AUTORADIOGRAPHIC AND CYTOCHEMICAL EVIDENCE FOR SYNTHESIS OF A LYSINE-CONTAINING RIBONUCLEOPROTEIN IN NUCLEOLI INHIBITED BY ACTINOMYCIN D

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Biochemical evidence for protein synthesis on nuclear ribosomes has accumulated in several laboratories in recent years (1, 2, 5). The morphologic sites of this activity in the intact mammalian cell, in particular the respective roles of the nucleolus and chromosomes in nucleoprotein and nucleic acid synthesis, remain unclear (6–12).

A quantitative autoradiographic and cytochemical study of this question has been reattempted by comparing the relative uptake of H^3-lysine and H^3-uridine or H^3-cytidine in untreated HeLa cells with that in cells in which nucleolar RNA synthesis was selectively inhibited by low concentrations of Actinomycin D according to the method of Perry (13). In parallel, some cellular sites of ϵ-amino groups were stained with Biebrich scarlet (14, 15).

It was found that lysine is rapidly incorporated into a nucleolar material solubilized either by agents which hydrolyze RNA or by the proteolytic enzyme trypsin. This incorporation is inhibited by concentrations of Actinomycin D which also selectively inhibit the synthesis of nucleolar RNA; but lysine incorporation is not inhibited in extranucleolar chromatin and cytoplasm. When stained for basic protein with Biebrich scarlet under selective conditions of fixation, nucleoli showing lysine incorporation present a characteristic granular substructure not exhibited, or severely altered, in actinomycin-treated cells (16) and in cells treated under conditions of RNA extraction.

MATERIALS AND METHODS

Hela cells[1] grown in Leighton tubes, maintained in Eagle's medium (17) supplemented by 10 per cent horse serum, were labeled in growth phase for 5 or 15 minutes with H^3-lysine (164.5 mc/mmole[2], 10 μc/ml) in Eagle's medium lacking lysine, and with H^3-uridine (2.60 c/mmole, 10 μc/ml) or H^3-cytidine (2.52 c/mmole, 10 μc/ml).[3]

The cells were washed 5 times in Hanks' balanced salt solution and fixed for 30 minutes at 0°C in 3.7 per cent formaldehyde (Fisher Scientific Company, New York, certified reagent) in Millonig's phosphate buffer (18) at pH 7.3. The cells were washed 3 to 5 times in cold distilled water, followed by 1 per cent perchloric acid at 0–4°C for 30 minutes, and again washed 3 to 5 times in cold distilled water, then air-dried and coated with liquid autoradiographic emulsion (Kodak NTB-3, diluted 1:1) according to standard techniques. The exposure times at −20°C were selected so that approximately the same nuclear grain counts were obtained with H^3-lysine (26 days) as with H^3-uridine or H^3-cytidine (6 days). The autoradiographs were developed in Ficq's diaminophenol developer (19) and stained through the emulsion with Harris' hematoxylin and eosin.

The relative uptakes of lysine and RNA precursor over the nucleolus, chromatin, and cytoplasm were then determined by mean grain count of groups of 20 technically clear, randomly selected cells and by comparison of the mean of the ratios of nucleolar to total

[1] Horse serum adapted, obtained from Microbiological Associates, Bethesda.

[2] Radiochemical Centre, Amersham, England.

[3] New England Nuclear Corporation, Boston.

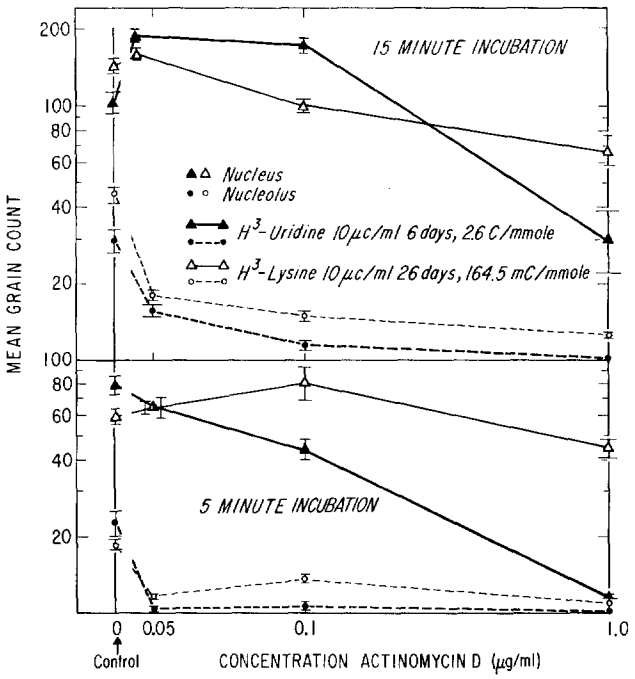

FIGURE 1 Effect of concentration of Actinomycin D on mean nuclear and nucleolar grain count of groups of 20 HeLa cells labeled with H^3-lysine and H^3-uridine.

nuclear counts. Grain counts were made at least in triplicate with the aid of an ocular micrometer grid (0.25 mm_2) and a Zeiss apochromatic 100/1.32 objective. The ratio of volumes of nucleolus to nucleus was approximated by the ratio of areas calculated as idealized rectangles.

Before labeling, some cells were pretreated with Actinomycin D[4] (0.05 to 1.0 μg/ml) for 3 hours followed in most experiments by a "chase" in maintenance medium for 3 to 4 hours (13).

Extraction and hydrolysis procedures for basic proteins and nucleic acids were carried out with 0.5 N $HClO_4$ and with 0.2 N HCl for 30 to 60 minutes at 25° and 0°C (20), with 1 N HCl at 60°C for 10 minutes as in the Feulgen procedure, and with 0.3 N trichloracetic acid at 90°C for 30 minutes.

Enzymatic digestion was performed with: (*a*) Ribonuclease, (5 times crystallized, Worthington Biochemicals Corp., Freehold, New Jersey and Pentex Inc., Kankakee, Illinois) 1 mg/ml in 0.016 M sodium phosphate buffer (McIlvaine), at pH 7.0 for 60 minutes at 37°C. (*b*) Desoxyribonuclease, (Sigma Chemical Company, St. Louis, 1 time crystallized) 0.5 mg/ml in 0.002 M tris(hydroxymethyl)aminomethane (Sigma 121, Sigma Chemical Company), 0.005 M $CaCl_2$ and 0.45 M $MgCl_2$ at pH 7.3 for 60 minutes at 37°C. (*c*) Trypsin, (Fisher Scientific Company, Nutritional Biochemicals Corporation, Cleveland) 0.5 mg/ml in distilled water adjusted to pH 6.5 at 0°C for 30 minutes (21).

Control cells were stained with Biebrich scarlet at pH 9.5 in 0.5 N glycine buffer (14) after controlled fixation and partial deamination as above.

RESULTS

As previously observed (22) uptake of tritiated lysine, unlike nucleoside label, occurs rapidly in both cytoplasm and nucleus of HeLa cells, a fact which complicates dynamic studies of localization. Comparison with RNA precursors shows parallel lysine labeling over the nucleus as early as 5 minutes and evident concentration over nucleolar areas at 15 minutes (Figs. 1 and 3). About 28 per cent of the nuclear uptake of lysine (Figs. 1 and 2 and Table I) and RNA precursors (Figs. 1 and 2) is concentrated in the nucleolus, a proportion 2.29 times[5] in excess of the nuclear uptake per unit area. Lysine incorporation in nucleoli is significantly smaller after removal of ribonucleic acid or total nucleic acids either by a Feulgen type hydrolysis, by hot trichloracetic acid extraction (Fig. 2 and Table I), or by ribonuclease digestion (Fig. 2). After trypsin digestion, which

[4] Kindly supplied by Merck, Sharpe & Dohme Co., West Point, Pennsylvania.

[5] Uncorrected for β-ray self-absorption.

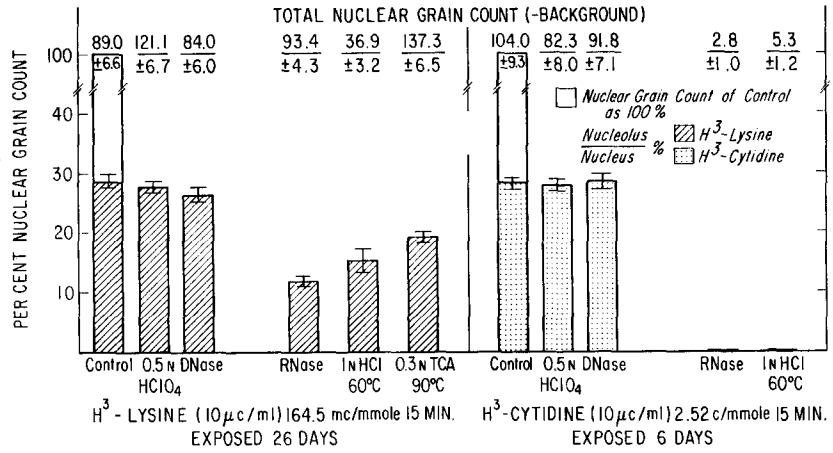

FIGURE 2 Percentage histogram of nucleolus/nucleus. Mean grain counts in groups of 20 HeLa cells labeled with H^3-lysine and H^3-cytidine.

TABLE I

Mean Grain Counts of 20 HeLa Cells Labeled with H^3 Lysine (10 μc/ml) for 15 Minutes and Exposed 26 Days

Treatment after fixation	Nucleolus / Nucleus	Nucleus (— background)	Cytoplasm (+ background)	Background 1/75 mm² (nucleus)
		Untreated		
None	0.2797 ± 0.0158 [0.120] ± [0.006]	85.9 ± 4.3	140.7	6.7
HCl O_4, 0.5 N	0.2782 ± 0.0126 [0.115] ± [0.010]	105.2 ± 6.2	147.8	5.0
HCl 0.2 N	0.2736 ± 0.0117 [0.129] ± [0.010]	96.2 ± 6.6	126.0	2.9
Average	0.2771 ± 0.0135 [0.121] ± [0.008]	95.7 ± 5.7	138.1	4.9
HCl 1 N, 60°C, 10 min. (Feulgen)	0.1261 ± 0.0065	88.2 ± 4.7	138.8	10.5
Trypsin 0.5 mg/ml 0°C, 30 min.	0.1552 ± 0.0087	90.1 ± 5.2	137.7	11.4
TCA 0.3 N, 90°C	0.1133 ± 0.0060	109.0 ± 8.0	130.4	5.7
Actinomycin D, 0.05 μg/ml, 3 hrs., followed by maintenance medium, 3 hrs.				
None	0.1252 ± 0.0067 [0.107] ± [0.008]	113.9 ± 8.4	155.6	4.7
HCl 1 N 60°C, 10 min. (Feulgen)	0.1252 ± 0.0101	84.5 ± 5.2	116.0	7.3
Trypsin	0.1230 ± 0.0058	73.5 ± 3.4	147.8	8.6
TCA 0.3 N, 90°C	0.1122 ± 0.0056	133.1 ± 7.6	154.2	6.0

Numbers in brackets are ratio of nucleolar to nuclear volume projected as rectangles.

hydrolyzes principally arginine and lysine peptide bonds (21), the grain count per unit area is equivalent for nucleolus and nucleus (Table I). The extent of nucleolar lysine incorporation is not reduced by extraction and partial hydrolysis with 0.5 N perchloric acid (Fig. 2 and Table I), 0.2 N hydrochloric acid (Table I) or by enzymatic digestion with desoxyribonuclease (Fig. 2).

Evidence that a portion of the nucleolar lysine incorporation may be chemically and kinetically associated with a rapidly labeled RNA fraction was obtained by pretreatment with low concen-

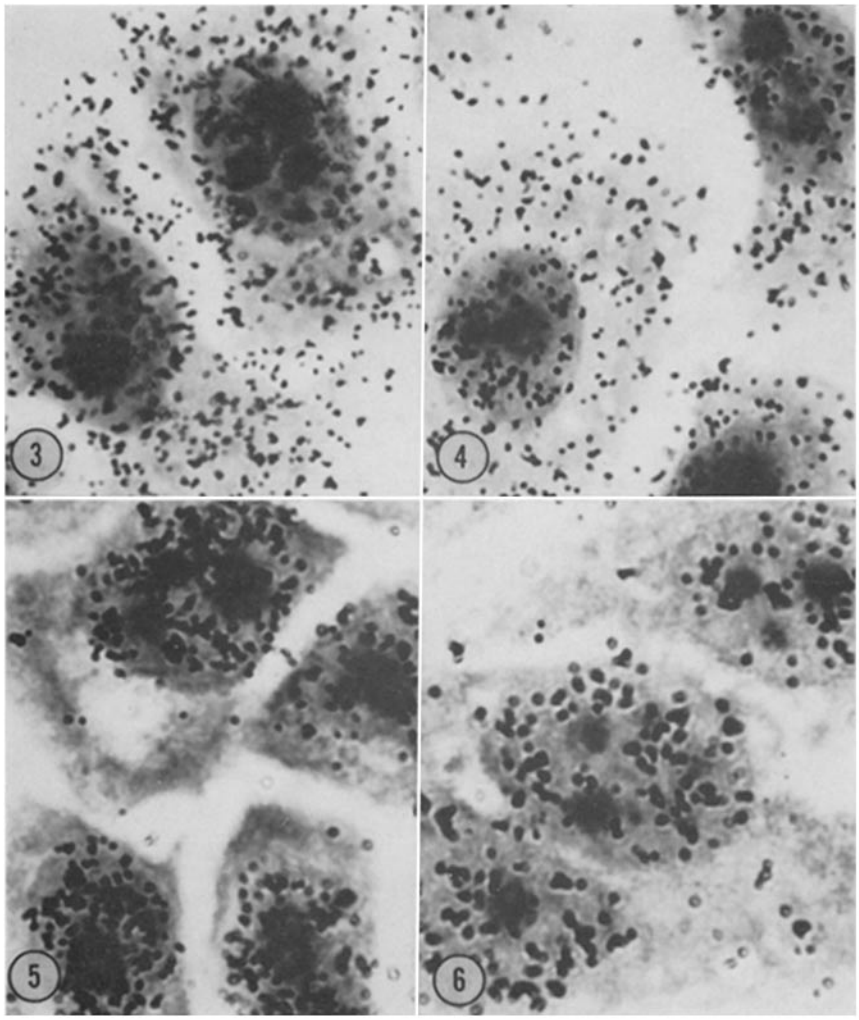

FIGURE 3 Autoradiograph, H^3-lysine (10 μc/ml, 15 minutes), HeLa cells, untreated. Note numerous silver grains over nucleoli, × 1050.

FIGURE 4 As above, pretreatment with Actinomycin D (0.05 μg/ml). Note sparse silver grains over nucleoli, but labeling over chromatin and cytoplasm. × 1050.

FIGURE 5 Autoradiograph, H^3-uridine (10 μc/ml, 15 minutes), untreated. × 1050.

FIGURE 6 As above, pretreatment with Actinomycin D. Note selective inhibition of uptake over nucleoli. × 1050.

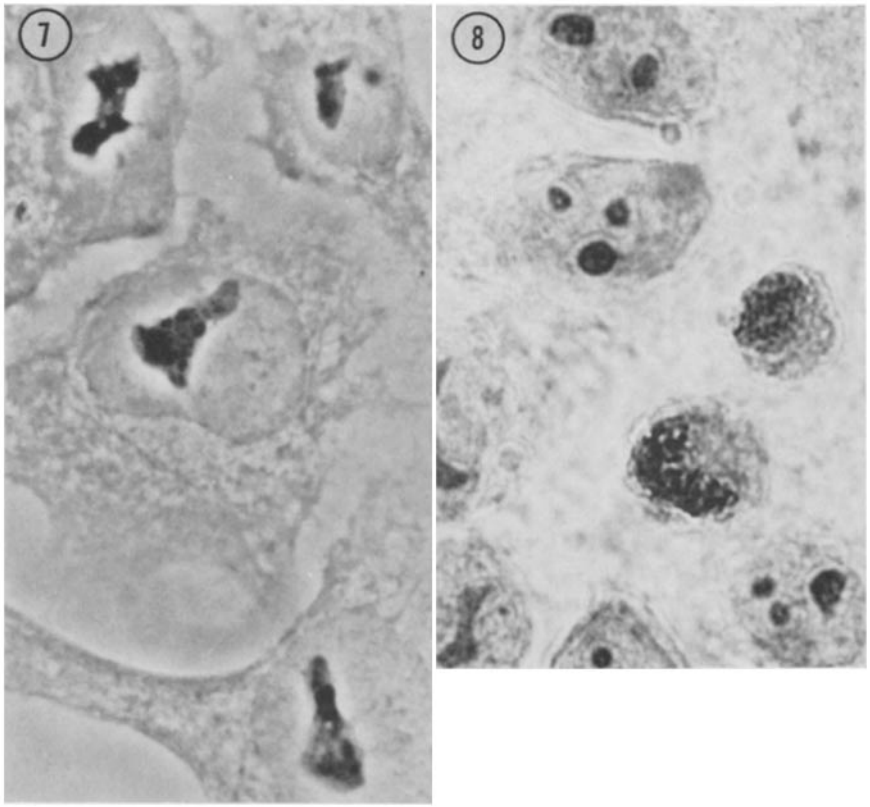

FIGURE 7. Biebrich scarlet, phase. Note intensely stained granules in nucleoli surrounded by lighter stained *pars amorpha*. × 1200.

FIGURE 8 As above, transmitted. Note granules associated with prometaphase chromosomes. × 1050.

trations of Actinomycin D (Figs. 1, 4, and 6 and Table I).

As shown in Figs. 1, 3 to 6, cells incubated with lysine in parallel with H^3-uridine show a concomitant depression of nucleolar grain count when pretreated with actinomycin at concentrations of 0.05 μg/ml. The proportion of nucleolar lysine uptake is reduced from 27.7 per cent of the total nuclear uptake in untreated controls to 12.5 per cent ($\alpha > 0.001$) in actinomycin-pretreated cells (Table I). This level is not significantly higher than the ratio of the area of nucleolus to nucleus and corresponds to the proportional uptake obtained after nucleic acid extraction of untreated cells. It could be interpreted both as nucleolar and as chromatin label overlying the nucleolus, an interpretation supported by the fact that no further reduction of lysine incorporation into nucleoli was observed after nucleic acid extraction or weak trypsin digestion of actinomycin-treated cells. Grain counts over the extranucleolar chromatin of both lysine and uridine (Fig. 1) fail to show a decrease at concentrations below 1 μg/ml. Similar observations with H^3-uridine and H^3-cytidine have been previously reported by Perry (13). The cytoplasmic lysine uptake is not significantly affected by actinomycin in this dosage (Table I).

The morphologic localization of this rapidly labeled, lysine-containing ribonucleoprotein in nucleoli was sought by parallel staining with the acid dye Biebrich scarlet at pH 9.5 for which Spicer has adduced histochemical and spectrophotometric evidence of specificity for ε-amino groups of polylysine (14, 15). Selective staining of nucleoli was obtained for HeLa cells after fixation and partial deamination for 30 minutes at 0°C with 3.7 per cent formaldehyde buffered at pH 7.3 in Millonig's phosphate buffer (18). Nucleoli

of growing HeLa cells then contain intensely stained granules or beaded filaments, surrounded by lighter stained areas (Fig. 7). With the disappearance of nucleoli during prophase these granules appear in the nucleoplasm associated with chromosomes (Fig. 8) and reappear in the nucleoli during telophase. Ribonuclease treatment in these experiments, while not removing acidophilia of nucleoli, results in prominent vacuolation (Fig. 9). Similar vacuolation is seen after

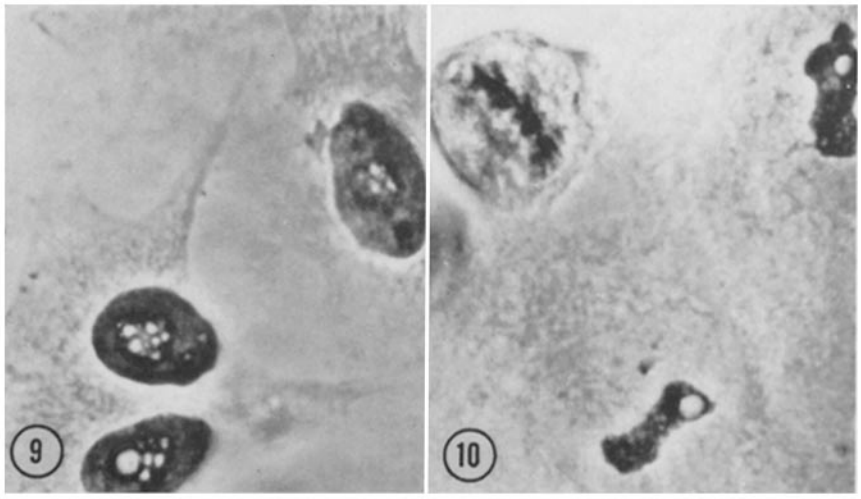

FIGURE 9 Biebrich scarlet, phase. HeLa cells digested with ribonuclease. Note prominent vacuoles in nucleoli. × 1050.

FIGURE 10 As above, after Feulgen hydrolysis. × 1050.

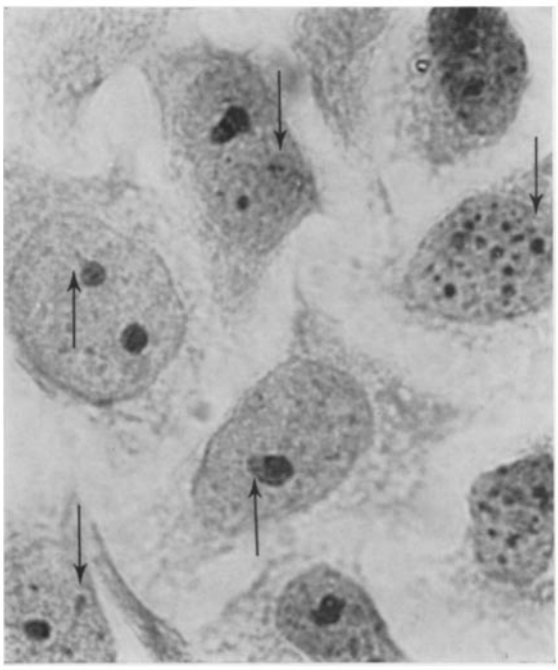

FIGURE 11 Effect of Actinomycin D, Biebrich scarlet, transmitted. Note lack of granular substructure of nucleoli and margination by weakly stained areas (↑). Chromatin granules (↓). × 1050.

Feulgen hydrolysis (Fig. 10) and hot trichloracetic acid extraction, whereas trypsin digestion also reduces nucleolar staining. The nucleolar granules appear resistant to DNase digestion, although some vacuolation is noted. Actinomycin treatment results in reorganization and ultimate disintegration of nucleoli. Stained with Biebrich scarlet, nucleoli of cells so treated have an amorphous appearance. Faintly stained areas often marginate the nucleolus as nucleoli diminish in size. Concomitantly, extranucleolar granules become progressively more numerous (Fig. 11). These observations suggest that the nucleolar granules may be a morphologic site of lysine incorporation.

Similar morphologic observations in actinomycin-treated cells have been made by Reynolds (23) and Clark (24). The Biebrich scarlet–reactive granules appear to resemble morphologically structures described in various studies with the light and electron microscopes (25–29). They vary in size and their demonstration depends on selective conditions of fixation. Their relationship to "nucleolar chromatin" (29) or to nucleolonemata and their function in nucleic acid and protein synthesis must await further cytochemical and autoradiographic characterization with the aid of the electron microscope.[6]

DISCUSSION

In the present study nucleolar granules containing basic protein, stained with the acid dye Biebrich scarlet at strongly alkaline pH, were observed in apparent association with rapidly labeled RNA precursors and lysine and are not observed or are severely altered under conditions of RNA extraction. Pretreatment with Actinomycin D resulted in inhibition of nucleolar lysine uptake but in continued uptake in chromatin and cytoplasm and in a loss of distinctive nucleolar granulation. As a working hypothesis it is suggested that these nucleolar structures may include sites of nuclear ribosomes or ribosomal precursor involving synthesis of ribonucleoprotein (3, 4, 6, 30).

Observations seemingly contrary to these findings and to such a hypothesis have, however, been reported by Errera *et al.* (22) in studies with ultraviolet-irradiated nucleoli. These authors have failed to demonstrate a significant reduction of amino acid uptake in nucleoli when nucleolar RNA synthesis was inhibited about 90 per cent. The proportional uptake of lysine in nucleoli relative to nuclei in controls of these experiments appears to be, however, considerably below that observed in the present experiments, suggesting that perhaps some soluble peptides were lost under different conditions of fixation (31, 32), or that possible differences in growth phase of cells (8), as well as differences of action of the inhibitory agents employed, might perhaps account for seeming discrepancies. An alternative interpretation might assume that the reduced incorporation of both nucleic acid and protein precursors is secondary to a general disintegration of the nucleolus following actinomycin treatment. This, however, would appear less likely in view of the late occurrence of such disintegration, the rapid biochemical effect of actinomycin in inhibiting DNA-directed RNA synthesis (34), and the reported observation that lysine incorporation in nucleoli is reduced under conditions of RNA extraction and trypsin digestion in control cells, but not in actinomycin-treated cells. Such a hypothesis would also presuppose that early alterations in the morphology of the nucleolus (23, 24) are independent of observed biochemical effects of actinomycin. The loss of lysine incorporation after ribonuclease treatment and acid hydrolysis could be due to soluble or nascent polylysine peptide chains thought to be linked by ester linkage to transfer RNA and non-specifically bound to ribosomes (32). Hydrolytic or enzymatic removal of RNA, or fixation at suboptimal pH or ionic strength would then result in solubilization of these peptide chains in aqueous media. In preliminary experiments similar observations to those reported here were noted under conditions of pulse labeling; further experiments to clarify the extent of protein or amino acid labeling in the nucleolus are in progress. A direct incorporation of lysine label into nucleotides *via* the glycine pathway would appear unlikely.

The continued lysine uptake in chromatin and cytoplasm in actinomycin-treated cells (4, 33) in the absence of nucleolar uptake supports the conclusion (8, 13) that at least some nucleolar protein and RNA is synthesized independently of chromosomal RNA in growing cells of human origin. It would seem that Actinomycin D at the concentration used may permit differentiation and localization of two types of lysine-containing

[6] Since this paper was submitted such a study has been reported by Schoefl, G. I., *J. Ultrastruct. Research*, 1964, **10**, 224.

protein or polypeptides; one which appears to be localized only in the nucleolus and which is removed under conditions of RNA extraction and linked metabolically or chemically to it and, a second found in the nucleus and cytoplasm, which is not removed under conditions of RNA extraction and which may be metabolically and chemically independent.

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