EFFECT OF TEMPERATURE OF ALDEHYDE FIXATION ON THE RADIOAUTOGRAPHIC LOCALIZATION OF RIBONUCLEOPROTEIN IN NUCLEOLI OF HELA CELLS

Inhibition by Puromycin and Actinomycin D

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ABSTRACT

In efforts to clarify the role of the nucleolus and substructures thereof in the assembly or synthesis of protein associated with formation of the complete ribosome, the effect of variation of some conditions of aldehyde fixation on the intranuclear distribution of lysine-^{3}H, arginine-^{3}H, and uridine-^{3}H was studied by differential grain count in radioautographs of PPLO-free HeLa cells. It was found that the nucleolus is a site of rapid assembly or synthesis of a protein, the synthesis of which is inhibited equally by puromycin (200 μg/ml) and by actinomycin D under conditions inhibitory for ribosomal precursor RNA synthesis ($P < 0.01$). This protein is fixed by phosphate-buffered formalin or glutaraldehyde at pH 7.3, but the label is diminished by fixation in customarily employed acetic ethanol or in formalin at acid pH. Elevation of temperature of formalin or glutaraldehyde fixatives to 37°C consistently reduces the nucleolar protein label, but not the RNA label, by a proportion identical with that incurred by puromycin or actinomycin inhibition. This proportional reduction of nucleolar protein label occurs without evident loss of total grain count and is independent of length of fixation between 30 min and 4 hr, but it is not observed at 23°C. The data support the interpretation that the proportion of nucleolar protein not fixed at 37°C is associated with nucleolar ribosomal RNA but that it is dissociated at 37°C in formalin or glutaraldehyde fixatives, probably on the basis of ionic dissociation of a conjugated ribonucleoprotein.

Biochemical evidence for the participation of nucleoprotein in the regulation (1–7) or stabilization of ribosome synthesis (8–12) is accumulating in several laboratories. The morphological sites of protein synthesis in the nucleus, and especially the function of the nucleolus and substructures thereof in the assembly of the ribosome complex, remain uncertain. Biochemical studies with isolated nucleoli point to a nucleolar synthesis of ribosomal or ribosome-associated protein (6; 18–22) in addition to synthesis of ribosomal RNA (13–17). Those observations appear to differ from some morphological observations with radioautographic techniques (23–27). Although data obtained by quantitative radioautography are of lesser biochemical specificity, this technique does allow the study of intact cells and a correlation with fine structure without introducing problems of contamination or mechanical artifact (22).

In a previous report (28), it was shown that under specific conditions of fixation tritiated lysine as well as tritiated nucleotide bases are rapidly incorporated into a nucleolar material solubilized by ribonuclease, Feulgen-type hydrolysis, or trypsin digestion. This incorporation is inhibited by low concentrations of actinomycin D which also selectively inhibit the synthesis of nucleolar ribosomal RNA (17, 29, 30) but not the incorporation of lysine into extranucleolar chromatin and cytoplasm. When stained for basic protein with alkaline Biebrich scarlet (31, 32), the nucleoli reveal that their rapid incorporation of lysine is associated with the appearance of a granular substructure which is inapparent, or severely altered, in cells treated either under conditions of RNA extraction or with actinomycin. These findings suggested that nuclear ribosome or ribosomal precursor synthesis and a concomitantly labeled, basic ribonucleoprotein may be associated with these granular sites (15, 32–35, 59).

The experiments to be reported were designed to corroborate these findings and the consistency of such a hypothesis by determining whether the basic amino acid, arginine, and lysine, are incorporated as nucleolar protein or as unbound amino acid (3, 5, 19, 27, 36, 37). In order to determine this point, especially in the face of discrepant observations concerning amino acid uptake in the nucleolus (23–26), the effect of some conditions of fixation, notably of temperature, on the radioautographic localization of amino acid and nucleotide label in nucleoli was investigated. Evidence will be presented that the nucleolus of HeLa cells is a site of rapid incorporation of both arginine and lysine into a nucleoprotein the synthesis of which is inhibited by puromycin as well as by actinomycin D. When the temperature of fixation in aldehyde fixatives is elevated to 37°C, this protein label in the nucleolus is dissociated from RNA label and is reduced proportionally to the same extent as by puromycin- and actinomycin D inhibition. These data are consistent with the interpretation that the proportion of nucleolar protein which is not fixed at the elevated temperature is conjugated with nucleolar ribosomal RNA (38).

MATERIALS AND METHODS

PPLO-free HeLa cells[1] grown for 48–96 hr in Leighton tubes containing Eagle's medium (39) supplemented by 10% human serum were treated in part with puromycin (200 μg/ml)[2] and in part with actinomycin D[3] (0.05 μg/ml) under conditions previously employed (28, 29), and were labeled for 15 min with 10 μc/ml of DL-lysine-^{3}H (201 mc/mmole)[4] or (L-arginine-G-^{3}H^3 (106 mc/mmole),[4] or uridine-G-^{3}H (290 mc/mmole, 270 mc/mmole).[4] The cells were washed five times in Hanks' balanced salt solution, with or without a previous rinse in growth medium, and were fixed for 30 min–4 hr either with 3.7% formaldehyde[5] in 0.136 M sodium phosphate buffer containing 10% glucose (40), or with 5% glutaraldehyde[6] in the same buffer. The pH and temperature of the fixative were adjusted to pH 7.3 and 0°C, or as specified below. The addition of Ca^{++} and Mg^{++} ions in some experiments did not significantly alter the results. Formaldehyde in 0.147 M sodium acetate buffer (pH 7.3) and Carnoy fixation (one part glacial acetic acid: three parts ethanol pH 3.0) were used for comparison in some experiments. In one experiment (Fig. 6), Tris buffer, 1.8 M, pH 8.0 at 37°C (41),[7] was applied as an eluent for 60 min to glutaraldehyde-fixed cells and compared with 0.5 M glycine buffer, pH 9.5.

After fixation the cells were again rinsed five times in cold distilled water. In the case of uridine-^{3}H label, acid-soluble nucleotides were removed by extraction with 0.5 N perchloric acid at 0°–4°C for 20–30 min, followed by rinsing in distilled water. Such extraction does not, however, alter significantly the amino acid label under these conditions of fixation (28) and was, therefore, not employed to distinguish the amount of label incorporated into free amino acid or into macromolecular protein. The cover slip preparations were either air-dried or briefly rinsed with ethanol (95%). They were coated with fine grain, radioautographic, liquid emulsion (Ilford L-4, diluted 1:1.6) at 43°C, were dried vertically, and were exposed at −20°C for 20–21 days (lysine-^{3}H and arginine-^{3}H preparations) and for 4–5 days (uridine-^{3}H preparations). The radioautographs were developed in Ficq's diaminophenol developer (42) and were stained with Harris' hematoxylin and eosin.

The mean grain count over nucleolus,[8] total nucleus, and cytoplasm of 25 or more randomly selected

[1] Microbiological Associates, Inc., Bethesda, Md.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio

[3] Generous gift of Merck, Sharpe & Dohme, West Point, Pa.

[4] Nuclear-Chicago Corporation, Des Plaines, Ill.

[5] Certified reagent, Fisher Scientific Company, New York.

[6] Polysciences, Inc., Rydal, Pa., and Union Carbide Corporation, New York.

[7] Trizma Base, Sigma Chemical Co., St. Louis, Mo.

[8] Not corrected for β-ray self absorption.

interphase cells per slide was then determined from five or more separate counts per cell within an approximate range of 10%. The background label was determined for the approximate nuclear area ($1/75$ mm^2) and was substracted from the nuclear and nucleolar grain counts, but the cytoplasmic grain counts were not corrected because of marked variation in the projected cytoplasmic area. Phase-contrast optics, with a Zeiss Neofluar 100/1.30 objective at a magnification of 1200, aided substantially in distinguishing nucleoli in stained preparations and gave adequate resolution and heightened contrast to the small silver grains. As an approximation of nuclear and nucleolar volumes, the relative areas of nucleolus and nucleus, calculated as idealized rectangles, were determined for each cell with the aid of an ocular micrometer grid. Because of a resulting reduction in the standard error relative to the mean (see below), the ratio of nucleolar to total nuclear grain count and the ratio per unit area was determined for each cell. Statistical comparison of the means was performed by variance analysis.

RESULTS AND DISCUSSION

In an attempt to clarify the interpretation of nuclear and nucleolar ultrastructure with respect to localization and synthesis of basic protein, the effect of variation of some conditions of aldehyde fixation on the intranuclear distribution of lysine-^{3}H and arginine-^{3}H was studied by differential grain count in radioautographs of PPLO-free HeLa cells (Figs. 1–6; Table I). Samples of 25 randomly selected cells were used.

The mean grain count over subcellular components of nonsynchronized HeLa cells labeled briefly with tritiated amino acids or nucleotide bases fluctuates, as expected, within a fairly wide range and shows an average standard error relative to the mean of 8.24% for the nucleolus and 9.54% for the nucleus (Table I, columns 3, 4; Fig. 1). However, the means of the ratio of nucleolar to nuclear grain count have more than twice this consistency, with an average relative standard error of 4.15% (Table I, column 2; Fig. 1). When this ratio of nucleolar to nuclear grain count per cell is expressed as ratio per unit area of nucleolus to nucleus (Table I, column 1; Fig. 1) there is a slight, but tolerable, decrease in the consistency of the mean (average relative standard error, 5.53%. Presumably this decrease occurs as a result of the introduction of a small, but fairly constant, error in the calculation of the relative areas of nucleolus and nucleus. This error is introduced by treating the relative area of nucleolus to nucleus as a ratio of two rectangles the areas of which were calculated from the products of their respective diameters; whereas a ratio of two ellipsoids would described these parameters more accurately, although more laboriously. Because they allow statistically significant inferences from a smaller sample of cells than the separate mean grain counts, these ratios of intranuclear amino acid metabolism are compared as percentage histograms in Figs. 1–6. In cells labeled for 15 min with lysine-^{3}H or arginine-^{3}H the nucleolus accounts for 34–36% of the nuclear grain count or 12–17% of the total amino acid label, when cells are fixed in phosphate-buffered formalin or glutaraldehyde (Figs. 1–4, 6; Table I).

In order to distinguish macromolecular protein label from unbound amino acid (35), the cells were pretreated with puromycin (200 μg/ml). This inhibitor has been shown to interfere with the formation of complete peptide chains on ribosomes, but is not known to reduce the cellular uptake of amino acid (43–46). An increment of amino acid label per unit area in untreated cells, compared to cells pretreated with puromycin may then be interpreted as due to protein synthesis, and residual label in puromycin-treated cells may be interpreted either as unbound amino acid and/or as an accumulation of incomplete peptide chains formed in the presence of puromycin (45, 46). Puromycin was thus employed in place of "pulse-labeling," which is a difficult technical procedure when one is studying labile peptides with a rapid turnover (19, 20, 28, 47, 48) that might readily be chased away. After puromycin treatment, cells show a significantly reduced absolute and proportional nucleolar amino acid incorporation under some conditions of fixation (Figs. 1–4, 6, Table I). The proportion of nuclear label counted over nucleoli of treated cells is almost one-half that of untreated control cells. If puromycin is removed from the growth medium, this loss of nucleolar protein label is reversible, and the proportion of nucleolar label is equivalent to or greater (49) than that of untreated cells. Despite some shrinkage of the nucleolus under these conditions (Table I), the grain count over the nucleolus per unit area in puromycin-treated cells is 46% of that of untreated controls and 69% of that of cells reversibly inhibited. The grain count of the nucleolus relative to the nucleus when adjusted to unit area, is about 27% below that of untreated controls ($P < 0.01$) and about 35%

Mean grain count of 25 HeLa cells lab

T65/122 inhibitor	3.6% Formaldehyde, pH 7.3, fixation time	Temperature	(Nucleolus/nucleus) / unit area	Nucleolus / nucleus	Nucleolus (−background
		°C			
Untreated	30 min	0	2.680 ± 0.188	0.3493 ± 0.0129	36.46 ± 3.3
	2 hr	0	2.633 ± 0.165	0.3609 ± 0.0168	32.61 ± 3.2
	4 hr	0	2.463 ± 0.128	0.3742 ± 0.0126	42.82 ± 2.7
	Avg 0°	(η = 75)	2.592 ± 0.160	0.3615 ± 0.0141	37.30 ± 3.1
	30 min	23	2.529 ± 0.175	0.3420 ± 0.0112	29.03 ± 2.1
	2 hr	23	2.641 ± 0.129	0.3689 ± 0.0111	43.73 ± 2.7
	4 hr	23	2.594 ± 0.121	0.3643 ± 0.0132	33.11 ± 2.8
	Avg 23°	(η = 75)	2.588 ± 0.142	0.3584 ± 0.0118	35.29 ± 2.5
	30 min	37	2.040 ± 0.086	0.2400 ± 0.0136	30.59 ± 3.1
	2 hr	37	1.989 ± 0.117	0.2774 ± 0.0155	25.56 ± 2.1
	4 hr	37	1.930 ± 0.091	0.2589 ± 0.0118	31.05 ± 2.6
	Avg 37°C	(η = 75)	1.986 ± 0.098	0.2588 ± 0.0136	29.07 ± 2.6
Actinomycin 0.05	30 min	0	1.857 ± 0.129	0.1939 ± 0.0140	10.81 ± 1.2
μg/ml 3 hr, growth	30 min	23	1.743 ± 0.109	0.1853 ± 0.0086	12.16 ± 0.8
medium 3 hr	4 hr	37	1.922 ± 0.083	0.2021 ± 0.0085	14.96 ± 1.2
	Avg 0° + 23° + 37°C	(η = 75)	1.841 ± 0.107	0.1938 ± 0.0104	12.64 ± 1.1
Puromycin 24 hr,	30 min	0	1.744 ± 0.124	0.2010 ± 0.0135	13.98 ± 1.2
200 μg/ml	4 hr	0	2.045 ± 0.128	0.2010 ± 0.0109	11.36 ± 0.9
	4 hr	23	1.742 ± 0.122	0.1754 ± 0.0117	11.22 ± 1.2
	30 min	37	2.011 ± 0.108	0.2220 ± 0.0130	14.07 ± 0.9
	4 hr	37	1.850 ± 0.095	0.1969 ± 0.0094	15.07 ± 1.0
	Avg 0° + 23° + 37°C	(η = 125)	1.878 ± 0.115	0.1993 ± 0.0117	13.14 ± 1.0
Puromycin 24 hr,	30 min	0	3.000 ± 0.131	0.3023 ± 0.0142	20.57 ± 1.2
200 μg/ml +	4 hr	0	2.941 ± 0.126	0.2909 ± 0.0118	15.97 ± 1.2
growth medium	30 min	23	2.804 ± 0.136	0.3009 ± 0.0168	16.53 ± 1.9
8 hr	4 hr	23	2.749 ± 0.143	0.2859 ± 0.0140	18.81 ± 1.2
	Avg 0° + 23°C	(η = 100)	2.874 ± 0.134	0.2950 ± 0.0142	17.97 ± 1.2
	30 min	37	2.035 ± 0.088	0.2192 ± 0.0116	10.44 ± 0.7
	4 hr	37	1.979 ± 0.099	0.1938 ± 0.0100	18.95 ± 2.2
	Avg 37°C	(η = 50)	2.007 ± 0.094	0.2065 ± 0.0108	14.68 ± 1.

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ie Intranuclear Distribution of Lysine ^3H

Lysine-^3H (10 μc/ml), 15 min)			Volume projected as rectangles (units)		
Nucleus (−background	Cytoplasm (+background)	Nucleus + cytoplasm	Nucleolus	Nucleus	Nucleolus / nucleus
4.37 ± 8.79	127.67 ± 13.34	232.04	58.92 ± 5.46	423.60 ± 26.18	0.1407 ± .0084
0.36 ± 16.27	90.31 ± 11.82	180.05	57.48 ± 4.60	398.44 ± 23.77	0.1441 ± .0078
4.45 ± 17.81	120.00 ± 8.58	223.06	55.72 ± 2.98	354.60 ± 13.96	0.1591 ± .0081
3.06 ± 14.29	112.45 ± 11.25	211.72± 25.54	57.37 ± 4.35	392.21 ± 21.30	0.1480 ± .0081
4.87 ± 6.24	99.45	184.32	56.48	389.52	0.1443 ± .0042
8.52 ± 6.62	137.24	255.76	58.92	409.04	0.1462 ± .0070
0.89 ± 7.45	87.30	178.19	51.76	358.92	0.1450 ± .0059
8.09 ± 6.77	108.00	206.09	55.72	385.83	0.1452 ± .0057
7.49 ± 11.36	126.14 ± 12.35	253.63	53.60 ± 5.48	440.96 ± 32.77	0.1196 ± .0076
2.14 ± 6.03	63.48 ± 4.70	155.62	59.56 ± 5.89	416.64 ± 40.15	0.1476 ± .0088
9.92 ± 9.27	107.91 ± 11.27	227.83	54.64 ± 3.77	394.52 ± 21.14	0.1402 ± .0083
3.18 ± 8.89	99.18 ± 9.61	212.36± 18.50	55.93 ± 5.05	417.37 ± 31.35	0.1358 ± .0082
5.77 ± 2.96	90.55 ± 7.33	146.32	43.48 ± 3.73	393.64	0.1078 ± .0063
5.64 ± 3.63	73.63 ± 6.64	139.27	34.53 ± 1.96	318.52	0.1140 ± .0077
4.00 ± 6.24	68.14 ± 8.94	142.14	44.20 ± 3.42	418.52	0.1072 ± .0045
5.14 ± 4.28	77.44 ± 7.64	142.58± 11.92	40.74 ± 3.04	376.89	0.1097 ± .0062
9.56 ± 4.17	66.29 ± 3.85	135.34	38.92 ± 2.28	334.72 ± 17.51	0.1178 ± .0054
6.54 ± 4.38	88.52 ± 7.82	145.06	46.12 ± 2.94	463.96 ± 24.68	0.1019 ± .0057
3.93 ± 4.55	72.45 ± 6.36	136.38	47.56 ± 4.34	435.84	0.1069 ± .0072
3.40 ± 3.40	67.90 ± 6.02	131.30	42.28 ± 2.91	375.04	0.1152 ± .0079
6.53 ± 5.13	80.60 ± 7.18	157.13	49.04 ± 4.20	446.20	0.1136 ± .0093
5.99 ± 4.34	75.15 ± 6.25	141.04± 10.59	44.78 ± 3.33	411.15	0.1111 ± .0071
8.06 ± 3.90	103.13	171.19	48.00	475.88	0.1023
4.89 ± 3.26	71.91	126.80	41.60	407.12	0.1036
4.96 ± 2.44	59.90	114.86	39.64	372.95	0.1093
5.78 ± 5.32	58.59	124.37	38.56	359.56	0.1070
0.92 ± 3.73	73.38	134.30	41.95	403.88	0.1056
7.49 ± 2.52	46.52	94.01	40.74	375.20	0.1097
7.78 ± 8.78	106.25	204.03	54.00	541.40	0.1006
2.64 ± 5.65	76.39	149.02	47.37	458.30	0.1052

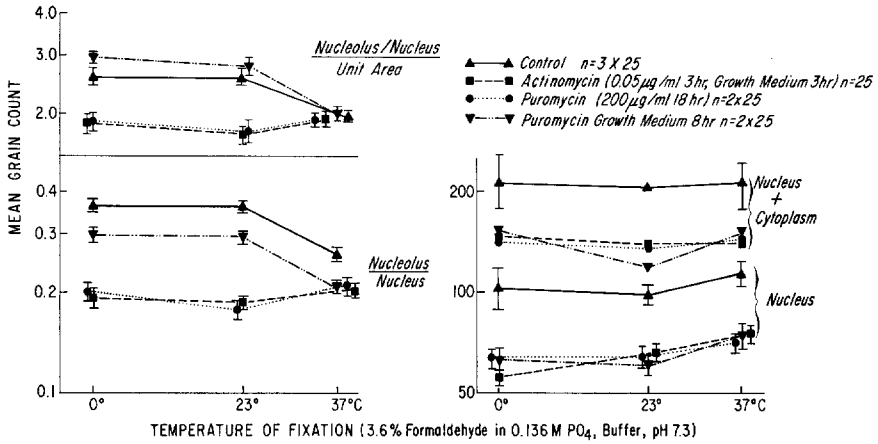

FIGURE 1 Effect of actinomycin and puromycin on the intranuclear distribution of lysine-^{3}H (10 μc/ml, 15 min) in HeLa cells at varying temperatures of formaldehyde fixation.

lower than that of cells reversibly inhibited ($P < 0.001$) (Table I; Fig. 1). In the total nucleus and cytoplasm, only about 30% of the amino acid label is inhibited by extended puromycin administration (Table I). The high residual grain count in the nucleus and cytoplasm is interpreted as unbound amino-acid and/or as incomplete puromycin peptide label fixed under these conditions (28). Similar results are shown in three other experiments with lysine-^{3}H label (Figs. 2, 4, 6) and with arginine-^{3}H label (Fig. 3). No such inhibition of nucleolar uptake was observed when uridine-^{3}H was employed as a brief label of RNA in stationary phase cells under these conditions (Fig. 5) (10). Puromycin has, however, been shown to affect the maturation of ribosomal RNA (8, 50) from a ribosomal precursor RNA and to depress nuclear RNA synthesis under conditions of logarithmic growth (10, 51–53).

A selective inhibition of nucleolar RNA synthesis can be produced, however, by low doses of actinomycin D (0.05 μg/ml) (28, 29), as shown in Table II. The nucleolar RNA inhibited under these conditions has been characterized as a rapidly sedimenting, guanosine- and cytosine-rich, ribosomal precursor RNA (17, 24, 29, 30). When the effect of actinomycin D (0.05 μg/ml) on the uptake of lysine-^{3}H in the nucleolus is compared with that of puromycin in the same experiment, an identical inhibition is observed, with results that appear to be superimposed on a logarithmic scale (Fig. 1, Tables I and II). These and previously reported data (28) are consistent with the interpretation that the nucleolus of HeLa cells is a site of rapid incorporation of amino acid into a basic protein, associated with guanosine- and cytosine-rich sites (54) of nucleolar ribosomes (or ribosomal precursor) (19), and not an exclusive site of unbound amino acid label (36) or of amino acyl *s*-RNA (19, 24).

Discrepant radioautographic observations (23–26) concerning a rapid concentration of protein label in the nucleolus may perhaps be related to the fact that these data are tied to specific conditions of fixation rarely employed in previous radioautographic studies (47). As shown in Figs. 2 and 3, Carnoy-type fixation, widely employed in such studies (23–26), or formalin fixation with varied pH or buffer species (55) results in a significantly lower concentration of amino acid label in the nucleolus than fixation in phosphate-buffered formalin or glutaraldehyde at a slightly alkaline pH (7.3) (28). The relative amount of nucleolar protein label eluted under those conditions of fixation was, however, not determined.

A rapid increase in binding of formaldehyde to *s*-RNA at 37°C in 0.1 M phosphate buffer at pH 7.4 has been reported and has been shown to be the result of thermal denaturation with consequent removal of steric hindrance from some amino groups of nucleotide bases that are thus freed to form additional Schiff bases with formaldehyde (56). Recently, similar observations have been made with nucleolar ribosomal and ribosomal precursor RNA; this points to a different degree of hydrogen bonding (16). Preliminary

TABLE II

Effect of Temperature of Formaldehyde Fixation on Intranulear Distribution of Uridine-^{3}H

T65/122 inhibitor	3.6% Formaldehyde, pH 7.3, fixation time	Temperature	Mean grain count of uridine-^{3}H (10 μc/ml, 15 min) labeled HeLa cells				
			(Nucleolus/nucleus) / unit area	Nucleolus / nucleus	Nucleolus (−background)	Nucleus (−background)	Cytoplasm
		°C					
Untreated	30 min	0	2.662 ± 0.097	0.3655 ± 0.0103	29.90 ± 1.75	81.75 ± 5.17	0
	2 hr	0	2.636 ± 0.117	0.3647 ± 0.0141	32.14 ± 2.61	88.85 ± 7.96	0
	4 hr	0	2.765 ± 0.117	0.3792 ± 0.0138	37.63 ± 2.88	97.76 ± 6.61	0
	30 min	23	2.936 ± 0.129	0.3726 ± 0.0158	36.79 ± 3.16	97.99 ± 6.21	0
	Avg 0° + 23°C	(η = 100)	2.750 ± 0.115	0.3705 ± 0.0135	34.12 ± 2.60	91.59 ± 6.49	0
	30 min	37	2.540 ± 0.135	0.3228 ± 0.0131	25.77 ± 2.59	79.35 ± 6.46	0
	2 hr	37	2.454 ± 0.126	0.3064 ± 0.0103	41.15 ± 3.09	136.14 ± 9.98	0
	2 hr	37	2.258 ± 0.092	0.2838 ± 0.0119	30.11 ± 2.59	106.26 ± 10.37	0
	Avg 37°C	(η = 75)	2.417 ± 0.018	0.3043 ± 0.0118	32.34 ± 2.74	107.25 ± 8.94	0
Actinomycin 0.05 μgm/ml, 3 hr, growth medium, 3 hr	30 min	0	1.938 ± 0.100	0.1639 ± 0.0086	10.17 ± 0.89	62.67 ± 5.15	0
	4 hr	0	1.914 ± 0.112	0.1528 ± 0.0110	14.98 ± 1.45	97.78 ± 6.38	0
	30 min	23	1.967 ± 0.126	0.1501 ± 0.0077	13.21 ± 0.94	87.50 ± 4.10	0
	30 min	37	1.862 ± 0.132	0.1686 ± 0.0092	9.05 ± 1.32	50.73 ± 5.98	0
	Avg 0° + 23° + 37°C	(η = 100)	1.920 ± 0.118	0.1589 ± 0.0091	11.85 ± 1.15	74.68 ± 5.38	0

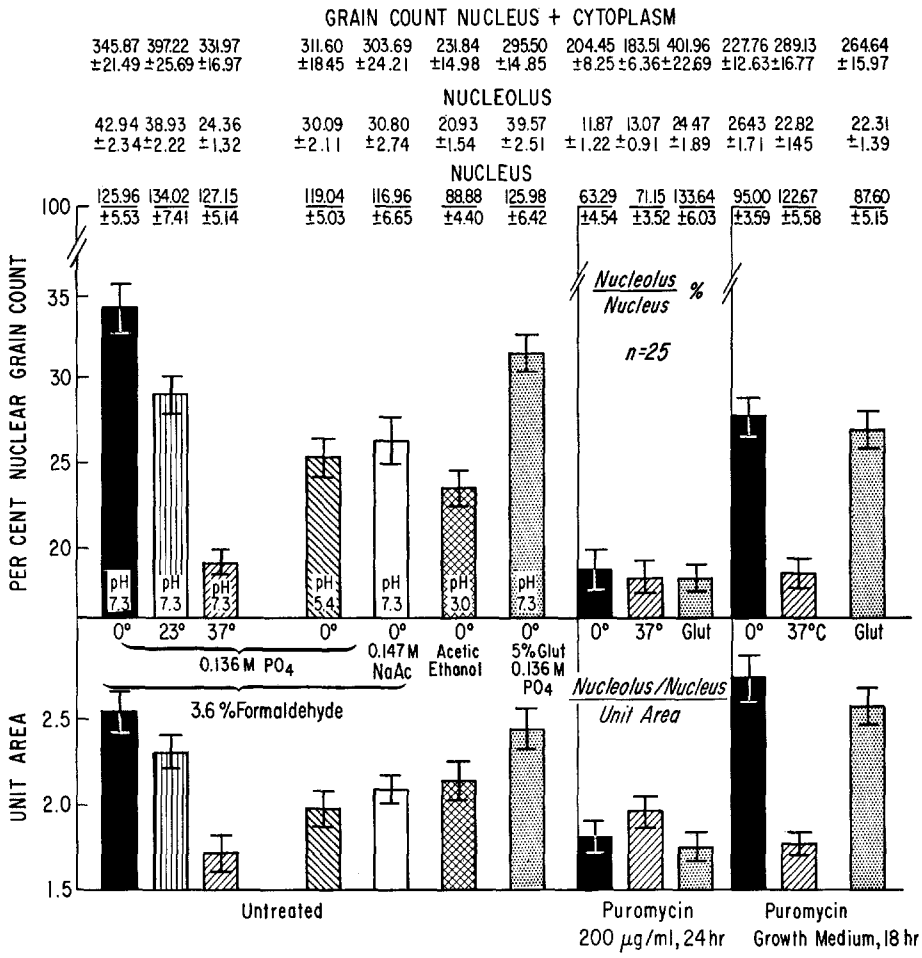

FIGURE 2 Effect of fixation on intranuclear distribution of lysine-^{3}H (10 μc/ml, 15 min) in HeLa cells compared with cells pretreated with puromycin.

cytochemical observations have suggested that the fixation of nucleolar protein might also be affected by such an increase in temperature of formalin, perhaps by a similar steric alteration of the binding sites of nucleolar RNA (16, 58). Experimental attempts to test such a hypothesis were made by comparing amino acid incorporation and nucleotide base incorporation at varying temperatures of aldehyde fixation.

When the temperature of formalin or glutaraldehyde fixatives of lysine-^{3}H- or arginine-^{3}H-labeled cells was elevated to 37°C and the grain count compared with grain counts of cells fixed at 0° or 23°C, a highly significant shift of intranuclear distribution of amino acid label was observed (Table I; Figs. 1–4). A diminution of about 28% ($P < 0.001$) of the ratio of nucleolar to nuclear label and of about 23% ($P < 0.01$) of the nucleolar to nuclear ratio per unit area under these conditions is shown in columns 1 and 2 of Table I. Comparable results with similar levels of significance for the proportional nucleolar label were obtained in four separate experiments (Figs. 1–4); but for reasons discussed above significant changes in the absolute nucleolar grain count ($P < 0.05$) were found only in two out of four experiments (Figs. 2, 3). This diminution of nucleolar amino acid incorporation at 37°C fixation occurs without demonstrable change in nuclear or nucleolar volume (Table I, last three columns), and is, therefore, unlikely to be produced by changes in β-ray self-absorption of the nucleolus

at 37°C. Furthermore, it is observed without significant change in either total nuclear or total cellular grain count (Table I, Figs. 1–4). When adjusted to unit area, the relative nucleolar amino acid label of untreated cells fixed at 37°C is almost identical with that of cells pretreated with puromycin and low doses of actinomycin D fixed in cold formalin or glutaraldehyde (Table I; Figs. 1–4). But puromycin- or actinomycin-pretreated cells fixed at 37°C show no significant changes in absolute or relative grain count when compared to cells fixed in the cold; and by removing the inhibitor the effect of puromycin on nucleolar protein label can be reversed only when cells are fixed at the lower temperatures (Table I; Figs. 1–4).

On the other hand, with uridine-^{3}H as RNA label (Table II; Fig. 5) no significant differences in nucleolar incorporation were observed at different temperatures of fixatives when expressed as ratio per unit area. A slight difference (12% $P \lesssim 0.01$) in the ratio of absolute nucleolar to nuclear grain count observed at 37°C fixation in one experiment (Table II, column 2) would appear to be the result of a nonrandom selection of cells with large nucleoli, in view of the proximity of the area adjusted and absolute grain counts.

These observations support the interpretation that a nucleolar protein not fixed by formaldehyde or glutaraldehyde at 37°C is associated with nucleolar ribosomal or ribosomal precursor RNA but that at 37°C it is dissociated from such RNA. Because of the wide variance of absolute grain counts and the resulting impracticality of obtaining a reliable dissociation curve, it was not determined conclusively whether this dissociation of nucleolar protein label at 37°C is the result of nonionic dissociation and solubilization in the fixative or of thermal denaturation in formaldehyde or glutaraldehyde of nucleolar RNA (16, 56) and

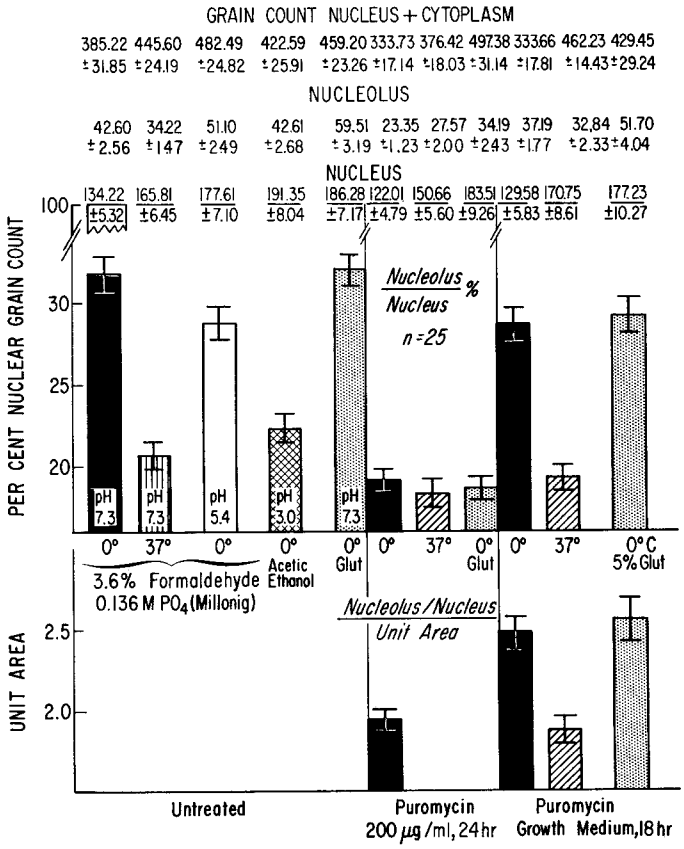

FIGURE 3 Effect of fixation on intranuclear distribution of arginine-^{3}H (10 µc/ml, 15 min) in HeLa cells compared with cells pretreated with puromycin.

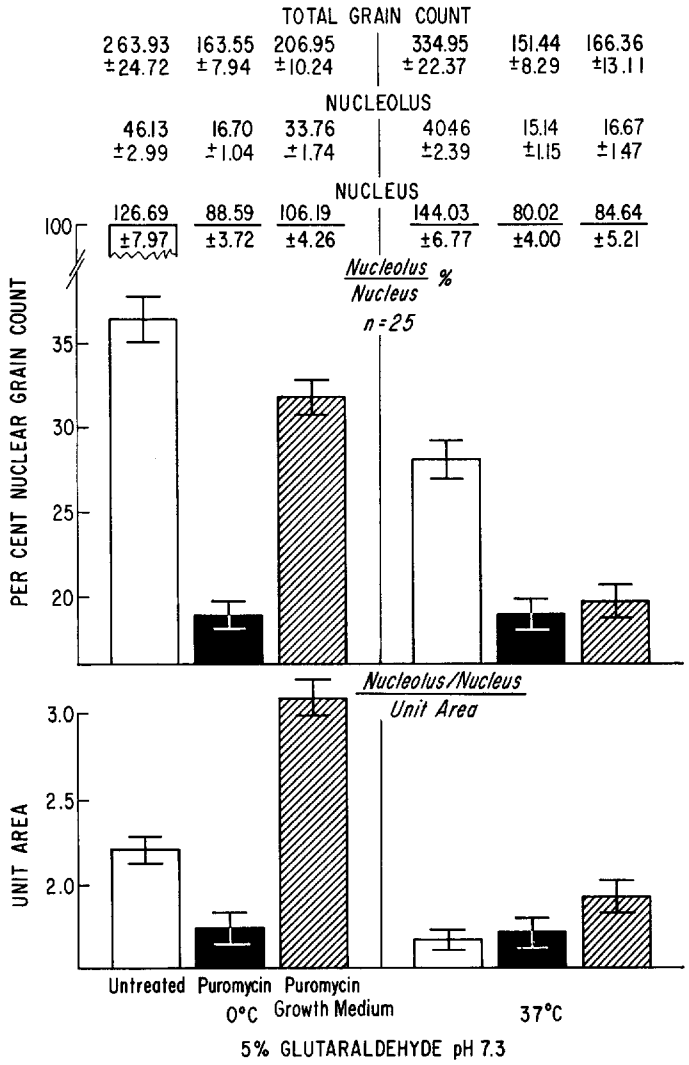

FIGURE 4 Effect of temperature of glutaraldehyde fixation on intranuclear distribution of lysine ^{3}H (10 μc/ml, 201 mc/mM, 15 min) compared with cells pretreated with puromycin (200 μg/ml, 18 hr).

ionic dissociation of a conjugated protein. Circumstantial evidence favors the latter alternative. If a nonionic dissociation were the only underlying mechanism of this phenomenon, the amount of evident label in nucleoli would probably vary with length of fixation time or with an intermediate temperature of fixation. As shown in Table I, Figs. 1 and 2, the relative amino acid label of the nucleolus at 0° or 37°C is not significantly altered by varying the fixation times between 30 min and 4 hr; and no significant differences were observed in relative or absolute nucleolar grain counts between specimens fixed at 0°C or at an intermediate temperature of 23°C.

The previously reported reduction of nucleolar amino acid label by a high concentration of ribonuclease (1 mg/ml in 0.016 M sodium–phosphate buffer) as well as by Feulgen-type hydrolysis with 1 N NCl at 60°C and by dilute trypsin is also consistent with the interpretation that this nucleolar material is a conjugated ribonucleoprotein (28). The experiment in Fig. 6 shows that analogous changes are produced when glutaraldehyde-fixed cells are eluted in hypertonic Tris

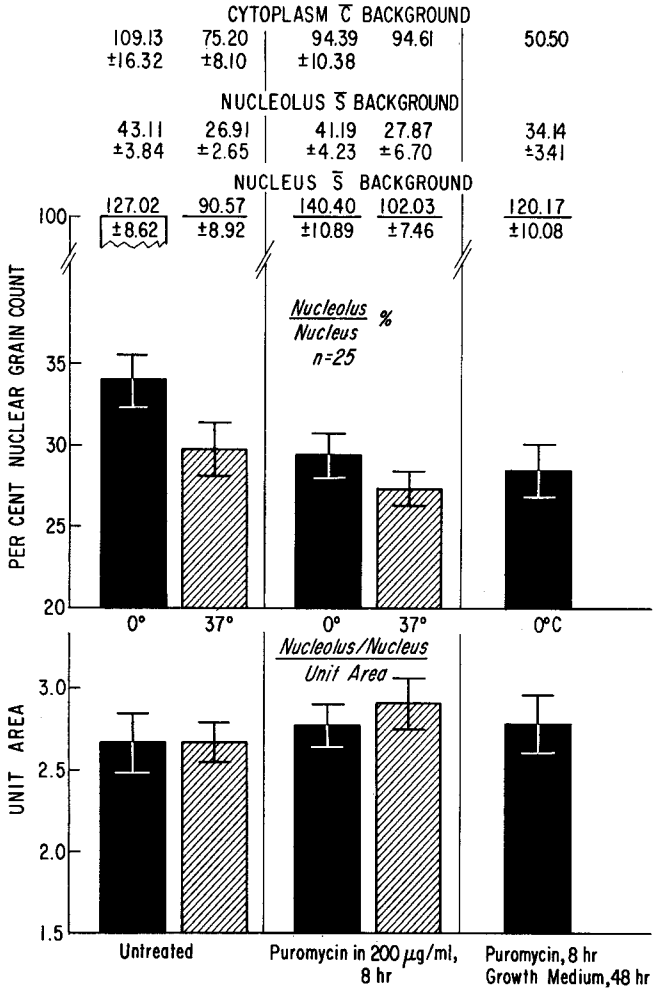

FIGURE 5 Effect of puromycin treatment on intranuclear distribution of uridine-^{3}H (10 µc/ml, 2.9 c/mM, 15 min) in HeLa cells fixed in formalin at 0° and 37°C.

buffer at pH 8, under conditions employed in vitro for stripping amino acid from *s*-RNA (41).

The biochemical nature of this nucleolar protein must remain speculative. Although it may be termed "basic" because of staining properties with the acid dye Biebrich scarlet at alkaline pH (28), the fact that it is not removed by 0.2 N HCl and is rapidly labeled may indicate that it is nonhistone (1, 3) and possibly of the residual or acidic type (7, 18, 20, 57). Lack of data precludes further speculation as to its relationship to nuclear proteins affecting RNA-polymerase activity (10) or as messenger RNA-associated "informosomes" (11), or to structural ribosomal protein affecting ribosome stabilization (9, 12).

The site of synthesis of this protein also is not conclusively resolved by these experiments. The rapid labeling of this protein in the nucleolus within 5 min or less (18, 20, 28), although consistent with a nucleolar site of synthesis, does not preclude the rapid transfer of the protein from other sites. The greater consistency of the means of the ratio of the nucleolar to total nuclear grain count than of the separate mean grain counts suggests the possibility of a constant intranuclear transfer. Several recent exceptions have cast doubt on the original premise that the effect of actinomycin on protein synthesis is necessarily related to its effects on template RNA synthesis (60–62), but these exceptions do not apply in the present experimental conditions. Nevertheless the inhibitory effect of actinomycin D on amino acid incorporation into a nucleolar protein is consistent, but not conclusive, evidence of in situ synthesis.

It may be concluded from these experiments that the nucleolus is a site of rapid assembly or

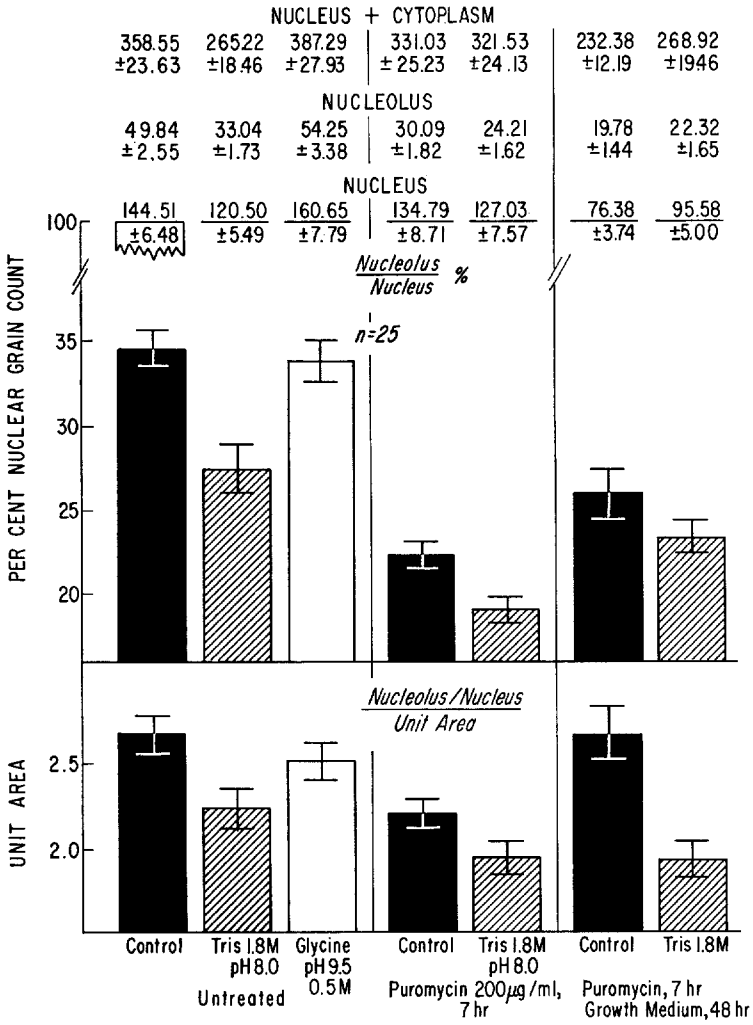

FIGURE 6 Effect of elution with Tris buffer (1.8 M, pH 8.0) on intranuclear distribution of lysine-^{3}H (10 μc/ml, 15 min) in HeLa cells fixed with glutaraldehyde.

synthesis of a protein inhibited identically by puromycin and low doses of actinomycin D. Thus this protein is labeled by the basic amino acids, lysine and arginine, as polypeptide and appears to be labeled in association with nucleolar ribosomal RNA or ribosomal precursor RNA. It can be fixed in cold, phosphate-buffered formalin or glutaraldehyde at pH 7.3 but apparently not in Carnoy-type fixatives or aldehyde fixatives at acid pH; and for this reason it may have escaped detection in some radioautographic studies (23–26). When the temperature of fixation in aldehyde fixatives is elevated to 37°C this protein label, but not the RNA label, is proportionally reduced in the nucleolus to the same extent as by puromycin and actinomycin D inhibition, but without evident loss in total nuclear or cellular grain count. These observations support the interpretation that the nucleolar protein not fixed at 37°C is associated with nucleolar ribosomal RNA, but that at 37°C it is dissociated from the RNA label and from the nucleolus. Although a definitive conclusion as to the nature of this dissociation cannot be made from these data the findings that such dissociation is independent of length of fixation time and is not observed at an intermediate temperature of fixation suggest that this is an ionic dissociation of a conjugated ribonucleoprotein produced on the

basis of thermal denaturation of associated nucleolar ribosomal RNA in formaldehyde or glutaraldehyde fixatives. Study of the ultrastructural sites of this nucleoprotein may be facilitated by these findings.

SUMMARY

A relative ignorance of the molecular basis of fixation is a major obstacle to the correlation of information gained by methods of the newer molecular biology with the structure and function of intact mammalian cells. The foregoing radioautographic experiments represent an attempt to study the effects of aldehyde fixation on nucleolus-associated protein and RNA in a fairly well-described, molecular model system affecting the formation of nuclear ribosomes. It was hoped, thereby, to facilitate a functional interpretation of nucleolar ultrastructure and to help clarify discrepant observations concerning the role of nucleolar protein in the assembly of the ribosome complex.

It has been shown that the nucleolus, in addition to being a site of rapidly labeled RNA synthesis, is a site of rapid assembly or synthesis of a protein inhibited equally by puromycin and by low doses of actinomycin D. The reversible inhibition of lysine-^{3}H and arginine-^{3}H-incorporation in nucleoli by puromycin supports the conclusion that the rapid concentration of amino acid in nucleoli is not due to unbound amino acid label, but to polypetide label; and the identical inhibition by low doses of actinomycin D under conditions inhibitory also to the synthesis of a nucleolar ribosomal precursor RNA supports our previous data which pointed to an association of this protein with a nucleolar ribosomal RNA (28). These radioautographic findings are in agreement with conclusions reached by more precise biochemical techniques involving isolated nucleoli (7, 18, 19), but differ from some of the previous radioautographic observations (23–26). Such discrepancies would appear to be related to differences in methods of fixation, as the nucleolar protein label is diminished in acetic ethanol fixative or in formalin at acid pH but is fixed in phosphate-buffered formalin or glutaraldehyde at pH 7.3. Concordant radioautographic findings with the use of "neutral" formalin have been previously reported (47).

Attempts to explore the nature of the association of nucleolar protein label with RNA by variation of the temperature of fixation led to the surprising observation that fixation at 37°C in formaldehyde or glutaraldehyde consistently reduces the nucleolar protein label, but not the RNA label, by a proportion identical with that incurred by puromycin or actinomycin inhibition; but this reduction in nucleolar protein label occurs without evident loss of nuclear or total grain count. These findings imply that the proportion of nucleolar protein not fixed at 37°C is associated with nucleolar ribosomal RNA but that at 37°C it is dissociated from such RNA and from the nucleolus. The variance of absolute grain counts does not permit a definitive conclusion as to the nature of this dissociation of nucleolar protein. However, the observation that an intermediate temperature of fixation or extended fixation time does not produce this phenomenon suggests that this is an ionic dissociation of a conjugated ribonucleoprotein.

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REFERENCES

1. HUANG, R. C., and J. BONNER. 1967. *Proc. Natl. Acad. Sci. U. S.* **48**:1216.
2. HUANG, R. C., and J. BONNER. 1965. *Proc. Natl. Acad. Sci. U. S.* **54**:960.
3. ALLFREY, V. G., and A. E. MIRSKY. 1964. *In* The Nucleohistones. J. Bonner and P. Tso, editors. Holden-Day, Inc., San Francisco. 267.
4. ALLFREY, V. G., V. C. LITTAU, and A. E. MIRSKY. 1963. *Proc. Natl. Acad. Sci. U. S.* **49**:414.
5. LIAU, M. C., L. S. HNILICA, and R. B. HURLBERT. *Proc. Natl. Acad. Sci. U. S.* **53**:626.
6. HNILICA, L. S., M. C. LIAU, and R. B. HURLBERT. 1966. *Science.* **152**:521.
7. BUSCH, H. 1965. Histones and Other Nuclear Proteins. Academic Press Inc., New York.
8. TAMAOKI, T., and G. C. MUELLER. 1965. *Biochim. Biophys. Acta.* **108**:73.
9. TAMAOKI, T., and G. C. MUELLER. 1965. *Biochim. Biophys. Acta.* **108**:81.
10. SUMMERS, W. P., W. D. NOTEBOOM, and G. C. MUELLER. 1966. *Biochem. Biophys. Res. Commun.* **22**:399.

11. SAMARINA, O. P., A. A. KRICHEVSKAYA, and C. P. GEORGIEV. 1966. *Nature.* **210**:1319.
12. PERRY, R. P., and D. E. KELLEY. 1966. *Biochem. Biophys. Res. Commun.* **24**:459.
13. MCCONKEY, E. H., and J. W. HOPKINS. 1964. *Proc. Natl. Acad. Sci. U. S.* **51**:1197.
14. BIRNSTIEL, M. L., M. I. H. CHIPCHASE, and B. B. HYDE. 1963. *Biochim. Biophys. Acta.* **76**:454.
15. RITOSSA, F. M., and S. SPIEGELMAN. 1965. *Proc. Natl. Acad. Sci. U. S.* **53**:737.
16. MURAMATSU, M., J. L. HODNETT, and H. BUSCH. 1966. *J. Biol. Chem.* **241**:1544.
17. RO, T. S., K. S. NARAYAN, and H. BUSCH. 1966. *Cancer Res.* **26**:780.
18. BIRNSTIEL, M. L., and W. G. FLAMM. 1964. *Science.* **145**:1435.
19. VINCENT, W. S. 1964. *Proc. Intern. Congr. Genet. 11th The Hague 1963.* 343.
20. STEELE, W. J., and H. BUSCH. 1963. *Cancer Res.* **23**:1153.
21. GROGAN, D. E., R. DESJARDINS, and H. BUSCH. 1966. *Cancer Res.* **26**:775.
22. MAGGIO, R. 1966. *Biochim. Biophys. Acta.* **119**:641.
23. ERRERA, M., A. HELL, and R. P. PERRY. 1961. *Biochim. Biophys. Acta.* **49**:58.
24. PERRY, R. P. 1964. National Cancer Institute Monograph No. 14. 73.
25. STENRAM, V. 1964. *Exptl. Cell Res.* **36**:242.
26. LEBLOND, C. P., and M. AMANO. 1962. *J. Histochem. Cytochem.* **10**:162.
27. SIRLIN, J. L. 1962. *Progr. Biophys.* **12**:22.
28. SUSKIND, R. G. 1965. *J. Cell Biol.* **24**:309.
29. PERRY, R. P. 1963. *Exptl. Cell Res.* **29**:400.
30. SCHERRER, K., H. LATHAM, and J. E. DARNELL. 1963. *Proc. Natl. Acad. Sci. U. S.* **49**:240.
31. SPICER, S. S. 1962. *J. Histochem. Cytochem.* **10**:691.
32. WINKELMAN, J., and S. S. SPICER. 1963. *J. Histochem. Cytochem.* **11**:489.
33. LOVE, R., and R. G. SUSKIND. 1961. *Exptl. Cell Res.* **22**:193.
34. LACOUR, L. F., and J. W. C. CRAWLEY. 1965. *Chromosoma.* **16**:124.
35. WALLACE, H., and M. L. BIRNSTIEL. 1966. *Biochim. Biophys. Acta.* **114**:296.
36. BRUNISH, R., and J. M. LUCK. 1952. *J. Biol. Chem.* **197**:869.
37. WOODS, P. S., and G. ZUBAY. 1965. *Proc. Natl. Acad. Sci. U. S.* **54**:1705.
38. SUSKIND, R. G. 1966. *Federation Proc.* **25**:233.
39. EAGLE H. 1953. *Science.* **130**:432.
40. MILLONIG, G. 1962. *In* Electron Microscopy: Fifth International Congress on Electron Microscopy Held in Philadelphia, Pennsylvania August 29th to September 5th, 1962. S. S. Breese, Jr., editor. Academic Press Inc., New York. **2**:P8.
41. SARIN, P. S., and P. C. ZAMECNIK. 1964. *Biochim. Biophys. Acta.* **91**:653.
42. FICQ, A. 1959. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press Inc., New York. **1**:77.
43. YARMOLINSKY, M. B., and C. L. DE LA HABA. 1959. *Proc. Natl. Acad. Sci. U. S.* **45**:1721.
44. NATHANS, D. 1964. *Federation Proc.* **23**:984.
45. RABINOWITZ, M., and J. M. FISHER. 1962. *J. Biol. Chem.* **237**:477.
46. MORRIS, A., S. FAVELUKES, R. ARLINGHAUS, and R. SCHWEET. 1962. *Biochem. Biophys. Res. Commun.* **7**:326.
47. MATTINGLY, A. 1963. *Exptl. Cell Res.* **29**:314.
48. WANG, T. Y. 1963. *Biochim. Biophys. Acta.* **68**:52.
49. SELLS, B. H., AND K. CRUDUP. 1966. *Biochim. Biophys. Acta.* **123**:253.
50. TAMAOKI, T., AND G. C. MUELLER. 1963. *Biochem. Biophys. Res. Commun.* **11**:404.
51. HOLLAND, J. J. 1963. *Proc. Natl. Acad. Sci. U. S.* **50**:436.
52. STUDZINSKI, G. P., and R. LOVE. 1964. *J. Cell Biol.* **22**:493.
53. STUDZINSKI, G. P., and L. G. JACKSON. 1966. *Nature.* **212**:194.
54. SOBER, H. A., S. F. SCHLOSSMAN, A. YARON, and G. W. RUSHIZKY. 1966. *Biochemistry.* **5**:3608.
55. HOLTZMAN, E. 1965. *J. Histochem. Cytochem.* **13**: 318–327.
56. MARCIELLO, R., and G. ZUBAY. 1964. *Biochem. Biophys. Res. Commun.* **14**:272.
57. WANG, T. Y. 1965. *Proc. Natl. Acad. Sci. U. S.* **54**:800.
58. LAMM, M. E., L. CHILDERS, and M. K. WOLF. 1965. *J. Cell Biol.* **27**:313.
59. FREEDMAN, M. L., A. R. HONIG, and M. RABINOVITZ. 1966. *Exptl. Cell Res.* **44**:263.
60. REVEL, M., H. H. HIATT, and J. P. REVEL. 1964. *Science.* **146**:1311.
61. HONIG, G. R., and M. RABINOWITZ. 1965. *Science.* **149**:1504.
62. WIESNER, R., G. ACS, E. REICH, and A. SHAFIQ. 1965. *J. Cell Biol.* **27**:47.