiments	Subfractions	Per cent of total nuclear mass§	Distribution‖		
			RNA	DNA	Protein
1*	Nucleolar	9.8	17.2	7.5	10.0
	Nucleoplasmic I	33.2	28.8	34.0	33.0
	Nucleoplasmic II	57.0	54.0	58.5	57.0
3‡	Nucleolar	6.3	11.5	4.9	6.3
	Nucleoplasmic I	40.7	40.0	46.0	50.0
	Nucleoplasmic II	53.0	48.5	49.1	53.0

* Subfractionation after 20-minute sonic treatment.

‡ Subfractionation after 27-minute sonic treatment.

§ Calculated as percentage of the sum of RNA, DNA, and protein of whole nuclei.

‖ Calculated as percentage of the total RNA, or DNA, or protein in each of the three subfractions.

chromatin, and since the nucleoplasmic subfractions contain a sizable amount of salt extractable RNA (see below), it is conceivable that the small amount of RNA extracted from the nucleolar subfraction is entirely due to nucleoplasmic contamination. Using the salt extraction data in Table III and assuming that truly nucleolar RNA is not extractable by salt, it can

be calculated that 25 to 40 per cent of the RNA in the nucleolar subfraction is of nucleoplasmic origin. This figure agrees with other estimates of nucleoplasmic contamination: ~30 per cent on the basis of microscopic observations, and ~40 per cent on the basis of DNA distribution.

The salt extract of the pellet obtained by centrifuging (105,000 *g* for 60 minutes) the combined nucloplasmic fractions I and II accounted for 15 to 20 per cent of the RNA, 60 to 80 per cent of the DNA, and about 35 per cent of the protein of the nuclei (more precisely of the total RNA, DNA, and protein recovered in all nuclear subfractions). The final supernate of the common pellet contained ~10 per cent of the nuclear RNA and DNA together with 10 to 20 per cent of its protein. The figures given concern the whole supernate, for in this case the salt insoluble fraction appears to be negligible.

COMMENTS: Our failure to extract a significant amount of nucleolar RNA by salt can be correlated with the results published by Zbarskii and Georgiev (22): using a lower salt concentration, they obtained from a hepatic nuclear fraction (rat) a residue in which they recognized, by light microscopy, intact nucleoli in addition to residual chromosomes.

3. Nucleotide Composition of Various Nuclear RNA's

RESULTS: Table IV gives the nucleotide composition of the RNA's of the nucleolar and nucleoplasmic fractions and of the salt insoluble and salt extractable RNA's obtained from some of these fractions.

TABLE III

The RNA, DNA, and Protein Content of NaCl Extracts and Residues of Nuclear Subfractions

Extraction with 1 M NaCl was carried out overnight in the cold with stirring

Nuclear subfractions	RNA		DNA		Protein	
	mg	*per cent‡*	*mg*	*per cent‡*	*mg*	*per cent‡*
Nucleolar						
NaCl residue	0.066	18.1*	0.065	4.2	1.50	9.5
Nucleoplasmic						
Pellet						
NaCl residue	0.187	51.8	0.175	11.3	6.73	42.8
NaCl extract	0.077	21.1	1.200	78.0	5.62	35.9
Final supernate	0.032	9.0	0.100	6.5	1.85	11.8
Nucleolar						
NaCl residue	0.108	16.3*	0.245	8.0	1.72	7.5
Nucleoplasmic						
Pellet						
NaCl residue	0.409	61.5	0.480	15.8	8.84	38.5
NaCl extract	0.097	14.6	1.950	64.0	7.89	34.5
Final supernate	0.051	7.6	0.375	12.2	4.49	19.5

* In other experiments it was found that only 5 to 10 per cent of the total nucleolar RNA (*i.e.* 1 to 2 per cent of total nuclear RNA) could be extracted with 1 M NaCl.
‡ Percentage of RNA, or DNA, or protein in the total recoverable material of the subfractions.

The salient finding is the peculiar base composition of the RNA of the nucleolar fraction: it is characterized by a high GMP, and a low AMP and CMP content, and as such it is different from the RNA's of all the other cell fractions examined, nuclear as well as cytoplasmic (*cf.* Table IV in reference 8). Its AMP/CMP ratio, for instance, is close to 1, whereas the corresponding value for nucleoplasmic RNA's is nearly double. The salt extract of the nucleolar RNA retains this peculiar composition. The two nucleoplasmic subfractions, already known to be similar in their gross chemistry, are also similar in the nucleotide composition

of their RNA's. This finding reinforces our contention that the two subfractions do not represent distinct nuclear components, but reflect differences in the degree of fragmentation of chromatin. In other experiments the two nucleoplasmic fractions were combined, and centrifuged to yield a supernate and a pellet which were subjected to salt extraction (Table IV). The data on the nucleotide composition of the NaCl insoluble and NaCl extractable RNA's are hard to interpret because they concern only two experiments and show relatively small differences in base composition. Tentatively they suggest that the two RNA's are different. One could point out in this respect that in both experiments the UMP/GMP ratio was close to 1 in the RNA of the residue and considerably smaller than 1 in that of the extract. Other differences between these two RNA's will be discussed in the next section.

TABLE IV

Nucleotide Composition of the RNA's Extracted from Nuclear Subfractions

Nuclear subfractions	Moles/100 moles of total nucleotides				
	UMP	GMP	CMP	AMP	A+C/G+U
Nucleolar*	22.4	50.8	14.5	12.3	0.37
Nucleoplasmic					
I (middle interface)*	28.7	33.8	14.9	22.6	0.60
II (upper layer)*	26.4	34.9	14.8	23.9	0.63
Nucleoplasmic pellet					
NaCl residue‡	32.0	33.5	12.0	22.5	0.53
NaCl extract‡	25.2	35.2	15.6	24.0	0.66
Final supernate	26.0	33.0	14.0	27.0	0.69

* Average of three experiments.
‡ Average of two experiments.

The nucleotide composition of the RNA of the final supernate is quite similar to that of the RNA of the salt extractable nucleoplasmic fraction. Since the final supernatant RNA is almost completely salt extractable, the former finding suggests that the non-sedimentable nucleoprotein represents that finely dispersed chromatin of the nucleoplasm in which salt extractable RNA predominates.

COMMENTS: Some of the features of the RNA's of our nuclear subfractions are similar to those described in the literature for comparable materials isolated from other sources. The occurrence of ribonucleoproteins in chromosomes and chromatin, corresponding to our nucleoplasmic fractions, has already been studied and discussed by Kaufmann *et al.* (23), Mirsky and Ris (24), and Taylor and McMaster (25). Vincent (7) has mentioned the high GMP content of nucleolar RNA as compared to cytoplasmic RNA in starfish oocytes. Edström *et al.* (26) also found that nucleolar RNA has a higher GMP and a lower CMP content than cytoplasmic RNA, again in *Asterias rubens* oocytes. To this extent we confirm their results, but the agreement is not general: they (26) did not find a low AMP content in the nucleolar RNA as we did, and conversely we did not detect a high AMP content in the nucleoplasm, as they did (26). From autoradiographic studies it was also inferred that the AMP/CMP ratio of nucleolar RNA is considerably lower than that of the RNA of the rest of the nucleus (27) (*cf.* Table IV). The salt insoluble RNA of whole nuclear fractions isolated from liver and other tissues (28) has a high GMP/UMP ratio, which might reflect the high GMP content of the NaCl-insoluble nucleolar RNA.

4. Pseudouridylic Acid Content

RESULTS: Only 0.020 μmoles of ψuridine were found in 4.46 mg total RNA extracted from our nuclear fraction. The amount is equivalent to 1.25 moles of ψ UMP per 100 moles of UMP. No traces of ψuridine were found in the RNA's prepared from nucleolar and nucleoplasmic fractions, the starting amounts of RNA being

0.63 mg and 2.39 mg, respectively. These negative results can not be ascribed to technical limitations, since the procedure used was capable of detecting ψuridine in the expected amounts in RNA's known to contain ψ UMP. For example, such results, expressed as percentage ψ UMP of UMP, were 12 per cent and 18 per cent for soluble RNA's from *E. coli* and guinea pig liver, respectively. The corresponding data in the literature are 14 per cent for the soluble RNA of *E. coli* (29), 25 per cent for the soluble RNA of rat liver (15), and 22 per cent for the "pH 5 enzyme" from the same source (30). For the RNA of both hepatic and pancreatic ribosomes we have obtained 4 per cent ψ UMP of UMP. There are no directly comparable data in the literature, but a close approximation is represented by the figures of 7.5 per cent (29) and 2.7 per cent (30) published for total microsomal RNA. For RNA isolated from *E. coli* ribosomes we obtained only 0.8 per cent ψ UMP of UMP as compared to 1.7 per cent found by Osawa (30).

COMMENTS: It is possible that the limited amount of ψ UMP found in the total RNA of our nuclear fraction represents a cytoplasmic contamination. If we assume that this is not the case, that the nuclei contain soluble RNA of their own, and that this RNA contains ψ UMP in the same concentration as its cytoplasmic counterpart, our data imply that only 5 per cent of the total nuclear RNA could be soluble RNA. This interpretation is in agreement with the conclusion that little or no soluble RNA occurs in neutral extracts of thymus nuclei preparations (31).

If we take into account the limit of sensitivity of our procedure and the necessarily small amounts of RNA available as starting material, and again assume an equal ψ UMP concentration in cytoplasmic and nuclear soluble RNA, our data further indicate that the concentration of transfer RNA must be below 10 per cent of the total RNA of the nucleoplasmic subfraction and below 20 per cent of that of the nucleolar subfraction to escape detection. Accordingly, our results do not exclude the presence of transfer RNA in liver nuclei, but are compatible only with the occurrence of small amounts therein. Metabolic evidence for the existence of transfer RNA in a thymic nuclei preparation has been reported (32).

5. Turnover of RNA's

RESULTS: Preliminary experiments with short time *in vivo* labeling (5, 10, and 30 minutes) showed that the hydrolysates of the RNA's of nuclear subfractions are contaminated, to a surprising extent, by non-nucleic acid phosphorus compounds. Although the incorporation of ^{32}P was very active, only 6 to 12 per cent of the counts was recovered in the isolated mononucleotides, the rest being present as inorganic phosphate. At later times after ^{32}P injection (1 to 4 hours), the contamination, although noticeably reduced, remained large: only ~50 per cent of the counts were recovered in mononucleotides. As a result of these experiments, the later time points were chosen for further work on RNA metabolism; the corresponding data are more reliable than at earlier time points, but the approach admittedly can not detect rapidly turning-over nuclear RNA's if such are present.

At the 1 hour point (Table V), the specific radioactivity of the RNA was similar in the nucleolar and nucleoplasmic subfractions; it increased in parallel in all subfractions between 1 and 2 hours, and leveled off in the nucleoplasmic subfractions between 2 and 4 hours. The specific activities of the 4 mononucleotides varied to a certain extent: those of AMP and UMP were similar throughout the nuclear subfractions; that of the CMP was generally above the AMP-UMP level, expecially in the RNA of the nucleolar subfraction; and finally, that of the GMP was below the same level in the nucleoplasmic subfractions.

Since no differences in the metabolic activities of the total RNA's of the nuclear subfractions were detected, the inquiry was extended to the salt extractable and salt insoluble RNA's obtained from the same subfractions. The time point selected for these experiments was 2 hours, since previous work had revealed maximum RNA labeling after this interval. The results showed (Table V) that the specific radioactivity of the salt extractable RNA of the combined nucleoplasmic subfractions was only half that of the salt insoluble RNA's of the same preparation. It was also half the specific radioactivity of the total RNA of the nucleolar subfraction. Further differences became apparent when the specific radioactivity of the various mononucleotides was considered. The lowest value was found for the CMP of the salt extractable RNA of the nucleoplasmic fractions, and the highest for the CMP of the salt insoluble RNA of the same fractions. The CMP of the total RNA of the nucleolar fraction showed an even higher specific radioactivity.

Since the phosphate of the CMP, as isolated from an alkaline hydrolysate, belongs to its next neighbor in the RNA chain, and since the salt insoluble RNA of the nucleoplasmic fraction and especially the RNA of the nucleolar fraction are characterized by a high GMP content, our results suggest a high frequency of CMP-GMP (rather than CMP-AMP, or CMP-UMP) sequences in the corresponding RNA chains. Conversely the low specific radioactivity of the CMP obtained from the salt extractable RNA of the nucleoplasmic fractions may reflect the high frequency of CMP-GMP and CMP-AMP sequences in this particular RNA. This interpretation assumes a uniform labeling of all triphosphate precursors of the mononucleotides incorporated in the RNA chains.

TABLE V

Specific Radioactivity of Mononucleotides Obtained from the RNA's of Various Nuclear Subfractions after Intravenous Injection of ^{32}P Inorganic Phosphate

The nuclear subfractions and extracts were obtained as described in the text; the RNA was extracted, digested, and the nucleotides isolated by paper chromatography and counted according to the methods described in the text.

Time after injection	Nuclear subfractions	Per cent of total RNA in fraction	Radioactivity (CPM)				
			RNA*	UMP	GMP	CMP	AMP
hrs.			*mg*	*μM*	*μM*	*μM*	*μM*
1	Nucleolar‡	11	70,000	21,900	24,400	30,000	21,300
	Nucleoplasmic						
	I (middle interface)	41.5	64,000	21,800	19,300	27,000	23,000
	II (upper layer)	47.5	64,000	20,600	19,500	33,000	20,900
2	Nucleolar‡	12.1	100,000	30,000	33,000	53,000	32,000
	Nucleoplasmic						
	I (middle interface)	39.3	93,000	32,800	27,700	36,500	33,700
	II (upper layer)	48.6	100,000	35,500	31,300	45,000	37,000
4	Nucleoplasmic						
	I (middle interface)	39.7	100,000	36,200	28,500	45,000	36,500
	II (upper layer)	49.1	90,000	32,900	25,000	37,000	33,000
2	Nucleolar§	17.2	99,000	35,000	25,600	61,600	34,800
	Nucleoplasmic pellet						
	NaCl residue§	56.7	96,800	32,740	26,100	47,900	36,800
	NaCl extract§	17.8	48,800	20,200	13,800	9,900	13,300
	Final supernate	8.3	99,500	35,700	28,000	44,000	35,500

* These values were obtained by dividing the sum of the counts in each of the four mononucleotides by the sum of the amounts of each of these nucleotides after converting μM nucleotide to mg RNA.
‡ In these experiments, the nucleolar fractions were obtained after 27 minutes of sonication of the nuclear fraction; in the other experiments sonication lasted for 20 minutes.
§ Average of two experiments

COMMENTS: The metabolic properties of the RNA of whole nuclear fractions have been repeatedly studied in the past. The results generally indicate that it has a greater turnover rate than the RNA of other cellular fractions (33–35). As a result of numerous autoradiographic studies it has also been inferred that nucleolar RNA is metabolically more active than any other cellular RNA (36–39), but the possible misleading effect of high RNA concentration in the nucleolus has been discussed by Harris (27).

Our results indicate clearly the metabolic diversity of nuclear RNA and confirm the concomitant incorporation of ^{32}P in chromosomal and nucleolar RNA's, but do not support the view that there is between these two RNA's a precursor-to-product relationship.

A detailed analysis of nuclear RNA types and metabolism has been carried out by Zbarskii and his collaborators (*cf.* reference 40), who found (41) that after 20 minutes' labeling *in vivo* with $^{32}P_i$, the fraction with the highest specific radioactivity was the residue of the extraction with 0.14 M NaCl, which they called the "nucleolar-chromosomal apparatus." The activity of the "high polymeric RNA" of the "nuclear sap" was half as high, but still far above that of various cytoplasmic RNA's (*e.g.*, soluble and ribosomal RNA). There are evident similarities between these findings and ours, although a clear appreciation of the extent of agreement is hampered by the fact that different isolation and extraction procedures were used in the two cases. At present, the functional role of the various nuclear RNA's is still poorly understood (*cf.* review by Sirlin, 42), because of the diversity of these RNA's, the difficulty of isolating nuclear subfractions and of interpreting kinetic data.

DISCUSSION

Our general results indicate that the nuclear components considered, *i.e.* the nucleoli and the nucleoplasm, contain different types of RNA characterized by different solubility in 1 M NaCl, different nucleotide composition, and different metabolic properties. At present we do not know to what extent our results are affected by the protracted procedure and extended sonication used for the isolation of our nuclear subfractions. Preferential damage of some RNA's either by shear or RNase activity is not excluded, especially during nuclear subfractionation. Partial answers to such questions may come from a comparison now under way between the physicochemical characteristics of RNA's extracted from whole nuclei and those of RNA's obtained from nuclear subfractions.

The RNA of the nucleolar subfraction is characterized by its insolubility in salt solutions, its high GMP and low AMP content, and its high turnover rate. The different turnover rates of its individual mononucleotides suggest a high frequency of CMP-GMP sequences in the corresponding RNA chains. There are reasons to believe that some of these peculiarities, especially the nucleotide composition, would become more pronounced if it would be possible to reduce the nucleoplasmic contamination of the fraction.

The RNA of the nucleoplasmic subfractions appears to be a mixture of at least two RNA species, one characterized by high solubility in 1 M NaCl and relatively low turnover rate, the other having converse features. The difference in nucleotide composition is not particularly pronounced but differences in the turnover rate of individual mononucleotides indicate rather different sequences in the corresponding RNA chains.

On the assumption that the subnuclear RNA's studied are homogeneous, the considerable differences in nucleotide composition revealed by our findings strongly suggest that a relation precursor → product cannot exist between the various nucleolar and nucleoplasmic RNA's on the one hand and the various cytoplasmic RNA's on the other. Moreover, if the nucleotide composition of liver DNA (43) is considered, there is again no extensive similarity between it and that of the RNA of any nuclear subfraction. Evidently complementarity as well as precursor → product relations are still possible if the various subnuclear RNA's are mixtures of two or more distinct RNA species. Recently published work suggests indeed that such may be the case with the nucleolar RNA. Georgiev *et al.* (41, 44–47) have fractionated nuclear RNA's by a combination of salt and phenol extraction procedures and have arrived at the conclusion that the salt insoluble RNA of the "nucleolo-chromosomal apparatus" contains a fraction with a particularly high turnover rate which is the precursor of some cytoplasmic RNA. The interpretation of their results is hampered by the fact that radioactivity was measured in RNA fractions rather than in separated mononucleotides, a circumstance which introduces a certain degree of uncertainty even when phenol-extracted RNA fractions are considered (*cf.* reference 48). Also recently, Sibatani *et al.* (49) and Hiatt (50) have reported the isolation of an RNA fraction from thymic (49) and hepatic (50) nuclear fractions by a modified phenol extraction procedure. This fraction has a higher turnover rate than any other nuclear RNA fraction. Moreover in both cases it was demonstrated that the radioactivity was in RNA and it was postulated that the new RNA fraction, supposedly the "messenger" RNA, is part of the nucleolar RNA.

Although our experiments have not yet succeeded in resolving the mixtures of nuclear RNA's into functionally meaningful types, we believe that they represent a step in the right direction because they put nuclear fractionation on a structural basis, and introduce more stringent criteria of morphological control. With further

refinements, based on such premises, the present nuclear RNA fractions may be resolved into homogeneous components of defined functional significance.

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